

Biosensor-Bearing Wound Dressings for Continuous Monitoring of Hard-to-Heal Wounds: Now and Next

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Abstract

Skin wound management, especially of hard-to-heal and burn wounds, has not only a major impact on the quality of life of patients, but also on society at large due to tremendous direct or indirect cost. A significant part of the nursing activities is actually attributed to wound management. State-of-the-art clinical protocols call for frequent removal and replacement of wound dressings, especially to allow clinicians to evaluate the state of the wound. However, in case of good healing progress frequent changing the wound dressing is not only superfluous but also potentially hampers the healing process. Wound dressings provided with point-of-care biosensors (able to periodically acquire and to provide the wound manager with information on the state of the wound) could significantly optimize and improve wound management and therapy. This review summarizes the latest developments in this field, highlights gaps that need to be filled and provides a foresight on future developments.

Keywords: Biosensor; Burn; Dressing; Hard-to-Heal; Smart; Wound

Introduction

Human skin has a remarkable capacity to repair or regenerate upon wounding. However, occasionally if wound size exceeds certain limits or if the wound aetiology gives rise to it dedicated

treatment by medical experts is required. In general, skin wounds are classified in acute and hard-to-heal wounds (Figure 1). Of the acute wound the burn wounds as well as hard to heal wounds not only have a tremendous impact on the quality of life of the patients, but also greatly affect healthcare systems due to high evoked costs [1]. Burn and hard-to-heal wounds often require intensive long-term treatment.

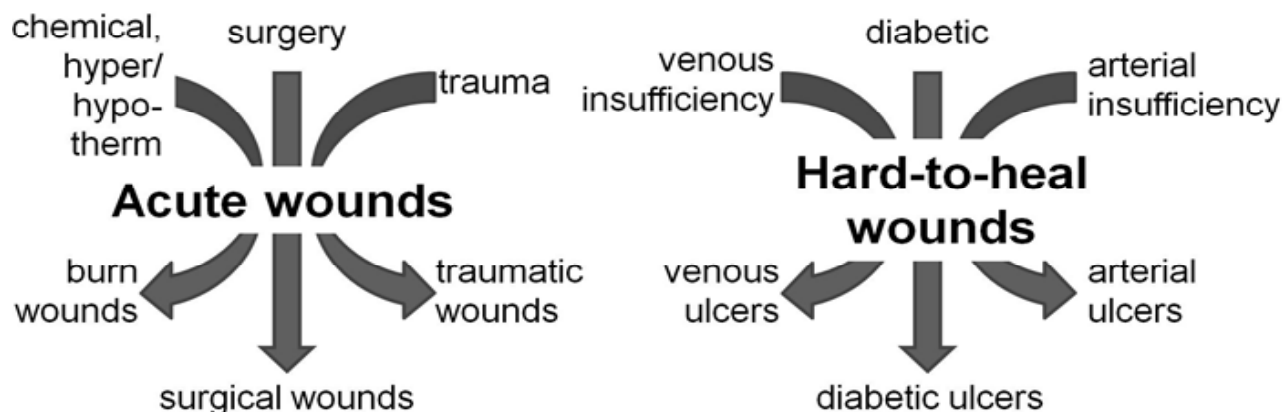


Figure 1: The schematic representation of the different common types of acute due to acute events and hard-to-heal wounds as symptom of the pathological state of the local tissue or patient.

A substantial proportion of the nursing activities are restricted to wound control in order not to miss the moment where additional treatment starts to be needed [1]. However, this is generally connected with frequent and often painful replacement of the wound dressing. Every unnecessary wound dressing replacement, however, impedes the progress of the wound healing process. The problem of unnecessary wound care interventions was shown in a recent report, where around 85% of the wounds that underwent a change of wound dressings were actually not infected [2]. Knowing the wound condition beforehand such unnecessary dressing replacements could have been avoided [3]. Currently, control of the wound generally occurs by visual inspection after removal of the non-transparent wound dressing. One way is to solve this is making wound dressing fully transparent [4]. However, even with making visual inspection possible, it may be too late. If the latter visual inspection justifies additional tests, wound alterations are already in a rather late pathological state [5]. A prompt intervention at the earliest time point, at which normal healing changes towards a pathological process, may overcome a tedious and costly wound care management procedure. Therefore, it is of paramount importance for optimal wound therapy, to know how wound characteristics change over time. For this, however, smart wound dressings that allow non-invasive (semi-) continuous monitoring of these properties are necessary. While it is still a long road before such dressings will be available for routine clinical application, recent publications [6,7] and patents (e.g. WO2015168720, US2014298927 and US2014298928) indicate that we are on the verge of developing the next generation of wound dressings, which not only support wound healing but which are also “Smart” due to the presence of sensors monitoring the state of the wound. For this however, such biosensors need to fulfil a range of specifications including: (i) high specificity for the target (specific protein, sugar, lipid, ECM component, etc.), (ii) sensitivity in the relevant concentration-range, (iii) stability for real-time applications over a period of days to weeks, (iv) self-regenerating to enable multiple measurements of the target at varying concentrations and (v) modular organisation to allow assemblies of multiple sensors as 2D-array. The latter is of crucial importance for most target parameters since the wound state may diverge between the locations within the wound.

In the present review, after a short overview of wounds including bacterial contamination, current concepts for smart dressings that monitor wound state characteristics are described. Additionally, some gaps are identified that need to be filled before major progress in this field can be made. Furthermore, a foresight is given on what developments we may expect in future.

Background

Wounds

Normal Wound Healing: The skin is a layered structure which can

be subdivided in: (i) the non-vascularized epidermis as top layer mainly consisting of keratinocytes, (ii) the dermis as vascularized connective tissue mainly with fibroblasts, macrophages, hair follicles, glands, lymphatic vessels and nerve endings, and (iii) hypodermis containing mainly large blood vessels, fibroblasts and adipocytes. The skin represents an important barrier protecting the body for microbial attack and fluid loss. After wounding these structures are locally (partially) disrupted and repair processes start. Whereas superficial-layer wounds still have the hair follicle reservoir to heal, in case of full-thickness wounds can only heal from the edges. As a result, in the latter case local differences in wound chemistry are expected at different locations within the wound. Normal wound healing represents an orchestrated cascade of cellular and biochemical processes. It can be divided in at least 4 overlapping phases, i.e., haemostasis, inflammation, granulation (also called proliferation or fibroblastic phase) and remodelling.

At the initial phases at which wound is not closed by a scab wound fluid will be secreted. Due to the injured vasculature or vascular leakage wound fluid composition of acute surgical and traumatic wounds reflects at the beginning largely that of blood serum. However, during and after closure of vascular defects the wound fluid content changes and is suggested to contain among others high levels of Interleukin (IL)-6 and IL-8, as well as elevated levels of Monocyte Chemoattractant Protein (MCP)-1 (also termed C-C motif-chemokine ligand 2 or CCL2), IL-1 Receptor Antagonist (RA), Platelet Derived Growth Factor (PDGF), interferon gamma-Induced Protein (IP)-10 (also termed C-X-C motif chemokine 10 or CXCL10), Interferon (IFN)- γ and Tumour Necrosis Factor (TNF)- α with relatively low levels of IL-1 β and IL-10 as measured 1 day after a surgical wounding [8]. After a first rise expression of cytokines is returning to normal levels during wound healing as for instance is shown for CCL2 and TNF- α [9]. Regarding ions it is reported that in rats during the first 5 days of normal healing after full thickness skin wounding Ca^{2+} concentrations are increasing to return to normal levels afterwards [10]. At day 5 after wounding also increased levels of Mg^{2+} and Zn^{2+} were found.

Burn Wounds: Persons with acute wounds including burn wounds are generally in a good healthy state at the moment of wounding. This is in contrast to patients with hard-to-heal wounds as discussed in the next chapter. Furthermore, in comparison to other acute wounds based on other impacts (e.g. cut, rupture) and also from hard-to-heal wounds, burn wounds behave different in several respects. Heat itself induces a set of general effects. For instance, due to heat capillary permeability largely increased resulting in a significant plasma loss. This fluid flow is mostly stopped until 48h after thermal wounding. The wound fluid composition also differs from that of other wounds or blood serum promoting not only the healing process [11] due to the higher levels of angiogenin, but also inhibits (or at least diminish) proliferation of the wound contaminating bacteria [12]. Additionally, Nissen and co-workers found significant lower levels of fibroblast growth factor (FGF)-2 in burn relative to that of surgical wound fluid both being collected 6-12 h after injury [13]. Furthermore, especially if affected areas

are large and deep (second-deep and third degree), immune system deregulations occur. These manifests as immunosuppression [14] in the beginning resulting in an increased likelihood of infection. Concomitant a hyper inflammatory response is seen with a chance of a shock [15,16]. This is not seen with other wounds. For instance, Mikhal'chik and co-workers [17] reported that before surgical treatment (around 4 days after burn) and relative to healthy children the blood plasma of children with uncomplicated burns exhibited increased concentrations of IL-6, IL-8 and MCP-1. In blood serum of children with complicated burns (septic toxemia, toxemia, and pneumonia) additionally the concentrations of IL-1RA, IL-10, TNF- α , IFN- γ and Granulocyte Colony-Stimulating Factor (GM-CSF) were increased. Plasma cytokine concentrations in the last group were generally much higher than in first group of patients. Interesting to note is that no correlation was found between the concentration of any of the evaluated 27 cytokines in blood plasma and exudate.

Hard-to-Heal Wounds: In most cases wounds are able to heal rapidly and completely within weeks. However, in some cases they need, if at all, a very long time to heal, do very often never really close and/or wounds rapidly re-occur [18]. The latter are termed hard-to-heal or chronic wounds. Although so far, no official definition exists for hard-to-heal wounds [19], it is general accepted that these wounds fail to heal with standard therapy in an orderly and timely manner. This stagnation of the healing process may occur at any for the sequential phases of normal wound healing. Commonly, a prolonged strong reduction of blood and oxygen supply is the cause. The latter may be due on the one hand on bad vasculature. The latter may be evoked by disturbed transport of blood away from the involved area giving rise to venous ulcers or by insufficient transport to this area causing arterial (insufficiency) ulcers. On the other hand, reduced blood circulation may be based on external pressure (e.g. limited change of position in bed or wheel chair resulting in decubitus ulcers or pressure ulcers). People with diabetes mellitus have a prevalence to develop a pressure ulcer

kind of ulcer that occur at the bottom of the feet and is termed Diabetic (Foot) Ulcer (DFU) [20]. The predisposition of diabetic in developing hard-to-heal wounds are based on various factors such as microcirculatory deficiencies [21], decreased Activated Protein C (APC) levels [22] or the presence of hyperglycaemia [23].

Several aspects may be mentioned that characterizes a chronic wound. One aspect is certainly the local tissue temperature which is increased at the wound site compared with peri-wound skin [24]. Increase of temperature is a sign of increased inflammation, critical microbial colonization or other factors disturbing the wound healing. As mentioned above, another characteristic of wounds is the production of wound fluid as reaction on tissue injury. The wound fluid is on the one hand defined by the amount that is produced per time unit and on the other hand by its pH and composition. Comparing acute normal healing and chronic wounds differences are seen in the change of wound fluid pH over time [25]. At wounding the acute wound pH is around 6.2 and rapidly go down to a pH below 6. During the inflammation phase the pH rises and reaches in the granulation phase a value above 7 during the granulation phase. During epithelization pH goes down again to around 6.2. In case of a hard-to-heal wound the latter decrease does not occur. It stays above pH 7. The pH has a large impact on wound healing mainly due to its effects on enzyme activity and bacterial colonization [25].

Besides the pH evolution also the wound fluid composition is different depending on the healing stage and wound type. It may be assumed that the wound fluid is directly reflecting the state of the wound bed physiology [26] and differs from that of the blood serum [27-29]. Most wound fluids of hard-to-heal wounds are characterized by increased proteolytic activity and increased levels of a large set of immune cells specific cytokines and other molecules (Table 1(A, B)).

A. Diabetic foot ulcers

Component and levels in non-healing wound	Group size and kind	Remarks	References
lactate \uparrow	45 pa with infected DFU, 64 non-infected DFU and 15 non-infected healed DFU, 20 pe	lactate concentration is correlated with wound severity of DFU and presence of infection	[30]
IL-6 \uparrow , CRP \uparrow , fibrinogen \uparrow , MIP1 α \uparrow , MIF \uparrow , IP-10 \uparrow , RANTES \downarrow upon infection	170 DFU pa, 140 pe	associated with the severity of diabetic foot ulcerations; no difference in IL-8 and MCP-1	[31]
MMP-9 \uparrow , MMP-9:TIMP-1 ratio \uparrow	23 DFU pa with finally healed and 39 DFU pa with non-healed ulcers	poor wound healing in diabetic foot ulcers versus good healing ones	[32]

tenascin↑, Serum amyloid P-component↑, collagens 1A1, 1A8, 3A6, 5A2, 6A3↑, ANX-A3, -A4, -A5, -A6, -A11↑, Protein S100A-4, -8, -9 & -P↑, Pyridoxal kinase↑, putative phospholipase B-like 1↑, Ke-I/14↑, soluble TGFβR3↓	10 pa with DFU, 6 pe	Comparison DFU versus acute wounds	[33]
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B. Venous ulcers

Component and levels in non-healing wound	Group size and kind	Remarks	References
urate↑, urate precursors (e.g. adenosine) ↓	29 pa	elevated uric acid and depletion of precursors correlate with wound severity	[34]
inactivation of α1-antichymotrypsin (by neutrophil elastase)	15 pa, 5 pe	inactivation is correlated with severity of wound healing	[35]
IL-6 ↑, TNF α↑	25 pa with non-healing venous ulcer, 20 pa with mixed venous/arterial ulcers	IL-6 was especially elevated at high bacterial load; CRP and LBP were not associated with the level of bacterial triggers in hard-to-heal wounds	[27]
MMP1,2,3,7,8,9,12,13↑	29 pa with new untreated venous leg ulcers	comparison ulcer versus healthy tissue; no increase in MMP7	[36]
IL-1α↓, IL-1β↓, IFN-γ↓, IL-12p40↓ and GM-CSF↓	29 pa with new untreated venous leg ulcers	comparison ulcer fluid: <40% healed vs. >40% healed	[36]
IL-8↓, MIP1α↓, Lipocalin-2↑	8 pa: 4 pa with healing and 4 pa with non-healing wounds	comparison non-healing vs healing wounds fluid	[37]
CRP↑	8 pa	non-healing vs healing wounds fluid	[29]
FC β↑, FC γ↑, COMP↓, vitronectin↑, tetranectin↓, olfactomedin-4↑, fibulin-1↓, HSP2↑, CAC 1(I)↓, CAC 2(I)↓, CAC 3(III)↓, MMP9↑, thrombin↓, elastase↑, PK3↑, Cystatin C & M↓, Cystatin A↑, SPI A1 & F1↓, SPI D1 & B4↑, adipsin↓, peroxiredoxin-2↑, azurocidin-1↑, myeloperoxidase↑, BPIP↑, lactotransferrin↑, lipocalin↑, dermicidin↓, annexin A1↑, SCBP A7-9↑	19 pa: 9 pe	comparison venous ulcer vs acute healing wound fluid	[38]
TGFβ1↓	80 pa	inversely correlated with venous ulcer size change; increased level in healing ulcers.	[28]
MCP: monocyte chemoattractant protein; MIF: macrophage migration inhibitory factor; CRP: creatinine reactive protein; IP: interferon-γ-inducible protein; LBP: lipopolysaccharide-binding protein; MMP: matrix metalloproteinase; TIMP: Tissue Inhibitor of matrix metalloproteinase; COMP: cartilage oligomeric matrix protein; PK: protein kinase; HSP: heparan sulfate proteoglycan; CAC: collagen α-chain; SPI: serpin peptidase inhibitor (clade and member); BPIP: bactericidal/permeability-increasing protein; SCBP: S100 calcium binding protein; FC: fibrinogen chain; TGFβR: TGF beta receptor; ANX: annexin; Ke-I/14: Keratin, type I cytoskeletal 14. DFU: hard-to-heal diabetic foot ulcer; pa: patient; pe: healthy person			

Table 1: Some wound fluid components which are significantly reduced or increased to a relevant extent in non-healing diabetic foot ulcer and venous leg ulcer wound.

Components that are released by bacteria (Table 2). Non-healing may be associated with persistent stimulation of the innate immune response as suggested by Pukstad and co-workers for hard-to-heal venous leg ulcers [37]. This may be based on the diseased state of the patient [39] and/or due to the presence of bacteria. Generally, a hard-to-heal wound is contaminated with bacteria. The inflammatory cells besides combatting the microbial contaminants also release proteases and actively degrade the provisional Extracellular Matrix (ECM) [40]. The latter is needed for starting tissue regeneration. Thus, inflammation seems to be a general aspect of the hard-to-heal wound. However, Liu and co-workers concluded after reviewing various publications regarding

venous ulcer pathogenesis that many of the statements made do not withstand a critical examination [41]. For instance, against current opinion, they suggest that there is no proven clear indication that pro-inflammatory cytokines and growth factors are increased in hard-to-heal venous ulcers or that TGF- β and TNF- α are actively involved in the chronicity of the disease. Due to unclear patient inclusion-exclusion criteria of the studies, by not taking confounding factors into account (like age, gender, and wound aetiology) and/or too small number of patients the base on which the statements are funded is rather weak. The same may be true for other types of ulcers. As a result, there is certainly a need for new studies which take the criticised aspects into account.

Bacterial species	Molecules	Remarks	References
<i>P. aeruginosa</i>	odd-carbon methyl ketones, particularly 2-nonanone and 2-undecanone, and 2-aminoacetophenone. Dimethyldisulfide and dimethyltrisulfide. Butanol, 2-butanone, 1-undecene, and isopentanol	Volatile molecules (partly responsible for the malodour)	[42]
<i>P. aeruginosa</i>	Rhamnolipid B	QS-regulated; Toxic for polymorphonuclear leukocytes	[43]
<i>P. aeruginosa</i>	Pyocyanin	QS-regulated; antimicrobial to <i>E. coli</i> ; cytotoxic	[44]
<i>P. aeruginosa</i>	NHLs (OdDHL and N-butanoyl homoserine lactone), 4-hydroxy-2-heptylquinoline, 3,4-dihydroxy-2-heptylquinoline	QS molecules. OdDHL have bactericidal activity toward gram-positive organisms, including <i>S. aureus</i> , but not toward gram-negative bacteria	[45-48]
Gram(+) and (-) bacteria	AI-2	QS group of furanones, resulted from spontaneous cyclization of DPD	[49]
Gram (-) bacteria	DSF, fatty acid methyl esters	QS molecules	[48]
<i>S. aureus</i> and <i>S. epidermis</i>	AIP molecules	AIP (QS molecules) activates the agr system controlling the expression of a series of toxins and virulence factors and the interaction with the innate immune system	[47,50]

DPD: 4,5-dihydroxy-2,3-pentanedione; NHLs: N-acylhomoserine lactones; DSF: cis-11-methyl-2-dodecenoic acid; OdDHL: N-3-oxododecanoyl homoserine lactone; AIP: gram-positive bacteria autoinducing peptides; agr: accessory gene regulator.

Table 2: Examples of Quorum Sensing (QS) signalling and other molecules released or produced by bacteria.

Bacterial Colonization and Its Effects on Wound Healing

Bacterial contamination of the wound is seen as a crucial factor in the delay of wound healing. It has been reported that more than 1000 bacterial species normally live on the human skin [51]. As a result, after wounding it is not a question if but a matter of time a wound gets contaminated by single planktonic bacteria. The bacterial composition of contaminated wounds is extremely complex and varies between patient and wound [52].

However, some similarities in bacterial composition seem to exist between the types of wounds. For instance, *Acinetobacteria* were mostly detected in acute wound infections of patients injured on the battle field [53]. This group of bacteria is also most prominent on normal skin [54]. In contrast, in hard-to-heal wounds (characterized by a chronic bacterial contamination) increased populations of *Firmicutes* (with *Staphylococcus aureus* as member) and *Proteobacteria* (e.g. *Pseudomonas aeruginosa*) and less *Acinetobacteria* are observed [54]. A colonization of the wound by methicillin-resistant *S. aureus* and *P. aeruginosa* are

suggested to delay wound healing [55]. The effect of products of *P. aeruginosa* (primarily Rhamnolipid B) is reported to inhibit bacterial clearance by polymorphonuclear leukocytes [43,56]. This may explain why low levels of *P. aeruginosa* facilitate *S. aureus* infection in a rat model of complex orthopaedic wounds [57]. However, bacteria may not only be detrimental for wound healing. It has been reported that inoculation of a sterile wound with low levels of *S. aureus* or of *P. aeruginosa* may even promote wound healing [45,58].

A wound represents an optimal environment for bacterial colonization and proliferation: It is warm, moist and nutritious. These bacteria release, among others, a set of so called, quorum signalling molecules. Above a certain threshold level these molecules modify bacterial gene expression (quorum sensing, QS) and the excretion of biofilm matrix components starts. Embedded in this matrix bacteria are protected from attacks by the environment. After biofilm maturation planktonic bacteria are released that may start a new colony elsewhere or invade the tissue as premise for infection. Given the importance attributed to QS for gene regulation in bacteria [48] it may be interesting to note that significant differences in gene expression of *P. aeruginosa* in burn and hard-to-heal wounds are observed [59]. A variety of molecules are known to serve as QS molecules with different degree of specificity, i.e. from bacterial class to species specific. However, also other components which do not serve as signalling molecules are synthesized and released, produced as metabolites or which formation are catalysed by bacteria. Biogenic diamines and thiol compounds and other organic species may be related to bacterial degradation of the tissues [60]. Some examples are listed in Table 2. Depending on their vapour pressure a part may also vaporize. They are responsible for the malodour which is characteristic for increased anaerobic bacterial species colonizing the hard-to-heal wound [61]. Evidence was found that alterations in the overall VOC (Volatile organic compound) profile rather than quantitative analysis of single specific VOC species may give the greatest insight into hard-to-heal wound metabolic processes [60].

Current Wound Management and Dressings

Burn and hard-to-heal wound management aims to stimulate wound closure by provide an optimal environment for healing, e.g. by giving optimal moisture balance, protect wound edges, remove dead cells, fibrin and biofilm by good debridement, and antimicrobial treatment [62-64]. Besides grafting of skin (temporary) skin substitutes in case of burn skin wounds [65], the currently used dressings and bandages focus predominantly on protection of the wound, exudate and moisture management, aid in debridement, combating bacterial contamination, reduction of malodour and wound pain [66-69]. Despite the proven positive effects of these a number of treatments [70] the closure of burn and

hard-to-heal wounds still remain extremely challenging [65,71]. Since failure of treatment may result in amputation and morbidity, currently a next generation of dressings are under development or already on the market focussing additionally on promotion of vasculature for instance by vacuum therapy, improving wound oxygenation by additional hyperbaric oxygen pressure or by adding oxygen delivering dressings, on stimulating wound regeneration by steering the wound pH, by adding therapeutic agents including antibiotics and/ or on delivery of an (artificial) degradable extracellular matrix backbone as intermediate solution for improved tissue regeneration [4,25,67,69,72-76]. Although the latter dressings positively affect wound regeneration, they are not able to provide the wound manager with information what the actual state of the wound below the dressings is. However, large efforts are currently made in developing high-tech dressings including sensors with which at least some wound parameters can be monitored, such as moisture, pH, oxygen tension and/or temperature [3,77-79].

Monitoring the State of Wounds by Means of Biosensors

In the last decade an increasing number of biosensors have been developed. Sensors can be classified according the technology used (optical, electrochemical, thermometric, piezoelectric or magnetic) or according the parameter which is measured. In relation to wounds these parameters are for instance temperature, moisture, pH, oxygen tension and wound fluid and volatile components including bacterial factors. In the next few chapters of each characteristic type some latest examples in biosensor development are given.

Temperature

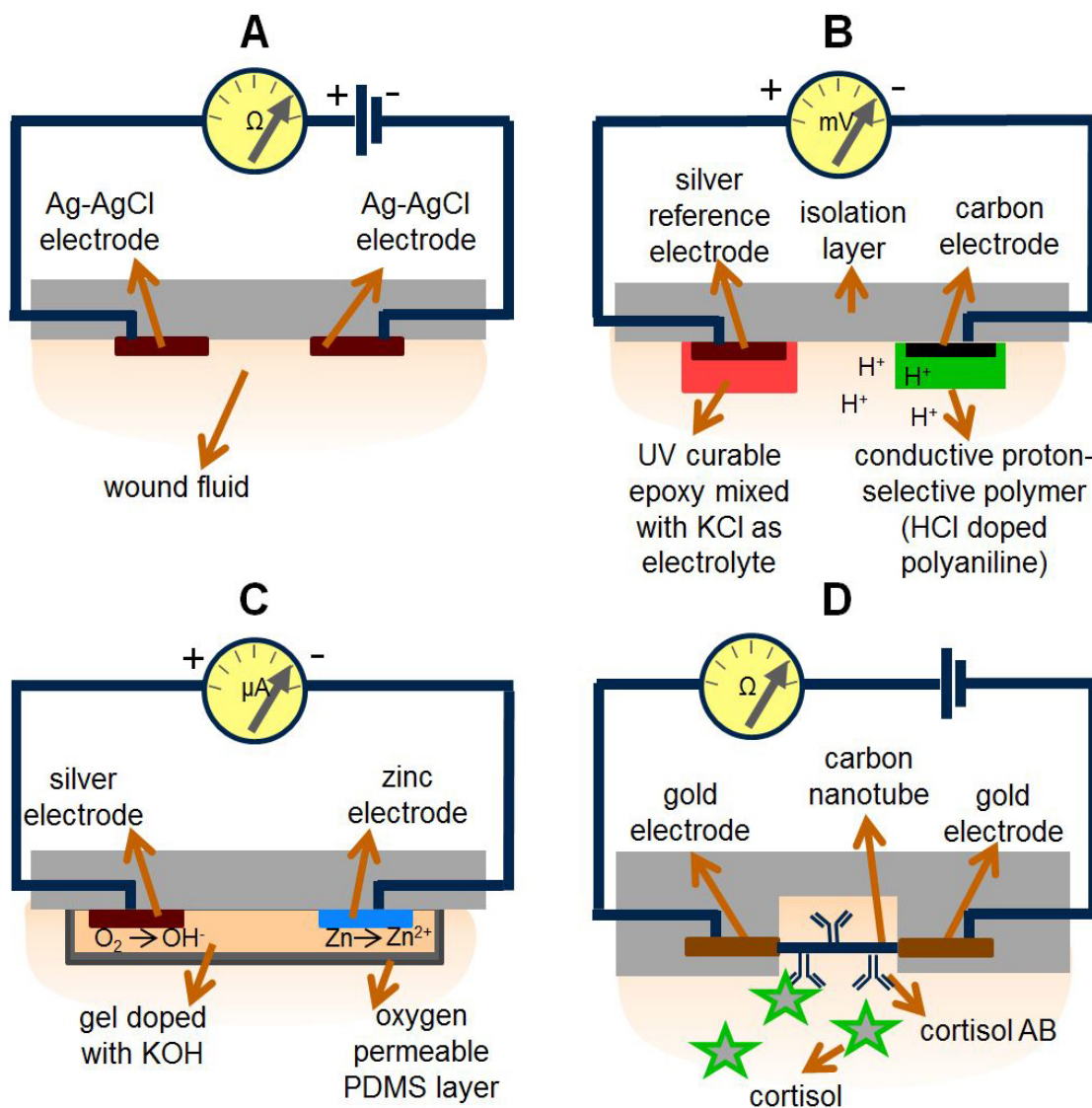
One characteristic of hard-to-heal wounds is the inflammation-based local increase in temperature. High temperature gradients between feet of diabetic type 2 patients may predict onset of neuropathic ulceration [80] or to predict healing of venous ulcers [24]. Some efforts were made to assess the surface temperature, on the one hand by infra-red images [24,81] The key limitations of the infra-red images are that the dressing has to be removed for this and the absence of on-line monitoring possibilities. Their advantage is certainly the high 2 D resolution. On the other hand, biosensors have been developed based on the use of thermistor materials (materials which resistance varies with temperature and the latter reproducible). These materials are usually films which can be made of metal (such as platinum [82] or gold [83] or carbon (graphite or multiwall Carbon Nanotubes (CNT) [84,85]. These biosensors make an on-line monitoring possible and organized in an array they deliver a low resolution 2 D picture of skin surface temperature [85]. To improve the flexibility of the sensor (array) recently an interesting concept was developed by Chen and co-

workers [83]. The sensor is based on a highly meandering gold lane which is placed between water repellent breathable flexible semipermeable membranes with flexible CNT films for the wiring. The challenges of this kind of sensors still represent the signal to noise ratio, for obtaining a high-resolution picture of the temperature distribution the size of the sensors and the wiring organisation of the array of sensors.

Moisture

For a good wound healing a correct moisture balance is needed. This is between insufficient, resulting in a drying out of

the wound killing the ingrowing cells, and excessive, which may lead to tissue maceration. Various methodologies to measure moisture have been described [86,87], However, only one is so far implemented for moisture measurements in wound dressings. In 2013 a commercial product came on the market (Wound Sense of Ohmedics) with which moisture condition at the wound site can be monitored. It is based on 2 parallel electrodes with a defined distance measuring the impedance between them [3,77] (Figure 2A). Recently also another promising approach was described to assess moisture in bandages [88]. It makes use of the capability of graphene to bind water molecules. The latter affect its conductivity which can be measured [89].

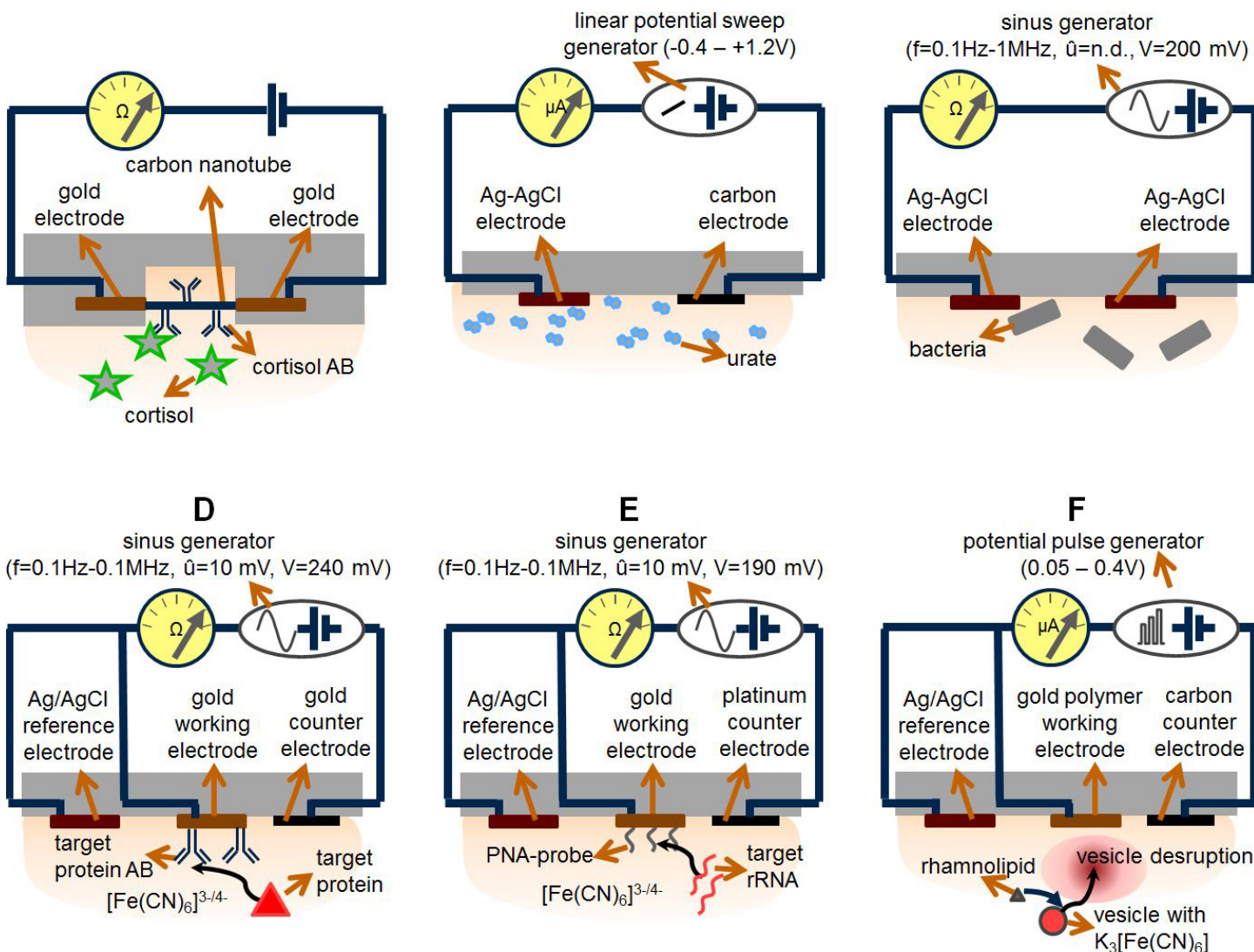


Figures 2(A-D): Schematic representation of various potentiometric kind of biosensors. **A:** moisture biosensor based on resistance measurements [3]. **B:** pH biosensor based on surface charge change depending on proton concentration [90]. **C:** oxygen biosensor based on reduction of Zn to Zn²⁺-ions [91]. **D:** Cortisol biosensor based on change in resistance [92]. AB: antibody.

pH

The pH evolution of a wound is dependent on its state of healing and the phase of wound regeneration [25], the tissue type and bacterial colonization [93]. By that the pH cannot be used as sole parameter to assess the wound condition. Nevertheless, biosensors have been developed enabling a monitoring of the wound pH. For instance, a sprayable one which is based on the use of amino cellulose particles functionalized with fluorescein isothiocyanate as pH indicator and [ruthenium(II)-tris-(4,7-diphenyl-1,10-

phenanthroline as reference dye. pH pattern of the wound is visualized by processing pictures taken using two wavelengths, one to determine the pH-dependent luminescence of the pH indicator and one to relate this to the pH independent intensity of the reference dye [94]. A limitation of this concept is its unknown long-term effect of the molecules used on the tissue (cytotoxicity and the inability for on-line monitoring. More applicable seem to be in this regards the concepts based on electrochemical measurements. For instance, recently Rahimi and co-workers described an inexpensive, flexible array of potentiometric kind



Figures 3(A-F): Schematic representation of various voltammetric kind of biosensors using 2 (A_C) or 3 (D-F) electrodes. **A:** pH biosensor based on square wave voltammograms [95]. **B:** urate biosensor based on change of linear sweep voltammograms [96]. **C:** Bacterial biosensor based on change in frequency sweep impedance relationship [97]. **pH biosensor based on surface charge change depending on proton concentration [90].** **D:** protein biomarker biosensor based on impedance change [98] and **E:** bacterial rRNA [99] biosensor based on change of frequency (cyclic) sweep impedance patterns. **F:** bacterial toxin (rhamnolipid) sensitive biosensor based on change in differential pulse voltammogram patterns [100]. f= frequency; \hat{u} = amplitude; n.d.: not defined; AB: antibody.

(the difference in electrode potentials is measured) of pH sensors which can be integrated in a wound dressing [90] (Figure 2B).

The operation of the pH sensors is based on the protonation and de-protonation of nitrogen atoms by the H^+ -ions of the wound fluid in the polymer chains of the polyaniline layer around the carbon electrode. The resulting change at the electrode surface charge relative to the reference electrode is taken as an index of wound fluid pH. One limitation of application is the period of use since KCl concentration of the reference electrode cover will assimilate to that of the wound fluid. It must be noted that a drift in signal was already seen after 5 h of use. Beside potentiometric also voltammetry (the cell's current is measured over time) methodologies have been pursued. A recent example is given by Mc Lister and Davis [95]. They used a 2-electrode based system composed of a silver chloride reference electrode and a poly-L-tryptophan modified carbon fibre-mesh electrode (Figure 3A).

The quinoid moieties of the tryptophan serve herein as pH sensitive redox system. They found that the shape of the square wave voltammograms is directly related with the pH value. A further technology to measure the pH is the ISFET (ion sensitive field effect transistor) (Figure 4). Here the interaction with H^+ -ions with the pH sensitive layer changes the current flow between source and drain which is taken as index of the pH [101].

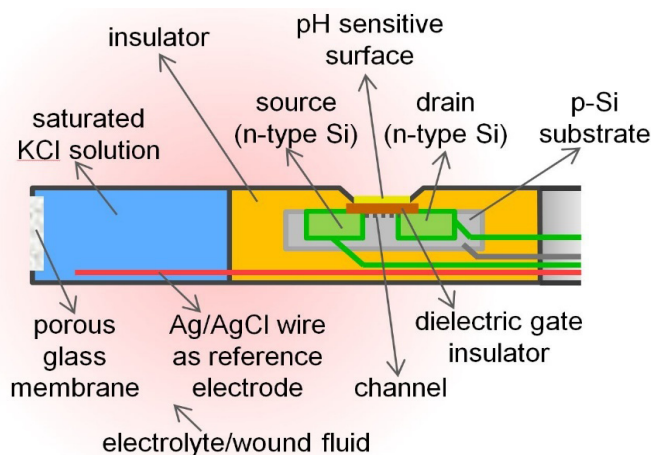


Figure 4: Schematic representation of an ISFET pH sensor with the various elements (e.g. source, gate, drain and reference electrode) (modified from [101,102]).

Oxygen Tension

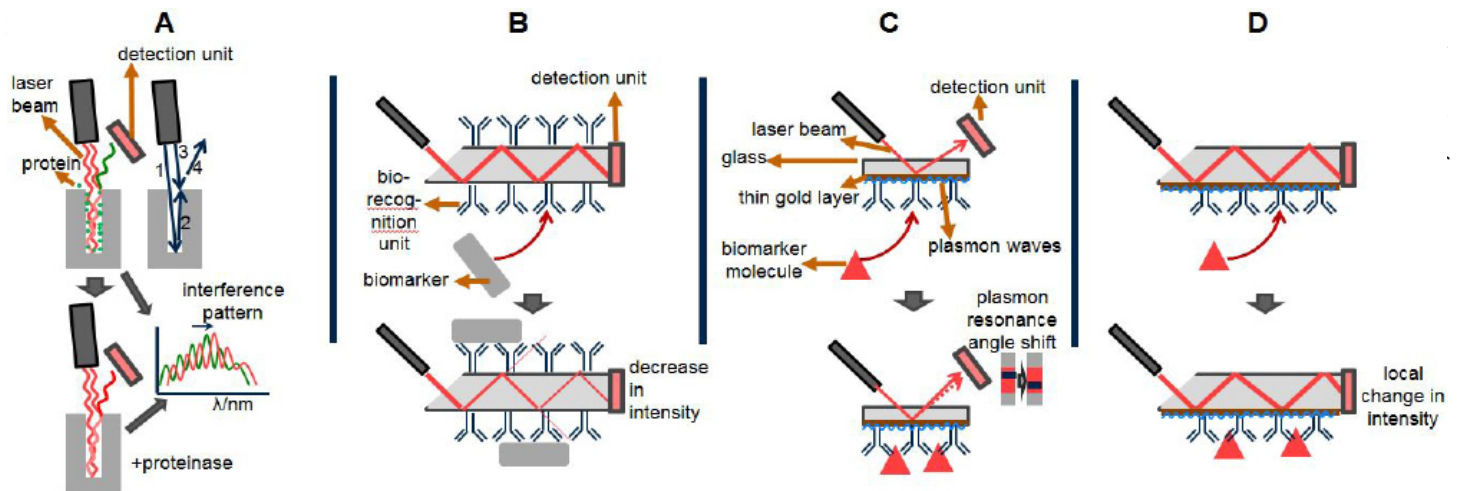
The oxygen tension in wounds is dramatically reduced. For instance, in hard-to-heal wounds it is only in the range of 0.6-2.6% whereas that of normal subcutaneous in the range of 4-7% [103]. Different methods to measure oxygen tension have been described. Mostafalu and co-workers developed a bandage based

on a flexible galvanic oxygen sensor to on-line monitor the wound oxygen tension [91] (Figure 2C). The measurement is based on the reduction O_2 at a silver electrode delivering OH^- as product. At the same time a zinc atom of the zinc electrode is reduced forming Zn^{2+} . The oxidation velocity is directly related to the oxygen concentration of the fluid that contacts the sensor. So far, the system has only been evaluated using a simulated wound set-up. The system has the potency to be organised as array but like all electrochemical set-up that were developed so far still large efforts must be put in the wiring concept and the miniaturization of the analysis device. Another type of sensor based on oxygen-dependent quenching of luminescence of metal porphyrin complexes has been described by Babilas and co-workers [104] and more recently by Wisniewski and co-workers [105]. With this immobilized in a polystyrene matrix as transparent planar sensor the surface pO_2 distribution can be mapped with a high temporal resolution of approximately 100 ms and a spatial resolution of at least 25 μm . The sensor as designed by Wisniewski and co-workers is integrated in an injectable tissue-integrating hydrogel with a size of 0.5X0.5X5 mm with which oxygen tension can be monitored for month to years inside the body.

Wound Fluid Components Including Bacterial Factors

The concentration of large set of molecules is highly dependent on the state of the wound. These are not only released or modified by cells of the patients but might also be synthesized or produced as metabolites by bacteria. All of these represent potential diagnostic marker molecules characterizing the wound state. Furthermore, their change in concentration may be used as an indication for the impact of wound therapy. Regarding the detection of specific components in wound fluids two different philosophies are currently pursued. One is to analyse the fluid after sampling in a specialized laboratory or using a kind of a test strip, and this with smallest volume, lowest costs, increasing specificity and highest sensitivity. The other one is to analyse it continuously directly within wound dressings. It can be recognized that the methodologies which are developed for external analysis are used as base or toolbox for the development of biosensors which can be integrated in wound dressings. Therefore, both kinds of biosensors are mentioned below.

Dressing External Optical Analysis: Several optical biosensors have been proposed for the detection of biomarkers wound fluid samples. Here one can distinguish label-free and label-based set-ups. Label-Free Optical Set-Up. One example of a label-free set-up is the one proposed by Krismastuti and co-workers for the detection of bacterial colonization of hard-to-heal wounds [106]. It is based on the interferometric reflectance at the Nano porous aluminium oxide surface due to difference in light path of laser in and outside the pores (Figure 5A). Here the pores are filled with a poly-lysine based substrate. The latter can be digested by



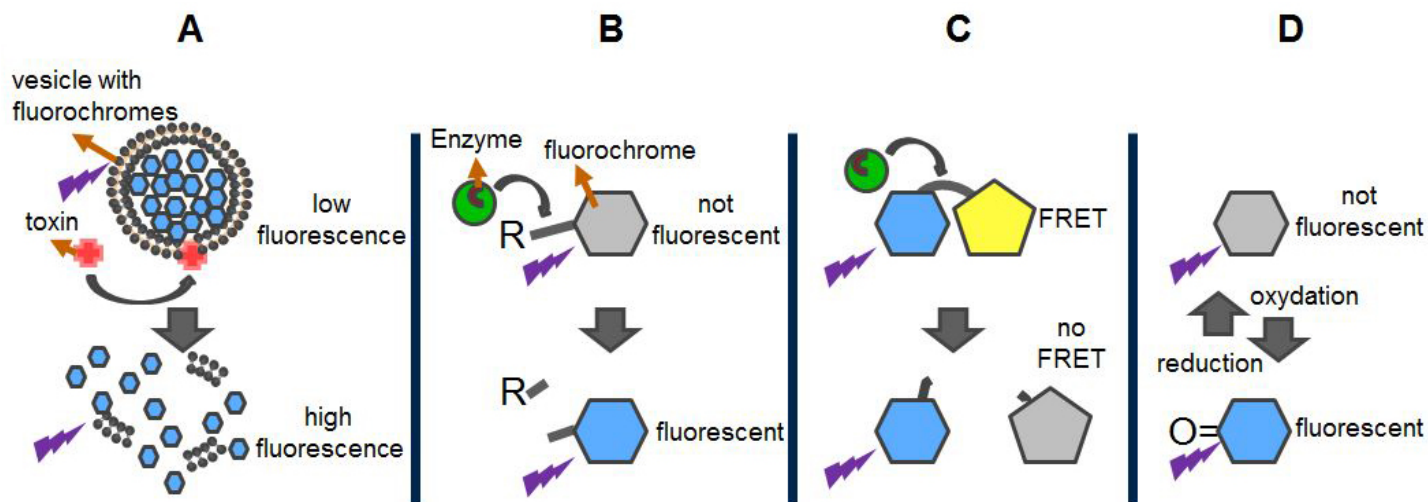
Figures 5(A-D): The schematic representation of the principle of an interferometric reflectance (A), evanescent wave fluorescence biosensor (B), surface plasmon resonance (C) and a combination of evanescent and plasmon resonance biosensor (D). In (A) part of the laser light goes to the bottom of the pores (1) and is reflected (2). This light will meet the other part of the light (3) which is reflected at the top surface. The combination of this reflected light and the light coming from path 3 will result in an interference pattern which is shifted if light (1) has to go through a protein layer. In (B) the binding of bacteria or marker protein as antigens to the antibodies will result in a coupling out of the evanescent wave and a decrease in light intensity at the end of the optical fibre. In (C) a laser beam is directed under a defined angle. Within the reflected light beam one angle exists at which due to the plasmon waves at the gold layer medium surface the light is annihilated representing the surface plasmon resonance angle. This angle is shifted if the boundary on the other side of the gold layer is changed for instance by proteins that are bound to it. The latter affect the plasmon waves and as a result the surface plasmon resonance angle. In (D) the principles of (B) and (C) are combined.

Its oscillations interfere with the laser light, i.e., it can enhance and reduce its intensity depending on the angle at which it hits the surface. The plasmon waves are sensitive to the adsorption of molecules of the medium. As a result, the interference is changed and by that the surface plasmon resonance angle as well as the light intensity at a certain reflectance angle. A slightly more sensitive and advanced set-up (Figure 5D) was developed by Liu and co-workers [109] combining the principles of plasmon resonance (Figure 5C) and evanescent wave biosensor (Figure 5B). For this a flow chamber was built around a light-guiding silica capillary that is stripped off its cladding and coated with a 50-nm gold film. The latter gold film is functionalized with antibodies against the biomarker of interest. The whole is connected to a smartphone, taking images and monitoring the change of relative intensity as index of the biomarker concentration in the perfusate.

Label-Based Optical Set-Up. The simplest and most common label-based assays for all types of antigens are the ELISA test and

immunofluorescence assay. Here biomarkers are quantified using specific antibodies which are linked to an enzyme catalysing the formation of a molecule with a specific absorbance, respectively, to a fluorochrome. The extent of absorption or fluorescence is directly related to the biomarker concentration of interest in the wound fluid. The immunofluorescence assay methodology was extended by using an evanescent set-up as described in figure 5B but labelling the antigen with a fluorochrome subsequently [110,111]. By being adsorbed the fluorochrome is located within the evanescence layer and excited as a result. Instead of measuring the light intensity at the end of the fibre, the fluorescence is measured which intensity is again reflecting the antigen concentration.

A more complex set-up is developed by Thet and Jenkins [100] for detecting bacterial toxins. Their sensor is based on toxin-sensitive vesicles which are filled with a fluorochrome (Figure 6A). Due to the presence of the toxin the vesicles break and the fluorochrome is released. As a result, the fluorescence intensity of the solution will increase.



Figures 6(A-D): The working principle of some fluorescence-based biosensors. **A:** Vesicles with fluorochromes are damaged by a biomarker (in this scheme a toxin). As a result, the fluorochrome is released resulting in an increase of fluorescence of the solution. **B:** A biomarker enzyme removes a part of the molecule (R) resulting in an increase of the fluorescence of the fluorochrome. **C:** A biomarker enzyme cleaves the connection between two fluorochromes resulting in the disappearance of the FRET signal. **D:** A non-fluorescent molecule is oxidized resulting in the formation of a highly fluorescent molecule.

Enzymes as biomarkers offer the possibility to take advantage of their characteristic to alter structures and to take the latter as index for their concentration. Here, specific moieties are connected to fluorochrome units suppressing its fluorescence capabilities. These moieties are designed in a way that they can be cleaved (removed) by the specific biomarker enzyme of interest. As a result, it becomes fluorescent again (Figure 6B). For example, Hasmann and co-workers developed optical sensors using this principle for assessing the activity of the serine protease neutrophil elastase (HNE) and cathepsin G (CatG) in the wound fluid [112]. These proteases are involved in the pathogenesis of a number of inflammatory disorders and are seen as markers for early infection. Another way of detection offers the linking of two different chromophores which in the linked situation deliver a FRET (Förster resonance energy transfer) signal (Figure 6C).

An enzymatic cleavage of this link increases the distance between the two resulting in the disappearance of the FRET phenomenon. This kind of set-up was used by Schulenberg and co-workers to quantify HNE activity [113]. Similarly, redox-responsive (and even reversible) near infrared small-molecule biosensors have been proposed which is based on the change in fluorescence intensity due to a redox induced structural change of the fluorochrome [114] (Figure 6D). On the one hand, since these chromophores as described are not bound to a surface and/or the assay is a multistep approach, these set-ups as proposed cannot be included as such as monitoring system in dressings. But even if so, the proposed set-up's (except the previous one) are designed for single measurement use and a fluorescence analysing unit

would have to be included in the dressing to be able to monitor fluorescence or FRET intensity speaking against the use of such sensor inside dressings. On the other hand, there are also clear advantages of these above mentioned-label-based optical set-ups for biomarker detection [113,115]. These assays are reported to be not only specific and sensitive but also can be done relatively quickly.

Dressing External Electrode-Based Analysis

Different kind of electrode-based biosensors have been developed (Figure 2D, 3, 4). For instance, a quick test for small wound fluid samples has been proposed for the detection of some key markers for inflammation (TREM-1, MMP-9 and bacterial HSL) [116]. This is done using Faradaic 3 electrode electrochemical impedance spectroscopy (for an in-depth description of the methodology see [98] (Figure 3D). For this antibodies against the marker molecule are bound to the working electrode and the change in impedance properties is reported to be related to the quantity of bound marker molecules. Because electrodes have to be treated with wound fluid before impedance measurement (for which a non-biocompatible $\text{Fe}(\text{CN})_6^{3-/4-}$ redox system is used) this set-up cannot as such be implemented for the use inside a wound dressing. A similar system is used by Henihan and co-workers for detecting bacterial rRNA [99] (Figure 3E). Here, Peptide Nucleic Acid (PNA) probe for the targeted rRNA is bound to the working electrode and after treating with solution containing the target rRNA again the change in impedance properties is measured. In this case the impedance characteristics are modified by the degree

of hybridisation. A variant of this method was described by Thet and Jenkins for detecting bacterial contamination (Figure 3F). They developed a 3-electrode electrochemical sensor concept for the detection of virulence factors from *S. aureus* and *P. aeruginosa*. It is also based on the use of non-biocompatible $K_3[Fe(CN)_6]$ but here biomimetic vesicles are containing it [100]. In the presence of toxins like rhamnolipids and delta toxin in the vesicles are damaged. The released content can be quantified by a change in redox current. An interesting new miniature biosensor approach for detecting ultralow quantities of biomarker molecules (1 pg/ml) in lowest volume samples was reported by Tlili and co-workers [92]. It is based on connecting two gold electrodes with a single walled carbon nanotube which is functionalised with an antibody against the biomarker of interest (in this study cortisol) (Figure 2D). In the presence of the biomarker the resistance is found to be concentration dependently strongly reduced. So far, this sensor has not been adapted for and evaluated with wound fluids. For analysing fluid compositions also, field effect transistors have been developed which are similar as the one shown in figure 4 (and to a certain degree the example shown in figure 2D) but with an analyte-specific modification of the dielectric gate insulator such as a specific antibody, enzyme or other reactant [102,118,117]. Unfortunately, all of these sensors are for single use only and not made for integration in wound dressings. It may be noted that the sensor platforms such as presented in figures 2D and 3A, D, E and 4 are strongly dependent on the design of the electrode surfaces (in case of field effect transistor: of the dielectric gate isolator outer surface) and its specificity to react with solely the biomarker of interest. In the meantime, numerous surfaces have been made and their sensitivity tested (See: [117,119,120]), However, although a high sensitivity (taking detection limit as index) could be shown for most applications, challenges which still remain is the interference with other components of the fluid including biofouling effects and reliable scalable fabrication methods for mass production of these sensors [117].

Dressing Internal Optical Analysis

The optical biosensors designed to be included in dressings are to a large extent similar to the ones used for external analysis. The difference is that the fluorochrome is more or less linked to the wound dressing. For instance, like the set-up used by Thet and Jenkins [100] (Figure 6A), Zhou and co-workers [121] used toxin-sensitive vesicles filled with fluorochrome to detect bacterial contamination. In case of Zhou and co-workers these vesicles were attached to a fabric as representative for a dressing. Also, here the fluorochrome is released by the vesicles as result of the presence of toxins/virulence factors. The resulting increased fluorescence is visualized using a high intensity UV source and after taking an image the latter is analysed. Its applicability and specificity were proven using *S. aureus* and *P. aeruginosa* cultures with *E. coli* as non-pathogenic reference but unfortunately not its biocompatibility.

The latter is of course of key importance since the fluorochrome is released into the wound. The latter disadvantage is not present if the fluorochrome is directly coupled to the wound dressing. For instance, Derikvand and co-workers [122] (like the set-up used by Hasmann and co-workers [112] (Figure 6B) used moiety tethered fluorochromes and bound them to dressing components such as cellulose. Only in case these moieties were enzymatically removed (in this example by esterases) the fluorochrome gets fluorescent which is visualized like the previous one using an UV source.

Although by coupling the fluorochrome to the dressing one disadvantage (possible cytotoxic effects) could be eliminated, others are still not solved. Like the dressing external optical biosensors, the disadvantage of these kind of sensors is that it is only applicable if the marker enzyme is negligible present in the correctly healing wound but which concentration is strongly increased in case the dressing has to be changed or if wound needs additional therapy. Furthermore, fluorescence has to be judged by eye (or from an image taken from the wound or dressing) and is not quantified by an equipment that is integrated in the dressing.

Dressing Internal Electrode-Based Analysis

Several electrode-based sensors have been proposed to be integrated in wound dressings or which integration is possible. One example is the one developed by Sharp and Davis for sensing bacterial wound contamination taking urate as an index [96]. Urate is normally present in the wound at concentrations of 190-420 μ M and bacteria, especially *P. aeruginosa*, are able to metabolise it resulting in a significant wound fluid concentration reduction [96]. Their biosensor with a two-electrode configuration is to a certain degree similar to the one described for measuring pH (Figure 2B). However, in this case the conductive proton selective polymer is omitted and instead of measuring at one specific voltage voltammogram are made in the range of -0.4 to +1.2 V (Figure 3B). The wound fluid urate is quantified by the typical shape of the square wave voltammogram taking the maximum electric flow in μ Ampere around 0.2 V as index. Similarly, but using 3 electrodes with a bias potential of 350mV and measuring the resulting current, Liu and Lillehoj could reproducibly monitor urate concentrations [123]. Unfortunately, both set-up was only tested using different urate concentrations in simulated body fluids but were not evaluated under real conditions using wound fluids.

Another example of electrode-based sensor is described by Farrow and co-workers for measuring bacterial load of the wound fluid [97]. With their sensor (Figure 3C) impedance profiles are assessed during frequency sweeps from 1 MHz to 0.1 Hz at a root mean square voltage of 200 mV. With this they were able to correlate the obtained pattern with the bacterial load. Similar has been described by Sheybani and Shukla [124]. However, unfortunately both were only evaluated using artificial fluids and again not using wound fluids. Other examples of optical and electrode-based biosensors and their limitations for the specific

detection of pathogenic microorganisms and other parameters can be found among others in reviews of Silva and co-workers [125] Damborsky and co-workers [126] and by You and Lee [127].

Odour

So far malodour's are analysed and characterized using large external devices such chromatography-mass spectrometry [60]. To circumvent this disadvantage Lu and co-workers developed a promising electrochemical set-up in which electrodes were functionalized with odorant-binding proteins from the honeybee [128]. Binding of the odorant resulted in a different impedance spectrum. Recently, Dieffenderfer and co-workers developed another and new kind of small mass-based biosensor for volatile compounds in the exhaled air of asthma patients. It is founded on the use of a capacitive micro-machined ultrasonic transducer which acts as an electrostatically actuated mechanical resonator. Thanks to its nature, specific volatile compound(s) adhere to the coating resulting in a change of mass bound and by that in a change in mechanical resonant frequency. Its principle can probably also be used for wound volatile compounds. Thanks to its reduced size it may be integrated into wound dressings in future.

Knowledge Gaps

Wounds are complex and the kind and state of the wound determines which treatment is optimal. For a proper classification multiple parameter have to be evaluated. Although significant progress has been made in the development of biosensors for wound monitoring, the development of high-tech sensors for wound dressings is still in its infancy stage. This may be traced back on various knowledge gaps of which some are mentioned below:

- (i) A solid knowledge base is still missing regarding molecules which may be used as sensor key target representatives. Not all reported studies can withstand a critical evaluation and should be interpreted with care. Furthermore, the representatives should not only enable an early prognosis regarding the fate of the wound with the applied therapy but also recognize early changes as effect of additional or changed therapy.
- (ii) One key factor of most of the current biosensors is that the analysis of obtained images or wound fluids/exudate cannot be performed at the point-of-care but still must be done in a laboratory with often long assay times. Here new wearable microdetectors and/or rapid analysing units have to be developed that uses a different kind of detection method enabling a (nearly) on-line monitoring.
- (iii) The state of the wound differs within the wound. Therefore, it is important to get a high resolution 2D picture of the state of the wound. With some exceptions, current sensors are too large for this and if miniaturized the wiring represent a challenging issue.
- (iv) Another key limiting factor for the use of most above-mentioned

biomarker molecule biosensors is the fact that they can only be used once (and by that can deliver only a single snapshot of the current state) and are not able to monitor the wound state. The latter is especially relevant for targeting declining marker component concentrations or which concentrations elevation is relative small relative to normal levels. For this, biosensors are needed that are able to regenerate between the measurements. Some possible strategies are listed in a review of Goode and co-workers [129].

- (v) Limited specificity and sensitivity as well as biofouling are for some biosensors key limiting factors and ways have to be found to solve this [117]. Furthermore, many of the proposed set-up were only evaluated using artificial fluids and not under real conditions, i.e. on the wound or using wound fluids.
- (vi) Material inertness a key issue, especially for biosensors based on materials that are sprayed into the wound or which are known to release constituents. Unfortunately, for many compounds the long-term biological effects are not known.
- (vii) The delayed healing of wounds is considered as a disease symptom. Therefore, knowledge of the disease, the underlying mechanism by which they induce hard-to-heal wounds and the relation between disease therapy and wound healing process are of key importance. However, in most cases this is only rudimentary known and in addition partially patient (and by that wound) specific. With the development of new biosensors this knowledge will increase which on their turn will help to improve biosensor target definitions.

The above-mentioned knowledge gaps represent currently to our opinion the key bottle necks in the development of sensitive and prognostic biosensors for high-tech wound dressings. Therefore, filling these white pages should have first priority as premise for the development of the next generation high-tech wound dressings.

Foresight of Future High-Tech Dressings

The removal of the above-mentioned bottlenecks certainly represents a big challenge for the future. However, the reward will be commensurate. The list below represents certainly only a tip of potential improvements which could be foreseeable.

Telemedicine

Since tethered monitoring technology is cumbersome to the patient and also because it strongly restricts the behaviour of the patients, wireless solutions may be preferred. This also enables remote patient monitoring of the patients living at home (Figure 7A). In case wound dressings needs to be replaced or treatment has to be adapted an alert will be given to the patient and practical nurse. This will enable a therapy or wound management intervention at the correct time point ensuring that care givers do not interact with patients more than they otherwise would, but also that a critical change in wound state is not missed. Additionally,

based on the wound values the correct diagnosis can be given and evolved treatment initiated on the earliest possible stage. As consequence life quality of the patient may be increased as well as costs and resources may be saved. Currently, telemonitoring is already discussed for wound care and other health issues [79,130-133].

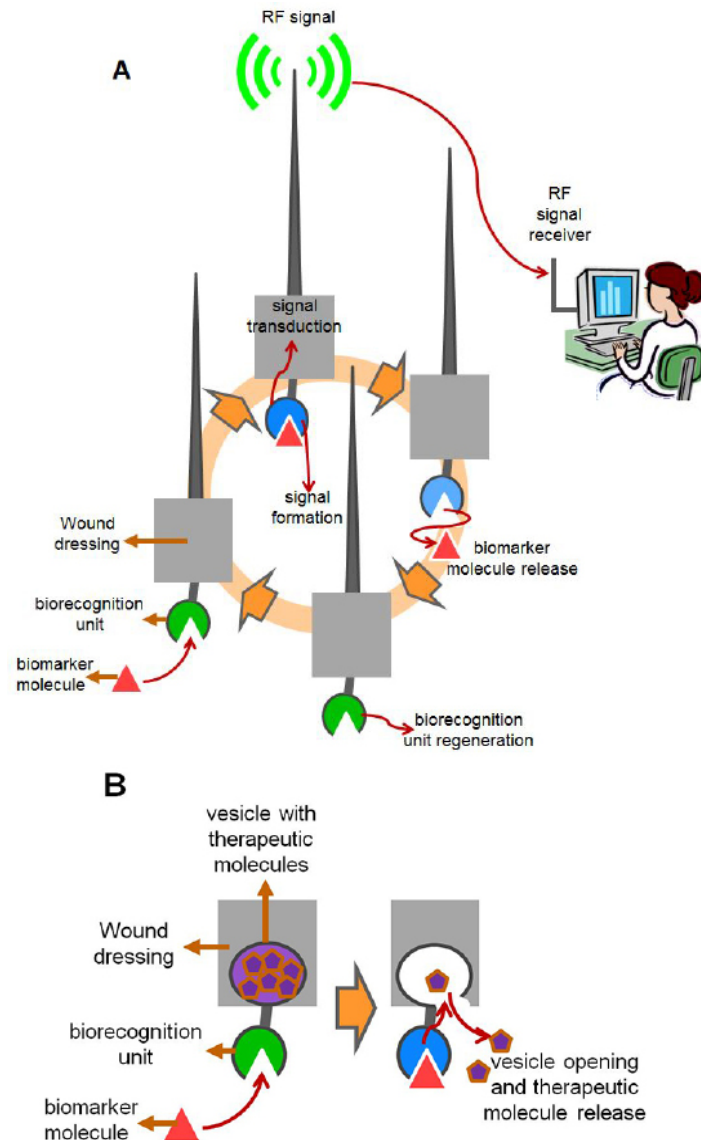


Figure 7: Wound dressings with wound status monitoring capacity based on integrated biosensors. A: Schematic representation of a wound dressing with biosensor unit able to repetitively measuring the concentration of a biomarker molecule transforming this to a signal which can be wirelessly transmitted to a data analysis device. B: In this schematic representation the binding of the biomarker molecule results in the release of specific therapeutic molecules enabling to redirect and/or steer the wound healing process.

Holistic Approaches

Since the way wound heals is directly affecting the extent of scar formation, dressings will be developed that addresses wound closure and scar formation. For instance, to address the effect of increased tension on scar formation promotion, future dressings may include force modulating properties to control the wound mechanical environment [134,135].

Personalized Medicine - Patient Specific Dressings

The need for personalised medicine and based on this patient specific dressings is unquestioned and will be common in future. First steps in this direction are made. For instance, it could be shown that an adaptation of the wound care based on the characterization of the bioburden by molecular diagnostics could significantly improve the treatment outcome [136]. Not only the bacterial background also differences in wound healing based on the genetic background of the patient and based on the prevalence for the development of certain disorders may require a personalised approach for wound treatment. For instance, for therapeutics that are metabolized by the CYP2D6 or CYP2C19 gene product a test came on the market as an aid to clinicians to analyse of the CYP2D6 and CYP2C19 genotype of the patient in order to adapt accordingly therapeutic strategy and treatment dose for these therapeutics [137].

Theranostics

Wound therapeutic wound dressings will become available which include on the one hand biosensors sensing the wound state and on the other hand therapeutic compounds. The capability of dressings to release specific therapeutic compounds depending on certain most common pathological wound progress patterns, would have a tremendous potential for most optimal point-of-care wound care management (regarding time point, location of treatment and compound dose) (Figure 7B). These can be like the sensor proposed by Zhou and co-workers for bacterial detection [121] but instead of releasing a fluorochrome a drug or antibiotic would be released. The premise for this is beside the availability of a complete set of highly specific markers, the availability of a dense high-resolution biomonitoring and data analysis unit. Furthermore, the development of a set of controllable matrices of miniaturized compound release units for the local delivery of the various compounds is crucial enabling to redirect wound healing process from the not preferred /unwanted pattern towards the rapid healing state.

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