Hydrogels as Intrinsic Antimicrobials

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Abstract:

Hydrogels are currently applied as the vehicles for drugs or active substances, rather than the active contributors to therapeutic activities. With increasing challenges related to antimicrobial resistance against many of antibiotics, natural-origin hydrogels become attractive as more than just vehicles. Although many of natural polymers exhibit intrinsic antimicrobial properties, these properties were not fully exploited up to now. This chapter provides an overview of hydrogels prepared from natural origin with intrinsic antimicrobial properties. The hydrogels are summarized as i) vehicles for active substances (active substances-in-hydrogels) or as ii) vehicles for delivery systems for active substances (active substances-in-delivery system-in-hydrogels). Both types of formulations offer synergistic activities of both drug/active substances and hydrogel, often covering broader antimicrobial spectra and means to combat antimicrobial resistance. Focus is put on the hydrogels for therapy of skin and vaginal infections, however some other administrations sites are included. The factors affecting the antimicrobial activity of hydrogels are discussed in more details. The toxicity, potential local irritancy as well as stability issues of novel hydrogels are given required attention. Perspectives on wider use of hydrogels as active pharmaceutical ingredients are proposed.

Key words: hydrogels; antimicrobial therapy; skin therapy, vaginal therapy; chitosan; nanoparticles

Introduction

In spite of numerous technological advances in the medical field, the 21st century brought comeback of many of infectious diseases earlier considered treatable; these infections are now presenting a clear health treat due to increased antimicrobial resistance and reduced choice of available antibiotics to combat the resistant infections successfully. Antimicrobial resistance emerged as one of the greatest challenges in current drug therapy (Tacconelli and Pezzani, 2019). Recent population-leveled modelling analysis in the European Union and European Economic Area indicates that burden of infections caused by antibiotic-resistant bacteria increased since 2007 and is the highest among infants and elderly, most vulnerable patient populations (Cassini et al., 2019). Microorganisms are continuously developing mechanisms of resistance against antimicrobials; yet the process for discovery and development of novel antimicrobials is lengthy and expensive, often lasting over 10 years and having a very limited success yield. New antimicrobials must overcome several types of resistance mechanisms that already exist against established antimicrobials and, simultaneously, avoid the development of novel resistance mechanisms. To add to the already serious situation is the rather limited understanding of the resistance mechanism. It is worth mentioning that antibiotics found in nature have often large, complex structures and multiple interaction sites, making them less prone to resistance development (Fernandez, 2015).

Academia, research institutions and pharmaceutical industry have addressed the increasing concern related to limited options in antibiotic use by different approaches and means. One of the approaches relies on re-using the old antibiotics packed in novel dosage forms and delivery systems that act on improving their efficacy (Cassir et al., 2014; Ingebristen et al., 2017). Rather extensive efforts have been put in search for novel antimicrobials of natural, semi-synthetic and synthetic origin. However, as mentioned earlier, the processes are lengthy and often with very limited success. Unfortunately, some of industrial partners opt for even giving up on antibiotic research and development, which can be considered the worst option (Shlaes, 2015).

Systemic exposure to antibiotics leads to faster development of antimicrobial resistance and one of the means to reduce the resistance development would be to treat localized infections locally rather than systemically. We have in past decades focused on localized therapy as a mean to assure improved therapy efficacy of various site of diseases, including infections (Basnet and Škalko-Basnet, 2013; Škalko-Basnet and Vanić, 2017). We have been interested in the skin infections (Škalko et al.; 1992; Škalko et al.; 1998; Hurler and Škalko-Basnet, 2012; Hurler et al., 2012; Vanić et al.; 2015; Palac et al.; 2014; Ingebrigsten et al.; 2017; Rukavina et al.; 2018); and treatment of vaginal infections (Pavelić et al., 1999; Pavelić et al., 2005a; Pavelić et al., 2005b; Vanić et al., 2014; Jøraholmen et al.; 2014; Andersen et al., 2015; Jøraholmen et al.; 2015; Vanić and Škalko-Basnet, 2017).

In recent years, and responding to the increasing problem of antimicrobial resistance to antibiotics, we have looked for the material of natural origin with intrinsic antimicrobial properties as the building blocks for our delivery system. The synergy between the antimicrobial, either of natural or synthetic origin and the building material with intrinsic antimicrobial properties, is expected to lower the doses required to achieve efficient

antimicrobial treatment. Applied topically, avoiding the systemic absorption, it limits the chances of resistance development

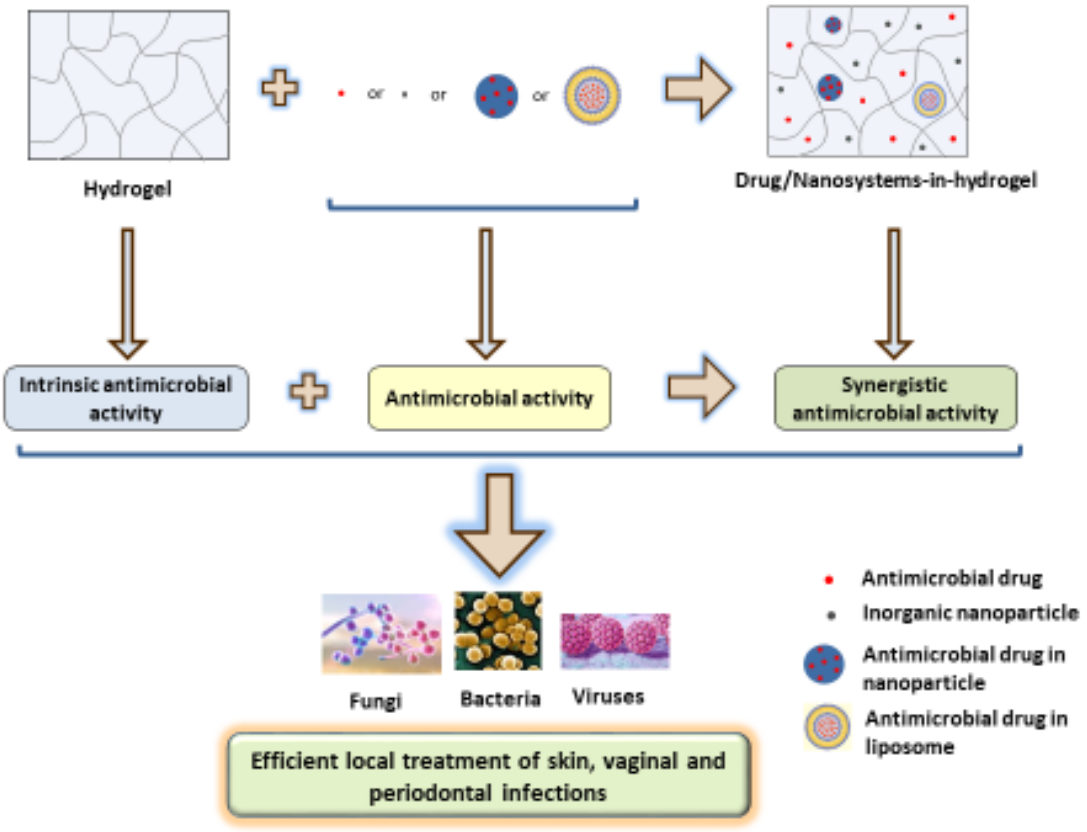
One of the most promising material with intrinsic antimicrobial properties is chitosan. Chitosan can be used as a building block of various nanosystems as well as vehicle. We have focused on chitosan as building block in hydrogels utilizing its antimicrobial activity and contributing to the antimicrobial potential of the final formulation.

Chitosan forms hydrogels which are mucoadhesive and have great potential in application on wounded skin and vaginal site (Hurler et al., 2013; Vanić and Škalko-Basnet, 2017).

Hydrogels have for long been used as basic building blocks in tissue engineering and regenerative medicine (Dimatteo et al., 2018). However, in spite of tremendous development of polymer chemistry and innovation pressure on pharmaceutical industry, only a relatively few "*smart-materials*" have been translated into clinical use (Cooke et al., 2018). There are different reasons behind the lack of a success; however, most of the polymers used as hydrogels were serving only as vehicles and not as active ingredients.

Since this book is dedicated to hydrogels as dosage form, we have focused this chapter mostly on hydrogels made of material with intrinsic antimicrobial properties, rather than discussing the characteristics of hydrogels. Since chitosan is the most studied polymer with intrinsic antimicrobial properties, we have discussed the mechanism of its antimicrobial action in more details.

The simplified concept discussed in this chapter is presented in Figure 1.



Intrinsic antimicrobial properties of chitosan

Chitosan. [α(1–4) 2-amino 2-deoxyβ-D glucan], is a cationic polysaccharide produced from the deacetylation of chitin, a natural element abundantly sourced from the shells of crustaceans. Over the last 200 years since its discovery, the study and application of chitosan has taken on many different forms (Dai et al., 2011). Its biocompatibility, biodegradability, low toxicity, mucoadhesive and antimicrobial properties are reason for its use in a wide range of skin products and biomedical applications. Chitosan is considered to be one of the most successfully developed biodegradable polymers.

Chitosan can be dissolved in various organic and inorganic acids. Its reactive amino groups are a good target for possible modifications with different ligands, functional groups and moieties (Naskar et al., 2019). Furthermore, the wound healing property of chitosan, which is dependent on the degree of deacetylation and molecular weight, has been utilized in as patches, scaffolds, bandages, and bioadhesive gels where chitosan enhances the function of inflammatory cells, therefore accelerating the wound healing (Amidi et al., 2010: Dai et al. 2011). This chapter focuses on chitosan as polymer with pronounced antimicrobial effects. The effects are contributed to its ability to destabilize the outer membrane of Gram-negative bacteria (Rabea et al., 2003) and permeate the microbial plasma membrane (Tang et al., 2010). The molecular weight, degree of acetylation and the ionic strength and pH of the dissolving medium will affect its antimicrobial properties. By tailoring its formulation (gel, film, etc.) it is possible to optimize

its antimicrobial potential (Dai et al., 2011). Although the exact mechanisms of the antimicrobial actions of chitosan remains discussed, it has been proposed that the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes results in the disruption of microbial membrane, and consequent leakage of proteinaceous and other intracellular constituents (Kong et al., 2010). It was suggested that at a lower chitosan concentration (<0.2 mg/ml), the cationic groups of chitosan bind to the negatively charged bacterial surface to cause agglutination, while at higher concentrations, the larger number of cationic groups form a net positive charge to the bacterial surfaces forming a suspension (Rabea et al., 2003).

It is well established that polycationic structure of chitosan contributes to its antimicrobial activity. A higher positive charge density is expected to lead to stronger activity, suggesting that positive charge is associated with degree of deacetylation (DD) or degree of substitution (Kong et al., 2010). Regarding its molecular weight, rather contradictory results were published on the correlation between the chitosan MW and bactericidal activity. However, there is a consensus that hydrophilicity of chitosan is crucial for its antimicrobial potential. Due to a limited space, we have not go deeper into various chitosan derivatives and possible tailoring of its properties by chemical modifications.

The mechanism of antibacterial properties of chitosan remains to be discussed and deeper insight as well as proposed mechanisms need to be conformed. At pH below its pKa (6.3) the electrostatic interactions between its polycationic structure and predominantly anionic parts of the surface of Gram-negative lipopolysaccharides and cell surface proteins are considered major contributors. The number of amino groups linking to C-2 on chitosan backbones is important in electrostatic interaction. The native chitosan with higher DD should exhibit stronger inhibitory activity than chitosan with lower DD (Kong et al., 2010). However, at the environmental pH above its pKa, hydrophobic and chelayting effects are dominated over its electrostatic interactions (Kong et al., 2008a).

Low molecular weight chitosan hydrogel has shown promising anti-*Candida* activity in an *in vivo* catheter mouse model (Silva-Dias et al., 2014). Chitosan was also proposed as possible prevention or treatment of fungal biofilms on central venous catheters (Martinez et al., 2010). Chitosan at very low concentrations (0.0313 %) could kill more than 50 % of *C. albicans* cells in the early and intermediate phases of biofilm, formation whereas higher concentrations were required to kill cells in mature biofilms (Pu et al., 2014).

Particularly attractive is the proven ability of chitosan to disrupt bacterial biofilms in bacterial vaginosis. Bacterial vaginosis is one of the most recurrent infection of genital tract and most of the therapy fails to completely disrupt the persistent bacterial biofilms. Negatively charged polysaccharide matrix covers biofilm bacteria and prevents/restricts the penetration of applied antimicrobials. Chitosan gels were able to disrupt *Pseudomonas aeruginosa* biofilms in a pH-independent manner. Considering that pKa of chitosan is 6.3, more than 99 % of its amino groups will be protonated at pH 4, and only about 60 % at pH 6; however, at both pH the activity was similar and possibly independent of the cationic charge density (Kandimalla et al., 2013). The chitosan concentration required to achieve this effect was rather low (0.13 %).

Its relevance has been confirmed in clinical settings as well. Akincibay and colleagues (2007) reported the clinical effectiveness of chitosan in the treatment of chronic periodontitis contributing the observed therapeutic efficacy to antimicrobial action of chitosan. Similarly, Boynuegri and co-workers (2009) reported that chitosan gel either alone or in combination with demineralized bone matrix/collagenous membrane has beneficial effects on periodontal regeneration.

Chitosan has been used intensively to form nanocomposites with other known antimicrobials such as silver for example (Arjunan et al.; 2016); however, as the focus is on hydrogels with intrinsic antimicrobial potential, we have not discussed nanocomposites further.

Antimicrobial hydrogels for wound therapy and treatment of skin infections

Hydrogels as wound dressings can play a major role in wound healing process due to their physical properties allowing absorption and retention of wound exudate thus promoting fibroblast proliferation and keratinocyte migration; both events necessary for complete epithelialization of the wound and reduced scarring (Hurler and Skalko-Basnet, 2012). However, this hydrated, water-rich, environment can also facilitate microbial infections. Therefore, hydrogels capable of imparting antimicrobial action in addition to their primary role as wound dressing are of high importance (Madaghiele et al., 2014; Veiga and Schneider, 2013). In that context, the hydrogels constituted of polymers with intrinsic antimicrobial properties, such as chitosan, are of great relevance, especially considering the treatment of acute infected wounds, or as dual-acting formulations when incorporating either antimicrobial drugs or nanoparticles, for the treatment of chronic, deep wounds (Dai et al., 2011). Namely, the tight mesh network within the hydrogel allows incorporation of antimicrobial substances or nanoparticles loaded with antimicrobials; the active ingredients are then gradually released to the infected wound as hydrogel absorbs the exudate and swells (Madaghiele et al., 2014). Additional advantage of the hydrogel as wound dressing lies in its cooling effect contributed to high water content, assisting in pain relieve (Zarrintaj et al., 2017).

Chitosan-based hydrogels

Chitosan-based hydrogels are considered superior as wound dressings because they induce faster wound healing, protect from secondary infection and minimize scarring. Moreover, chemical modifications of chitosan leading to derivatives such as N, N, N-trimethyl chitosan, N-succinyl chitosan, N-carboxymethyl chitosan, and thiolated chitosan or combinations of chitosan/chitosan modifiers with other polymers or nanoparticles improve the hydrogel properties related to wound healing (Liu et al., 2018). For instance, carboxymethyl chitosan is a water-soluble polymer and its antibacterial activity is found to be superior to that of unmodified chitosan (Anitha et al., 2009).
Hydrogels composed of O-carboxymethyl chitosan embedding lincomycin exhibited good pore structure and swelling characteristics. Compared with plain O-carboxymethyl chitosan gel, the

in vitro antibacterial activities of lincomycin O-carboxymethyl chitosan toward Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* significantly improved with the increase in lincomycin concentration within the hydrogel. Interestingly, increase in the content of O-carboxymethyl chitosan and crosslinking agent in the gel formulation have resulted in decrease of antimicrobial activities against the both tested strains (He et al., 2016). These results are not so surprising considering that it has been earlier confirmed that, at higher chitosan concentrations (> 0.2 mg/ml), the large number of positive charges imparted a net positive charge to the bacterial surfaces to keep bacteria in suspension. However, at lower concentrations, the polycationic chitosan is bounded to the negatively charged bacterial surface causing agglutination (Rabea et al., 2003; Sudarshan et al., 1992).

Targeting the treatment of infected wounds, Hurler et al. (2013) have developed mupirocin-loaded liposomes incorporated into chitosan hydrogel composed of 2.5% high molecular weight (HMW) chitosan. The formulation was found to be nontoxic toward keratinocytes *in vitro* and exhibited anti-biofilm activity against *S. aureus* biofilms, although the effect was more pronounced toward planktonic bacteria and prevention of biofilm formation than against the mature biofilms. The *in vivo* wound healing study performed in mice burn model during 28 days demonstrated that the mupirocin-loaded liposomal hydrogel was safe for use and exhibited the same healing effect as the registered topical formulation of mupirocin. The histological evaluations revealed complete re-epithelialization of all wounds, whereas the healing time for the liposomal formulation was shorter in comparison with the marketed formulation. However, due to the limits of the used *in vivo* method, the authors suggested additional *in vivo* evaluations to prove the potential of the novel liposomal formulation clinically.

Many of the chitosan-based hydrogels investigated for the treatment of infected wounds contained incorporated metallic nanoparticles such as silver, gold or zinc. Among these metals, silver is the most commonly used because of its good antibacterial properties and relatively low toxicity (Yang et al., 2018).

Presence of silver nanoparticles inside the matrix of chitosan hydrogel contributes to the hydrogel hardness and therapeutic efficiency. Using the chitosan powder with degree of deacetylation of 80-95% and alkali-urea solutions, Xie and collaborators (2018) synthesized the hydrogels with integrated silver nanoparticles, which exhibited pronounced hardness and antibacterial activities. The inhibition rates of the blank chitosan gel against *S. aureus* and *E. coli* were 27 and 12%, respectively, while incorporation of silver nanoparticles resulted in superior antibacterial efficiency that was confirmed *in vivo* (rat model). Namely, the wound healing effect was significantly increased and accelerated in comparison with the plain chitosan gel.

Zhou and colleagues (2012) prepared series of gelatin/carboxymethyl chitosan hydrogels comprising silver nanoparticles by radiation induced reduction and crosslinking at ambient temperature. The hydrogels had interconnected porous structure and could absorb 62-108 times of deionized water to its dry weight. Silver nanoparticles contributed to the antibacterial efficacy of the hydrogels assuring the controlled release and erosion of the hydrogel. The composite hydrogel incorporating 2 and 5 mM nanosilver inhibited more than 25% and 50% of *E. coli*, respectively, while the maximal activity was obtained with 10 mM nanosilver. However, the cytotoxicity studies were not conducted to prove the biocompatibilities and lack of toxicity of the developed formulations.

Chitosan hydrogels prepared from HMW chitosan at different concentrations of the polymer (3.5, 1.75 and 0.875%, w/w) and incorporating silver or gold nanoparticles, were evaluated for

the rheological, thermal, antibacterial and biocompatibility properties. Chitosan-gold nanoparticles hydrogels demonstrated better thermal stability than chitosan-silver nanoparticles hydrogels, while the viscosities of the both types of hydrogels were affected by the concentrations of the embedded nanoparticles in the hydrogel. *In vitro* antibacterial studies performed against reference *Streptococcus mutans, S. aureus* and *E. coli* strains as well as four clinical isolates of *S. mutans,* demonstrated similar efficiency of the hydrogels containing either silver or gold nanoparticles against the tested reference strains. Generally, the activities of the formulations against clinical isolates of *S. mutans* were significantly lower than toward reference *S. mutans* strains. Testing of the plain 3.5% chitosan hydrogels prepared with different concentrations of acetic acid showed the highest efficiency for the hydrogel prepared with the highest concentration of acetic acid (6 vol%), which was similar to efficiency of the hydrogels incorporating nanoparticles prepared with 4 vol% acetic acid. *In vivo* wound healing evaluation in a rat model performed with the hydrogels containing the highest concentrations of either silver or gold nanoparticles, demonstrated normal healing at 28 days. Interestingly, the rats treated with chitosan-gold nanoparticles hydrogel exhibited scarring at the wound site. The histopathological evaluation of the same group of rats showed a moderate infiltration of leukocytes as compared to the light infiltration in groups of the animals treated with chitosan-silver nanoparticles or plain chitosan hydrogels. The findings suggest more delayed wound healing probably as a result of the toxic effects produced by the contact of gold nanoparticles with the skin cells (Samano-Valencia et al., 2014).

Sundheesh Kumar and coworkers (2012) incorporated zinc oxide nanoparticles into chitosan hydrogel prepared from LMW chitosan for the possible treatments of infected wounds and diabetic foot ulcers. This flexible and microporous composite bandage displayed controlled degradation, excellent platelet activation ability, enhanced blood clotting and *in vitro* antibacterial activities against *S. aureus* and *E. coli*. However, the plain chitosan gel (without ZnO nanoparticles) have not revealed antibacterial activities possibly due to the neutral pH of the chitosan as a vehicle. The observed antibacterial effect was therefore attributed to only ZnO nanoparticles, which are expected to produce reactive oxygen species. These species, together with zinc ions, attacked the negatively charged bacterial cell wall and caused cell wall leakage. The higher activity attained against *E. coli* than *S. aureus* was ascribed to the thick layer of peptidoglycans in the cell wall of *S. aureus*. The cytotoxicity evaluation on a human dermal fibroblast cells demonstrated that plain chitosan hydrogel did not exhibit any toxicity during 24, 48 and 72 h of incubation. However, the presence of ZnO nanoparticles in the chitosan hydrogel affected the cell viability, which was between 30 and 60% after 24 h, depending on the concentration of ZnO incorporated in the chitosan hydrogel. Interestingly, after longer period of incubation, the remaining viable cells began to multiply and the cell viability increased, suggesting proliferative effect. *In vivo* wound healing assessment in a rat wound model proved the enhanced healing effect with faster re-epithelization and collagen deposition. Moreover, even plain chitosan hydrogel demonstrated a good healing effect after one and two weeks. The superiority of the formulation was confirmed by *in vivo* antibacterial evaluation against *P. aeruginosa*, *Staphylococcus intermedius* and *Staphylococcus hyicus*, the bacteria isolated from the rat wounds, thus proving the potential of the nanocomposite chitosan hydrogel for the treatment of infected wounds and diabetic foot ulcers.

Wahid and colleagues (2016) have proposed similar approach for the treatment of infected wounds. It was based on the *in situ* incorporation of ZnO nanorods (160-190 nm) into the crosslinked carboxymethyl chitosan hydrogel. The nanocomposite-based hydrogel exhibited excellent antibacterial activity against *E. coli* and *S. aureus* bacteria, with *S. aureus* being more

reposnive to the formulation. However, the hydrogel without ZnO nanorods showed poor antibacterial effect probably due to the crosslinking of carboxymethyl chitosan contributing to a decrease of positive charge on the formed hydrogel, or due to the poor dispersion of hydrogel in PBS solution. Biodegradability and biocompatibility studies have not been performed to evaluate possible physiological acceptability of the formulation.

The properties of the chitosan hydrogels as wound dressings can also be improved by preparing their combination with other polymers of either natural of synthetic origin, to exert the advantages of each component and enhance the therapeutic effect of the final wound dressing. For example, alginate exhibits low cell adhesiveness due to its poor protein adsorption to the hydrophilic surfaces; however, its mixture with chitosan enables improved cell interaction, adhesion and proliferation (Liu et al., 2018). Accordingly, the wound dressing based on the alginate/chitosan hydrogel promoted the cell proliferation *in vitro* and accelerated the wound closing rate (Alsharabasy et al., 2016). In a relevant study, a hydrogel composed of a mixture of chitosan, alginate, silk and dextrin, containing recombinant human epidermal growth factor was prepared and evaluated for *in vivo* wound healing effect. Although antimicrobial studies were not encompassed, the results demonstrated that this multicomponent natural origin hydrogel could promote the healing process and recovery of deep diabetic wound in the rats (Sukumar et al., 2015).

To develop a wound dressing with intrinsic antimicrobial properties, Straccia et al. (2015) have designed the alginate hydrogel coated with water-soluble chitosan hydrochloride. *In vitro* antibacterial studies against *E. coli* confirmed pronounced bactericide effect of the chitosan-coated gel, while the non-coated, i.e. alginate gel, failed to show any zone of inhibition.

A biodegradable hydrogel dressing with high antibacterial efficiency prepared by the reaction between aldehyde and amino groups of oxidized alginate and carboxymethyl chitosan and incorporating tetracycline microspheres has been recently developed. The hydrogel was evaluated for swelling, degradation, compressive modulus, rheological properties and the drug release profile. Increasing the ratio of microspheres incorporated within the hydrogel contributed to the shorter gelation time, lower swelling rations and higher strength of the formulation. The composite hydrogel containing 30 mg/ml microspheres showed optimal mechanical properties for wound healing allowing sustained tetracycline release. The plain alginate/carboxymethyl chitosan hydrogel (without tetracycline) exhibited very small inhibition zones for both *S. aureus* and *E. coli*. However, the hydogels containing either free tetracycline or tetracycline-loaded microspheres demonstrated stronger antimicrobial activities for the both tested strains. The inhibition zones for hydrogel containing tetracycline-in-microspheres against *E. coli* and *S. aureus* were slightly reduced as compared to those determined for tetracycline-in-hydrogel, probably being a result of the slower drug release from the microsphere-based hydrogel (Chen et al., 2017).

Noppakundilograt and collaborators (2013) synthesized chitosan grafted poly[(acrylic acid)-co-(2-hydroxyethyl methacrylate)] to prepare nanocomposite hydrogels incorporating mica. The higher mica loading produced a rougher surface of the nanocomposite hydrogel, and the water absorbency decreased with increasing levels of mica loading. The *in vitro* antimicrobial testing against *S. aureus* demonstrated that the presence of mica inside the hydrogel had no influence on antibacterial efficacy. Regardless of the mica loading levels in the hydrogel the minimum inhibitory concentration (MIC) was constant at 12.5 mg/ml and was significantly higher that the MIC value of the chitosan grafted poly(acrylic acid) gel (1.56 mg/ml).

Gomez Chabala and collaborators (2017) recently presented an interesting approach in design of novel wound dressing. It proposes development of porous chitosan/alginate matrices embedding *Aloe vera* gel and silver nanoparticles. Namely, *Aloe vera* gel has been well known for its anti-inflammatory, anti-tumor, immodulatory and antibacterial properties. A porous structure with interconnected pores inside the chitosan/alginate matrices showed great capacity for absorbing *Aloe vera* gel, which could be gradually released from the formulation following its application. Moreover, the presence of silver nanoparticles, chitosan and *Aloe vera* gel enabled improved antibacterial activities against *S. aureus* and *Pseudomonas aeruginosa*; these effects were comparable to those achieved with classical antibiotics such as tetracycline and gentamicin.

Chronic wounds, commonly associated with ischemia, venous stasis diseases and diabetes mellitus, require specifically designed treatment protocols. They are difficult to cure due to a lack of the growth factors required for the naturally occurring healing process. Moreover, chronic wounds are usually infected with bacteria that may form biofilm. Therefore, their management require simultaneous administration of antimicrobial drugs and tissue grow factors such as platelet-reach plasma (PRP) (Hirase et al., 2018; Saghazadeh et al., 2018).
Targeting the treatment of chronic wounds infected with *S. auerus*, Nimal and co-workers (2016) developed injectable nanocomposite hydrogel by incorporation of tigecycline-loaded chitosan nanoparticles (93 nm) and activated PRP powder into chitosan hydrogel prepared from LMW chitosan. The formulation demonstrated shear thinning property, thermal stability, injectability, biocompatibility, and enabled sustained release of tigecycline, which is considered beneficial to reduce inflammation phase at the wound site. Additionally, nanoparticle-loaded hydrogel could induce the proliferation of the fibroblasts at the wound site and fasten the proliferative phase of wound healing *in vitro*, while cell migration studies confirmed that PRP containing gel system was more effective in the migration of fibroblasts compared to the gel system without PRP. *In vitro*, *ex vivo* (porcine skin) and *in vivo* (*Drosophila melanogaster* infection model) antibacterial studies revealed that the chitosan-PRP gel system containing either free or nanoparticle-loaded tigecycline inhibited bacterial growth to a greater extent, while the plain gel system (without tigecycline) failed to show any antibacterial activity.

Hydrogels destined for administration to vaginal site

Hydrogels are one of the most common dosage forms/delivery system for the topical vaginal administration of various drugs and active substances, including antimicrobials. They are easily spread over the vaginal surface allowing good distribution, and retention of the active ingredients inside vaginal cavity, while their high water content assures a moist microenvironment and an lubrication effect that help improving the symptoms associated with vaginal dryness (Palmeira de Oliveira et al., 2015; Vanić and Škalko-Basnet, 2017). Hydrogels can be prepared from different polymers of natural, semi-synthetic and synthetic origin. However, among various natural origin hydrogels with relevant intrinsic antimicrobial activities the carrageenan, chitosan, and gels from plant extracts are especially attractive.

Carrageenan is a natural, sulfated, anionic polysaccharide obtained from edible red seaweeds, which is typically used in pharmaceutical and food industries as a gelling, thickening and stabilizing agent (Necas and Bartosikova, 2013). Due to pronounced intrinsic antimicrobial properties, carrageenan-based gels have attracted considerable attention as microbicides in prophylaxis of sexually-transmitted infections caused by *Herpes simplex* virus (HSV), *Human papilloma* virus (HPV) and Human immunodeficiency virus (HIV). Its antimicrobial mechanism of action lies in the prevention of the attachment of viruses to target cells via electrostatic interactions with viral gp120 (Trapp et al., 2007; Pirrone et al., 2011). Carrageenan-based gel containing a mixture of lambda- and kappa-carrageenan (Carraguard®) reached and was investigated in Phase III clinical trial on more than 3000 South African women, which were followed for up to 2 years. Although Carraguard® was found to be safe for human use, unfortunately, its efficacy as a single microbicide in preventing vaginal transmission of HIV has not been confirmed (Skoler-Karpoff et al., 2008).

The approach has been further exploited in investigations with carrageenan-based gels, namely a prototype zinc acetate carrageenan gel, designed and challanged *in vivo* on the macaques and mice infection model (Fernández-Romero et al., 2011; Kenney et al., 2011). It is known that the zinc salts exhibit antiviral activity against a broad range of viruses, including HIV and HSV (Arens and Travis, 2000). Therefore, topical application of formulations containing low-dose zinc salts represents a promising strategy for preventing the sexual transmission of HSV-2 and potentially HIV, without the systemic use of antiretroviral drugs. The performed animal studies have proven the safety and high efficacy of formulation against both high-dose vaginal and rectal HSV-2 infections, respectively (Fernández-Romero et al., 2011). The formulation was further improved by addition of non-nucleoside reverse transcriptase inhibitor MIV-150 (PC-1005) and clinically tested on 20 healthy, HIV-negative, abstinent women. PC-100 used vaginally for 14 days was found to be well tolerated with low systemic levels of MIV-150 observed, while plasma zinc levels were unchanged. Post-dose cervicovaginal lavages demonstrated both anti-HIV and anti–HPV activities, thus proving a platform for the novel formulation to be applied in prophylaxis of sexually-transmitted viral infections (Friedland et al., 2016).

Chitosan-based hydrogels are considered to be very attractive vehicles for vaginal drug delivery due to their lower pH (4-5), high water content, biodegradability and pronounced mucoadhesiveness (Bonferoni et al., 2006; Frank et al., 2017; Perioli et al. 2008). However, up to now, there is a rather limited number of studies evaluating antimicrobial activities of chitosan hydrogels (without or with incorporated antimicrobial drug, either in a free or nanoparticle-encapsulated form) for the topical vaginal therapy of bacterial, fungal and viral infections.

Kandimalla et al. (2013) investigated the efficacy of medium molecular weight (MMW) chitosan hydrogel incorporating metronidazole against *Pseudomonas aeruginosa* biofilms for the possible treatment of recurrent bacterial vaginosis. The biofilms were treated with various concentrations of chitosan- and polycarbophil-based gels at pH values 4 and 6. Interestingly, chitosan gels containing metronidazole were shown to be more effective in disrupting integrity of *P. aeruginosa* biofilms at the both pH values and concentration of chitosan as low as 0.13% w/w. Polycarbophil-based hydrogel has shown comparable anti-biofilm activity at pH 4 and at

substantially increased polymer concentration (above 1%), while its efficacy was significantly reduced at pH 6. Since bacterial vaginosis is characterized with vaginal pH higher than 5, it is hypothesized that metronidazole containing chitosan gels could be more effective for the topical therapy of bacterial vaginosis than the commonly used polycarbophil gels.

In another study, the 2 % w/w chitosan hydrogels, prepared from the low molecular weight (LMW), MMW or high molecular weight (HMW) chitosan and embedding miconazole or econazole within the polymer matrix, were assessed for the topical therapy of vulvovaginal candidiasis. The *in vitro* antifungal testing performed by applying the agar well diffusion method proved the efficacy of all tested gels containing antifungal drugs against *Candida albicans*. The most pronounced inhibition zone was determined for miconazole embedded within the LMW chitosan gel (33.6 mm), followed by econazole LMW gel (31 mm), while the activities of miconazole within MMW and HMW chitosan gels were slightly less potent than miconazole in LMW chitosan. Surprisingly, the empty chitosan hydrogels (without the antimicrobial drugs) have not produced any zone of inhibition (Senyigit et al., 2014). The authors suggested that the absence of the antifungal activities of the empty hydrogels could be favorable for vaginal administration; they raised concerns regarding a possible negative impact of chitosan on vaginal microflora. However, these results are not in an agreement with other published findings and could be a consequence of the experimental setup and pH values of the gels, which varied between pH 3.8 and 5.4. Generally, the gels incorporating miconazole were of lower pH (4.5-4.8) than the corresponding empty gels (5.0-5.4), but exhibited higher pH values than the gels containing econazole (3.8-3.9).

Very recently Perinelli et al. (2018) evaluated the anti-Candida efficacy of 1% w/w LMW chitosan incorporated into 5.5 % w/w hydroxypropylmethylcellulose (HPMC) gel, either as free polymer or assembled in nanoparticles (400-900 nm). While 1% w/w chitosan dispersion in buffer pH 4.5 displayed good activity against all tested Candida spp. strains, the chitosan nanoparticles in buffer pH 4.5 were ineffective against all tested *C. albicans* strains, whereas a comparable inhibition growth effect to 1% chitosan dispersion was observed against the tested non-albicans strains. Slightly higher activity of nanoparticles prepared at the ratio chitosan/sodium tripolyphosphate 12:1 was assumed to be a consequence of their smaller size as compared to the nanoparticles prepared at 6:1 ratio. When 1% chitosan was incorporated into HPMC gel as free polymer, a variable degree of antifungal activity was obtained against all tested *Candida* spp. Overall, a slightly lower antifungal effect with respect to 1% chitosan dispersion was observed, which could be explained by the slow diffusion capacity compared to chitosan dispersion in buffer. The hydrogels incorporating 1% chitosan nanoparticles, in contrast to 1% chitosan nanoparticles in a form of liquid dispersion (active only on non-albicans strains), have demonstrated activity against all tested *Candida* spp. strains. In addition to the hydrogels free of the antimicrobial drug, the authors have also prepared the hydrogels loaded with 0.75% w/w metronidazole aiming at the formulation with improved antimicrobial activity. Metronidazole has already been encapsulated in chitosan-containing liposomes (chitosomes) to achieve a dual effect of the mucoadhesive nanoformulation on both vaginal bacterial and fungal infections (Anderson et al., 2017). However, the presence of metronidazole in the hydrogels containing either 1% chitosan in a form of polymer or as nanoparticles dispersion has not

increased the antimicrobial activity against all tested *Candida* spp. strains, proving that metronidazole has no intrinsic anti-*Candida* effect of chitosan (Perinelli et al., 2018).

The search for hydrogels with intrinsic antimicrobial properties involved not only polymers of natural origin but also the plants extracts which could form hydrogels while exhibiting intrinsic antimicrobial activities. This approach represents a novel strategy for the topical vaginal therapy. For instance, *Sophora flavescens* alkaloid gel has been investigated *in vivo* in a rat model for the therapy of aerobic vaginitis. In comparison to placebo, i.e. Carbomer-based gel, the plant gel exhibited much higher antibacterial effects toward *Staphylococcus aureus* and *Escherichia coli*. Additionally, the number of vaginal *Lactobacilli* significantly increased and the plant gel was well tolerated in contact with vaginal tissue. The finding is highly relevant considering the importance of maintaining the naurally occurring flora within the vaginal cavity. The authors suggested that the increased efficacy of the *Sophora flavescens* alkaloid gel was a result of the combined events involving high antimicrobial activity, immune regulation and enhancement of *Lactobacilli* growth supported by the moisture environment of the gel as well as its prolonged retention inside vagina (Xiu et al., 2017).

Periodontal diseases

Periodontal disease is defined as a chronic infection caused by accumulation of bacteria in dental plaque which produces localized inflammation of the periodontium. It is commonly associated with prevalence of Gram-positive aerobic and Gram-negative anaerobic bacteria and is highly prevalent in spite of increased efforts in development of products for improved oral hygiene (Ji et al., 2010; Joshi et al., 2016).

Biodegradable and injectable thermosensitive hydrogels are particularly attractive for the treatment of often unreachable periodontal pockets; termosensitive hydrogels are liquefied at room temperature and form gel at the site of application (over 30 °C) enabling sustained release of incorporated antimicrobial substance(s).

Ji et al. (2010) designed injectable 0.1% (w/w) chlorhexidine-loaded thermosensitive hydrogel using chitosan, quaternized chitosan and α,β-glycerophosphate. Upon administering the hydrogel in liquid form, the gelation occurred at simulated *in vivo* condition (37 °C) after only 6 minutes, further allowing sustained release of chlorhexidine over 18 h. The formulation exhibited excellent inhibitory activity against periodontal pathogens, namely *Porphyromonas gingivalis*, Prevotella intermedia and *Actinobacillus actinomycetemcomitans*. The experiments clearly confirmed the crucial role of chitosan for antimicrobial activity of novel formulation; the MIC of chlorhexidine significantly decreased in the presence of chitosan solution and especially in the form of chitosan-based thermoresponsive hydrogel, thus proving the pronounced intrinsic antimicrobial effect of chitosan as well as quaternized chitosan. The inhibition zones obtained for the chitosan-based thermoresponsive gels revealed the strongest activity against *A. actinomycetemcomitans*, followed by *P. intermedia* and *P. gingivalis*. The

great potential of the novel formulation for the local treatment of periodontal infections was further supported by the lack of acute toxicity in *in vivo* evaluation of the gel in rats.

The effectiveness of chitosan, both as a vehicle for metronidazole in a form of hydrogel and as an active antimicrobial agent on its own, was confirmed in a clinical study involving 15 patients suffering from chronic periodontitis. Because of chitosan's pronounced mucoadhesive properties as well as its antimicrobial activity, the required administration time for the 1% chitosan hydrogel was only twice a week; moreover, metronidazole could have been incorporated within the hydrogel at a concentration of 15% rather than commonly used 25% (w/w). Patients were randomly divided into three groups. The first group received chitosan gel after scaling and root planning (SRP) treatment, while the second group received metronidazole-containing chitosan gel after SRP, and the third group (control) received only SRP treatment. The clinical parameters such as the probing depth, clinical attachment level, amount of gingival recession, plaque index, gingival index, and gingival bleeding time index were recorded at the baseline and at weeks 6, 12, and 24. The effect of gel formulations on the reduction of the gingival inflammation markers was found to be better than in the control group. In all groups, significant improvements were observed in clinical parameters between baseline and week 24. The reductions in probing depth values were 1.21 mm for blank chitosan hydrogel, 1.48 mm for metronidazole-containing chitosan gel, and 0.94 mm for control. The authors confirmed that chitosan itself as well as in combination with metronidazole are effective in the treatment of chronic periodontitis due to intrinsic chitosan's antimicrobial properties (Akncbay et al., 2007).

We attempted to provide the summerized overview of the relevant findings on chitosan-based hydrogels as intrinsic antimicrobials (Table 1). We included, in our opinion, the relevant references for readership to get an overview of the state-of-the art in this emerging field. We have to mention that we did not include the patent-protected work and opted to focus on scientifically proven and widely accessible research opus.

INSERT TABLE 1.

Conclusions

Hydrogels with intrinsic antimicrobial properties are gaining more attention with increasing treat of antimicrobial resistance. Biopolymers used to form these hydrogels are attractive building blocks as they often exhibit multitarget properties besides its antimicrobial action; currently chitosan-based hydrogels are widely studied for improved wound healing based on the ability of chitosan to act on enhanced healing and its intrinsic antimicrobial potential. However, other localized infections, such as genital, open up opportunities for novel strategic

approaches and formulations. Those formulations bear high innovative potential and are often comprising delivery-system-in-hydrogel concept. Moreover, the opportunity to chemically modify the properties of natural origin polymers, tailoring of polymers, is one of the research lines emerging recently. So far, very limited concerns have been raised regarding potential toxicity of the final formulations.

Perspectives

Understanding the mechanisms behind the polymers, including chitosan, exhibiting antimicrobial action would not only help us to optimize the formulation properties to maximize the efficacy but also address the possible resistance development. To achieve this, it is important to standardize the methodologies applied to evaluate and optimize the activity. Multidisciplinarity might be a shorter path to better understanding of the mechanisms and optimization process. Tailoring of the polymer properties would allow us targeting specific infections sites as well as formulation properties. It is very clear that biomaterials with intrinsic antimicrobial properties are offering many opportunities in product development and their full utilization is yet to be acknowledged.

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Table 1. Overview of chitosan-based hydrogels with intrinsic/synergistic antimicrobial activities

Target	Type of chitosan	Antimicrobials / nanoparticles / plant extract	Bacteria/ fungi	Study	Major findings	Reference
Skin infections (wounds)	Chitosan hydrochloride	-	*E. coli*	*In vitro*	Bactericide effect	Straccia et al., 2015
	Chitosan-grafted poly(acrylic)-co-(2-hydroxyethyl methacrylate)	Mica	*S. aureus*	*In vitro*	Mica does not affect the antimicrobial activity of the hydrogel	Noppakundilograt et al., 2013
	Chitosan from shrimp shells (≥ 75% deacetylated)	*Aloe vera* + silver nanoparticles	*S aureus, P. aeruginosa*	*In vitro*	Antibacterial activities comparable to tetracycline and gentamycin	Gomez-Schabala et al., 2017
	O-carboxymethyl chitosan	Lincomycin	*S. aureus, E. coli*	*In vitro*	antimicrobial efficiency proportional to lincomycin content; increase in chitosan content reduces antimicrobial activities	He et al., 2016
	Chitosan powder (80-95% deacetylated)	Silver nanoparticles	*S. aureus, E. coli*	*In vitro, In vivo*	Synergistic antibacterial effect of chitosan and silver nanoparticles; faster wound healing effect (rats)	Xie et al., 2018
	HMW chitosan	Silver nanoparticles, gold nanoparticles	*S. mutans, S. aureus, E. coli*	*In vitro, In vivo*	Plain chitosan gel prepared with 6% acetic acid better than chitosan gels prepared with 4% acetic acid and embedding silver or gold nanoparticles; slower wound healing effect with gold nanoparticles-in-gel *in vivo* due to toxicity of the gold nanoparticles	Samano-Valencia et al., 2014

	LMW chitosan (85% deacetylated)	Zinc oxide nanoparticles	*S. aureus*, *E. coli*, *P. aeruginosa*, *S. intermedius*, *S. hyicus*	*In vitro*, *In vivo*	Negligible antibacterial effect of plain chitosan gel toward *S. aureus* and *E. coli* due to neutral pH of gel; significant increase in activity by incorporation of zinc nanoparticles; effectiveness confirmed *in vivo* against *P. aeruginosa*, *S. aureus* and *S. hyicus*	Sundheesh Kumar et al., 2012
Vaginal infections	LMW chitosan	-	*Candida* spp.	*In vitro*	Antifungal activity varied among the tested *Candida* spp.; enhanced activity with chitosan nanoparticles in gel	Perinelli et al., 2018
	MMW chitosan	Metronidazole	*P. aeruginosa* (biofilm)	*In vitro*	Better anti-biofilm activities in comparison to poly(acrylic)-based gel at pH 6	Kandimalla et al., 2013
Periodontal diseases	Chitosan (Mw 108 kDa), quaternized chitosan	-	*P. gingivalis*, *P. intermedia*, *A. actinomyce-temcomitans*	*In vitro*	Presence of quaternized chitosan inside the thermoresponsive gel significantly increased antibacterial activity compared to pure chitosan	Ji et al., 2010
	HMW chitosan	Metronidazole	-	Clinical	The significant reduction in probing depth values with blank chitosan gel and drug-loaded gel	Akncbay et al., 2007

A. actinomycetemcomitans, Actinobacillus actinomycetemcomitans; *E. coli*, *Escherichia coli*; *P. gingivalis*, *Porphyromonas gingivalis*; *P. intermedia*, *Prevotella intermedia*; *P. aeruginosa*, *Pseudomonas aeruginosa*; *S. aureus*, *Staphylococcus aureus*; *S. hyicus*, *Staphylococcus hyicus*; *S. intermedius*, *Staphylococcus intermedius*; *S. mutans*, *Streptococcus mutans*.