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Understanding vaginal biofilms: The first step in harnessing antimicrobial nanomedicine

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ABSTRACT

In spite of multipurpose technologies offering broad-spectrum prevention for sexually transmitted infections (STIs) and contraception, the STIs incidences rise worldwide. The situation is even more alarming considering continuous rise in antimicrobial resistance (AMR) that limits therapy options. In this review we address the specific challenges of efficiently treating vaginal infections locally, at the infection site, by understanding the underlying barriers to efficient treatment such as vaginal biofilms. Knowledge on vaginal biofilms remains, up to now, rather scarce and requires more attention. We therefore propose a 'back to basics' insight that seeks to probe the complexity and role of the vaginal microbiota, its relationship with vaginal biofilms and implications to future therapeutic modalities utilizing advanced nano delivery systems. Our key objective is to highlight the interplay between biofilm, (nano)formulation and therapy outcome rather than provide an overview of all nanoformulations that were challenged against biofilms. We focused on the anatomy of the female reproductive organ and its physiological changes from birth, the unique vaginal microenvironment in premenopausal and postmenopausal women, vaginal biofilm infections and current nanomedicine-based approaches to treat infections in the vaginal site. Finally, we offer our perspectives on the current challenges associated with vaginal delivery and key considerations that can aid in the design and development of safer and potent products against persisting vaginal infections.

1. General introduction

Despite impressive development in the medical field in past decades, some of the health areas remain both stagnant and are even facing additional challenges. Although multipurpose technologies offering broad-spectrum prevention for sexually transmitted infections (STIs) and contraception is more than ever widely available [1], efficient, cost-effective and user-friendly formulations remain scarce while STIs incidences rise [2]. A very recent report (May 21st 2024) by World Health Organization (WHO) highlights that case notifications of STIs are increasing in many regions in the world, with four (curable) STIs—syphilis, gonorrhea, chlamydia, and trichomoniasis—accounting for more than 1 million infections each day. The report concluded that "the global response is currently off-track" to meet the 2025 targets to reduce new infections and decrease disease burden [3]. Sexually transmitted infections (STIs) predominantly spread via unprotected sexual contact and can be transmitted during pregnancy, childbirth and breastfeeding through infected blood or blood products [4]. Trichomonas, chlamydia, gonorrhea and syphilis are the most common and curable STIs. On the other hand, viral STIs including HIV, genital herpes simplex virus (HSV), viral hepatitis B, human papillomavirus (HPV) and human T-lymphotropic virus type 1 (HTLV-1) lack or have limited treatment options. To prevent infection that can cause liver and cervical cancers, vaccines are currently available for hepatitis B and HPV infections. While HIV, HSV and HTLV-1 are lifelong infections, available treatments can suppress HIV and HSV viruses. However, rapidly increasing cases of antimicrobial resistance are a growing threat for untreatable gonorrhea. It is therefore fair to state that the need for better infectious disease prevention and treatment is increasing rather than decreasing, especially in an era of antimicrobial resistance (AMR). If the need for better treatment is increasing and technology is advancing, why do we still lack efficient therapy and broader therapy options? In this review we attempt to provide some answers and propose pathways to address the unsatisfactory situation in the field. We address the specific challenges of efficiently treating vaginal infections by understanding the underlying barriers to efficient treatment of vaginal biofilms, offering state-of-the-art formulations overview with focus on nanoformulations for treating vaginal biofilm infections. We propose a '*back to basics*'

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insight that will probe the role of vaginal microbiota, its relationship to vaginal biofilms and the implications to future therapeutic modalities utilizing advanced drug delivery systems. We will highlight the role of vaginal microbiota and biofilms, that rather surprisingly, remains underestimated despite biofilms being responsible for more than 80 % of all chronic infections [5]. Recent review by Sousa and colleagues discusses the reasons behind failed therapy against bacterial vaginosis (BV), highlighting the roles of both the polymicrobial nature of BV and the biofilm challenge [6]. Moreover, Swidsinski and colleagues [7] propose that both diagnostics and therapeutic options for BV are suboptimal, and that the development of advanced molecular genetic testing in synergy with novel therapeutics effective against biofilms are required. Although the authors propose different approaches in combating biofilms, the role of nanoformulations were not discussed. Additionally, these reviews focus on BV and do not consider the challenge of vulvovaginal candidiasis (VVC). Our review brings deeper insight on biofilm complexity, challenges of vaginal site and design of nanoformulations to assist in optimizing nanoformulations for vaginal infections that are beyond BV. Even more surprisingly, a rather tiny proportion of research addresses vaginal biofilms as an obstacle to better therapy of STIs. We therefore aim to offer better understanding of vaginal microbiome in health, its changes in infection and biofilm formation, to correlate with development of novel targeted antimicrobial therapy. The key objective is to highlight the interplay between biofilm, (nano)formulation and therapy outcome rather than provide an overview of all nanoformulations that were challenged against biofilms. We refer to extensive reviews in the field that can assist the readers in choosing the nanoformulation [2,8,9].

2. What are biofilms and their link to vaginal infections?

Biofilms are nature's most successful life model. First described in 1683 by Antoni van Leewenhoek, the virulent nature and clinical relevance of biofilm-related infections was recognized about 300 years later in the sputum of cystic fibrosis patients [10]. According to current scientific evidence and figures from the National Institute of Health, up to 80 % of microbial infections are caused by biofilms [11]. Biofilms are described as adherent or free-floating microbial communities (or 'aggregates') that are embedded in an extracellular polymeric matrix [12]. Exopolysaccharides, extracellular DNA, lipids, and/or proteins are common components of the biofilm matrix, but the actual composition and structure within biofilms can vary greatly depending on various factors (e.g., the causative microorganism, nutrient availability, physiological status, local shear stress, the interface/site on which they form and host environment) [13,14]. The matrix is the hallmark of biofilm formation and has been intimately linked with the emergent properties of biofilms [15]. As biofilms mature, embedded cells exhibit very different physiological state and phenotypes [16]. This heterogeneity is highly associated with differential gene expression and metabolic activity of the embedded cells and arises from inherent variations in the local microenvironment generated within the matrix [17]. It is recognized that microbial cells are exposed to gradients of nutrients, pH, oxygen, and metabolites within the biofilm structure [12]. The three-dimensional nature of biofilms is a barrier against host immune or inflammatory response and effective antimicrobial treatment [18]. Because of this, microorganisms within biofilms have higher tolerance or resistance to mechanical and chemical assaults compared to their planktonic form [19].

Biofilms formation is well documented on numerous tissues including the vaginal epithelium. Among curable STIs highlighted by WHO, the role of biofilm in gonorrhea infection, especially as an obstacle to efficient therapy, has been well established [20,21]. Interestingly, treatment of chlamydia and *Trichomonas* infection are hampered by vaginal biofilm formed by other microorganisms; recent evidence suggest that *Chlamydia trachomatis* infections are greatly impacted by *C*andida *albicans* or *Gardnerella vaginalis* made biofilm that accompany infection. Namely, the biofilm serves as a reservoir of *C. trachomatis* enabling its survival and propagation into the upper genital tract, worsening the outcome of the disease while increasing the risk of developing severe reproductive sequelae [22]. Similarly, recent data indicate that *G. vaginalis* biofilm provides attachment site for Trichomonas *vaginalis*, enhancing its pathogenic abilities [23]. Additionally, the role of biofilm in BV and VVC has been extensively studied [24–27]. Vaginal infections are highly prevalent gynecological concerns in all women of reproductive age [28]. These infections affect millions every year and can be symptomatic or asymptomatic [29]. The inherent fluctuations in the vaginal microenvironment during sexual activity, pregnancy, and breastfeeding has great impact on the vaginal microbiota and enhances the risk of infection [30]. Besides formation on tissues (i.e., living, and dead), several studies have also identified adherent bacteria and biofilms on removable medical devices such as pessaries, vaginal rings, tampons, and menstrual cups [31]. Without treatment, vaginal infections can trigger other infectious diseases and cause infertility, pre-term birth or miscarriages. This has dire epidemiological, clinical, social, and psychological consequences on women [32]. Research also shows accumulating evidence of resistance in a significant proportion of drugs widely used in the management of vaginal infections (e.g., BV) [33,34]. Due to the current burden of AMR coupled with the high recurrence rates of vaginal infection [2,35], the development of new treatment options based on nanomedicine has received the much-needed attention.

Antimicrobial resistance is a challenge for pathogens within the biofilm. Greater resistance to antimicrobials were reported by multidrug resistant (MDR) bacteria during biofilm formation [36]. Common defense strategies that control resistance in biofilms include quorum sensing and horizontal gene transfer. Quorum sensing mechanisms mediates chemical signals and are involved in biofilm formation. Accumulation of these signals can favour biofilm formation by promoting the overexpression of resistance genes, regulating drug efflux pumps and modifying antimicrobial targets [37,38]. In biofilms, higher rate of horizontal gene transfer occurs with easy transfer of plasmids which disseminate resistance to antimicrobials [39]. Colonization with MDR bacteria can complicate or prolong antibiotic treatment and can be transmitted to neonates during labour and delivery [40]. Extended-spectrum beta-lactamase (EBSL)-producing *Enterobacteriaceas*, carbapenem-resistant bacteria, methicillin resistant *Staphylococcus aureus*, vancomycin-resistant *S. aureus*, vancomycin-resistant *Enterococcus spp* are examples of MDR strains. In a recent study, 68 % of *G. vaginalis* isolates were reported to be resistant to metronidazole [41]. Among patients with recurrent BV infections, more than 58.8 % showed resistance against metronidazole. The US Centres for Disease control and prevention (CDC) has highlights increasing reports of AMR in Candida infections including the emergence of the deadly *Candida auris*. A recent study by Mesini and colleagues reported the first case of *C. auris* colonization in a preterm baby after vaginal delivery [42]. Although the authors could not discriminate the colonization route, the difficulty in eradication and the limited therapeutic options available for *C. auris* is a problem. A considerable number of patients with refractory VVC were caused by fluconazole-resistant *C. albicans* [43]. Interestingly, a dramatic increase in fluconazole resistance was seen from 20 % to 52 % at pH 7 and 4.5 respectively. Resistance of STIs such as *Neisseria gonorrhoeae* to several classes of antibiotics (sulfonamides, penicillins, tetracyclines, macrolides, cephalosporins) has been documented and prioritized by the World Health Organization. In 2022, antimicrobial susceptibility testing for Gonococcal in the EU showed two isolates that displayed resistance to ceftriaxone and were extensive drug resistance (XDR) and multidrug resistant (MDR) strains. In England, cases of *N. gonnorrhoeae* rose by 128 % between 2013 and 2019. The *N. gonorrhoeae* FC428 clone has been associated with ceftriaxone resistance and shown to have reduced susceptibility to azithromycin in numerous countries [44].

Despite the dramatic anatomical, physiological, and hormonal

changes that the genital track undergoes during the life cycle of a woman, its impact in nanomedicine design is not fully considered. Recently, interest in biofilms skyrocketed resulting in several extensive reviews that summarize the mechanisms, applications, and prospects of advanced nanotechnologies and their impact on biofilm formation by clinically relevant pathogens [45–48]. However, up to the best of our knowledge, no review focused on combating vaginal biofilms. This review will therefore focus on the anatomy of the female reproductive organ and its physiological changes from birth, the unique vaginal microenvironment in premenopausal and postmenopausal women, vaginal biofilm infections and current nanomedicine-based approaches to treat infections in the vaginal site. Finally, we will provide our perspectives on the current challenges associated with vaginal delivery and key considerations that can aid in the design and development of safe and potent products against persisting vaginal infections.

3. Vagina as a site for drug action

The female vagina, a component of the reproductive organ aids sperm migration, assists childbirth and removal of blood and tissue during menstruation. The vagina undergoes distinct morphological and physiological changes during puberty, menstruation, pregnancy, and menopause [49]. It is described as a distensible muscular canal which is between 6 and 12 cm long [50]. The vaginal wall consists of an inner mucosal layer supported by a thick lamina propria, muscularis layer and adventitia layer [51,52] (Fig. 1). The inner surface of the vaginal epithelium is covered by a series of prominent ridges termed rugae [53]. The human vaginal epithelium is the first line of defense against invading pathogens. It comprises of four distinct layers; the cuboidal basal layer, mitotically active parabasal layer, glycogen-rich intermediate layer and flattened non-cornified superficial layer [54]. The vaginal stratum corneum lacks intercellular junctions that do not keratinize or form a complete lipid envelope, permitting penetration of microorganisms and/or molecular mediators of immune response [55]. To maintain a characteristic acidic pH in the vagina, the shedding of cells in the epithelium promotes glycogen release which serves as a substrate for resident lactobacilli [56]. Sex hormones (e.g., estrogen) play an essential role in maintaining vaginal health by maintaining lubrication, strength, elasticity, and thickness of the epithelium [57]. Although the high permeability of the vaginal epithelium makes it an attractive target for drug delivery, its inherent physical (epithelial and immune cells), chemical (mucus, antimicrobial peptides etc) and biological (microbiome) barriers pose as a challenge to effective therapy.

In addition to challenges related to anatomical and physiological features of vagina, the complexity of its barriers, unique microenvironment and changeability of conditions within the microenvironment further complicate the understanding and design of successful therapy. We provide herewith a deeper insight into the barriers that need to be overcome for successful therapy.

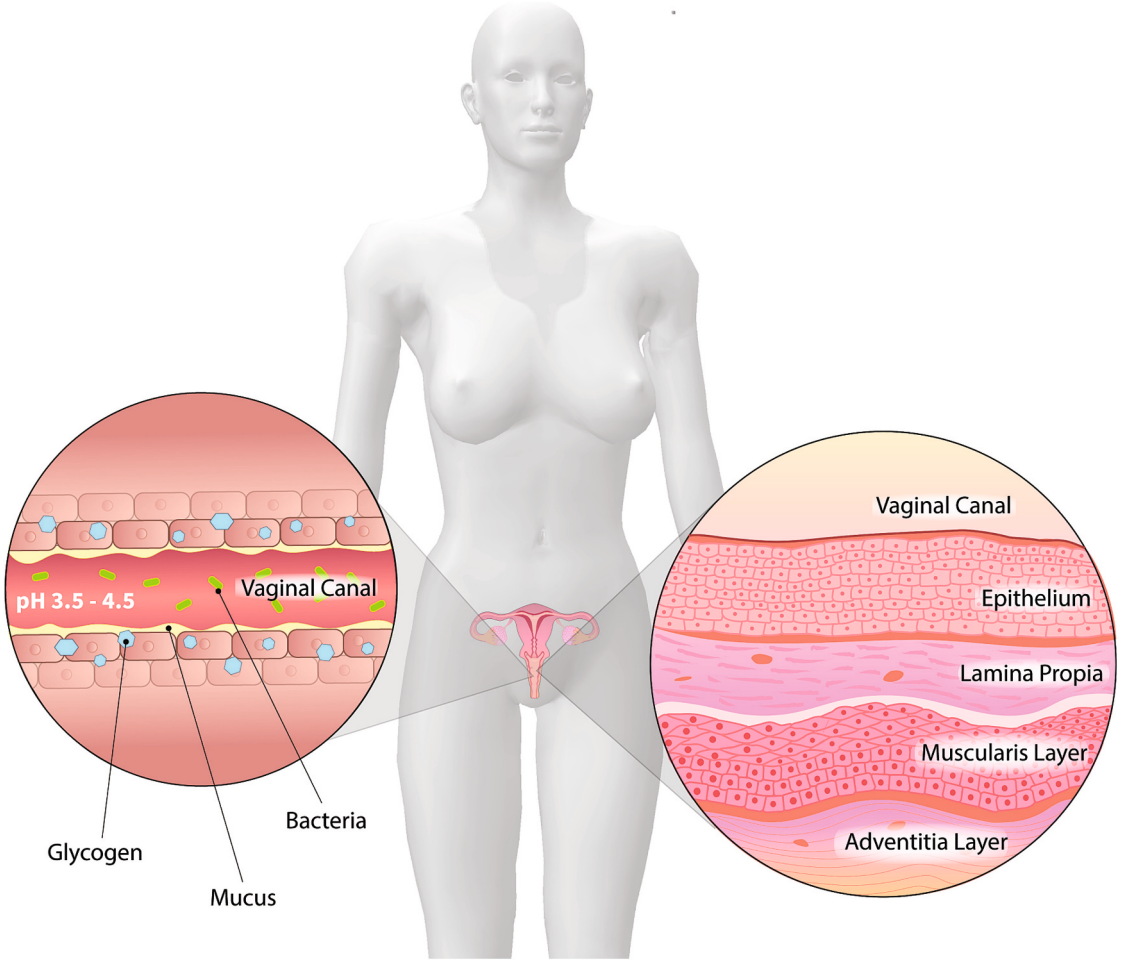

Fig. 1. Anatomical and physiological characteristics of vaginal site. Anatomical illustration of the three main layers includes the mucous membrane (epithelium, lamina propria), the muscular layer (muscularis) and the adventitia layers. Within the vaginal canal, the low pH, presence of mucus and glycogen are the physiological features of the site.

3.1. Vaginal microenvironment – Barriers to drug delivery

Any formulation destined for vaginal sites will face cervico-vaginal mucus and underlying epithelium. It is particularly relevant for nanoparticulate delivery systems that need to be optimized for their size, surface properties, stability and composition. The lack of easily translatable animal models as well as reliable *in vitro/ex vivo* models additionally slows down product development [55].

3.1.1. The mucosal barrier

Mucus is a complex biological fluid comprising water (95 %), mucins (drives the viscoelastic property of mucus), lipids, salts, protein, growth factors, enzymes, and immunoglobulins [58]. It protects the epithelium by lubricating it, creating an unstirred layer (through which pathogens must pass) and physically trapping pathogens [59]. It is therefore a major hurdle for drug absorption in the vagina, as well as penetration of drugs/active molecules in deeper epithelial layers (Fig. 2). The consistency, compositions, and glycosylation status of mucus is highly regulated by hormonal changes over the life course of women: during menstruation, pregnancy, and menopause [60]. Mucin, the main component of mucus, is a highly glycosylated protein primarily secreted by cervical goblet cells [61]. The two main classes of mucins are secreted mucins (e.g., MUC2, MUC5B) and the membrane-spanning mucins (MUC1, MUC4, MUC16) [62]. The polymer network of mucin is highly heterogeneous and stabilized by intermolecular and intramolecular disulfide bridges, calcium crosslinks, and hydrogen bonding interactions [63]. With an average pore size of approximately 350 nm, the microstructure of mucus minimizes penetration of nano- and microscale objects via physical (steric) and adhesive (electrostatic, and hydrophobic) interactions [64]. Mucins provides nutrition, protection, and housing to commensals and are integral to their maintenance within the vaginal cavity while resisting the colonization of pathogens [65,66]. To gain time for local immune response, mucins capture pathogenic microorganisms and slow down their diffusion speed [67]. Although nanocarriers improve the delivery of therapeutics, potential interaction with mucus may influence stability and drug release [68]. Therefore, the

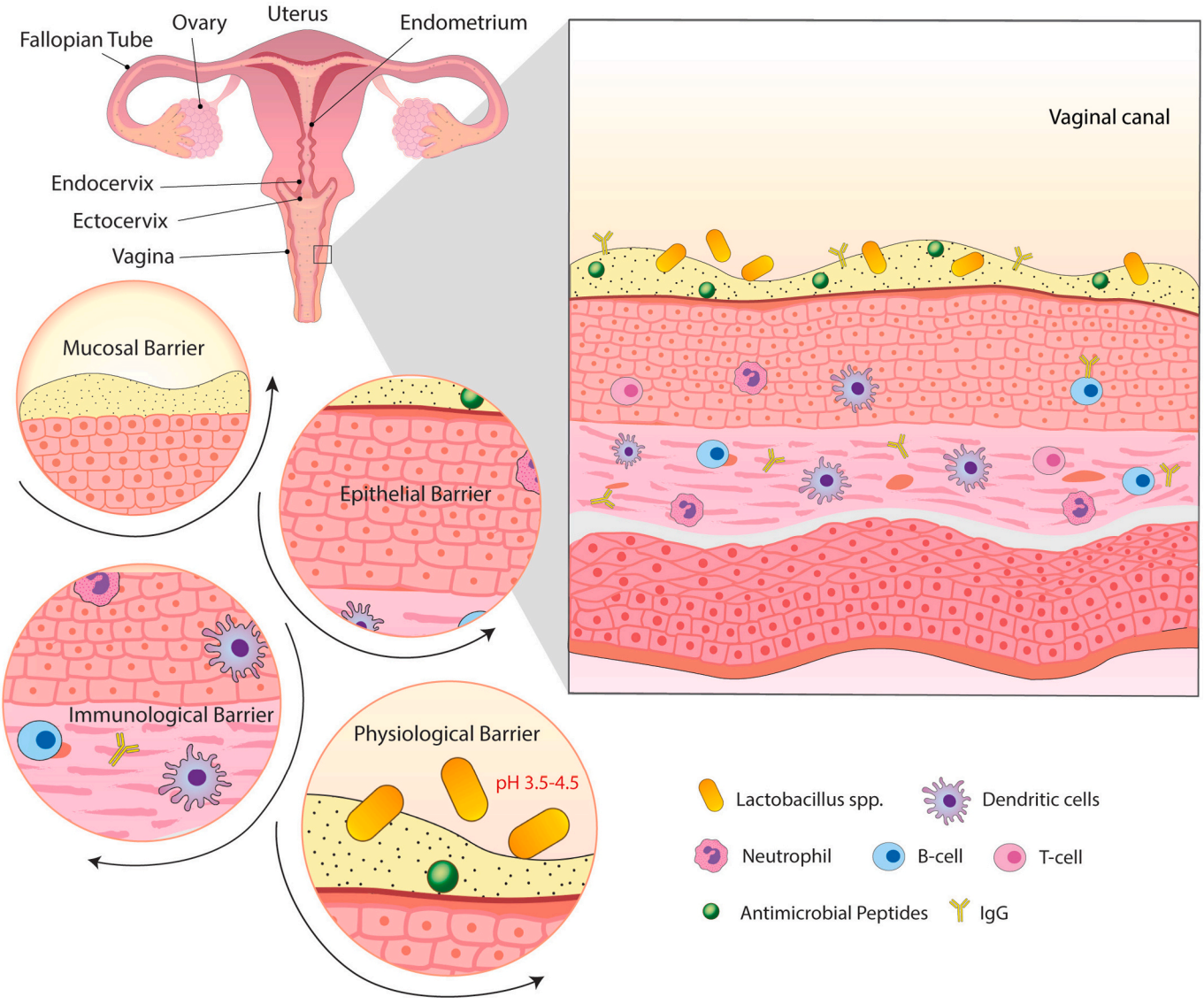

Fig. 2. Overview of the various barriers to drug delivery and their location within the vaginal site. The immunological, epithelial, mucosal and physiological barriers are depicted. The physiological barrier encompasses the vaginal discharge and its residence time, the enzymatic activity and the local pH. The other barriers can be viewed as physiological barriers. The immunological barrier comprises immune cells (e.g., Langerhan cells, dendritic cells and macrophages), antimicrobial peptides and immunoglobins. The epithelial barrier is the physical barrier provided by the epithelium. It varies in thickness during a woman's life. The mucosal barrier comprises mucus, a complex biological fluid that provides protection to the epithelium.

impact of mucus is a key consideration in the development of drug delivery systems (DDS) for local vaginal therapy. In-depth discussion on mucosa as a barrier to drug delivery to vagina, including the interaction between mucin fibers and nanoformulations is offered by das Neves et al. [69].

3.1.2. The epithelial barrier

During a woman's life, the vaginal epithelium undergoes varying structural changes [70]. Prior to puberty, the epithelium is thin but in the reproductive years, it thickens and develops distinct cornified layers [71]. On the other hand, menopause is characterized by diminishing glycogen stores and thinning of the vaginal epithelium [72]. The epithelial barrier is a physical barrier comprising multi-layers of stratified squamous cells with tight junctions that restricts permeation of free molecules (e.g., drugs and nanoparticles) through the paracellular space [73,74] (Fig. 2). Additionally, its highly folded and collapsed nature can impede uniform drug distribution [55]. The integrity of the epithelial barrier is further enhanced via the production of lactic acid from healthy resident microbiota [75,76]. Dysbiosis, a term often used to define a disbalance in the microbiota composition, alters microbial metabolites production which increases local inflammation and may damage the epithelium [75,77]. Furthermore, thinning of the epithelium predisposes women to infectious diseases [78].

3.1.3. The immunological barrier

The vagina is equipped with a full spectrum of immune cells that provide protection against invading pathogens such as Langerhans cells (LCs), dendritic cells, and macrophages [79] (Fig. 2). The LCs maintain peripheral tolerance and immune homeostasis at the epithelial surface [80,81]. Emerging research demonstrate that other immune cells can populate the reproductive tract during an infectious challenge [79] and in relation to miscarriage [82]. For instance, natural killer cells (NK) have been reported to be present in the vaginal mucosa [83]. They aid macrophage activation and generation of a pro-inflammatory and cytotoxic T cell response [84]. During infection, damage-associated molecular patterns termed DAMPs (e.g., heat shock proteins, DNA, ATP, formyl peptides and fibronectin) are released into the extracellular fluid [85]) in response to vaginal epithelium damage. Following their release, abundant quantities of neutrophils penetrate the vaginal epithelium to phagocytise the pathogen under the influence of chemotactic cytokines [84]. Neutrophils respond to pathogen invasion by producing reactive oxygen species (ROS) which aids in recruiting other immune cells and in the clearance of invading pathogens [86]. Other chemical components used by immune cells against invading pathogens include AMPs [87]. While AMPs are primarily expressed in neutrophils and epithelial cells, smaller fractions are reportedly found in dendritic cells, macrophages, and natural killer cells [84]. Among the many reported AMPs, defensins, protease inhibitors, lysozyme and lactoferrins are the most important AMPs in the vaginal innate system [88]. The vaginal epithelium is also equipped with local immune players such immunoglobulins [89]. High concentrations of immunoglobulins in the mucus further strengthen the mucosal barrier against infection [65]. Considering that vagina is coated with mucosa, its role needs to be understood beyond protection against pathogens. However, we are lacking deeper understanding of immune responses within genital tract in comparison to other mucosal surfaces. Deeper understanding of immune responses can guide interventions to prevent the spread of STIs. The vaginal microbiome is a critical modulator of inflammation in the reproductive tract that remains to be explored considering its impact on susceptibility versus protection against infection. The immune response in the lower reproductive tract fluctuates over the menstrual cycle opening a 'window of susceptibility' for STIs [79]. However, the complexity of type of microorganisms needs to be accounted for. For example, VVC bears unique features considering the protective immunity to opportunistic fungal infections. The inflammatory response is associated with symptomatic disease, not a pathogen clearance. Therefore, VVC can be defined as an inflammatory disease, that follows a cascade of events starting with activation threshold established by vaginal epithelial cells that drive neutrophil recruitment and dysfunctional neutrophil-mediated inflammation. Consequently, anti-inflammatory cytokines that protect against inflammatory pathology should be explored as therapy targets [90].

3.1.4. The physiological barrier

All barriers mentioned earlier can clearly also be seen as physiological barriers. However, to highlight the complexity of the microenvironment the formulation faces once administered into the vaginal cavity, we summarized the features that make vaginal site a unique site (Fig. 2). In brief, the first obstacle is the residence time at vaginal site that is hampered by vaginal clearance and discharge. Vaginal discharge, a rather complex mixture of epithelium transudates, cervical mucus, exfoliating epithelial cells, secretions of the Bartholin's and Skene's glands, leukocytes, endometrial and fallopian' tubes fluids, can be seen as a variable feature that impacts performance of any formulation accommodated within vaginal cavity. Its volume and composition are affected by the stages of the menstrual cycle, hormones and sexual arousal. In addition, enzymatic activity needs to be considered as potential obstacle as well. The mildly acidic pH can hamper efficient drug delivery in the vagina. For more details the readers are referred to numerous reviews in literature [2,8,9].

3.2. The vaginal microbiome

The vaginal microbial niche is dynamic and recognized as a critical determinant of vaginal health [91]. It is thought that the vaginal microbiota acts as the first line of defense against pathogens via competitive exclusion and direct killing [92]. A significant portion of this protective role is attributed to *Lactobacillus spp* which typically dominates (70 %) the vaginal microbiome [93]. This microbe is considered the hallmark of a healthy vaginal microbiome and has marked interspecies diversity in the vagina [94] to ensure microbiome community stability. It maintains an acidic microenvironment (pH 3.8–4.5) via fermentation of glucose and maltose to produce lactic acid [95] (Fig. 2). Lactic acid constrains the growth of many pathogenic microbes. Compounds which play a secondary role in the control of the vaginal microbiome include hydrogen peroxide and bacteriocins which also play essential protective roles against pathogens [96]. There is a consensus that *L. crispatus* dominated communities have a more protective impact on the host than communities dominated by L. *iners* which fail to provide sufficient protection against vaginal dysbiosis [97]. The presence of non-Lactobacillus-dominant communities in vaginal flora has been linked to an increased risk of adverse health outcomes which indicate that these communities are less protective against spontaneous preterm birth and infectious diseases [91]. Although the contribution of fungi species to our immune defenses is acknowledged, very little is known about their role in vaginal health. The fungal community that colonizes the female reproductive tract include yeast and filamentous fungi [98]. Candida is the predominant strain with *C. albicans* making up 70 % of fungi in the vaginal mycobiome. Other non-albicans species detected include *C. alimentaria*, *C. dubliensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis* [97].

The taxonomic composition of the vaginal microbiota is subdivided into five specific configurations termed community state types (CSTs) [99]. Specific *Lactobacillus* strains have been found to dominate four of these subtypes [100]. On the other hand, CST IV often manifests as a dysbiosis state with multiple obligate and facultative anaerobes. Changes in the microbial composition of the vaginal microbiota is influenced by the age, ethnicity, pregnancy, and internal factors such as hormonal status as well as the immune system. External factors such as antibiotic use, contraceptive use, infections, douching, diet, and stress have been reported to impact the microbial composition [101,102]. The onset of menstruation can alter the microbial communities while

unprotected vaginal intercourse can introduce new microbial species or strains from penile microbiota [103,104]. Dysbiosis due to variations between the internal and external factors has been linked with preterm birth, bacterial vaginosis, vulvovaginal candidiasis, and an increased risk of STIs [105].

We know that the healthy vaginal microbiome is often dominated by one of four *Lactobacillus* species: *L. crispatus*, *L. iners*, *L. paragasseri*, and L. *mulieris*. Among these, *L. crispatus* has been associated with optimal health while the dominance of L. *iners*, is related to an enhanced risk for developing BV [106,107]. Although Srinivasan et al. [106] mapped the metabolic signatures of BV across multiple metabolic pathways, indicating that these signatures are associated with the presence and concentrations of particular bacteria, relatively little attention has been given to mapping the healthy vaginal microbiota. Recently Jimenez et al. [108] identified 95 significantly altered metabolites between individual lactobacilli, representative of healthy flora and mock controls. Metabolic analyses revealed distinct profiles of each *Lactobacillus* species. However, no comparison between healthy and infected microflora, especially in biofilm formation, is available that would be straightforward, reproducible, and insightful. Currently used conventional assays such as the disc diffusion test and broth micro-dilution can only define pathogen susceptibility and determine the minimum inhibitory concentration of antimicrobial agents. Moreover, they are time consuming, unreliable, and limited when dealing with biofilms. However, no single microbe seems sufficient to cause BV, and many BV associated bacteria are also found in healthy women. Therefore, it is essential to gain insight on the interplay between bacteria present in vaginal microbiota both in healthy and infected women.

3.3. Vaginal microbiome vs vaginal biofilm

3.3.1. Vaginal microbiome

Vaginal site can be seen as a highly dynamic ecosystem, comprising numerous (200) microbes that are impacted by the genes, but also ethnicity, and environmental-behavioral factors. Moreover, it is really an interplay that needs to be understood and followed in this dynamic setting. The microorganisms are highly diverse; ranging from the commensal, symbiotic to pathogenic organisms covering the vaginal surfaces, that are well interconnected [109]. The species of lactic acid bacteria (LAB) are predominant in healthy premenopausal women. In recent years, the field focused on gaining a deeper insight on the role of race or ethnicity, accompanied by patients' food habits, intake of probiotics, hygienic and sexual practices, as well as the use of lubricants. Currently, we may claim that we acknowledge the complexity of the

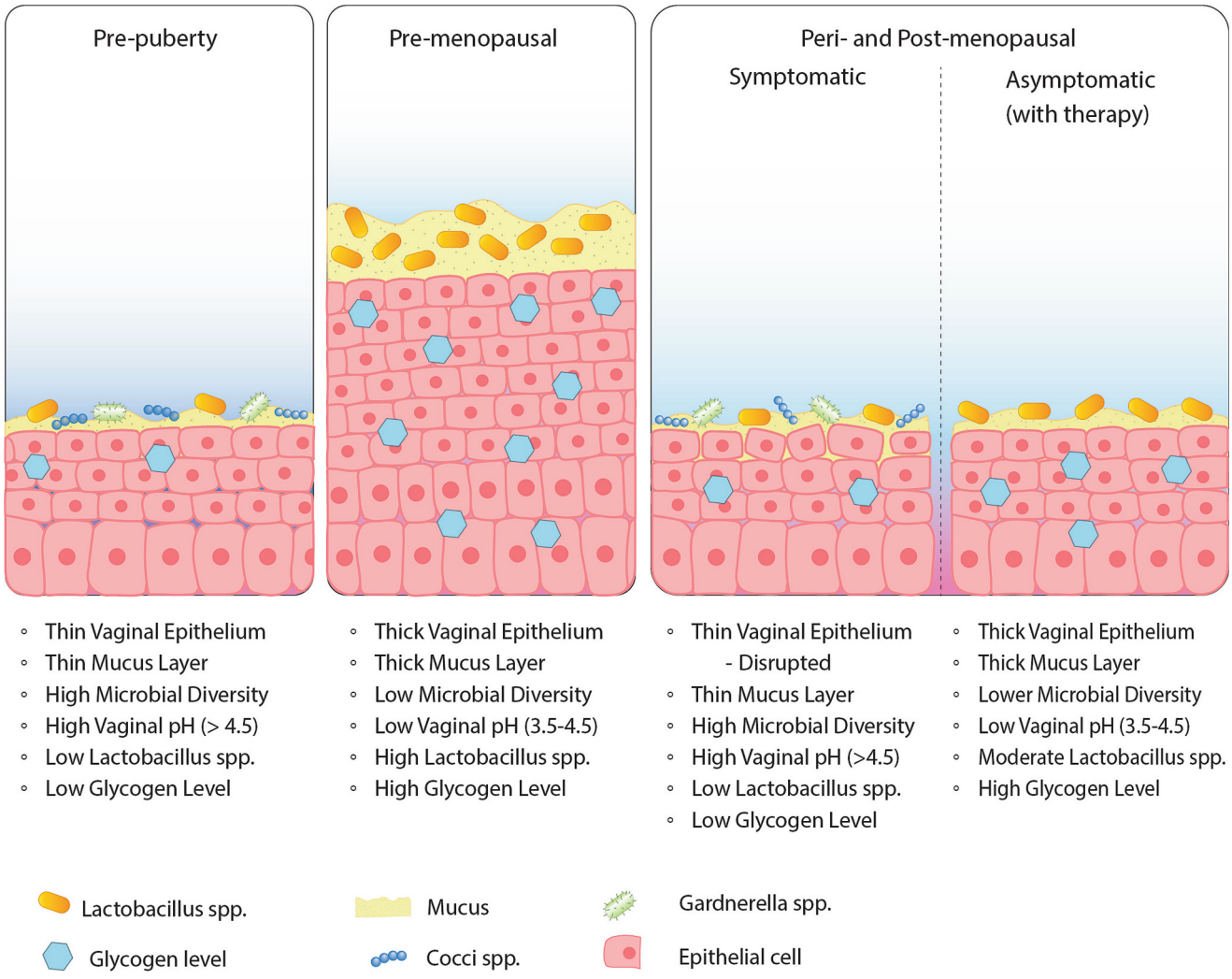

Fig. 3. Age-related changes in vaginal microenvironment (modified with permission from Shapiro et al., 2022). Changes in the thickness of the vaginal epithelium, lactobacillus composition, glycogen levels and mucus layer are dependent on the age of the woman. The pre-puberty stage is characterized by a thin epithelium with thin mucus, high microbial diversity and low levels of glycogen and lactobacillus. In the pre-menopausal stage, a thick vaginal epithelium, thick mucus layer, low microbial diversity with high levels of Lactobacillus spp. is observed along with low vaginal pH and high glycogen. Symptomatic women in the *peri* and post-menopausal stage show disrupted epithelium with thin mucus layer, high microbial diversity and reduced lactobacillus. This is modulated with therapy.

environment and understand its role in designing and tailoring efficient antimicrobial therapy [96]. However, as a common nominator, the *Lactobacillus* spp. species are playing an essential role in both vaginal health as well as wellness [110]. LAB are responsible for an acidic microenvironment relying on hydrogen peroxide and lactic acid production, impacted by hormonal variations, that control the growth of most pathogens through competitive exclusion [110]. A decrease in LAB levels permits overgrowth of the anaerobic bacteria resulting in bacterial vaginosis. *Prevotella,* one of the predominant genera [111], particularly *P. bivia,* appears to be connected to epithelial cytokine production, as detected in women diagnosed with pregnancy complications. Interestingly, synergistic relationships between *P. bivia* and *Gardnerella vaginalis* has been reported [112]. Moreover, vaginal microbiome undergoes continuous changes throughout woman's life (Fig. 3) [113].

The vaginal microenvironment is clearly everchanging environment that requires deeper understanding. The decrease in microbial diversity and consequent dysbiosis lead to vaginal infections, such as bacterial or yeast infections, STIs, urinary tract infections. Untreated or sub treated infections consequently negatively impact fertility. In addition, the vaginal microenvironment in women can be modified by her dietary habits, for example vitamin as well as iron deficiency impact bacterial vaginosis and *Candida* infection. Moreover, increased plasma glucose levels and obesity, smoking habits also reduce *Lactobacilli* count and are associated with higher Nugent scores [96].

Vaginal microbiome is directly responsible for the maintenance of woman's homeostasis and provides an insight on both women's health and wellbeing as well as critical changes due to infections. A deeper insight on complexity of vaginal microbiome composition, roles and modifications will lead to improved strategies for both infection prevention and efficient therapy [113]. The field of vaginal microbiome suffered for some misconceptions and controversies. For example, it was believed that throughout the pregnancy, a fetus grows in a sterile environment. However, there are rather convincing evidence obtained through next-generation sequencing utilizing placental and fetal samples that show that this is not the case [114]. However, it remains to be seen whether a concept of a sterile womb is correct and whether a transient exposure during gestation and at the time of delivery need to be accounted for [114]. Clearly, vaginal infections during pregnancy may induce changes in vaginal microbiome, yet the vaginal microbial composition and factors affecting its homeostasis need deeper understanding. The sudden cessation of transplacental estrogens during birth reportedly reduces concentration of vaginal glycogen and lead to neutralized or alkalized vaginal pH. Although during childhood, the pH of the vagina remains neutral or alkaline, the rise of estrogen and progesterone during puberty stimulate the colonization of lactobacillus throughout the reproductive age of women [113]. In addition to the predominant *Lactobacillus* species found in the vagina, other anaerobic bacteria and some traces of *Prevotella, Gardnerella and Peptoniphilus* accompany bacterial vaginosis [113].

During reproductive years, the vaginal microbiome changes due to influence by both estrogens and progestin, leading to lowering of local pH (below 4.5) due to glycogen metabolization (Fig. 3). This change enables limited/restricted growth of many pathogens. Simultaneously, elevated estrogen promotes hyperplasia of the vaginal mucosal epithelium and the rise in cellular glycogen. Enzymatic degradation of glycogen to produce maltose, α-dextrines and maltotriose by α-amylase are further metabolized by *Lactobacillus* leading to lactic acid. These further decreases vaginal pH to 3.5–4.5. The acidic environment enables better adherence, colonization and overall survival of *Lactobacilli* [115].

Dysbiosis may lead to bacterial vaginosis. In praxis, the Nugent score, providing a quantitative measure of *Lactobacillus* and other microorganisms (*Gardenerella vaginalis*, *Prevotella* spp., *Mobiluncus*) presence in Gram-stained vaginal smear is used to determine level of dysbiosis [116,117]. However, the original methods rely on the culture protocols and were often oversimplified. To provide a deeper understanding of the microbiome complexity, advanced culture-independent assays such the use of 16S rRNA gene sequencing reveal a myriad of resident bacteria. It is important to remember that interindividual variability and complex interactions between microbial species and vaginal microenvironment need to be accounted for [113]. Among *Lactobacillus* species, the role of L. *iners* in maintaining vaginal ecosystem is controversial, it has been isolated in both healthy vaginal microbiome and in vaginal microbiota of bacterial vaginosis [113]. There is a factor that is often neglected and that is an impact of race. While race has been included as a confounding variable, contrasting results from several studies on CST-race specificity suggest that genetic/immunological cues rather than social/behavioral habits influence the vaginal microbiome composition [118]. Therefore, vaginal site is unique in comparison to other sites of the body, namely its microbial variability is associated with a disease state rather than healthy state [113]. Lower levels of sialic acid and high-mannose glucans are found in cervicovaginal lavage fluid of BV-positive women; mucin degradation decreases the viscosity of lavage fluid, while adhesive barrier properties become impaired [55].

Although most attention was given to the role vaginal microbiome plays in women of reproductive age, postmenopausal women also are impacted by dysbiosis; it may drive vulvovaginal atrophy, vaginal dryness and impair their sexual health [119]. In the postmenopausal stage, the reduction in LAB levels is characterized as a physiologic change. For example, compared to pre-menopausal women, it is known that *Lactobacillus* and other bacteria associated with vaginosis have a lower presence in postmenopausal women. It is therefore conclusive that the composition of the vaginal microbiome undergoes a physological changes in postmenopausal women [113].

3.3.2. Biofilms in the vagina

Biofilms have in recent years gained well-deserved attention as a key obstacle in antimicrobial therapy, regardless of the infection site. Although their complexity, multispecies environment and interconnective relationship remain a challenge, there are at least theoretical means that can assist in understanding the biofilm challenges from the perspective of infection site. Considering vagina, the first step is usually to define the biofilm-forming culprits. Bacterial biofilms were the first to gain attention within the scientific community. The matrix of bacterial biofilm is organized through a complex internal architecture comprising channels that permit nutrients circulation [113,120].

However, knowledge of the composition matrix and triggers of biofilm formation is still scarce. Due to the specificity of different devices that will be inserted in vagina, biofilms can expand to vaginally inserted devices, such as tampons, intra-uterine devices and vaginal rings, further providing attractive environment for biofilm persistence. We have not included the discussion on devices-related biofilms in the review. To understand the biofilms in depth, it is important to, once again, highlight the complexity of microorganisms and their interrelationships that often limit therapy success, including biofilm eradication. Very often in literature we use the term vaginitis, referring to recurrent female infections. However, clinically, vaginitis is categorized into bacterial vaginosis, vulvovaginal candidiasis (VVC), and trichomonas vaginitis (TV) based on the nature of the infection [2]. However, some patients may exhibit multiple types of vaginitis simultaneously, which then erases the boarders between bacterial and candidal biofilms and limits the usefulness of classification. The occurrence of mixed vaginitis involves the presence of at least two types of vaginitis and formation of polymicrobial biofilms. Common symptoms associated with BV include pain/itching and an unusually foul discharge with a strong fish-like odour [121]. (See Fig. 4) The characteristic vaginal discharge is due to the acquisition of strict anaerobic and facultative bacteria. Alongside the identification of 20 % clue cells, mucin-degrading enzymes that are produced by the anaerobic bacteria reduce the viscosity of the vaginal fluid. BV bears a stigma in many countries; however, the consequences of untreated/sub-treated BV are much more severe. For instance, BV is

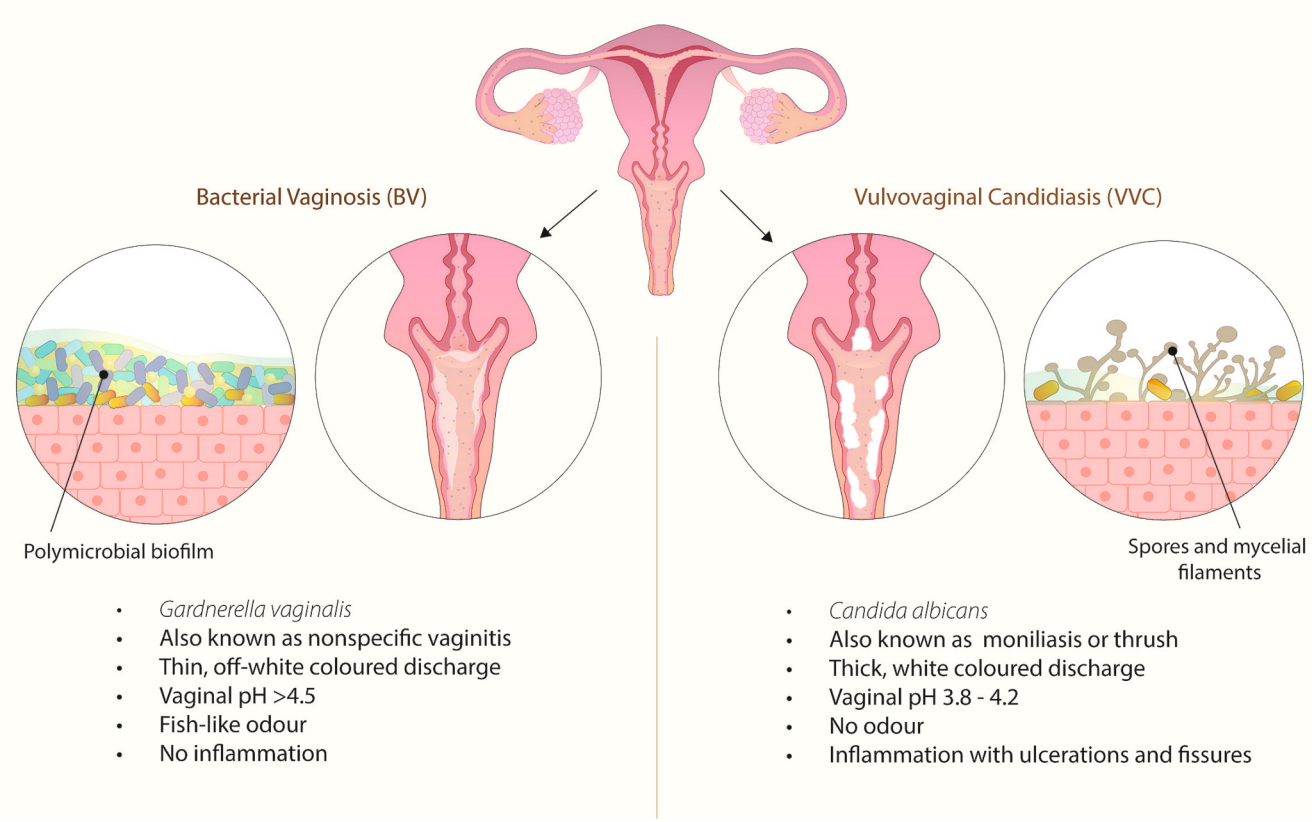

Fig. 4. Microbial biofilm infections caused by bacteria and fungi, and their symptoms. Illustration of polymicrobial biofilms causing bacteria vaginosis is characterized by a thin, off-white coloured discharge, high vaginal pH above 4.5, a fish like odour and the absence of inflammation. In vulvovaginal candidiasis infections, a thicker white coloured discharge is observed with low vaginal pH and the absence of odour. Inflammation causing ulcers and fissures can be seen.

linked to infertility and risk of preterm birth [122]. Provocatively, the recommended treatment of BV remains the same for decades. The absence of BV symptoms in certain patients was associated with host tolerance [123].

Vulvovaginal candidiasis (also referred as vulvovaginal candidosis, VVC) is the second most prevalent infection of the female genital tract (after BV) mainly caused by *C. albicans* (80–90 %) [9]. Other *Candida* species can also exist in VVC infections including *C. glabrata* (5–10 %), *C. tropicalis* (5 %) and *C. krusei* (1 %) The ability of *Candida* to form and organize in biofilms, can be seen as one of the explanations for underperforming therapy and high recurrence rates. Biofilms are able to obstruct the sufficient penetration of antifungals through their organized matrix, thus preventing complete eradication and potentially contributing to increased antimicrobial resistance [25]. Approximately 75 % of all women develop VVC at least once in their lifetime and recurrent vulvovaginal candidiasis (RVVC) is reported in up to 9 % of the global population [124]. Common presenting symptoms include vaginal/vulvar pruritis, burning/irritation and abnormal vaginal discharge [125]. While VVC infection does not alter vaginal pH, its polymicrobial nature such as co-occurrence with bacterial vaginosis, may lead to an elevated pH. The prolonged or repeated use of a single antifungal agent can be associated with disease recurrence often due to the development of drug resistance. Indeed, this has led to an increased predominance of non-albicans *Candida* species. It is well recognized that microbial communities within the vagina undergo shifts in their representation and abundance over time and during infections [126]. Typically, the total loads of bacteria during BV for instance are recently reported to be one or two orders of magnitude higher than in healthy patients [127]. Compared to the normal vaginal microbiota known for its enrichment of *Lactobacillus*, depletion of this species is seen during microbial infections with a corresponding overgrowth of various obligate anaerobic species [128]. For instance, during BV, depletion of Lactobacillus (except for L. *iners*) is observed [129]. It is worth noting that in previous studies, *L. iners* was not shown to have the same benefits as other Lactobacillus species from the vagina [130]. Recent mapping of microbial samples from 41 patients with different types of vaginitis reveal an abundance of Gardnerella (55.34 %) during BV [129]. In contrast, during VVC, the vaginal flora is seen to be dominated with Lactobacillus (65.52 %) while Gardnerella depletion (21.41 %) is commonly observed. In VVC + BV polymicrobial infections, despite the slight fall in Lactobacillus, it continues to dominate (50.76 %) with a slight increase in Gardnerella (28.81 %) compared to VVC.

Lactobacillus sp. in healthy vaginal microbiome dominate and produce various unique antimicrobial compounds [131]. This unique microbial population and metabolome has been reported in up to 70 % of healthy women [132]. On the other hand, in BV, the sharp decline in the total number of *Lactobacillus* alongside a corresponding increase in anaerobic microbes is observed. Another well-known difference between the microbiome and biofilm is the non-inflammatory vaginal environment associated with *Lactobacillus* dominance while a pro-inflammatory milieu and compromised genital epithelial barrier is associated with BV biofilms which leads to an increased acquisition of HIV and other STI infections [133]. Despite this information, the use of *in vitro* models in mapping and studying these dynamics has been rather challenging and some conflicting reports have been made. For instance, *in vitro* assessment of the biofilm forming properties of 24 clinical isolates of *Gardnerella sp* showed that only 11 of these species formed biofilms *in vitro* [134]. This makes it rather challenging to model biofilms from patients. A recent study accessed the biofilm formation of 16 vaginal *Lactobacillus* strains and the influence of the culture supernatants in a panel of *Candida* clinical isolates [135]. The anti-candidal activity of these strains were then correlated with the metabolome of

the culture supernatant where they exhibited strong fungistatic profiles. This demonstrates the interdependence between growth mode and metabolism of *Lactobacillus* in the vagina and their functional properties in maintaining vaginal health. In another study, the exopolysaccharides from *Lactobacillus* were tested for their effects on biofilms formed by opportunistic vaginal pathogens and on Lactobacillus strains [136]. The exopolysaccharides were shown to inhibit biofilm formation from bacterial and fungal pathogens in a dose dependent manner. Additionally, advanced vagina on chip preclinical models were used to measure associated host-immune responses following colonization of *Lactobacillus* and *Gardnerella vaginalis*-containing consortia [137]. Colonization by *Lactobacillus* led to the production of lactic acid and lower levels of inflammatory causing cytokine molecules than in control groups. On the other hand, damage to the epithelial cells, an increase in pH and a significant increase in multiple proinflammatory cytokines was observed following co-culture of *G. vaginalis*-containing consortia. This agrees with our current knowledge as observed in human patients with BV.

Despite a progress in different fields focused on understanding and successfully treating vaginal infections and inflammations, and vaginal microecology, the defined composition and functionality of different types of vaginitis remain to be agreed upon. To address this rather burning issue, Song and colleagues applied metagenomic sequencing to gain insight on the vaginal flora in patients with various types of vaginitis [138]. The authors characterized the dynamic changes in vaginal flora between patients infected with BV and healthy individuals; they were able to identify different bacterial biomarkers corresponding to different vaginitis types. This is a clear step forward in both understanding of vaginitis and targeted treatment of vaginitis. [2]

3.3.3. Approaches to restore microbial homeostasis in vagina

Considering the importance of maintaining acidic environment, one of the research pipelines focused on role and use of probiotics. Although numerous groups proposed probiotics as an effective means to tackle dysbiosis in women of reproductive age, their benefit remains not fully understood. Some studies highlight the beneficial influence of prebiotics and probiotics in restoring the vaginal microenvironment whereas others remain less convincing. For extensive overview the readers should refer to [113]. Interestingly, novel research line suggests rather daring approach of vaginal microbiome transplantation (VMT) from healthy donors. Four out of five patients suffering from symptomatic and recurrent BV exhibited a full long-term remission for up to 5–21 months upon VMT, that was attributed to the reconstitution of a *Lactobacillus*-dominated vaginal microbiome [139]. LAB bacteria adhere well to epithelial surfaces and can rather easily form biofilms. The use of biofilms produced by LAB against pathogenic bacteria has also recently be suggested as rather promising [140]. However, there is a fine line between the benefits of supplemented LAB biofilms, and possible harmful effects; the approach requires more in-depth analysis. Cohen and co-authors suggested the *Lactobacillus crispatus* strain CTV-05 as a means to reduce BV [141]. The compositional distinction between LAB and pathogenic biofilms remains unsolved. The fact that the biofilm composition changes depending on the biofilm maturity, the LAB strain involved, and environmental conditions such as oxygen, temperature, pH, nutrient availability, and desiccation can be explored to assist in distinguishing this [140].

Since our focus was on nanoformulations that can target vaginal biofilms, we did not include in-depth discussion on formulations designed to restore vaginal microenvironment.

4. Targeting vaginal biofilms

Conventional vaginal products still suffer leakiness, low residence time and often exhibit limited efficacy at the site of application [142]. To enable high local concentrations of the active substances, these topical products are often semi-solid or solid dosage forms and include gels, tablets and films. Polymeric excipients with hydroxyl groups or unionized carboxylate groups can be incorporated to promote mucoadhesive properties and to better enhance contact with the vaginal surfaces [143]. The high-water content of gel-based products is advantageous in the moisturization of the vaginal walls and can aid combating vaginal dryness in menopausal women [143]. Other properties such as buffering the vaginal environment to restore vaginal pH have been shown. The use of vaginal tablets such as fast disintegrating tablets which integrate mucoadhesive polymers have been reported. In a recent report, an osmotic pump system that delivers a viscous mucoadhesive gel was demonstrated to improve the drug distribution within the vagina [144]. For these products, it is important to consider the low amount of fluid available in the vaginal environment compared to other routes e.g., the oral route. Films are another example of vaginal products highlighted with mucoadhesive properties [145]. Compared to other pharmaceutical forms, the high contact time and lower weight of films provides generally better mucoadhesive properties. The reader is invited to read more extensive reviews that discuss mucoadhesive systems for vaginal drug delivery [142]. Vaginal retention influences the therapeutic effectiveness of these products and can be affected by the product properties or modified during infections. Long retention is advantageous in maximizing the effectiveness of drugs. A key parameter in retaining vaginal gels within the vaginal canal is the viscosity of the product. Therefore, selection of formulations with adequate viscosity is decisive for enhancing retention within the vagina as gels with low viscosity are prone to leakiness. Bioadhesive films based on Hydroxypropyl Methylcellulose (HPMC) and xantham gum were reported for the treatment of BV demonstrating vaginal retention for 8 h [146]. In another study, optimized bioadhesive films were retained in the vaginal compartment of pigtailed macaques for up to 7 days [147]. Sodium alginate-based hydrogels were retained in the vaginal tissues for more than 8 h [148]. Medium viscosity grade chitosan gels were shown to have high mucoadhesiveness and good vaginal retention [149]. Vaginal gels with lower gel viscosity are advantageous but are challenged by their faster leakage over time, lower coating integrity in the epithelium and a feeling of messiness following use of these products. The retention of vaginal tablets has been shown to persistent up to 24–48 h in sheep and women [150]. Vaginal retention can be tailored based on the choice of polymers, its viscosity and rheological behaviours. The pH and temperature were reported to significantly affect the gel viscosity of four commercial spermicidal gel and demonstrate the effect of vaginal fluid on the viscosity of gels [151]. Additionally, the *in vivo* model selected for the retention studies may have an impact. Despite formulation strategies such as mucoadhesion to enhance the retention time of vaginal products, these strategies rarely provide information on targeting of the drug to the invading pathogen.

Although nanocarriers could improve the therapeutic efficacy, translational challenges associated with the anatomy, unique microflora, mucus barrier, epithelial changes and enzymatic action of the vagina must be surmounted. Assuring high concentration of antimicrobial at the site of infection (vagina) is the prerequisite for efficient therapy/eradication of infection. Moreover, it is considered safer and suitable for pregnant patients as well [2]. However, understanding the complexity of microbial challenge and interplay with microenvironment need to be inbuilt into nanocarrier design. Unravelling the relationship between the changing vaginal microenvironment during biofilm infections and its impact on the release kinetics of drug delivery systems is crucial in achieving this but remains understudied. Without applying engineering principles, the complex vaginal environment during biofilm infections can further hamper coverage and residence time of nanocarriers within the vaginal tract. The next section will elaborate on literature reports on nanocarriers applied for the control of vaginal biofilm infections and what strategies were employed to achieve this effect.

4.1. Nanomedicine to treat vaginal biofilms

Engineered nanoparticles are promising platforms for the secure loading of antimicrobial agents. Literature reports demonstrate their exploitation as formulations with high biofilm penetration and the capacity to improve drug potency [152]. In the treatment of vaginal infections, local application offers lower drug doses which minimizes drug toxicity, avoids hepatic first-pass metabolism, and improves bioavailability. Considering the increased clearance and discharge during vaginal infections, current efforts focus on improving the residence time of nanocarriers within the vaginal tract via design principles that enhance muco-adhesion or muco-penetration (Fig. 5) [153,154].

This approach can also promote uniform distribution of nanoparticles over the surface of the vaginal epithelium. However, mucoadhesive nanoparticles can be hindered in the outer layer of the mucus which excludes them from reaching the biofilm matrix [154]. Furthermore, during biofilm infections, the invading pathogen influences the consistency of the vaginal discharge (e.g., thin consistency in BV and thicker consistency in VVC) [155,156] which may also alter the transport kinetics of nanocarriers to the epithelial surface. Elucidating these changes and their impact on nanomedicine transport can improve our understanding on the underlying kinetics and how we can enhance drug potency within biofilms associated with different pathogens on the vaginal site. In addition to achieving fast penetration across the mucus barrier (i.e., muco-penetrating effect), efficient penetration of nanoparticles into the dense protective layer of the biofilm matrix is crucial to disperse and eradicate biofilm [157]. To ensure that the biofilm matrix does not further delay the release of the antimicrobial agent, on-demand release of antimicrobials from nanocarriers is an attractive strategy commonly associated with stimuli-responsive systems. These stimuli-responsive nanocarriers can take advantage of the difference within the biofilm matrix and the external environment during infection to achieve targeted drug release. For instance, the pH of the vaginal environment during BV infection is less acidic (pH value of 7 or higher) than the normal pH value of the vagina (approximate pH of 4). Considering that the pH within biofilms has been reported to be acidic [158], nanocarriers that release their load under acidic environments may be beneficial in treating BV biofilms. Materials selected in designing these nanocarriers should contribute to the retention of the carrier's structural properties within the vaginal tract but promote the degradation/release of its contents within the biofilm. Such materials may be of lower benefit in the treatment of VVC, since symptomatic VVC is associated with normal vaginal pH (pH <4.5). However, there may still be use in instances where VVC co-occurs with BV. Another approach that may minimize drug loss within the vaginal tract is to take advantage of enzymes released from the invading pathogens. Enzymes (e.g., lipases and hyaluronidase) have been proposed in biofilm treatment as triggers to activate responses from nanocarriers (e.g., degradation and drug release) due to enzymolysis. However existing challenge of this approach lies with how to demonstrate degradation of the nanocarriers within the biofilm matrix and not by host enzymes or other enzymes outside the biofilm. Additionally, vaginal secretions have demonstrated

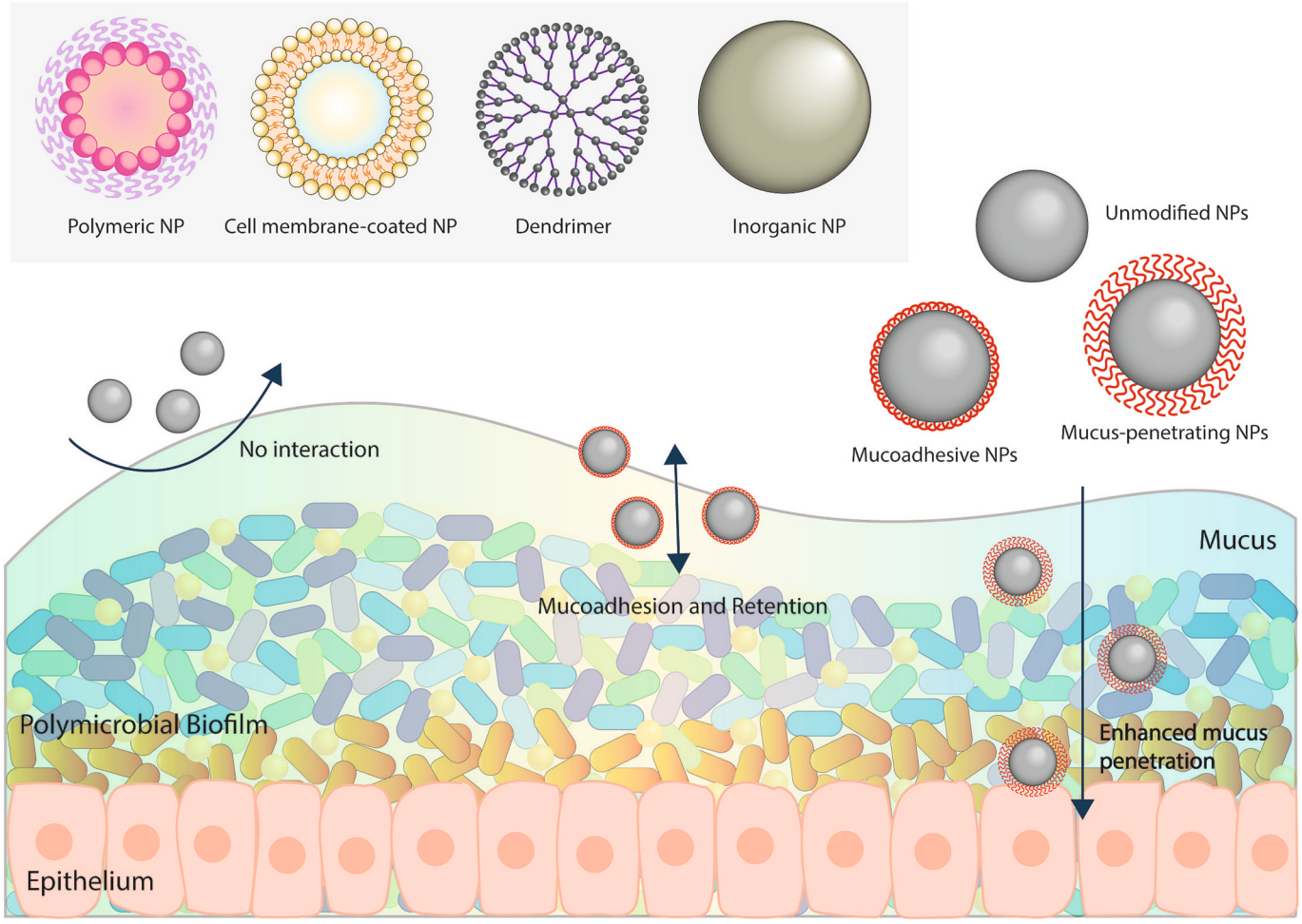

Fig. 5. Summarized illustration on engineered nanoparticles currently reported for targeting vaginal biofilms. The main materials reported include polymeric nanoparticles, dendrimers, inorganic nanoparticles and cell membrane coated nanoparticles. The mucoadhesive or mucopenetrating properties may promote retention in the mucus or enhance their mucus penetrating properties. A detailed overview of the reports are further summarized in Section 4.1.

enhanced enzymatic activity during infections [159] which might prove challenging for the success of this strategy. Still, careful consideration of the vaginal microenvironment can guide the rational choice of enzymes that enhance the selectivity and targeted release of antimicrobials in vaginal biofilms. Biofilm infected sites have been reported to have higher levels of reactive oxygen species (ROS) than their surrounding tissues. By exploiting hydrogen peroxide as a trigger, ROS-responsive nanocarriers have been proven to control the release of drugs against biofilms [160]. Despite the promising results, the benefit of this approach is yet to be explored in the treatment of vaginitis. Remote control of the physical properties of nanocarriers via the application of an external trigger (e.g., ultrasound) is attractive in the control of biofilms [161]. Such approaches may benefit from being combined with mucus and biofilm penetration to achieve maximum therapeutic effect.

Current advances for biofilm control exploit the use of organic, inorganic and hybrid materials in nanoparticle synthesis. Organic nanoparticles such as poly (lactic-*co*-glycolic acid) (PLGA) and chitosan nanoparticles offer high biocompatibility and low toxicity [162–164]. On the other hand, inorganic nanoparticles are considered for their ease of manufacturing, physical (e.g., magnetic and optical properties) and added chemical properties (e.g., stability, ease of functionality and stability) [165]. Hybrid materials combine the interesting properties of inorganic and organic nanoparticles to achieve novel properties such as catalytic activities. In the context of vaginal biofilm infections, we will discuss current efforts attempted by the scientific community with inorganic or organic nanoparticles [161,166]. (See Table 1)

Particulate drug delivery systems based on PLGA are commonly utilized as platforms for their sustained delivery of drugs and suitability for a wide range of applications. The popularity of PLGA stems from its biodegradability/ biocompatibility, ease of surface modification, capacity to protect drugs from degradation and ability to release drugs in a controlled and stable manner [167]. As a US FDA and EMA approved polymer, it has been used for the development of a variety of DDS in humans. *In vivo* hydrolysis of PLGA is associated with the release of non-toxic lactic acid and glycolic acid, two monomers that can be metabolized by the Krebs cycle [168]. In biofilm therapy, the use of plain PLGA and polyethylene glycol (PEG) modified PLGA nanoparticles have been explored for treatment of *C. albicans* biofilms by delivering fluconazole or amphotericin B [161,169]. The use of PEG coating is effective in shielding nanoparticles from potential adhesive interactions with mucosal components in the body [170]. *In vitro* experiments have demonstrated that PEGylation enables rapid diffusion of PLGA nanoparticles between mucin fibers [171]. The application of such mucus-penetrating strategies has been proposed to achieve closer proximity to epithelial cells [154]. The diffusion of plain PLGA and PEG-PLGA nanoparticles were studied in cervicovaginal mucus sampled from women with lactobacilli-dominated vaginal flora. The study demonstrated that while PLGA-PEG exhibited a Brownian-like time-lapse trajectories over 20s, the PLGA nanoparticles were strongly hindered and demonstrated highly constrained non-Brownian traces [172]. This observation was thus associated with the PEG coating since the two nanoparticles were of similar sizes and were considerably smaller than the average pore size of the CVM. Mucoadhesive formulations between 50 and 300 nm were shown to present better characteristics to transport through the mucus and to bind mucosal tissues. Despite this report on the hindered transport of plain PLGA nanoparticles, Ustun and colleagues explored their use by fabricating fluoconazole loaded PLGA nanoparticles using rhamnolipids as a co-surfactant [169]. The nanoparticles had sizes of 265.4 ± 7.3 nm an encapsulation efficiency of 85 ± 2 % which led to efficient inhibition of *C. albicans* biofilm formation. However, the study did not study the efficacy against mature biofilms and did not report on the biofilm penetrating properties of the nanoparticles. Furthermore, considering the poor mucus-penetrating features previously demonstrated for PLGA nanoparticles, this system may fail to offer translational benefits against vaginal biofilms. Yang and co-authors addressed the double barrier (i.e., mucus and biofilm) via a mucus-permeable sonodynamic therapy based on low-intensity US-mediated PEGylated PLGA nanoparticles for the delivery of amphotericin B (AmB) [161]. The nanoparticles had a mean diameter of 252.25 ± 4.59 nm and an anionic surface charge which is essential to minimize trapping in mucus. The PEGlyated PLGA nanoparticles effectively reduced *C. albicans* colonization (3.5-log reduction) in the vagina of rabbits after US application. Additionally, *in vivo* assessment of the permeability on mucus and vaginal tissues were conducted. Prior to US activation, the nanoparticles aggregated in the cavity of the mucus layers. After US irradiation, a continuous particle layer coated the entire vaginal mucosal surface. This was supported by quantitative fluorescence assessment showing that US increased mucus penetration. Finally, the authors further probed the cytotoxicity of the formulations against *Lactobacillus* and revealed lower toxicity compared to the free drug. This observation is advantageous and could promote the maintenance of vaginal homeostasis.

Chitosan is a naturally occurring cationic polysaccharide, approved by the FDA and extensively used in nanoparticle synthesis. It is composed of repeating units of *N*-acetylglucosamine and D-glucosamine, linked by β-(1–4) glycosidic bonds and is produced by deacetylation of chitin [173]. It is a basic constituent of the cell wall of fungi and the exoskeleton of insects and crustaceans [174]. Some common methods for preparing chitosan nanoparticles include ionic gelation, spray drying, emulsification and crosslinking [175]. Due to attractive functionalities such as pH sensitivity, biocompatibility, mucoadhesiveness, and bioactivity it is gaining popularity as building block of many drug delivery systems [176]. The molecular weight and degree of deacetylation has been shown to significantly influence the solubility and mucoadhesive properties of chitosan. For instance, high acetylation is associated with high solubility and high mucoadhesive properties [177,178]. Chitosan is easily degraded under physiological conditions by lysozyme (found in mucosal surfaces) and chitinase (found in the intestinal flora) [179]. The degradation products of chitosan are oligosaccharides which can be incorporated into the glycosaminoglycan and glycoprotein metabolic pathways or excreted in urine [179]. In biofilm therapy, chitosan is considered for its strong mucoadhesive properties. Although chitosan has been reported to disrupt intercellular junctions and increase the epithelium permeability, native and unmodified chitosan is not considered to be a mucopenetrating polymer [180,181]. A few reports from literature highlight the use of chitosan in vaginal biofilm therapy caused by *C. albicans*, *E. coli* and *P. aeruginosa* pathogens. Nayak and coauthors reported on three separate studies using chitosan nanoparticles for biofilm therapy. In the first, chitosan nanoparticles were prepared via ionic gelation using polyanionic phytic acid as a crosslinker [182]. Phytic acid has been shown to possess more phosphate groups than the tripolyphosphate (TPP), the most widely used polyanion in chitosan nanoparticle preparation. In biofilm therapy, phytic acid has been reported to possess chelating properties. The authors sought to explore this feature to chelate calcium ions within the biofilm matrix and alter the integrity of the biofilm. Experimental assessment revealed that following ionic crosslinking, phytic acid had a lower calcium chelating property (i.e., from 4.9 mol to 3.4 mol) due to its contribution in ionic gelation process. The anti-biofilm activity was monitored against *C. albicans* biofilms grown for 48 h and the biomass assessed via crystal violet staining. Significant reduction in fungal biofilm growth was observed at minimum inhibitory concentration (MIC) (1× MIC, 62.5 ± 1.2 %) concentrations of the phytic acid crosslinked chitosan nanoparticles (CPNP). Further assessment of the colony forming units (CFU) revealed approximately 3.7-log reduction in CFU following treatment. The authors also quantified the biofilm matrix revealing 35 %–55 % reduction after treatment with CPNP at sub-MIC (0.5×) and MIC (1×) concentrations. Finally, in an *in vivo* challenge experiment, the CPNP demonstrated 4-log reduction in CFU continuously until the 21st day after treatment. The continuous inhibition of biofilm growth was associated with the long residence of the chitosan nanoparticles in the vaginal tract and its strong mucoadhesive

Table 1
Overview of reported nanoformulations for treatment of vaginal biofilm (mono-species or polymicrobial) infections.(NA refers to not applicable).

No.	Composition	Delivered drug	Biofilm model (*in vitro*) and improvements	Biofilm model (*in vivo*) and improvements	Mucoadhesion or Mucopenetrating tests	Reference
1	Poly (lactic-co-glycolic acid), polyvinyl alcohol and rhamnolipid (Rh)	Fluconazole	*C. albicans* • 63 % biofilm inhibition • Not challenged in mature biofilms	NA	NA	[169]
2	Poly(lactic-*co*-glycolic) acid polyethylene glycol polymer (Ultrasound activated)	Amphotericin B	NA	*C. albicans* in rabbit • >3.5 log reduction	Mucopenetration (*in vivo*) • Higher fluorescence in vaginal mucosa.	[161]
3	Chitosan and phytic acid	Metronidazole	*E. coli* • 62 % biofilm inhibition • Not challenged in mature biofilms	*E. coli* in mice • 3-log reduction in CFU/mL	Mucoadhesive test (*in vitro*) • 72.4 ± 1.2 % mucoadhesiveness	[184]
4	Chitosan and phytic acid	NA	*C. albicans* • 62.5 ± 1.2 % reduction in mature biofilms • 35–55 % reduction in biofilm matrix	*C. albicans* in mice • 4-log reduction in CFU/mL	NA	[182]
5	Chitosan, borax and tannic acid	Metronidazole	*E. coli* • 72 % reduction in mature biofilms	*E. coli, C. albicans* in mice • 3-log reduction in *E. coli* CFU/mL • 1-log reduction in *C. albicans* CFU/mL	Mucoadhesive test (*in vitro*) • 78 ± 1.3 % mucoadhesiveness	[185]
6	Silver, L-carnitine	NA	*E. coli* and *S. aureus* • 90 % biofilm inhibition with 1000 ppm silver • Not challenged in mature biofilms	*E. coli, S. aureus* in mice • Macroscopic results showed infection was treated.	NA	[195]
7	Silver, L-carnitine	NA	*S. aureus* and *C. albicans* • 97.82 % biofilm inhibition with 1000 ppm silver	*S. aureus* and *C. albicans* • No colonies seen after 12 days.	NA	[196]
8	Copper sulfide, jacalin	Micafungin (MF) and amphotericin B (AMP B)	*C. albicans* and *C. glabrata* • 80 % biofilm inhibition with NPs alone • 80 % (AMP), 70 % (MF) biofilm eradication with drugs.	NA	NA	[166]
9	Vaginal epithelium coated nanoemulsion, IR780 iodide, perfluorocarbon, oxygen	NA	*C. albicans* • 80 % biofilm mass reduction when NP was co-delivered with candidalysin	*C. albicans* in mice • Significant reduction in *C. albicans* in the vaginal fluid after treatment for 24 h.	*In vivo* vaginal tissue penetration • NPs had deep penetration in epithelial lining	[190]
10	Mesoporous polydopamine, polyethylene glycol, a-cyclodextrin, pentaethylenehexamine	clotrimazole and nitric oxide (NO)	*C. albicans* • >70 % reduction in residual biofilm when NPs were co-treated with NO and clotrimazole	*C. albicans* mouse model • 98 % reduction in survival ratio when NPs were co-treated with NO and clotrimazole	NA	[187]
11	Astodrimer	NA	NA	BV in patients • Phase 2: 74.1 % for 1 % astodendrimer dose at 9–12 days • Phase 3: 50.4 % cure rates for 1 % astodendrimer at 9–12 days	NA	[198,199]

properties. It is worth noting that although chitosan is largely known for its mucoadhesive properties, a recent study reported on mucopenetrating properties of ionically crosslinked chitosan nanoparticles prepared with sodium carboxymethylcellulose and phytic acid [183]. However, the mucopenetrating properties of the phytic acid crosslinked properties were not directly studied in the work by Nayak and coauthors. In a subsequent study using the CPNP loaded with metronidazole (500 μM), the authors assessed the *in vivo* antimicrobial efficacy in mice infected with *E. coli* and reported approximately 3-log reduction in bacterial CFU on the 7th day following treatment [184]. Towards BV treatment, sodium tetraborate (BX) and tannic acid (TA) were explored as inorganic and organic antimicrobial crosslinkers in the formation of the nanoparticles [185]. Compared to TPP and TA crosslinked nanoparticles, BX crosslinking led to significantly higher increment in nanoparticle size. Although the authors did not assess biofilm eradication after the nanoparticles were loaded with metronidazole, the biofilm inhibitory assessments revealed 72 % reduction in biofilm growth compared to the free drug alone (19 % reduction). The authors reported a modest fluctuation (from 82 ± 1.7 % to 78 ± 1.3 %) in mucoadhesive strength for the BX crosslinked nanoparticles due to the reduction in surface charge after drug loading. A 2-log reduction in bacterial CFU was reported in *E. coli* infected mice while a 1-log reduction was observed in *C. albicans* infected mice after treatment with 5 mg of the BX crosslinked nanoparticles. To assess the retention of the nanoparticles *in vivo*, X-ray investigations were performed. Strong nanoparticle adherence was observed between 0 and 1 h around the application site and the intensity steadily reduced between 2 and 24 h due to self-cleaning in the vagina.

The use of mechanically interlocked molecules such as polyrotaxanes possess interesting topological features and have attracted attention in drug delivery. Common components include long chain polymers such as polyethylene glycol which acts as the axle component and ring or bead molecules which include cyclodextrins [186]. Due to the molecular dynamic properties of polyrotaxanes, Li and colleagues explored their utilization as carriers of nitric oxide and antifungal drugs for efficient drug synergy against biofilms [187]. In their work, a polyrotaxane decorated mesoporous polydopamine nanoparticle was synthesized for the codelivery of clotrimazole and nitric oxide. The authors demonstrated that the polyrotaxane decoration effectively enhanced the adhesion to *C. albicans* biofilms and had higher penetration/distribution. A 4.19 times higher fluorescence intensity of the polyrotaxane decorated nanoparticles was observed compared to the plain mesoporous polydopamine nanoparticles. Treatment of mature biofilms revealed approximately 70 % reduction in residual biofilm for the polyrotaxane coated nanoparticles loaded with nitric oxide and clotrimazole. Approximately 20 % and 30 % reduction in residual biofilm was observed when the polyrotaxane coated nanoparticles were loaded with clotrimazole alone or with nitric oxide alone thus highlighting the synergistic activity of co-delivery the two agents together. Finally, the co-delivery system was tested in the *in vivo C. albicans* mouse model. The authors reported a dramatic reduction in the amount of *C. albicans* on the vaginal tissue (no survival of *C. albicans* seen after plating). Finally, the number of *Lactobacillus* in formulation-treated group was almost equal to that of the mice in the healthy group. This clearly demonstrates the re-establishment of vaginal homeostasis after successful eradication of the biofilms. Unfortunately, the mucopenetration properties of these nanoparticles were not explored in this study and thus there is a need to address this feature in further work.

Combined photothermal and photodynamic therapy can be leveraged in biofilm care but there are challenges with off-target heat production and toxic ROS exposure to healthy cells. To address this challenge, cell-membrane coated nanoparticles have drawn attention for their cell-like functionalities (e.g., long blood circulation time and specific molecular recognition) [188]. Due to their ability to mimic the surface properties of the source cells, they have been reported to promote specific adhesion to bacteria (e.g., macrophage-cloaked nanoparticles) for the targeted treatment of infections [189]. Other attractive features include their superior biocompatibility and reduced clearance by macrophage cells. Exploiting the natural binding ability of *C. albicans* to the vaginal epithelium and the cytolytic properties of candidalysin, Lin and colleagues reported on the development of vaginal epithelium cell membrane-based nanoparticles for phototherapeutic therapy against intravaginal infections caused by *C. albicans* [190]. The epithelial cell membrane was loaded with perfluorocarbon (PFA) and IR780 (photosensitizer) to enable the precise focus of phototherapeutic power on *C. albicans*. The cell membrane also served as a decoy for the neutralization of candidalysin to attenuate its toxicity on resident cells and to promote responsive release of the loaded phototherapeutic agents in response to candidalysin. The nanoparticles were applied to *C. albicans* biofilms and then irradiated under an 808 nm laser for 300 s. Approximately 30 % reduction in the biomass of mature biofilms was observed with XTT staining. When the nanoparticles were co-delivered with candidalysin, a significantly higher reduction in biofilm biomass (80 % reduction) was reported. In an *in vivo* model of *C. albicans* established for 3-days, the authors also explored the effect of the nanoparticles with or without candidalysin. Candidalysin associated enhanced antifungal activity was observed due to the on-demand generation of ROS under NIR irradiation. Furthermore, significant reduction in *C. albicans* was observed in the vaginal fluid after treatment for 24 h.

Inorganic metal nanoparticle include nanoparticles comprising of metals and metal oxides. They have drawn attention for their high stability, hydrophilicity and biocompatibility with living systems. Metal oxide nanoparticles are composed of metal atoms bonded to an oxygen moiety and comprise of iron oxide, silver oxide, zinc oxide and copper oxide which have been shown to demonstrated broad spectrum antibacterial properties [191–193]. They also possess attractive properties such as high surface reactivity, photocatalytic activity and magnetic properties. On the other hand, metal nanoparticles which are composed of pure metals includes silver, gold and iron have been applied to fight pathogens and as drug or gene delivery platforms. Due to the surface plasmon resonance of metal nanoparticles, unique optical and electrical properties have been reported [194]. In vaginal biofilm therapy, the use of copper sulfide and silver nanoparticles have been reported [166,195,196]. Silver nanoparticles have shown excellent synergistic effects with conventional antibiotics and capacity to control biofilm formation. Dehpahni and coworkers reported the use of silver nanoparticles and L-carnitine against vaginitis in two studies [195,196]. Both *in vitro* and *in vivo* assays assessed biofilm inhibitory effects of the formulations without evaluating efficacy against mature biofilms. Despite this, the authors showed strong inhibitory effects against biofilms of *S. aureus* and *E. coli*. For instance, 97.82 % inhibition of mixed *S. aureus* and *C. albicans* species biofilms was observed at 1000 ppm of silver nanoparticles. Copper sulfide nanoparticles were complexed with jacalin (JCuSNPs) and assessed for their biofilm eradication potential against mono-species and mixed-species biofilms caused by *C. albicans* and/or *C. glabrata* [166]. The use of the nanoparticles alone or in combination with standard antifungal agents like micafungin and amphotericin B was also assessed. The authors reported gradual reduction in biofilm formation in comparison with the control. Against *C. albicans* and *C. glabrata* mixed biofilms, the use of JCuSNPs alone led to 60 % biofilm eradication while in combination with Amphotericin B, approximately 80 % biofilm eradication was observed. It is worth noting that the treatment with AmB alone led to only 40 % biofilm eradication, thus highlighting the synergistic potential of this combination. Despite the obvious potential of metal nanoparticles, their mucoadhesive or mucopenetrating properties have not been assessed. Furthermore *in vivo* assessments on the efficacy of metal nanoparticles against mature biofilms are currently lacking.

Astodrimer sodium, formulated as a Carbopol-based mucoadhesive gel, was recently assessed for its efficacy against BV in clinical trials. The dendrimer (a highly branched 3-D architecture) comprises a benzhydrylamine amide of L-lysine core with four successive branching units of L-lysine [197]. The nanoparticle inhibits BV associated bacteria by

blocking its attachment to cells and can also disrupt mature biofilms. It is hypothesized that the nanoparticle disrupts electrostatic bonds holding the biofilm together and thus eliminates the biofilm to restore normal vaginal flora [197]. The penetration of these nanoparticles into BV associated biofilms is lacking. Nevertheless, in a phase 2 clinical trial with 132 women, its efficacy vaginally was explored for 7 days [198]. At earlier time points, i.e. on 9–12 days post treatment, clinical cure rates were 62.5 %, 74.1 % and 55.2 % for the 0.5 %, 1 % and 3 % astodrimer doses. Interestingly, for 0.5 %, 1 % and 3 % astodrimer doses, the clinical cure rates were only 28 %, 46.2 % and 23.3 % respectively on day 21–30. Although the higher dose (3 %) was initially selected for providing the broadest activity, it had lower cure rates than the 1 % dose. Furthermore, the poorer clinical cure rate at the later time points requires further investigation to understand how the formulation influences the recovery of vaginal homeostasis and lactobacillus. In a phase 3 clinical trial with a vaginal dose of 1 % astodrimer, the formulation demonstrated higher clinical cure rates of 50.4 % in comparison with the control group (16.5 %) at day 9–12 for the control [199]. A Phase 2 trial was conducted using colloidal nanosilver gel against BV, VVC and Trichomoniasis. The results were subsequently reported in an article by the authors [200] showing that a high proportion of patients achieved clinical cure compared to placebo. A cure rate of approximately 100 % and 85.19 % cure rates were reported for bacterial vaginosis and vulvovaginal candidiasis respectively.

5. Conclusions and perspectives

Gaining deeper insight on the challenges related to treatment of vaginal infections that comprise biofilms is the fastest and safest road to improved therapy of such infections. It requires interdisciplinary expertise as well as raising awareness on the extent of how alarming the problem is. Once understood, combating biofilms can be achieved through a smart arsenal of novel formulations and antimicrobials that mimic nature to a greater extent. Early focus on translational potential of such formulation should ensure easier translation into clinic. When optimizing formulations for vaginal administration the patient-friendly approach needs to be implemented at all stages of development, that is often not the case. Patient compliance is directly linked to easiness of administration. Moreover, it should address the cultural differences, climate challenges and overall accessibility. Misdiagnosis is often a major challenge to therapeutic success in BV management. Clinically, the Amsel criteria (determined by vaginal pH, vaginal discharge and whiff amine test results) and Nugent score (gold standard) are used in BV diagnosis [201]. Validity challenges associated with the Amsel criteria exist and include the impact of lubricants on vaginal pH. Additionally, both methods fail to underestimate the complexity of microbial alterations and thus may give inaccurate results [202]. While commercially available molecular diagnostic assays can detect multiple bacterial species and are beneficial in elucidating the invading pathogen, their high costs currently preclude use as point-of-care tests [203,204]. Still, whether the acquisition of a keystone pathogen or a polymicrobial consortium of bacteria is responsible for the development of the BV is important to decipher [126,205]. Despite the controversial aetiology of BV, occurrence of multispecies biofilms with an abundant *G. vaginalis* community during infection is well acknowledged as a key factor in BV [206,207]. Additionally, the recurrence of BV has been linked to the persistence of *G. vaginalis* after treatment [208] From this perspective, treatment strategies that aim to diminish this pathogen can be beneficial. Current recommended therapeutic regimens utilize multidose oral and intravaginal antibiotics or single-dose treatment options which can also impact the vaginal microflora [208]. These broad-spectrum antibiotics are often associated with long treatment times and adverse effects (e.g., metronidazole) which can lead to patient nonadherence and the recurrence of BV. Furthermore, restoring microbial microflora after treatment is crucial in preventing the recurrence of BV. Therefore, targeted treatment can be beneficial in sustaining the cure and effectiveness of antimicrobial therapies and in limiting recurrence by sparing the microbiome. Still further studies are necessary to first understand the dynamics within bacterial biofilms during BV infections and the response of the individual species (including Lactobacillus) following treatment. New techniques such as the use of advanced calorimetric methods based on isothermal calorimetry may be used to map different organisms within BV biofilms and in complex models. This label-free technique relies on the unique heat signature produced by different organisms and can monitor these changes in real time in the presence and absence of treatments. This technique is rather fast and simple compared to the use of RNA-based 16S qPCR methods and fluorescent in situ hybridization (FISH) techniques.

The cost of novel nanotechnology-based formulations remains a topic for societal discussions; however, innovative approaches are expected to deal with this challenge as well. Fig. 5 provides a summarized overview of current reports applying nanoformulations in the treatment of vaginal infections. It is worth noting that many of the *in vitro* experiments fail when the nanoparticles are challenged against mature biofilms and there is limited knowledge regarding their long-term safety profiles. Hence, despite some demonstrated *in vivo* benefit of the reported formulations, it is unsurprising that the current status of nanoparticles in clinical testing remains low. Considering the formulation, it is also important to critically evaluate the features bearing in mind that no ideal nanoformulation to combat all vaginal infections exists or could be developed. The tailoring of nanoformulations should address the specific target, even when targets are as complex as biofilms. We see the optimization process as an interplay (Fig. 6) that is rooted in smart design and approaches to efficiently target the infection complexity.

However, a remaining issue that needs solving is the modelling of biofilms, from in silico to *in vitro/in vivo* models. There are almost no models available for vaginal biofilms as per now. Very recently [209] scientists proposed a model dealing with vaginal permeability, but the model does not consider vaginal microbiota. On the other hand, several reports on vaginal microbiome are available, cited through the review, that can serve as a solid base for further model developments. Forging close collaboration between microbiologists, nanotechnologist and product developers can be seen as a move in the right direction. Considering the interplay between the vaginal microbiome, persistent biofilm infections and the vaginal barriers, development pipeline for new nanoparticles should clearly define the muco-penetrating/mucoadhesive properties of the formulation, their biofilm penetrating potential, the effect on mature biofilm infections (*in vitro* and *in vivo*) and finally *Lactobacillus* renewal after treatment. Despite the positive therapeutic implication of biofilm penetration and *Lactobacillus* renewal for nanoformulations developed against vaginal biofilm infections, only one of the reviewed studies [187] examined these aspects. In model development, considerations of the complexity of BV and VVC infections should be considered. Nevertheless, relying on animal models of vaginal infection become increasingly limited, especially in Western world with strict regulations on use of animals in research and 3Rs principles. The time to tackle alarming rise in STIs and AMR is now, and it requires multidisciplinary approaches accompanied by rising awareness. We have earlier proposed [2] pathways to tackle the AMR by i) expanding the choice of antimicrobial that would include new substances, phytochemicals, biomimetics, siRNA-based, as well as “recycling” of antimicrobials that are currently administered via other drug administration routes; ii) utilizing pharmaceutical excipients with intrinsic antimicrobial properties that can act in synergy to antimicrobial such as chitosan; iii) enhancing the user-friendliness of formulation, that would improve patient compliance and iv) improving the safety profiles of novel vaginal formulations with focus on pregnant patients.

Considering translation of novel formulations to clinic, researchers are often facing the barriers that are much more complex than originally anticipated and often bearing underlying issues. STIs affect women of all economic status but the access to treatment options remains rather limited to economically sound countries. Solving problems at one site

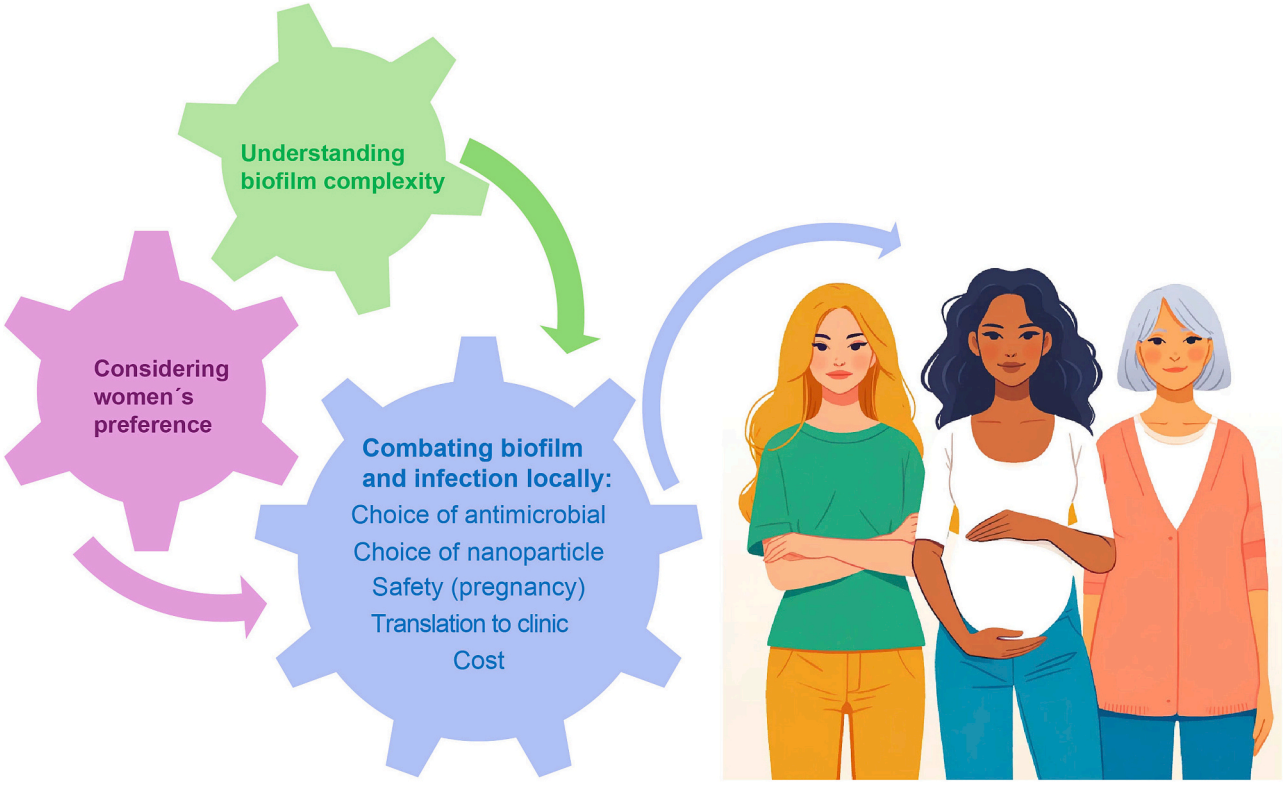

Fig. 6. Optimizing nanomedicine for biofilm targeting. Key considerations in designing nanoformulations to combat vaginal health include an understanding of the complexity of biofilms while considering the preferences of women. Additionally, there is a need to carefully select the antimicrobial agent, the carrier/nanoparticle, consider safety (including pregnancy), cost and the clinical translation (Image of women generated with DALL-E).

does not eradicate the problems world-wide, therefore the cost and accessibility needs to be implemented at early stages of development. Last few years witnessed an increase in STIs that led to limited increase in awareness and, in some parts of the world, also increasing attention within funding organizations. It remains to be seen whether pharmaceutical industry is willing/interested to catch up with this trend and increase focus on user-friendly, efficient and affordable formulations for tackling of STIs.

CRediT authorship contribution statement

Sybil Obuobi: Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Nataša Škalko-Basnet:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization.

Declaration of competing interest

None.

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Data availability

No data was used for the research described in the article.

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