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Nanomedicines for the topical treatment of vulvovaginal infections: Addressing the challenges of antimicrobial resistance

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ABSTRACT

Recent years have, surprisingly, witnessed an increase in incidence of sexually transmitted infections (STIs). At the same time, antimicrobial therapy came under the threat of ever rising antimicrobial resistance (AMR), resulting in STIs with extremely limited therapy options. In this review, we addressed the challenges of treating vaginal infections in an era of AMR. We focused on published work regarding nanomedicine destined for localized treatment of vaginal infections. Localized therapy offers numerous advantages such as assuring high drug concentration at the infection site, limiting systemic drug exposure that can lead to faster development of AMR reduction in the systemic side effects and potentially safe therapy in pregnancy. We provided a state-of-the-art overview of nanoformulations proposed to topically treat STIs, emphasizing the challenges and advantages of each type of nanocarriers, as well as issues of potential toxicity.

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Abbreviations: AIDS, acquired immunodeficiency syndrome; AgNCs, silver nanoclusters; AgNPs, silver nanoparticles; Ag-PVP, silver polyvinyl pyrrolidone; AMR, antimicrobial resistance; AV, aerobic vaginitis; AZT, azithromycin; BV, bacterial vaginosis; CFU, colony forming units; CNPs, chitosan nanoparticles; CSTs, community state types; CTAB, cetyltrimethylammonium bromide; CV, cytolitic vaginosis; DPGLs, deformable propylene glycol liposomes; EC_{90}, 90%-effective concentration values; EPSs, extracellular polymeric substances; HIV, human immunodeficiency virus; HPV, human papilloma virus; HSV-2, herpes simplex virus-2; IC_{50}, concentration that inhibit biofilm formation by 50%; IFN-α, interferon alpha; IL-10, interleukin 10; IL-12, interleukins 12; MIC, minimum inhibitory concentration; MIC_{50}, minimum concentration that inhibit bacterial growth by 50%; MNBA, 5-mercapto-2-nitrobenzoic acid; MP-NPs, mucus-penetrating nanoparticles; MW, molecular weight; NLCs, nanostructured lipid carriers; NPs, nanoparticles; PAMAM, polyamidoamine; PCL, poly(ε-caprolactone; PDGFR-β, platelet derived growth factor receptor-β; PEG, polyethylene glycol; PEI, polyethyleneimine; PEO, poloxamer 338 NF; PLGA, poly(lactide-co-glycolide acid); PVP, polyvinylpyrrolidone; Q-GRFT, oxidation-resistant variant of griffithsin; RES, resveratrol; ROS, reactive oxygen species; SFS, semen fluid simulant; siRNA, small interfering RNA; SLNs, solid lipid nanoparticles; SNAP-23, synaptose-associated 23-kDa protein; SPL 7013, sulphonate-terminated anionic poly(lysine) dendrimer; SLS, sodium lauryl sulfate; STIs, sexually transmitted infections; TNF-α, tumor necrosis factor alpha; VFS, vaginal fluid simulant; VLP, virus-like particles; VVC, vulvovaginal candidiasis (candidosis); WHO, World Health Organization.

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1. Introduction

This century brought an increased focus on women's health with emphasis on developing new means for efficient delivery of drugs locally within vaginal cavity. Many of the formulations and devices aim to prevent a range of health conditions women experience as well as efficiently treat infections. Emerging multipurpose technologies have been proposed to offer broad-spectrum prevention for sexually transmitted diseases and contraception [1]. However, it was assumed that infectious diseases are mostly treatable before emerging concerns related to antimicrobial resistance (AMR) reported for many of most powerful antimicrobials [2]. In the current review, we address the specific challenges of treating vaginal infections in an era of ever-growing AMR.

Different antimicrobials in novel delivery systems including nanosystems, have been proposed as formulations for localized therapy of vaginal infections [3–9]. Before discussing the specific situation of treating vaginal infections in an era of AMR, we summarize the advantage of treating vaginal infections locally, rather than systemically.

Topical treatment of vaginal infections could offer several advantages:

i) achieving high concentration of antimicrobials within vaginal cavity would lead to improved drug therapy at site of infection;
ii) reducing systemic exposure to antimicrobials would reduce the resistance development;
iii) reduced systemic exposure could lead to reduced side effects thereof possible treatment of pregnant women (Fig. 1).

Vaginal infections can be treated by various formulations; conventional, as well as advanced, that are not within nanorange such as hydrogels, films, etc [6,10]. We focused on localized treatment of vulvovaginal infections by utilizing nanomedicine. We structured the review to address first the alarming sexually transmitted infections (STIs) with most serious AMRs challenge, followed by most spread infections. Where available, we commented on the alternatives to conventional antimicrobials such as natural origin substances that might offer lesser potential for AMR.

Prior to discussing the current state-of-the art in antimicrobial nanomedicine-based topical vaginal therapy, the current rather alarming situation regarding STIs is summarized.

2. Treating sexually transmitted infections (STIs) in era of AMR

2.1. Vaginal microbiome and infection

In women of reproductive age, vaginal microbiome is a dynamic ecosystem affected by the estrogen levels, presence of lactobacilli and low pH. An ideal formulation for intravaginal delivery should not impair the vaginal environment and balance within normal microbiota [11]. It is worth noting that vaginal microbiome is much better defined than the vaginal mycobiome [12] although *Candida albicans* was the most frequently isolated fungus, which caused lower genital tract infections in about a third of women aged 15–24.

Vaginal dysbiosis, term describing abnormal vaginal microbiome comprising bacterial vaginosis and desquamative inflammatory vaginitis, has confirmed links to adverse pregnancy outcomes, pelvic inflammatory disease, an increased risk of STIs, and other reproductive health problems, such as a poor outcome of *in vitro* fertilization [13]. The pathological vaginal discharges can also be linked to the presence of lactobacillus overgrowth, known as cytolytic vaginosis (CV). The CV is becoming increasingly prevalent; moreover, it is frequently misdiagnosed. Treatment of CV requires clinical approaches that differ from those applied to other types of bacterial vaginosis (BV) [14,15]. The vaginal microbiome comprises

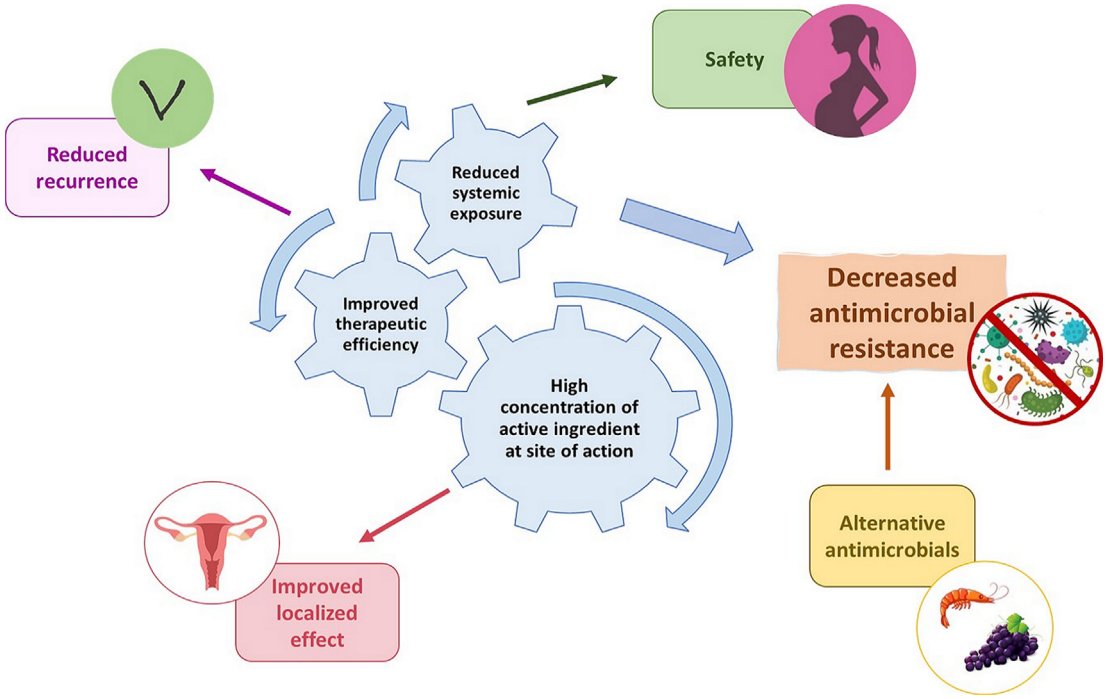


Fig. 1. Advantages of topical vaginal therapy in the era of antimicrobial resistance.

at least five community state types (CSTs). Four CSTs are dominated by a lactobacillus (lactic acid–producing) species: *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, or *L. jensenii.* Predominantly, *L. crispatus* was detected in healthy vagina, whereas *L. iners* dominated the flora prior to BV [16].

The hydrogen peroxide–producing lactobacilli are responsible for a low-pH environment, thereof inhibiting bacterial growth. Those lactobacilli contribute to 70 to 90% of the total microbiome in a healthy vagina [13,17].

Recently, polymicrobial biofilms, the structured groups of bacteria adherent to vaginal tissues, have emerge as a major contributor to chronic infections and alarming recurrence levels [11]. Biofilms represent a rather well-structured architecture comprising channels allowing nutrients circulation as well as divided areas that may contain genetically identical cells exhibiting different profiles of gene expression, as a consequence, the environment becomes strongly hostile against antimicrobial molecules as well as immune responses [18]. Virulence of bacterial biofilm can be contributed to the production of extracellular polymeric substances (EPSs); EPSs decrease the penetration of antimicrobial molecules through biofilms structure and reduce the action of human immune response cells. The extracellular matrix constitutes a physical barrier against all types of antibiotics [18].

2.2. Impact of STIs on women's health in the era of antimicrobial resistance

The effect of vaginal infections on woman's quality of life varies from personal experiences to health care costs related to the management of vaginal infections, as well as adverse reproductive health consequences [13]. Genital infections comprising STIs, endogenous infections such as vulvovaginal candidiasis (VVC), BV or aerobic vaginitis (AV) and healthcare-associated infections are raising serious concerns in era of AMR [19]. The infection-related complications in pregnant women may lead to premature rupture of membranes, miscarriages and premature birth [20]. It is important to consider that geographically, the resistance to STIs treatment can vary, for example, in Taiwan the resistance to tetracycline and erythromycin was found higher than in other regions of the world, while the resistance rate for levofloxacin was relatively lower [20]. Recent study conducted in Poland [19] indicated the highest resistance in *S. agalactiae*, namely the macrolide-linco samide-streptogramin B mechanism was identified in 38.6% of strains. It is important to highlight that the antibiotic consumption in Poland is much higher than the European average. Recent study conducted in Eastern Sicily revealed that *S. agalactiae* was the main pathogen responsible for early- and late-onset infections over the past 5 years [21].

The World Health Organization (WHO) periodically estimates health burden of four of the most common STIs: chlamydia (*Chlamydia trachomatis*), gonorrhea (*Neisseria gonorrhoeae*), trichomoniasis (*Trichomonas vaginalis*) and syphilis (*Treponema pallidum*) [22]. In 2016, estimated 376 million new infections of these four STIs were reported [23], corresponding to 1 million STIs per day, an alarming number [24]. In 2018 in the United States alone, more than 67 million prevalent and 26 million incident STIs were estimated, wherein chlamydia, trichomoniasis, genital herpes, and human papillomavirus (HPV) comprised 97% of all prevalent and 93% of all incident STIs [25].

Estimated 70% of all women will suffer from vaginal infections during their lifetimes, where VVC and BV are the most prevalent infections. Their treatments are hampered by high rates of resistance and recurrence, high probability of complications, and negative effects on the vaginal microbiota [12,13,26]. *Trihomonas vaginalis* is the most common nonviral STIs in the world [27]. The infection increases the risk of acquisition of other STIs, including human immunodeficiency virus (HIV) by almost three-fold but is considred curable.

BV is considered to be a polymicrobial disorder of the vaginal microbiome that is marked by the absence of vaginal lactobacilli [28]. Although it is rather challenging to estimate its real prevalence, estimates suggest that the rates are about 15% among pregnant women, 20 to 25% among young women, and up to 30 to 40% among women attending sexually transmitted disease clinics. BV

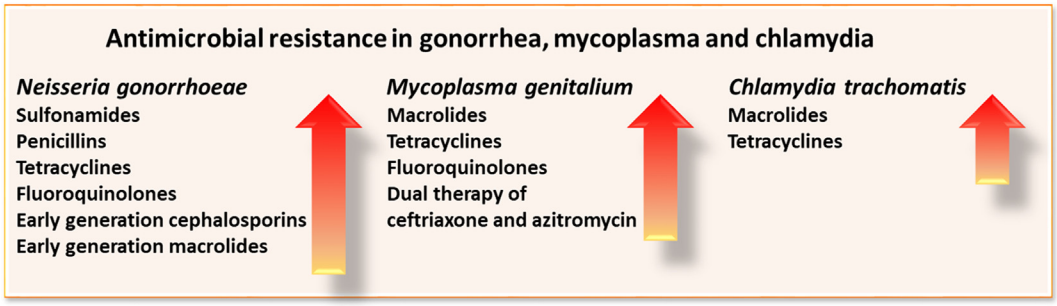


Fig. 2. Antimicrobial resistance identified in gonorrhea, mycoplasma and chlamydia (Based on references in respective chapters on nanoformulations). (*as per March 2021*).

represents an abnormal vaginal ecosystem, both qualitatively and quantitatively [13]. Moreover, BV is considered a biofilm infection, with a dense polymicrobial biofilm consisting primarily of *Gardnerella vaginalis* adhering to the vaginal epithelium. The strong polymicrobial biofilm reportedly present in 90% of women with BV and the high levels of drug-resistance found in *G. vaginalis* clinical isolates are considered major contributors to therapy failure [12]. Higher bacterial loads of *G. vaginalis* creates a favourable environment for *Atopobium vaginae,* further contributing to increased biofilm formation. Consequently, the biofilm creates a favorable anaerobic environment for other obligate anaerobic bacteria [29]. It is estimated that half of women with BV also have a BV–associated biofilm. However, better mechanistic insight on the direct link between biofilm and diseases is needed [30]. Women with BV exhibit a significant increase (1000x) in potentially virulent bacteria as compared to healthy woman [13]. BV is associated with both the acquisition and transmission of other STIs, especially HIV. Interestingly, the vaginal microbiome exhibited in women infected by BV, especially *G. vaginalis*, inactivates the topical microbicide tenofovir, widely used in the prevention of HIV transmission [31]. BV is also strongly linked to *C. trachomatis* infection.

Desquamative inflammatory vaginitis is a newly acknowledged clinical syndrome characterized by persistent purulent vaginal discharge and vaginal erythema, often with submucosal cervicovaginal petechiae [13]. Since the inflammation is the cardinal feature of this disorder, literature, although limited, often reports the term idiopathic inflammatory vaginitis [32]. The vagina becomes colonized with facultative bacteria, typically comprised of *Escherichia coli*, *Staphylococcus aureus*, group B streptococcus, or *Enterococcus faecalis.*

2.3. Threatening multidrug resistant STIs

To discuss the urgent need to treat increasing threat of STIs with limited treatment options, we attempted to categorize them based on the severity of the current resistance status. We are aware that the severity can also be categorized by the prevalence of a disease or available treatment options. We therefore propose the following severity ranking for three STIs with serious resistance against conventional treatment (Fig. 2).

2.3.1. Neisseria gonorrhoeae

In the current era of AMR, gonorrhea emerged as a priority pathogen [24]. It is caused by the bacterium *N. gonorrhoeae*; and is currently the second most common bacterial STI with an estimated yearly burden of 87 million cases worldwide [23]. Concomitant infections of *N. gonorrhoeae* with *C. trachomatis*, *T. vaginalis* and *M. genitalium* will occur in almost 30% of cases. Unsuccessful treatment may lead to pelvic inflammatory disease, ectopic pregnancy and infertility [24]. The choice of antimicrobials that can be used for treatment of gonorrhea is very limited, since resistance has even been reported to extended spectrum cephalosporins, which are currently recommended antimicrobial therapy. The first gonococcal isolates exhibiting ceftriaxone resistance as well as high-level azithromycin resistance were confirmed in England and Australia in 2018 [33]. After failures of dual-antimicrobial therapy approaches currently used to treat the infection, new antimicrobials, vaccines, novel technologies as well as diagnostic tools, repurposing of antimicrobials (spectinomycin, gentamicin, fosfomycin, and ertapenem) have been suggested as approaches to tackle the disease challenges [34–35].

2.3.2. Mycoplasma genitalium

M. genitalium is a bacterial STI linked to urogenital inflammation and symptoms in both sexes. Since *M. genitalium* is not a reportable illness, accurately assessing its prevalence and incidence is challenging [27]. Untreated infection can lead to cervicitis, pelvic inflammatory disease, preterm birth, spontaneous abortion, and infertility in women [36,37]. Its therapy is hampered by rapidly rising levels of resistance to azithromycin and moxifloxacin [27]. In a recent study in Nordic countries (except Island) macrolide and fluoroquinolone resistance-associated mutations were detected in 17.7% to 56.6% and 4.1% to 10.2% of *M. genitalium* infections, respectively [38]. Azithromycin resistance-associated mutations were identified in 57.0% of *M. genitalium*-positive participants [39]. Khosropour and co-workers [37] found that nearly all *M. genitalium* strains attested in Seattle, USA, exhibited a macrolide resistance mutation indicating a high previous exposure to azithromycin. Consequently, *M. genitalium* may become untreatable STI. Moreover, the infections are disproportionally affecting sexual and racial/ethnic minorities and socioeconomically challenged populations [27]. Pitt and co-workers [40] investigated the prevalence of AMR-conferring mutations in *M. genitalium* among the sexually active British general population and reported 16.1% macrolide resistance-conferring mutations and 3.3% fluoroquinolone resistance-conferring mutations. High prevalence of coinfection of azithromycin-resistant *M. genitalium* with other STIs, especially *Chlamydia trachomatis* has been reported [36].

2.3.3. Chlamydia trachomatis

Although the evidence that AMR is the reason behind unsuccessful treatment of *C. trachomatis* by conventional therapy remains a topic of discussion, there are rather clear proofs of *in vitro* heterotypic resistance to conventional antibiotics at high levels of organism load. A continuous rise in AMR is expected to further limit the available therapy [36,41–43]. *C. trachomatis* infections are often asymptomatic, and the reported number of 130 million new infections a year, is believed to be considerably underestimated [22]. Untreated infections can cause serious complications such as pelvic inflammatory disease, ectopic pregnancy, tubal infertility, preterm birth and increased risk of HIV transmission. Novel formulations for known antibiotics, novel antimicrobial

substances and localized vaginal therapy are all possible approaches for successful therapy.

3. Assuring efficient localized drug therapy

3.1. Understanding the challenges nanomedicine faces at vaginal site

Vagina as a site for drug administration and therapeutic effect bears unique challenges that need to be both understood and considered when designing and developing formulation destined for this site [3–9]. For nanomedicine to overcome the obstacles associated with the delivery of nanomedicine-associated drugs or active ingredients to vagina, careful tailoring of nanoformulation is a prerequisite. In particular, an interplay between the vaginal environment and nanocarriers' features need to be tweeted [8,44]. Some of the challenges are widely discussed in the literature, whereas other remain rather neglected.

One of the first challenges to consider is the residence time of the nanomedicine at vaginal site in spite of vaginal clearance and discharge. The mucoadhesive delivery systems were originally suggested to assure prolonged residence time within vaginal cavity [4]. However, the challenge was rather more complex than anticipated. To address the potential of mucoadhesiveness, it is important to gain deeper insight on the role of mucosa and mucus. The surface of mucosal sites, including vaginal, comprises nonkeratinized epithelium forming a most stringent hurdle to molecular and particle permeability/transport. This barrier exhibits selective permeability to both small molecules (e.g., water and ions) as well as large, supramolecular structures such as microorganisms, spermatozoa or nanoparticles [45]. The mucosal barrier is rather variable and highly dependent on specific functions related to mucosa location. In addition, enzymatic activity as a barrier to drug needs to be considered at the mucosal tissue. Although vaginal site does not exhibts equally strong enzymatic activity (in comparison to gastrointestinal tract) it has to be considered. The mildly acidic pH can hamper efficient drug delivery in the vagina. More detailed discussion on mucosa as a barrier to drug delivery, including the interaction between mucin fibers and nanoformulations can be found in the recent review by das Neves et al. [45]. Moreover, mucins interactions render the formation of a natural gel with variable viscosity and limited homogeneity. Vaginal mucosa responds to sexual stimulations and may change in viscosity and "wetness". Moreover, the change may affect the drug release from formulation applied intravaginally [46]. The role of mucoadhesiveness was challenged by the proposed role of the mucus-penetrating vehicles [47]. Regardless of the mucoadhesion or mucus-penetration, to achieve efficient localized drug effect, released drug needs to evenly distribute throughout the vaginal environment, often referred as pharmacokinetic functioning, to render its therapeutic action or pharmacodynamic functioning. Released drug overcoming dilution and barriers can reach the epithelium, underlying stroma and beyond [44]. Katz et al. [44] proposed simplified multi-compartment model comprising an interplay between the features of the delivery system, ambient vaginal fluid, semen, epithelium, stroma and the bloodstream, if systemic absorption takes place. However, the model requires additional complexity to address a challenge of nanoparticle shape, symmetry and softness/hardness on the elastic deformation of the mucus gel. In a recent review by das Neves and colleagues [45] the authors addressed the limitations of currently available models that mostly focused on the mucus-penetration or mucoadhesiveness of drug nanocarriers regarding pharmacokinetic performance without sufficient comprehensive understanding of molecular, physicochemical and mechanical interactions.

Vaginal discharge is additional challenge; it is a rather complex mixture of epithelium transudates, cervical mucus, exfoliating

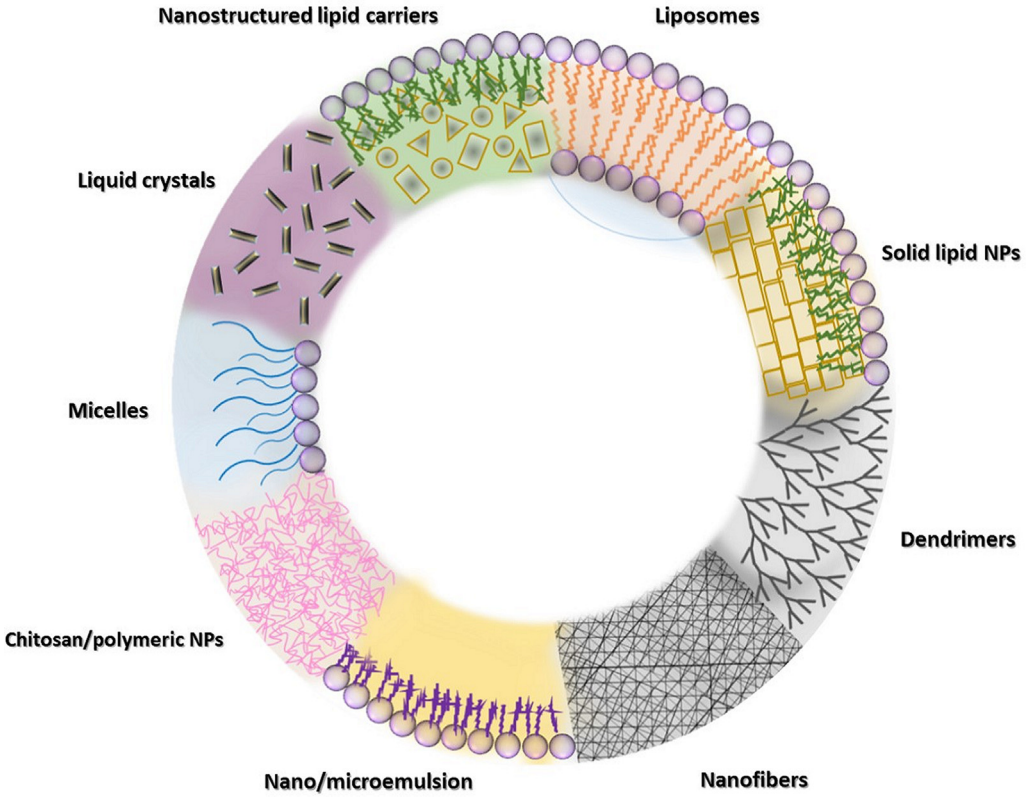


Fig. 3. Schematic summary of nanosystems proposed for topical therapy of vaginal infections.

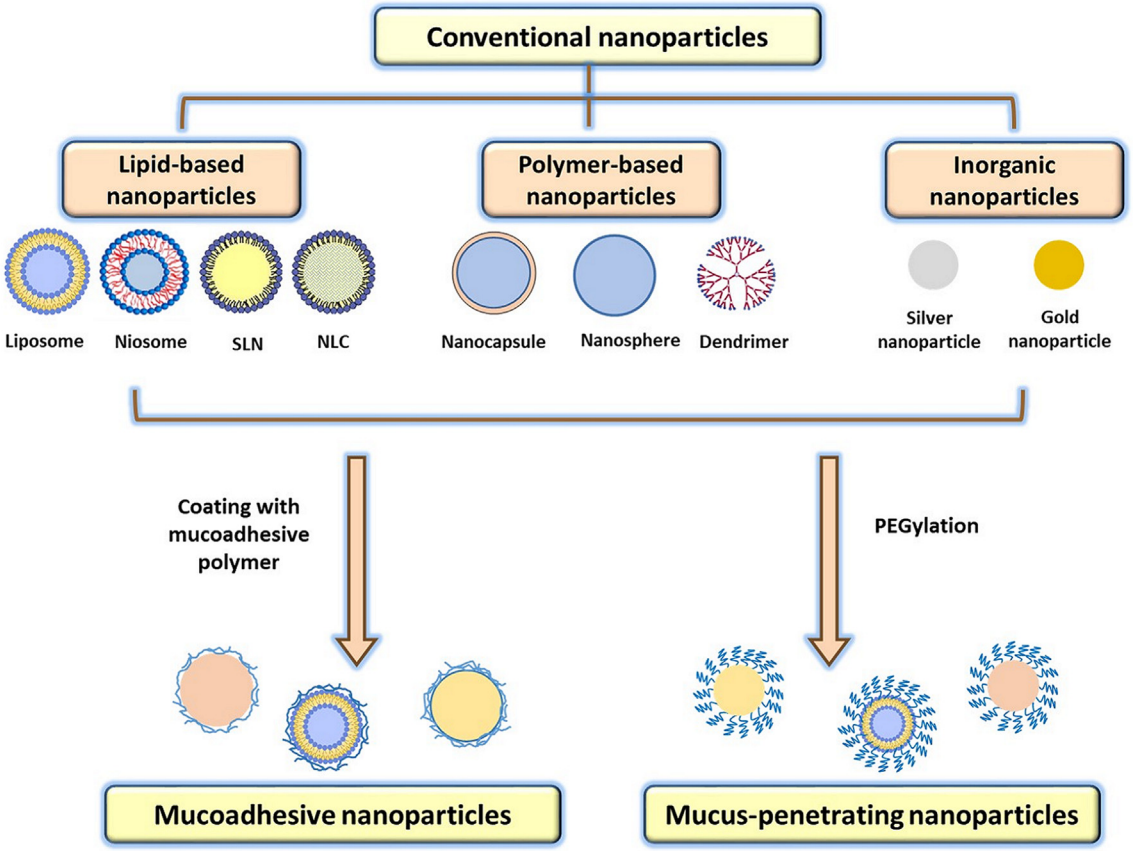


Fig. 4. Classification of nanoparticles, modified from [4] (Permission from Copyright Clearance Center, Elsevier).

epithelial cells, secretions of the Bartholin's and Skene's glands, leukocytes, endometrial and fallopian' tubes fluids. It comprises enzymes, enzyme inhibitors, proteins, carbohydrates, amino acids, glycoproteins, lactic acid, acetic acid, glycerol, urea, glycogen and various ions [48]. The volume and composition of vaginal fluid are affected by the stages of the menstrual cycle, hormones and sexual arousal [49].

The drugs will both diffuse faster through the vaginal fluids and disappear from the luminal fluids than from the underlying tissue. Diffusion and penetration of particles can be tailored by the particle size and surface properties. The size regulates the ability to fit into the mesh pores within mucin fibres while the surface properties control the interaction between the nanoparticles and mucin [5]. Utilizing polyethylene glycol (PEG) for PEGylation, the fate of NPs can be tuned to desired distribution [50].

The use of vaginal pH as an indicator of possible infections as well as pre-/post-menopausal status has been widely discussed [8,45,51]. Rather limited scientific data are available on the effect of overuse of hygienic products on the natural vaginal pH which serves as a first defense against microbial infections [8]. Candidiasis and BV elevate the vaginal pH above pH 5 due to reduction of *Lactobacilli* population [52]. Rather limited attention has been given to the effect of prolonged use of oral contraceptives or hormone replacement on the changes in vaginal pH and membrane thickness [53].

3.2. The role of nanomedicine in assuring localized drug therapy

This review focusses on utilization of nanomedicine for topical therapy of vaginal infections. Different type of nanoformulations have been proposed as vehicles for improved delivery of active ingredients to vaginal site. The types of nanomedicines (nanopharmaceuticals, nanoformulations, nanosystems, nanoparticles, nanocarriers) that are discussed in this review are schematically summarized in Fig. 3.

3.2.1. Overview of nanoformulations

The nanoformulations can be categorized in various manners, however, based on their ability to remain within vaginal cavity and permit the delivery of active molecules into the deeper epithelium, we propose, in Fig. 4, the classification based on both origin and surface characteristics relevant for system's mucoadhsiveness versus mucopentration.

In summarizing the reported data on nanomedicine for vaginal therapy of infections, we have mostly focused on classification according to the material used for nanoformulations. We also prioritized the type of nanocarriers which were more extensively studied.

3.2.2. Lipid-based nanoparticles

3.2.2.1. Liposomes. Liposomes are phospholipid-based nanovesicles consisting of one or more concentrically arranged bilayers enclosing inner aqueous compartment(s) (Fig. 4). Structural properties of liposomes allow entrapment/incorporation of drugs differing in molecular weights and lipophilicity. Their functionalization permits tailoring for different routes of drug administration [54–56], however, in comparison to their application via parenteral and (trans)dermal routes, their potential in vaginal therapy has been reported relatively late [57,58]. Liposomes can be classified as conventional and elastic vesicles including deformable liposomes, propylene glycol liposomes, and deformable propylene glycol liposomes (DPGLs) [4]. More than 50% of all the investigations on the

use of liposomes for vaginal administration refer to conventional liposomes. Elastic liposomes consist of phospholipids and the edge activators, i.e., single-chain surfactants (deformable liposomes), and/or polyols (propylene glycol) contributing to the bilayer flexibility [4].

The liquid nature of liposomes and leakage from the application site is considered their limitation as potential formulation for vaginal site, that can be overcome by:

i) coating/incorporating mucoadhesive polymers, such as chitosan, within liposomes [59,60],
ii) incorporation of liposomes within semisolid mucoadhesive vehicle, such as hydrogel [61–67], or
iii) embedding liposomes into solid vehicle, i.e. tableted preliposomes [68].

The issue of liquid nature and need for secondary vehicle to prolong their residence time at vaginal site is not unique to liposomal suspensions and applies to different nanocarriers suspensions discussed in the review.

3.2.2.2. Solid lipid nanoparticles. Solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are often found superior to liposomes for industrial scale manufacturing. SLN, made of solid lipids at room temperature, are spherical particles, accommodating a drug or active molecules between fatty acid chains or attached to the carrier matrix surface [69,70]. SLNs comprise surfactants which serve as stabilizing agents (Fig. 4). NLCs lipid core comprises a mixture of solid and liquid lipids, acting on improved stability [71,72]. Since vaginal mucosa is preferable environment for more hydrophilic vehicles, the evaluation of SLNs and NLCs in localized vaginal therapy remains limited. The studies are discussed in respective diseases overview.

3.2.2.3. Liquid crystals. Lipid crystals are unique structures comprising lipids that flow like liquids but maintain the structural characteristics of crystalline solids. Although they do not transfer directly from the liquid to solid state, under certain conditions lipid crystals exhibit molecularly organized intermediate phases (mesophases) with both liquid and solid properties, i.e. liquid crystalline phases. They have attracted considerable attention as drug delivery system enabling sustained release and protection of embedded sensitive drugs [73,74]. Moreover, their malleable structure allows easiness of topical application, including vaginal route of administration [75].

3.2.3. Polymeric nanoparticles

Polymeric NPs can be built of natural and synthetic polymers. Considering the biocompatibility and safety in regard to vaginal administration, the number of NPs build from synthetic polymers is rather limited [3]. The polymers poly-D,L-lactic acid (PLA), polyglycolic acid (PGA), poly(lactic-co-glycolic acid) (PGLA), poly-e-caprolactone (PCL), and poly(methylmethacrylate) (PMM) are considered safe for human use [76]. Due to extensive research efforts to develop safe and efficient microbicides against HIV, NPs proposed as microbicides dominate the literature (e.g. [77–79]). Moreover, a single NPs for combination of antiretroviral agents were also proposed [80,81]. Mucoadhesive NPs based on the natural polymer carrying tenofovir enabled a controlled release of this drug [82]. More polymeric NPs are discussed in chapters focused on the infection type the NPs were proposed for. We highlight here the dendrimers, one of the most successful polymeric nanocarrier destined for vaginal site, as well as nanofibers, emerging type of nanoformulations.

3.2.3.1. Dendrimers. Dendrimers are extensively branched polymeric macromolecules (nanoparticles) constructed through the sequential addition of branching units radiating out from an initiating point. Dendrimer comprise four major components: initiator (core), branching units, linkers and surface groups [83]. Highly branched 3D structure permits a high degree of surface functionality and ability to incorporate various drugs and active molecules (inside or on the periphery of dendrimer). The advantages of dendrimers consider their water solubility, defined molecular weight, nanometer size range (typically 5–20 nm) and narrow polydispersity, allowing easier transport across the biological barriers. The most studied dendrimers are based on poly(amidoamine), poly (propylene imine) and L-lysine, while novel approaches include hybrid dendrimers based on the assembly of dendrimers with NPs [84,85].

3.2.3.2. Nanofibers. Nanofibers are fiber shaped porous polymeric nanostructures, wherein two of their dimensions are in nanoscale. They are characterized by high surface area-to-volume ratio and ability to incorporate various pharmacologically active ingredients [86,87]. Mechanical properties, in addition to their large surface area, as well as solubility, stability, ability to be manufactured sterile, and potential for controlled drug release at different pH conditions, offer unexplored advantages as novel transmucosal nanoformulations [88].

There are other different types of nanocarriers which did not gain widespread attention such as inorganic NPs (silver, gold), micelles and similar (Fig. 3). Those are discussed in the chapters dealing with the respective infections.

However, considering the surface properties of NPs and their interaction with vaginal mucosa as a barrier to drug delivery, we separately address their interaction with vaginal mucus in section 3.3.

3.3. Mucoadhesive and mucus-penetrating NPs

Numerous work has been published on mucoadhesive formulations for vaginal delivery, especially mucoadhesive polymer-based formulation [10]. However, Lai et al. [89] challenged this concept questioning the ability of mucoadhesive nanocarriers to reach deeper epithelial layers, where the infection mainly resides. Utilizing viruses as biomodel, viral-like nanoparticles become widely studied NPs [4]. Experimental evidence proved that densely PEGylated large NPs (200–500 nm), diffused readily through undiluted human cervicovaginal mucus, unlike corresponding particles of smaller size (100 nm) due to the pore sizes within the mucus [89]. Moreover, it was proven that PEG-PLGA NPs (100 nm) with a neutral surface rapidly diffused through fresh, undiluted cervicovaginal mucus [90]. Ensign et al. [91,92] followed PEG-PLGA and polystyrene mucus-penetrating NPs through mouse cervicovaginal mucus in different medium and concluded that NPs in hypotonic solution penetrated deep into the vaginal folds within minutes and remain there for 24 h, whereas conventional NPs were captured in the thick mucus layer unable to reach the deeper tissue. These findings suggest that mucus-penetrating PLGA NPs, particularly PEG-PLGA, are safe and effective for the prevention and treatment of STIs [91,92]. Understanding the interplay between NPs and mucus (Fig. 5) while maintaining the native barrier of epithelium opens up a possibility to optimize the nanocarrier penetration into deeper layers [91–93].

4. Proposed nanomedicine to treat specific vaginal infection

We organized presenting the nanoformulations according to the urgent need to improve the therapy due to alarming AMR issues

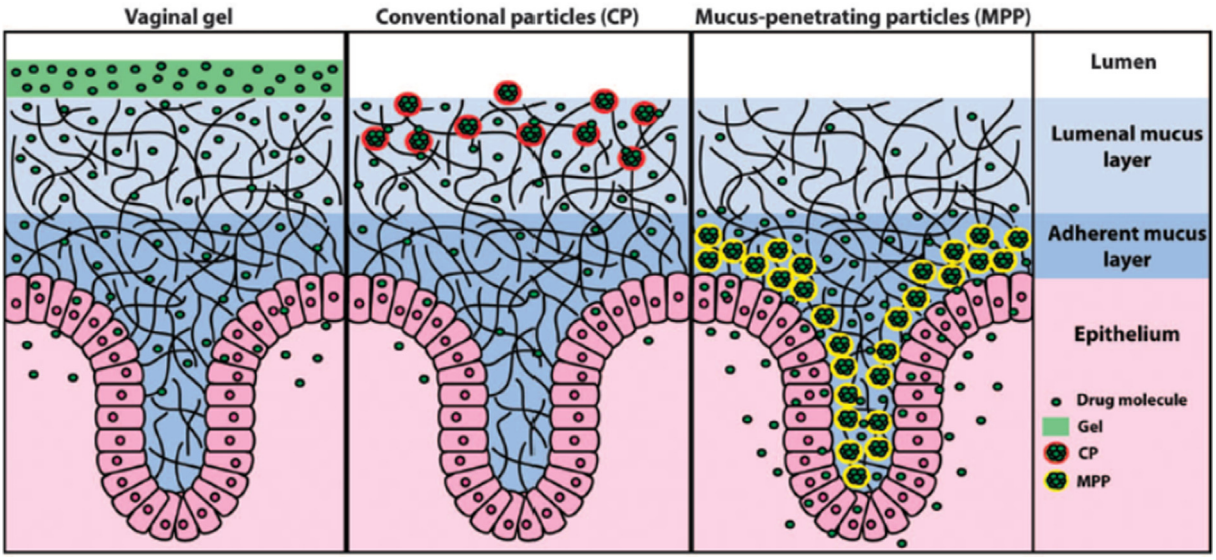


Fig. 5. Schematic drawing of interplay between formulation and vaginal mucus as presented for gel, conventional nanoparticles (CP) and mucus-penetrating nanoparticles (MPP). Adapted from Ensign et al. [92]. (Permission from Copyright Clearance Center, Elsevier).

(Fig. 2). Therefore, the first discussed are nanomedicine for treatment of gonorrhea.

4.1. Nanomedicine for the treatment of Neisseria gonorrhoeae

4.1.1. General considerations

Gonorrhea emerged as a major public health concern with an estimated global incidence of 86.9 million annually [94]. Infections caused by *N. gonorrhoeae* are often asymptomatic, and due to anatomical differences, infections are more complicated to diagnose in women [95]. *N. gonorrhoeae* is gram-negative, human obligate bacteria which pathogenesis involves transmission, adherence, colonization and invasion, ending in immune evasion (Fig. 6).

Initially, *N. gonorrhoeae* adheres to host epithelial cells of vaginal mucosa, replicates and forms microcolonies, subsequently entering the cells by transcytosis. Invasion triggers the release of inflammatory cytokines and chemokines, recruiting neutrophils that interact with the bacteria forming a purulent exudate that facilitates the transmission of *N. gonorrhoeae* [96]. *N. gonorrhoeae* generally causes mucosal vaginal tract infection; however, it can also attach to the epithelium of the ectocervix. Untreated or unsuccessful treatment of infection can ascend and cause serious complications as well as increased transmission risk of other STIs [94]. Oral or intramuscular administration of antibiotics is the first-line treatment; however, *N. gonorrhoeae* is rapidly developing resistance to every major class of antibiotics and even dual treatment often fails [9]. The recommended therapy is currently antibiotics ceftriaxone and azithromycin (AZT), generally as dual therapy [35]. However, the susceptibility is rapidly decreasing globally [97].

4.1.2. Choice of nanoformulation

Considering that *Neisseriaceae* bacteria has been co-evolving with its human host for centuries [94], the introduction of nanomedicines for the localized treatment of *N. gonorrhoeae* infection is rather recent. To our knowledge, the first nanoformulation for vaginal delivery targeting *N. gonorrhoeae* infection was a liposome microbicide formulation developed by Wang et al. [98]. The *in vitro* effect of liposomal formulation containing octylglycerol was compared to the microbicide effect of plain Carbopol® hydrogel containing octylglycerol. Liposomal octylglycerol formulation expressed a superior activity against *N. gonorrhoeae* compared to the conventional gel formulation; the activity was maintained for at least two months. *In vivo* safety evaluations in macaque model by rectal application, confirmed its safety. Moreover, *ex vivo* penetration studies on human ectocervical tissue demonstrated the lack of octylglycerol penetration, confirming localized effect. No toxicity was inflicted to the freshly excised ectocervical tissue [98].

The natural-origin polymer chitosan is shown to exhibit significant intrinsic antimicrobial activity [99], to efficiently disrupt vaginal biofilms [100] and is therefore considered highly attractive in the development of vaginal delivery systems. The potential of chitosan NPs (CNPs) in the localized treatment of *N. gonorrhoeae* has been recently evaluated *in vitro* [101]. The anti-gonococcal effect of CNPs was confirmed and shown effective against the strains with high-level resistance against commonly used antibiotics. Several mechanisms of antimicrobial action of chitosan were proposed, such as the interaction with cellular surface, interaction with targets within the cell, and antimetabolite action [102]. The antimicrobial activity of CNPs was clearly influenced by the intrinsic, environmental and microbial factors [103], however, the exact mechanisms is not yet known. The study by Alqahtani and colleagues indicated that the pH highly influenced the activity of CNPs; a superior antimicrobial effect was seen at pH 5.5, however, it was also expressed at neutral pH. Moreover, CNPs were confirmed to be non-toxic [101].

Li et al. [104] evaluated carbon nanotubes, silica, zinc oxide, and silver nanoparticles (AgNPs) for their anti-gonococcal activity *in vitro*. Standard *N. gonorrhoeae* strains were challenged against the nanomaterials of various particle size. A superior activity was seen for the 120 nm AgNPs. Several mechanisms for the antimicrobial activity were suggested, including the disruption of the cell membrane, induced production of reactive oxygen species (ROS), and disruption of DNA replication [105–107], however, the exact mechanism remains unclear. Further, the combination of AgNPs and cefmetazole, an antibiotic that is commonly ineffective towards *N. gonorrhoeae*, was challenged against resistant clinical isolates. Estimated MICs showed an increased effect of the antibiotic when in combination with the NPs [104]. In a more recent study, the anti-gonococcal effect of silver nanoclusters (AgNCs) was evaluated *in vitro* [108]. The AgNCs were coated with

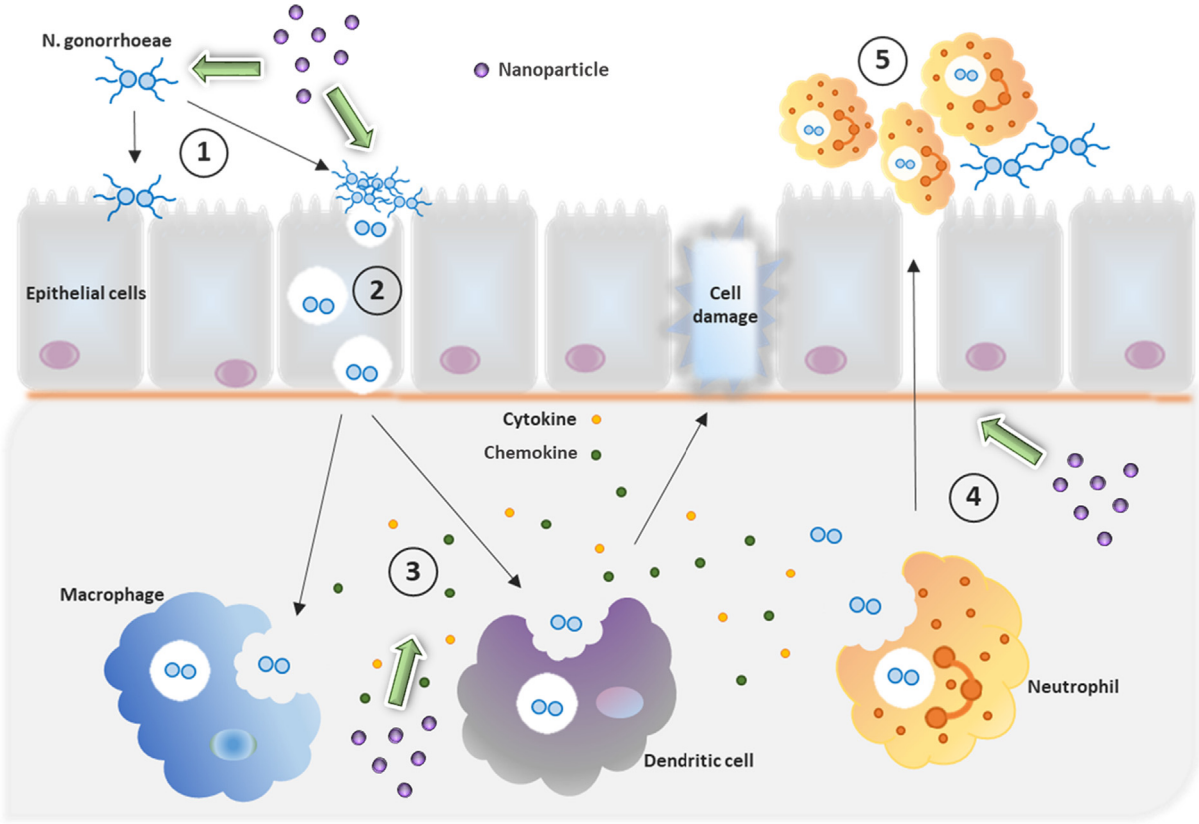


Fig. 6. Stages of *Neisseria gonorrheae* infection and targets for nanomedicines (green arrows). 1) *N. gonorrheae* adheres to epithelial cells then replicates and forms microcolonies. Nanoparticles can act as microbicides and avoid bacterial adhesion. 2) Bacteria crosses the epithelial barrier by transcytosis. Nanoparticles can act as microbicides and prevent bacterial colonization and invasion of epithelium. 3) *N. gonorrheae* is phagocytosed by macrophages and causes production of inflammatory cytokines and chemokines. Interaction with dendritic cells modulate apoptosis and also causes the release of inflammatory cytokines and chemokines. Nanoparticles can enable effective treatment of infection and/or inflammation. 4) Neutrophils are recruited to the infection site where they interact with and phagocytose *N. gonorrhoeae*. 5) Neutrophils migrate across the epithelium facilitating the transmission of *N. gonorrhoeae*. Nanoparticles can impair the bacterial transmission. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5-mercapto-2-nitrobenzoic acid (MNBA). Resistant *N. gonorrhoeae* strains were exposed to the MNBA-coated AgNCs and treatment compared to commonly used antibiotic ceftriaxone. A concentration- and time-dependent effect was seen for both MNBA-coated AgNCs and the antibiotic, however, the antibacterial effect of MNBA-coated AgNCs was superior to ceftriaxone. The study showed that the nanoformulation could effectively kill multidrug resistant *N. gonorrhoeae* within 1 h also at high level of organism load. Moreover, no *in vitro* toxicity was found [108].

In addition to the search for novel treatment options, the prevention of infection is of importance. The development of vaccines for multidrug resistant *N. gonorrhoeae* has resulted in four clinical trials, of which all failed to provide adequate protection from infection [109].

4.2. Nanomedicine for the treatment of Mycoplasma genitalium

4.2.1. General considerations

M. genitalium is an emerging sexually transmitted bacterial infection that is highly prone to the development of AMR [110,111]. *M. genitalium* is transmitted by direct mucosal contact and infections are often asymptomatic [112]. The life cycle of *M. genitalium* and the mechanism by which it causes inflammation and cellular damage is unknown [113]. However, infection can cause serious complications in women, such as pelvic inflammatory disease, cervicitis, preterm birth and spontaneous abortion [114].

M. genitalium was first isolated in 1981 and described as a mycoplasma [113]. Due to the lack of a peptidoglycan-containing cell wall, antibiotics that target the cell wall are ineffective against the pathogen and options of antibiotic treatment is limited [110]. Moreover, *M. genitalium* can easily develop single-nucleotide polymorphisms that can lead to AMR [115]. Currently the macrolide AZT is recommended as a first line, and the fluoroquinolone moxifloxacin as a second line treatment [116]. However, the prevalence of macrolide and fluoroquinolone resistance is rapidly increasing. *M. genitalium* is evolving into a superbug that is immensely difficult to treat, or even untreatable [116,117].

4.2.2. Choice of nanoformulations

To our knowledge, the use of nanomedicine has so far not been explored for improved therapy of vaginal *M. genitalium* infections. The need for new treatment options is evident [118] and nanomedicine can be an important tool to better understand the pathology of *M. genitalium* and identify promising strategies to better tackle the challenges of AMR. Advanced delivery systems have the potential to provide improved efficacy of currently used antimicrobials as well as intensify the potential of alternatives to existing antibiotics.

4.3. Nanomedicine for the treatment of Chlamydia trachomatis

4.3.1. General considerations

With more than 127 million new infections reported globally each year, *C. trachomatis* is a major cause of bacterial STIs [22]; it is particularly prevalent in young females with a four times higher infection rate compared to the general population [119]. Genital

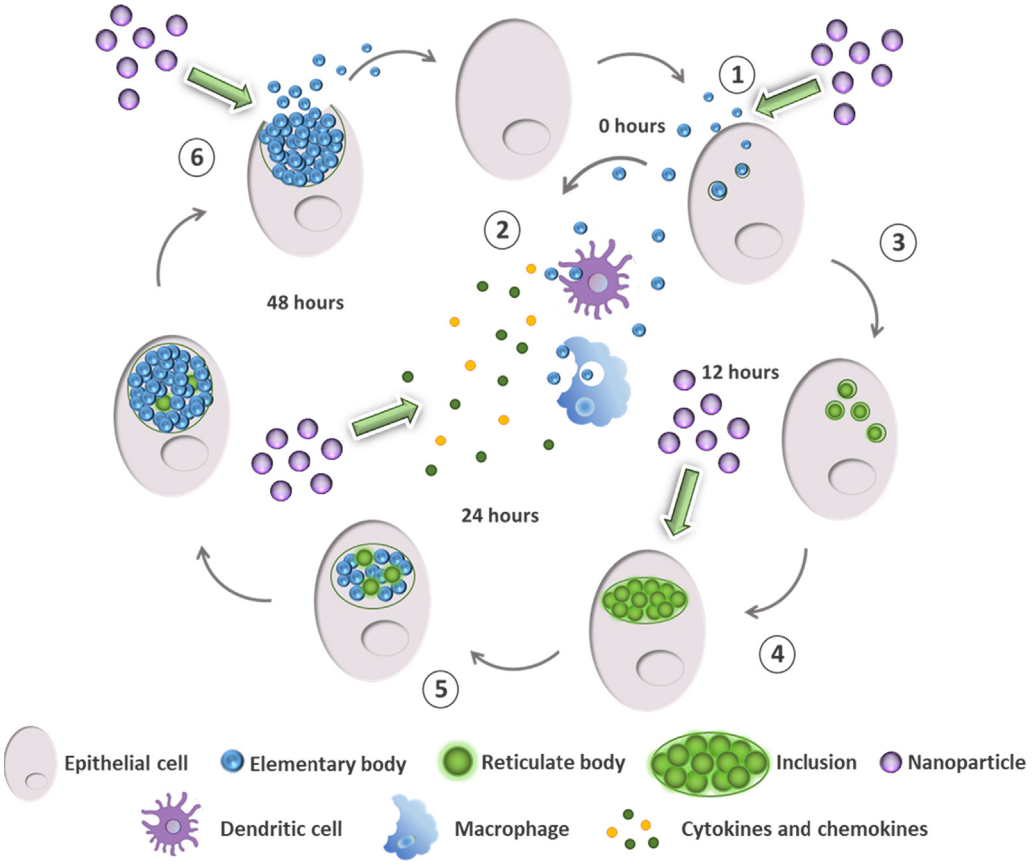


Fig. 7. The chlamydia life cycle and possible targets for nanomedicines (green arrows). 1) Elementary body, the infectious form of *C. trachomatis*, attaches to a host cell and is phagocytized ending up in a vacuole called inclusion. Nanoparticles can act as microbicides and avoid bacterial cell entry. 2) Immune cells activate the production of inflammatory cytokines and chemokines. Nanoparticles can enable effective treatment of inflammation. 3) Elementary body reorganizes to reticulate body. 4) Reticulate bodies replicate, producing several reticulate bodies within the inclusions. Nanoparticles can enable effective treatment of infection. 5) The reticulate bodies start to convert back to elementary bodies. 6) Infectious elementary bodies are released from the cell. Nanoparticles can impair the bacterial transmission. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

chlamydia infection is associated with minimal or even no symptoms in approximately two-thirds of women, and the reported number of infections is considered substantially underestimated. *C. trachomatis* is a small obligate intracellular, gram-negative bacterium with a complex biphasic life cycle (Fig. 7).

The bacterium exists in two forms; the infectious elementary body and non-infectious reticulate body. Elementary bodies enter the host cells and differentiates to reticulate body that replicates within an inclusion, concealing its antigenic profile from the immune system. The reticulate bodies start to convert back to elementary bodies and move between the hosts in the non-replicative form [120]. Oral antibiotics are available for the treatment of *C. trachomatis*; however, they are efficacious only when administered at an early stage of infection, and clinical failure and recurrence rates are high [121]. The lack of treatment or its failure, due to asymptomatic infections, affect the public health burden by favoring the onset of severe complications [122]. Moreover, persistent *C. trachomatis* infections enhance the risk of HIV transmission.

4.3.2. Choice of nanoformulation

Liposomes were the first and are most studied delivery system proposed for the treatment of chlamydia. However, dendrimers, PLGA NPs and other nanoformulations were also tested. Interestingly, natural origin substances such as resveratrol, were also proposed as alternative to antibiotic. Report on nanomedicines developed for the delivery of antibiotics targeting *C. trachomatis* was, to our knowledge, first published in 1992. The ability of liposomal tetracycline to inhibit the propagation of *C. trachomatis* in McCoy cells was evaluated [123]. McCoy mouse fibroblast is the commonly applied cell line for studying chlamydial infections under *in vitro* conditions [124–126]. Chlamydia infected cells were exposed to free tetracycline and tetracycline incorporated into cationic, anionic and neutral liposomes, and incubated for 48 h. When compared with free tetracycline, neutral liposomes containing tetracycline were the most efficient for inhibiting the growth of *C. trachomatis* [123]. In another early study, tetracycline and doxycycline were incorporated separately into cationic, anionic and neutral liposomes to evaluate the anti-chlamydial activity *in vitro* [127]. The results showed that the anti-chlamydial effect increased for both tetracycline and doxycycline when in liposomal formulation. Moreover, the ability of formulations to prevent the formation of inclusions after a second passage was evaluated. The superior activity of liposomally entrapped antibiotics was confirmed [127]. In further study, Sangaré and colleagues challenged liposomal doxycycline prepared as intramuscular injection against chlamydia infection *in vivo* [128]. Doxycycline was incorporated into cationic liposomes and injected into progesterone-treated, female mice infected with *C. trachomatis* serovar L_2; two days post-infection the treatment group were injected with the free and liposomal doxycycline (10 μg/g body weight) for 3 or 7 consecutive days. Results showed that the infection rates were lower in all treatment regimens compared to untreated mice, however, there were no significant difference in the free doxycycline and liposomal doxycycline treatment. In conclusion, the 7-day

treatment with liposomal doxycycline was proven the most protective against ascending chlamydial genital infection in mice [128]. Although not an example of localized drug therapy, the work is important to understand the potential of nanosystem to enhance potency of antibiotics.

Azithromycin (AZT) is a broad-spectrum macrolide commonly administered orally as *C. trachomatis* therapy, however, it is contraindicated during pregnancy as well as associated with subsequent side effects, clinical failure and high recurrence rates [129]. The localized delivery of AZT to the site of action at lower doses can prevent systemic antibiotic effects and reduce the side effects. Moreover, the risk of developing AMR can be reduced by the localized therapy. Recently, Vanić and colleagues developed AZT liposomes with varying bilayer elasticity and surface charge, to evaluate the effect of AZT in nanoformulations against *C. trachomatis* infection *in vitro* [130]. HeLa 229 cells were infected with *C. trachomatis* serovar D and exposed to free AZT and liposomal AZT. After 48 h of incubation, deformable propylene glycol liposomes (DPGLs) containing AZT was found the most promising among the liposomal formulations tested toward *C. trachomatis*. All liposomal formulations containing AZT expressed a lower efficacy compared to the free AZT. However, a free drug exhibited a high penetration through the vaginal tissue *ex vivo*, suggesting that an undesirable systemic effect can occur. Antibiotic treatment for pregnant patients suffering from vaginal infections is limited; assuring the efficient topical effect while avoiding systemic absorption is considered the best therapy option. Liposomal AZT enabled a retention on top and within the vaginal tissue, demonstrating the potential of liposomal AZT in the localized vaginal therapy. Moreover, DPLGs was found to express the strongest potential for the eradication of biofilms [130]. The ability of liposomes to enhance the delivery through the formed biofilms can generate a high local concentration of AZT, improving the therapeutic potential of the developed delivery system.

The disturbance of microbiota or the absence of *Lactobacillus spp*, caused by bacterial vaginosis, is associated with an increased risk of acquiring STIs, including *C. trachomatis*. [119]. Employing natural origin substances with multi-targeted pharmacological properties might be valuable tools to tackle AMR [67,131]. The liposome-in-hydrogel delivery system containing resveratrol (RES) for the localized treatment of *C. trachomatis* has been recently evaluated *in vitro* [126]. RES is poorly water-soluble and has rather limited stability, by liposomal entrapment, the solubility improved while the substance was protected from environmental and chemical changes. RES is naturally occurring polyphenol exhibiting many potential health benefits, and a wide range of bacterial, viral and fungal species are known to be susceptible to RES, including *C. trachomatis* [124,132]. McCoy cells were infected with *C. trachomatis* serovar E. Two hours post infection, the infected cells were exposed to free RES, liposomal RES and RES liposomes-in-hydrogel formulation and the effect on bacterial survival was evaluated by staining of *C. trachomatis* inclusions 48 h post infection. RES formulations reduced the number of *C. trachomatis*-infected cells, and RES liposomes-in-hydrogel expressed superior anti-chlamydial activity in the lower concentrations, compared to free RES [126]. The mechanism behind the antibacterial action of RES is not yet fully established. Several mechanisms are proposed, such as the inactivation of the efflux pump [133], inhibition of bacterial type III secretion system [134,135] and prevention of cell entry by covalently binding to the elementary body of *C. trachomatis* [136]. The superior effect of RES when in delivery system indicates the need for a delivery system to assure successful therapy. Although chitosan is known to exert intrinsic antibacterial activity [99], chitosan hydrogel had no effect on the anti-chlamydial challenge on its own [126].

Combination drug therapy can amplify the therapeutic effect of existing antibiotics; the co-delivery of rifampicin and AZT using polymeric NPs has been evaluated in *C. trachomatis* therapy *in vitro* [137]. NPs comprised biodegradable PLGA polymer, contained two antibiotics either encapsulated separately or in a combination. McCoy cells were infected with *C. trachomatis* serovar K and exposed to free and PLGA encapsulated drugs immediately, 24 or 48 h after infection. When treated immediately after infection, no difference in MIC_{50} was seen for free drug vs NPs. However, when treatment was introduced after 24 or 48 h, the encapsulation in PLGA NPs improved the effectiveness of both drugs. The co-delivery of antibiotics expressed a superior anti-chlamydial effect compared to individual drugs. Toti et al. [137] proved that PLGA NPs can efficiently accumulate in *C. trachomatis* inclusions and improve the effectiveness of drugs when in delivery system.

Efficient localized delivery of microbicide is another approach of great importance for prevention of STI transmission, including chlamydia infections. Recently, Yang et al. [138] investigated the encapsulation of small interfering RNA (siRNA) in PLGA NPs, targeting the knock down of platelet derived growth factor receptor-β (PDGFR-β) to reduce vaginal *C. trachomatis* infection. PDGFR-β is an important surface binding protein for *C. trachomatis*; the PLGA encapsulated siRNA enabled a reduced entry of *C. trachomatis* (serovar K) into McCoy cells as well as reinforcement of the host cells defense against the bacteria.

Dendrimers have also been studied for the localized therapy of genital chlamydia infections. Mishra et al. [139] developed dendrimers for the sustained delivery of AZT to infected cells. AZT was conjugated with the polyamidoamine (PAMAM) dendrimers and the effect on acute and persistent *C. trachomatis* infection in human bronchial epithelial cells evaluated *in vitro*. Infected cell cultures were pulsed with labeled dendrimer for 1 h at 24 or 48 h post infection. The fluorescence microscopy confirmed that the dendrimers clearly entered and accumulated in the inclusions. Moreover, AZT-dendrimers reduced the size and number of inclusions to a greater extent than free AZT, demonstrating the superior antimicrobial effect of AZT when conjugated with PAMAM dendrimers [139].

Dendrimers exhibit both intrinsic antiviral as well as microbicide properties. Patton and colleagues developed gel formulations containing the microbicide SPL7013 dendrimer to protect against *C. trachomatis* infections in pigtailed macaque models. The pigtailed macaques were not protected from *C. trachomatis* infection after treatment with a single dose of 3% SPL7013 [140]. Yet, both formulated and unformulated SPL7013 have shown the ability to protect against *Herpes simplex* virus type 2 infection in mouse models [141,142]. The formulation of dendrimers in hydrogels can offer the safe localized treatment of ascending genital infections in pregnant women avoiding adverse effects to the fetus [143] and has the potential to improve local therapy of vaginal infections.

C. trachomatis infection causes a continuous production of inflammatory mediators leading to severe complications, therefore, the control and regulation of inflammatory responses is subsidiary in successful therapy. Yilma et al. [121] developed silver polyvinyl pyrrolidone (Ag-PVP) NPs and evaluated their effect on inflammatory mediators triggered by *C. trachomatis* infection *in vitro*. The NPs controlled inflammatory mediators and inhibited cytokines and chemokines produced by the infected macrophages, confirming the potential of Ag-PVP NPs as regulators of inflammatory responses induced by *C. trachomatis* infection. Jøraholmen et al. [126] confirmed the superior anti-inflammatory activity RES when in liposomes-in-hydrogel formulation, suggesting that RES liposomes-in-hydrogel bears potential in the treatment of other types of vaginal infections and related inflammations.

Currently, there is no licensed vaccine for *C. trachomatis*. The search for effective vaccines is intensifying; liposomes [144], polymeric NPs [145–151] and dendrimers [152] are some of the promising candidates in the development of successful vaccines preventing *C. trachomatis* infections.

4.4. Nanomedicine for the treatment of bacterial vaginosis

4.4.1. General considerations

Bacterial vaginosis (BV) is one of the most prevalent genital infections commonly affecting women of reproductive age, although it has also been reported in menopausal women. BV represents disbalance in normal vaginal microbiota, namely, an overgrowth of anaerobic bacteria including *G. vaginalis*, *A. vaginae*, *Mycoplasma hominis*, *Ureaplasma urealiticum*, *Mobiluncus spp.*, *Bacteroides* spp. and *Prevotella* spp. Simultaneously, healthy vaginal microflora (lactobacilli) undergoes significant reduction/disappearance [153]. Currently recommended treatment regimens rely on both oral and local administration of metronidazole, clindamycin and/or tinidazol [154]. In the era of AMR, topical therapy become a preferable approach due to higher local drug concentrations and avoidance of antibiotic-related side-effects and resistance [7,155]. However, BV treatment is often challenged by the thick polymicrobial biofilm adhering to the vaginal epithelium [156]. Conventional antibiotic therapy often fails to completely eradicate biofilms [157]; thereof BV is characterized by a high rate of relapse and recurrence [154]. In addition, the link between BV and an increased susceptibility to STIs (particularly viral), spontaneous abortion, pre-term birth, pelvic inflammatory disease and endometritis become evident [156]. The last three decades witnessed an increased interest in vaginal nanomedicines for improved treatment of BV [8].

4.4.2. Choice of nanoformulation

Most of the nanoformulations for topical BV therapy encapsulated metronidazole, the most prescribed drug in standard (conventional) BV therapy. Majority of the research focused on liposomes (non-mucoadhesive and mucoadhesive), nanofibers, polymeric NPs and dendrimers.

One of the first studies on liposomes in vaginal therapy has focused on *in vitro* and *in situ* stability of metronidazole-containing liposomes for the treatment of BV. Lecithin-based liposomes (300–350 nm), bearing 6–8% metronidazole, were found stable in buffers pH 4.5 and 5.9, mimicking pre- and post-menopausal vaginal conditions. Burst release of metronidazole was detected in the first hour, followed by the sustained release in the following 23 h at low pH and the presence of cow vaginal mucosa (*in situ* stability studies). Liposomes would therefore permit a high initial and medium maintenance doses, a favorable approach in BV treatment [158]. To improve the penetration of liposomes into deeper mucosa assuring delivery of encapsulated metronidazole, the same researchers assessed deformable liposomes. Several edge activators including sodium deoxycholate, Tween 80 and Span 80 were tested to optimize the composition of deformable liposomes. Deformable liposomes comprising 15% deoxycholate demonstrated enhanced permeation of metronidazole through the *in vitro* model of the epithelial barrier as compared to conventional liposomes [159]. Further research efforts have been directed towards development of the new elastic liposomes with increased bilayer deformability, and increased drug load. This has been achieved by addition of propylene glycol, acting both as drug solubilizer and affecting bilayer elasticity. Thus, deformable propylene glycol liposomes (DPGLs) containing 10% (w/w) propylene glycol embedding sodium deoxycholate entrapped 14 μg of metronidazole/mg of lipid compared to deformable liposomes prepared without propylene glycol (7 μg/mg lipid). To assure the retention within vaginal cavity, liposomes were incorporated into Carbopol 974P hydrogel. The *in vitro* release studies at simulated vaginal conditions proved extended and diffusion-based release, which was affected by bilayers' fluidity. Approximately 80% of metronidazole has been released from elastic DPGLs [66], in comparison to 50% metronidazole from the conventional liposomes, characterized by rigid membranes [64]. The hydrogel vehicle both impaired the viscosity and favorably enhanced the storage stability by maintaining their original size distributions [62–64].

Prolonged retention of liposomes onto vaginal mucosa can be also achieved by utilizing mucoadhesive polymers either as a coating on the liposomal surface or building block of liposomal bilayers [59,160]. For instance, Andersen et al. [160] developed metronidazole-containing chitosomes and pectosomes, i.e. liposome-based vesicles comprising mucoadhesive polymer (chitosan or pectin) both on the liposomal surface as well as in the inner and outer aqueous phases of the vesicles. The mucoadhesive polymer enabled retention at the mucosal site and increased metronidazole encapsulation.

Alternatively, liposomal metronidazole could be applied to vaginal site as a solid formulation. This novel approach encompasses the use of tablets of pre-liposomes powder that, upon contact with vaginal fluid, disintegrate, subsequently forming liposomes. For this purpose, pre-liposomes were prepared by direct spray drying of dispersion containing soy lecithin, mannitol and metronidazole, which were then mixed with the different types of non-mucoadhesive and mucoadhesive (chitosan, pectin) fillers and compressed into tablets. Such solid liposomal formulation offered a unique synergy between the ability of liposomes to solubilize poorly-soluble drugs and increased stability provided by compressed solid formulations. In addition, all metronidazole was associated with the spray-dried powder, not only the liposomally-entrapped drug, leading to higher drug load [68].

Metronidazole has also been loaded in polyvinylpyrrolidone (PVP) nanofibers. It was demonstrated that PVP concentration influenced the size, mechanical and mucoadhesive properties of the nanofibers. Regardless of the PVP concentrations, fast release of metronidazole was obtained (greater than 95% within 2 h) due to the hydrophilic nature of the polymer. In addition, *ex vivo* permeation studies performed on cow vaginal mucosa revealed increased flux and permeability of metronidazole from the nanofibers as compared to the gel or solution [88]. The liposomally-entrapped drugs, in comparison to nanofibers, assured slower release and limited permeation across the vaginal mucosa, the features considered relevant in local vaginal therapy, especially considering pregnant patients [60,130,161].

Because BV is characterized by the diminished levels of lactobacilli and increased pH of vaginal fluid (greater than 4.5), administration of lactic acid favorably affects the re-establishment of normal vaginal microflora and BV treatment. Rajan et al. [162] developed poly(ethylene glycol) (PEG) nanocarrier-based degradable hydrogels for the controlled release of lactic acid. PEG-lactic acid nanocarriers were prepared by covalent attachment of lactic acid to 8-arm polyethylene glycol thiol (PEG-SH) which were then crosslinked with 4-arm N-hydroxylsuccinimide functionalized polyethylene glycol (PEG-NSH) to form nanocarrier-based hydrogel. The novel nanocarrier-based hydrogel enabled sustained release of lactic acid over several days. *In vitro* antibacterial studies against *G. vaginalis* showed complete bacterial growth inhibition within 48 h.

Among the nanomedicines studied for topical BV therapy, the dendrimers have indisputably showed superiority. Astodrimer (also known as SPL7013), a L-lysine dendrimer with a polyanionic surface charge developed by Starpharma Pty Ltd (Australia) was shown to inhibit growth of bacteria associated with BV via a novel

mechanism of action compared to conventional antibiotics. Dendrimer acted by blocking the attachment of bacteria to cells thereby inhibiting the biofilm formation [83]. Astodrimer was further incorporated into mucoadhesive Carbopol gel to assure sufficient retention in the vagina. In the Phase 2 clinical trial performed on 132 women, once daily application of Astodrimer 1% gel was proven to be effective for treating BV. Moreover, the formulation was shown to be safe and well-tolerated, acting only locally [163]. The superiority of the Astodrimer 1% gel has recently been confirmed in the Phase 3 clinical trial. Due to its innovative mechanism of action and safety, Astodrimer 1% gel offers great potential for therapy of recurrent BV, especially women who fail to respond to conventional therapy, or are intolerant to existing antibiotic therapy [164].

4.5. Nanomedicine for the treatment of aerobic vaginitis

4.5.1. General considerations

Aerobic vaginitis (AV) has been defined as a vaginal condition that is distinct from BV that requires different clinical treatment. It is characterized by a diminished dominance of *Lactobacillus* and consequent presence of abnormal vaginal microflora comprising aerobic, enteric commensals or pathogens like Group B *Streptococcus* (*S. agalactiae*), *Enterococcus faecalis*, *Escherichia coli* and *Staphylococcus aureus* [165]. Additionally, AV is accompanied with variable levels of vaginal inflammation and deficient epithelial maturation. The prevalence of AV is generally lesser than BV (typically 7–12%). Although both AV and BV share some common features, there are clear differences between them. For instance, BV is not accompanied by inflammation; vaginal tissue in AV is commonly thinner, red and edematous with possible small ulcerations. pH can be significantly higher in AV, and vaginal fluid is thicker, commonly yellow or green colored. On the other hand, discharge in BV is typically whitish or gray with fishy smelling and water consistency [32,166]. In spite of these differences, AV can be often falsely diagnosed leading to inappropriate therapy and severe complications particularly in pregnancy [166,167]. Therapy of AV typically encompasses oral and/or local administration of antibiotics, antiseptics, estrogens, non-steroidal anti-inflammatory drugs and probiotics. Among the antibiotics, those active against aerobic bacteria, preferably kanamycin, clindamycin and moxifloxacin, are used, while probiotics are beneficial to increase the lactobacilli levels. Vaginal atrophy requires hormonal therapy, wherein topically administered estrogens in combination with antibiotics are commonly applied [167,168]. If not completely eradicated during the treatment, AV-associated bacteria can form biofilm (particularly *E. coli*), similarly as in BV.

4.5.2. Choice of nanoformulation

Most studies on potential local therapy of AV involved liposomal antibiotics (chloramphenicol, azithromycin (AZT), ciprofloxacin), although natural-origin nanomedicines, such as liposomal resveratrol (RES) were also proposed as well as antiseptics (chlorhexidine digluconate chitosan/alginate complexes).

A very early research assessing the potential of liposomes in vaginal therapy was focused on chloramphenicol, broad-spectrum antibiotic active against AV-bacteria, although at the time AV has not been defined as specific type of vaginitis. Chloramphenicol has been sufficiently encapsulated in both conventional and elastic propylene glycol liposomes, wherein better entrapment was achieved with elastic liposomes because of a propylene glycol solubilizing effect. *In vitro* liposomes' stability in the buffers, pH 4.5 and 5.9, indicated greater stability in the pH 5.9 buffer [158], corresponding to pH value of the vaginal fluid often found in AV [166]. Better stability of the conventional liposomes was contributed to the bilayer rigidity reducing leakage of the encapsulated drug [158]. Conventional liposomes containing chloramphenicol were incorporated into 1% (w/w) Carbopol 974P gel to assure prolonged residence within vaginal cavity [63]. The *in vitro* studies were performed in buffer pH 4.5 in presence of vaginal fluid simulant (VFS, pH 4.5) [169]. The studies confirmed prolonged and diffusion-controlled release of chloramphenicol from the liposomal gel.

To treat *E. coli*-caused AV, Vanić et al. [130] proposed several types of AZT liposomes, differing in bilayer fluidity and surface charge. The (phospho)lipid composition, presence of the edge activator and propylene glycol influenced the encapsulation of AZT, liposomal size, surface charge, bilayer fluidity, storage stability and *in vitro* release under simulated vaginal conditions (VFS, pH 4.5). Optimized AZT liposomes were assessed for *ex vivo* vaginal deposition/penetration, *in vitro* antibacterial activity against several planktonic and biofilm-forming *E. coli* strains, as well as *in vitro* biocompatibility with the cervical cells. All liposomes were more effective than the free AZT against planktonic *E. coli* ATCC 700,928 and K-12 with minimum concentration that inhibited bacterial growth by 50% (MIC_{50}) approximately 3-fold lower than the MIC_{50} of the free AZT. Anti-biofilm activity of the AZT liposomes was dependent on the bacterial strain and type of the liposomes. Variability in the anti-biofilm activity of different AZT liposomes was a consequence of the liposomal bilayer properties affecting AZT release and the susceptibility of the different *E. coli* strains to AZT. The similar effect was confirmed in *ex vivo* permeability studies. All liposomes enabled localization of the AZT on/within the vaginal tissue. Hence, the conventional liposomes would be suitable for the treatment of superficial infections, while DPGs would be appropriate for treating complicated, biofilm-related, AV infections.

Pisano et al. [75] demonstrated potentials of ciprofloxacin loaded liquid crystals for topical AV therapy. Liquid crystal structures provided prolonged release of the encapsulated antibiotic; and were effective against *E. coli* with minimum inhibitory concentrations (MIC) 5-fold lower than those obtained for the free ciprofloxacin.

Since AV is characterized by vaginal inflammation along with increased levels of aerobic bacteria [32], the utilization of resveratrol, exerting both anti-inflammatory and antibacterial properties, seems to be a very promising strategy, especially when considering the safe treatment of pregnant women and growing problem of AMR. Following this approach, Jøraholmen et al. [161] prepared chitosan-coated liposomes with high RES loading (77%). In addition to assuring a sustained RES release and retention of nanoformulation at vaginal mucosa, liposomal formulation enhanced *in vitro* anti-oxidative and anti-inflammatory properties of RES.

Considering the treatment of non-complicated AV and mixed vaginal infections, antiseptics such as povidone iodine and chlorhexidine can also be used [170,171]. Chlorhexidine digluconate was loaded in chitosan/alginate complexes, which were subsequently freeze-dried to obtain the inserts suitable for vaginal application. The loaded insert demonstrated strong antibacterial activity against *E. coli*. Already after 6 h of the insert incubation with the bacteria, bacterial concentrations were below the limit of detection [172].

4.6. Nanomedicine for the treatment of vulvovaginal candidiasis

4.6.1. General considerations

Vulvovaginal candidiasis (also known as vulvovaginal candidosis, VVC) is the second most prevalent infection of the lower female genital tract (after BV) caused by yeast *Candida albicans* (80–90%), although other *Candida* species can be present, such as *C. glabrata* (5–10%), *C. tropicalis* (5%) and *C. krusei* (1%) [7,52]. Almost 75% of childbearing age women experience VVC at least once in their life, while half of them experience recurrent infections [173]. Even though *C. albicans* is a part of healthy vaginal microflora, the imbalance of vaginal microbiota and the progress of the yeasts'

colonization and adhesion to epithelial cells can lead to its overgrowth. As a result, the infection process transforms from asymptomatic to symptomatic, comprising virulence factors such as formation of blastoconidia and pseudohyphae capable of destroying the vaginal epithelium, as well as the production of enzymes, toxins and phospholipases [174,175]. Majority of all VVCs are non-complicated, caused by *C. albicans* and exhibiting mild symptoms. They are often triggered by exogenous factors, such as intake of antibiotics, and are typically occurring in pregnancy. On the other hand, complicated (recurrent) VVCs are linked to non-*C. albicans* species characterized by four or more recurrent incidents per year. Recurrent VVCs accompany uncontrolled diabetes mellitus, AIDS, immunosuppressive or hormone replacement therapy [52,173].

Therapy is commonly relying on oral and/or topical administration of antifungal agents including pyrimidine analogues, polyenes and mainly azoles, imidazoles and triazoles. However, the persistent strains (*C. parapsilosis*, *C. krusei*, *C. glabrata* and *C. tropicalis*), less susceptible to the antifungal agents, in addition to azoles' resistance to *Candida*, contribute to the treatment failures and recurrence [175,176]. Moreover, *Candida* is also able to organize in biofilms, obstructing the sufficient penetration of antifungals through the formed matrix, thus ensuing increased antimicrobial resistance and unsuccessful therapy outcome [174,175].

4.6.2. Choice of nanoformulation

Various antifungal-based delivery nanosystems have been proposed for improved VVC topical therapy. Typically, antifungal drugs used in conventional therapy were encapsulated in different lipid- and polymer-based nanomedicines (liposomes, solid lipid NPs, nanostructured lipid complexes, nanoemulsions, polymeric NPs and nanofibers), alone or incorporated in a secondary vehicle to improve localization of the drug at the site of infection. Novel approaches explore the potential of herbal- and seaweed-based nanomedicines.

Pavelić et al. [158] encapsulated an imidazole derivative, clotrimazole, in elastic propylene glycol liposomes and two types of conventional liposomes. Encapsulation of the drug inversely correlated to the liposomal bilayer fluidity. Inclusion of clotrimazole-containing liposomes in Carbopol 974 PNF gel positively affected their stability providing prolonged clotrimazole release [64]. A favorable balance between the hydrogel's cohesiveness and adhesiveness was achieved, allowing spreading and retention inside vagina [66]. Ning et al. [177] confirmed the extended release of clotrimazole from proliposomes. The proliposomes demonstrated potent antifungal activity without visible changes in the vaginal tissue.

Jøraholmen et al. [60] studied mucoadhesive, chitosan-coated clotrimazole liposomes for the VVC treatment in pregnancy. *Ex vivo* permeation studies performed on the vaginal tissue of pregnant sheep, demonstrated reduced clotrimazole permeation from the coated liposomes in comparison to the non-coated liposomes or the drug solution. Moreover, concentration of the chitosan used for the coating of clotrimazole liposomes affected their mucoadhesiveness; at the lower chitosan concentrations, mucoadhesiveness of the liposomes was enhanced.

Chitosan-based nanomedicines offer additional advantages in the treatment of vaginal infections due to chitosan's intrinsic antimicrobial properties and activity in slightly acidic environment [10,178]. For instance, the activity of empty chitosomes (without entrapped metronidazole) against *C. albicans* was comparable to chitosomes containing metronidazole, wherein metronidazole itself failed to show any anti-Candida effect [179].

In addition to chitosan, mucoadhesive liposomes can be prepared utilizing other mucoadhesive polymers, such as Eudragit, Carbopol and pectin. In a very recent study, pectin-coated cationic liposomes containing sertaconazole nitrate were prepared and assessed for local VVC therapy. Pectin-coating significantly reduced zeta potential of the cationic liposomes, increased encapsulation and permitted sustained release of the sertaconazole nitrate. The optimized liposomal formulation, coated with 0.1% pectin and incorporated into 1% hydroxypropylmethylcellulose gel showed reduced permeation of the drug into vaginal tissue in comparison to the control gel. The superiority of the formulation was confirmed *in vivo* on *C. albicans* infected rats, where sertaconazole nitrate liposomal gel enabled a significant reduction of microbial count and diminished inflammatory responses with the minimal histopathological changes of vaginal tissue in comparison to the control gel [180].

To enhance vaginal delivery of liposomal ciclopirox olamine, Karimunnisa and Atmaram [181] used poly(acrylic)acid-based gel. Viscosity measurements, as well as oscillatory stress sweep and frequency sweep tests, conducted under dilution of the liposomal gels with simulated vaginal fluid (VFS), proved pseudoplastic features of the formulation. Moreover, extended drug release from the liposomal gel determined its *in vitro* antifungal activity; *C. albicans* was eradicated after 6 h in comparison to 3 h for the free drug [181].

Contrary to the majority of the findings obtained with the liposomal gels, where sustained release of the encapsulated drugs was reported [61–66,181]. Kang et al. [182] demonstrated increased release of amphotericin B from the cationic liposomes incorporated into thermosensitive gel. The authors proposed that such findings could be a consequence of the amphiphilic nature of the drug and its high molecular weight.

In a study by Melo and collaborators [183], negatively surface charged Eudragit RL100 NPs coated with hyaluronic acid (<200 nm) allowed controlled and continuous release of amphotericin B in buffer pH 5.5 during 96 h following zero order kinetic profile. The released therapeutic doses of amphotericin B efficiently inhibited growth of *C. albicans in vitro* and permitted rapid and complete yeast elimination in *in vivo* VVC model (rats). Presence of hyaluronic acid on the nanoparticles' surface facilitated interaction with CD44 receptor in the membrane of vaginal epithelial cells permitting internalization by the cells *via* receptor-mediated endocytosis.

Amaral et al. [184] confirmed superior *in vivo* anti-Candida activity of miconazole-loaded chitosan nanoparticles (CNPs) in VVC murine model in comparison to conventional miconazole cream. However, the amount of the drug used in the chitosan-based NPs was almost seven-fold lower than in the conventional cream formulation. Moreover, cytokine production assessed by monitoring the levels of tumor necrosis factor alpha (TNF-α), and interleukins 10 (IL-10) and 12 (IL-12) demonstrated significant decrease for both empty and miconazole-loaded NPs, suggesting that CPNs-mediated cytokines suppression might be mechanism responsible for potentiating antifungal activity.

CPNs have also been studied as a carrier for thiosemicarbazide, a synthetic compound with multiple activities including antifungal activity. *In vivo* evaluation in VVC murine model showed similar efficacy of both free and chitosan-loaded thiosemicarbazide against *C. albicans*. However, histopathological evaluation displayed the absence or mild inflammation in the group treated with the thiosemicarbazide NPs, while treatment with the free drug revealed reduced inflammation in comparison to control [185].

Ravani et al. [186] demonstrated potential of clotrimazole-loaded nanostructured lipid carriers (NLCs) embedded in thermosensitive hydrogel for the local VVC therapy. Pluronic-based favorably affected *ex vivo* localization of the drug in vaginal tissue with increased *in vitro* antifungal activity. The authors postulated that the increased activity was caused by the hydrogel facilitating intimate contact of the drug-loaded NLCs with *C. albicans*. The hydrogel beneficially affected the HeLa cell viability, too.

Solid lipid nanoparticles (SLNs) have also been exploited for topical VVC therapy. SNLs-based Carbopol 940 gel provided sustained release of luliconazole with suitable anti-Candida activity *in vitro*. Physicochemical assessment confirmed the stability of the formulation during two months storage at room conditions [187].

In study by Nematpour and collaborators [188], anti-Candida properties of mucoadhesive clotrimazole-loaded nanofibers were compared to vaginal films. Although both formulations provided complete release of clotrimazole in 60 min, the nanofiber formulation exhibited greater mucoadhesiveness and stronger anti-fungal effect as compared to the vaginal film. On the contrary, PLGA nanofibers permitted delivery of therapeutic doses of amphotericin B over 8 days enabling effective *in vitro* antifungal activity; the single treatment with fibers eliminated *in vivo* vaginal fungal burden in the murine VVC model after 3 days [189].

Application of herbal- and seaweed-based medicines presents an alternative approach for the treatment of vaginitis as many of them exhibit multiple favorable activities including antimicrobial, anti-oxidative and anti-inflammatory. However, the challenge associated with their application lies in their poor solubility and bioavailability, which could be overcome by nanotechnology approach. In a very recent study clove essential oil was entrapped in nanoemulsion-based emulgel for the possible VVC treatment. Physico-chemical properties of the nanoemulsion contributed to its stability during 12 weeks storage. *In vitro* antifungal studies revealed lower activity of nanoemulsion compared to the free clove oil because lower liposolubility of nanoemulsion reflected to inferior penetration into the lipid membranes of the yeast. However, superior anti-oxidative activity of the nanoemulsion plays an important role in suppressing inflammation caused by *C. albicans* infection [190].

Arumugam and Rajendran [191] evaluated anti-Candida activity of callophycin A (seaweed derived metabolite) loaded in both CNPs and spicules from marine sponges. *In vitro* studies demonstrated better loading of callophycin A in CNPs and subsequently stronger activity against *C. albicans* than callophycin A-conjugated spicules, wherein the antifungal efficacy was almost comparable with 1% clotrimazole cream. *In vivo* studies performed on *C. albicans* animal model showed a significant reduction in the fugal burden of vaginal lavage by chitosan-based nanoformulation and protective effect to vaginal mucosa in infected animals.

Pronounced antifungal activity of chitosan has also been confirmed in a study by dos Santos et al. [174]. Chitosan hydrogel embedding nanoemulsion of *Peargonium graveolens* essential oil provided strong antifungal activity against *Candida* spp., with MIC values almost 64-fold lower than the MICs s for nanoemulsion of *P. graveolens* essential oil. Good mucoadhesiveness of chitosan hydrogel enabled prolonged retention of *P. graveolens* nanoemulsion on the vaginal mucosa and decreased its irritation score.

Interesting approach to treat candidiasis was recently proposed by Giordani et al. [26]. VVC is known to cause itching, pain and inflammation at vaginal site. The authors utilized antimicrobial polyphenols for multitargeted therapy, namely novel liposomes for simultaneous delivery of two polyphenols (quercetin and gallic acid) acted in synergy to eradicate infection while alleviating the symptoms of VVC. Quercetin was selected for its anti-itching and anti-inflammatory properties, while gallic acid for its anti-Candida activity. Liposomes bearing both polyphenols considerably reduced *C. albicans* growth *in vitro*.

4.7. Nanomedicine for the treatment of viral genital infections

4.7.1. Nanomedicine for the treatment of genital herpes

4.7.1.1. General considerations. Genital herpes is a common sexually transmitted disease, affecting 491 million persons worldwide [192]. The infection is caused by herpes simplex virus-2 (HSV-2), often leading to lifelong infection and periodic reactivations. The clinical signs of infection encompass single or clustered vesicles on the perineum, genitalia, upper thighs, buttocks, or perianal areas that ulcerate before resolving. The subsequent incidences are affected by reactivation of latent virus; and are usually milder [193]. The HSV-2 infection increases the risk of infections by HIV-1, as well as chlamydia and syphilis [193–195]. It is currently considered to be incurable disease, because of a lack of available vaccine, as well as the drugs that are fully effective in virus eradication or prevention of lifelong latency. Namely, HSV-2 effectively escapes the host immune system, remains latent in neurons, infects new individuals and cause multiple complications, such as aseptic meningitis, encephalitis, hepatitis, neonatal infection, pelvic inflammatory disease and pneumonitis. Neonatal herpes presents a substantial risk for morbidity and mortality in neonates, thereof efficient management of the infection in pregnant women is urgently required [196]. Therapy is commonly comprising oral nucleoside analogues such as acyclovir, famciclovir or valacyclovir, the drugs characterized by low bioavailability and numerous side effects. Moreover, their frequent and long-term clinical use results in development of drug-resistant strains [193]. Therefore, there is an urgent need for development of new topical therapeutic platforms enabling efficient antiviral therapy.

4.7.1.2. Choice of nanoformulation. Various nanotechnology strategies have been examined for prevention and eradication of HSV-2 infections. For instance, zinc oxide tetrapod micro-nanostructures were designed to prevent attachment of HSV-2 to the cells and neutralize the virions. When infected with HSV-2 virions that were pre-incubated with the nanostructures, human vaginal epithelial and cervical HeLa cells, exhibited significantly reduced infectivity due to ability of tetrapods to bind the HSV-2 virions. Additional increase of the antiviral activity was obtained following illumination of zinc oxide tetrapods with UV light [197]. In a study by Halder et al. [198] monodispersed gallic acid-stabilized gold nanoparticles (7 nm) strongly inhibited HSV-2 infection by preventing viral attachment and penetration into the Vero cells. Consequently, the EC_{50} was 38 μM in comparison to acyclovir (13 μM), while the cytotoxicity decreased. Following green synthesis approach, tannic acid, a plant-derived antiviral compound, was used as a functionalizing ligand in preparation of silver nanoparticles (AgNPs). Such antiviral nanoformulation exhibited multi-targeted antiviral mechanism that included blocking the viral attachment, preventing its entrance in the cells, and stimulation of antiviral cytokine and chemokine production. The HSV-2 infected mice, which were intravaginally treated with tannic acid-modified AgNPs (33 nm), demonstrated better clinical scores and lower virus titers in the vaginal tissues shortly after the treatment. Following recurrent infection, the vaginal tissues treated with the nanoformulation displayed a significant improvement of anti-HSV-2 immune responses by stimulating B cells' activation and plasma cells' homing. It was assumed that the developed NPs assisted in transfer of HSV antigens to draining lymph nodes, where naïve B cells can recognize and activate long-term anti-HSV-2 memory [199].

To enhance poor bioavailability of acyclovir several research groups suggested its encapsulation in liposomes, polymeric NPs and microemulsions. Encapsulation of acyclovir in propylene glycol liposomes increased its solubility, while the surface charge of the vesicles affected their stability at simulated vaginal conditions (VFS with addition of mucin, pH 4.5). The positively charged (cationic) liposomes were the most stable, probably because of their interaction with the negatively charged mucin, protecting the cationic liposomes from unfavorable low pH and vaginal fluid components. Incorporation of the acyclovir liposomes in the

mucoadhesive Carbopol 974P NF gel enabled retention at vaginal mucosa, preserved their original size distribution and additionally sustained release of acyclovir [65]. Ramyadevi et al. [200] developed thermosensitive gel incorporating cationic polymeric NPs that permitted controlled release of therapeutic doses of acyclovir. The formulation improved drug permeability through vaginal mucosa and extended its release. *In vivo* studies in the rat model, confirmed maintenance of the average therapeutic drug level for 24 h and 2–3-fold better tissue distribution in comparison to that obtained with the free drug. Significant enhancement in the mean residence time of the drug was obtained with a 2-fold increase in the relative bioavailability compared to that of the free acyclovir.

Due to the high ratio of surfactants in their composition, microemulsions can enhance the drug solubility and subsequently its bioavailability. In a study by Deshkar et al. [201], acyclovir was formulated in microemulsion-based thermosensitive gel. The optimized formulation exhibited acidic pH, enabled sustained drug release during 8 h, and provided shear-thinning behavior with the gelation temperature in the range 30–35 °C.

Neutral mucus-penetrating NPs (MP-NPs), are of particular importance for advanced drug delivery into the deepest layers of vaginal mucosa. Ensign et al. [92] demonstrated that MP-NPs provided uniform distribution over the vaginal epithelium, penetrated into the deepest mucus layers, and were retained for more than 6 h. Intravaginal application of acyclovir loaded MP-NPs prior HSV-2 infection protected 53% of mice, while only 16% were protected in the group treated with acyclovir solution, even at 10-fold higher drug concentration.

Novel approaches for the management of genital herpes involve advanced delivery of siRNA molecules and hyper-branched cationic polymers exhibiting antiviral activity. Namely, siRNA molecules can lower the level of proteins encompassed in HSV-2 infection and proliferation, but their use is restricted due to poor intracellular delivery and short-term activity [202]. To overcome these limitations, Steinbach et al. [203] developed PLGA NPs incorporating siRNA permitting high siRNA loading and controlled release *in vitro*. Intravaginal administration of NPs enabled effective prevention of genital HSV-2 infections in mice, by siRNA-mediated knockdown of the host cell protein (nectin). The animals infected with a lethal dose of HSV-2, and cured with siRNA-loaded PLGA NPs, demonstrated improved survival from more than 28 days (treated animals vs 9 days untreated animals) without signs of inflammation.

Vaginal administration of branched low molecular weight polyethyleneimine (PEI) has shown prophylactic activity against genital herpes in mice [204]. When incorporated in liposomes (<200 nm) and administered at a dose of 0.2 mg PEI and 0.4 mg lipid/mouse, the increased activity towards HSV-2 infection in comparison to acyclovir has been obtained, without acute lesions or toxicity before and after infection. Reduced cellular associations of PEI liposomes induced higher antiviral effect [205].

Dendrimers have been established as outstanding microbicides. Precisely, sulphonate-terminated anionic poly(lysine) dendrimer (SPL 7013), formulated as hydrogel (VivaGel™), have been clinically confirmed to be effective in prophylaxis of HIV and HSV-2 infections [206]. Other dendrimers, such as carbosilane dendrimers, have also revealed antiviral effect [207]. For instance, Ceña-Diez and collaborators [208] have assessed *in vitro* and *in vivo* anti-HSV-2 activities of several carbosilane dendrimers in preventing vaginal and rectal HSV-2 transmissions. G2-S16, G1-S4, and G3-S16 dendrimers exhibited the strongest activity toward HSV-2 *in vitro*. They inhibited binding and internalization of HSV-2 into Vero cells; the activity was most pronounced when the dendrimers were applied one hour post HSV-2 infection. The superiority of the carbosilane dendrimers has been proven *in vivo*; vaginal or rectal application of G1-S4 or G2-S16 inhibited almost 100% HSV-2 transmission in BALB/c mice. The carbosilan-based dendrimers exhibited *in vitro* synergistic activity against HSV-2 when applied together with acyclovir and tenofovir. This was because all tested antivirals acted at different phases of viral cycle; carbosilane dendrimers at entry level, acyclovir prevented viral replication, while tenofovir inhibited HSV-2 DNA polymerase, respectively.

4.7.2. Nanomedicine for treatment of human papilloma virus

4.7.2.1. General considerations. Human papilloma virus (HPV) is a causative pathogen responsible for a significant proportion of cancers and precancerous lesions in both women and men [209]. The HPV infections are among the most common STIs; approximately 75% of society will acquire this HPV infection at least once in their lifetime [210]. HPV infection is a highly contagious infection transmitted via mucosal surfaces [211]. The efficient anti-viral therapy to treat HPV infections remains a challenge [212]. The current treatment is painful, causing serious systemic and local adverse effects, while failing to eliminate the latent viral infection [213]. siRNA-based therapy presents a novel approach for the prevention and treatment of a wide range of diseases, including STIs [214]. However, vaginal siRNA nanomedicine remains, at least up to now, a rather neglected area in comparison to conventional approach established on encapsulation of antiviral drugs, but offers a great potential, especially for the treatment of HPV-caused cervical cancer [215]. The pioneering research by Wu et al. [216] demonstrated importance of PEGylated siRNA lipoplexes to allow sustained and effective delivery of siRNA to female reproductive tract. Nevertheless, the most success in prophylaxis of HPV infections has been attained by vaccines based on virus-like particles (VLP) that gained recognition as efficient preventive measure in combating cervical cancer [217].

4.7.2.2. Choice of nanoformulation. Although prophylactic vaccines gained wider acceptance and availability, there are limited options to treat HPV-associated lesions, while there are no antiviral therapies targeting HPV infections. Foldvari and Moreland [58] introduced nanomedicines in the search for localized treatment of genital HPV infections. In this preliminary clinical study, liposomal interferon α (IFN-α) was applied to genital warts caused by HPV in two patients, however, results were inconclusive. IFN-α is approved for intralesional injection in treatment of genital warts, however, it is painful and limited to visible lesions. In a later study, Foldvari et al. [218] tested the effect of locally applied IFN-α in patients with genital warts. IFN-α was encapsulated in biphasic vesicles comprising submicron emulsion and phospholipid-based vesicles. After 2 weeks treatment, wart size decreased, and resolution of smaller lesions was confirmed [218].

The use of PEG on nanoparticle surface has shown to enable a closer contact to mucosal tissue, potentially improving the antiviral effectiveness of drugs administered locally. Jøraholmen et al. [93] used PEG as coating material to obtain mucus-penetrating liposomes for improved delivery of IFN-α. The PEGylated liposomes avoided interactions with mucin and improved IFN-α penetration through vaginal tissue.

4.7.3. Nanomedicine for the prevention of HIV infections: Importance of nanomicrobicides

4.7.3.1. General considerations. Although great efforts are being made to prevent and combat the spread of a serious COVID-19 pandemic, sexually transmitted HIV infection continues to be a global challenge having claimed nearly 33 million lives so far [219]. Following transmission, the virus remains in the mucosal tissues and spreads to the lymphoid organs, where primarily infects the CD4 + T cells over envelop glycoprotein gp 120. It causes a progressive weakening of the immune system until the patient descent into an immunodeficient state called acquired

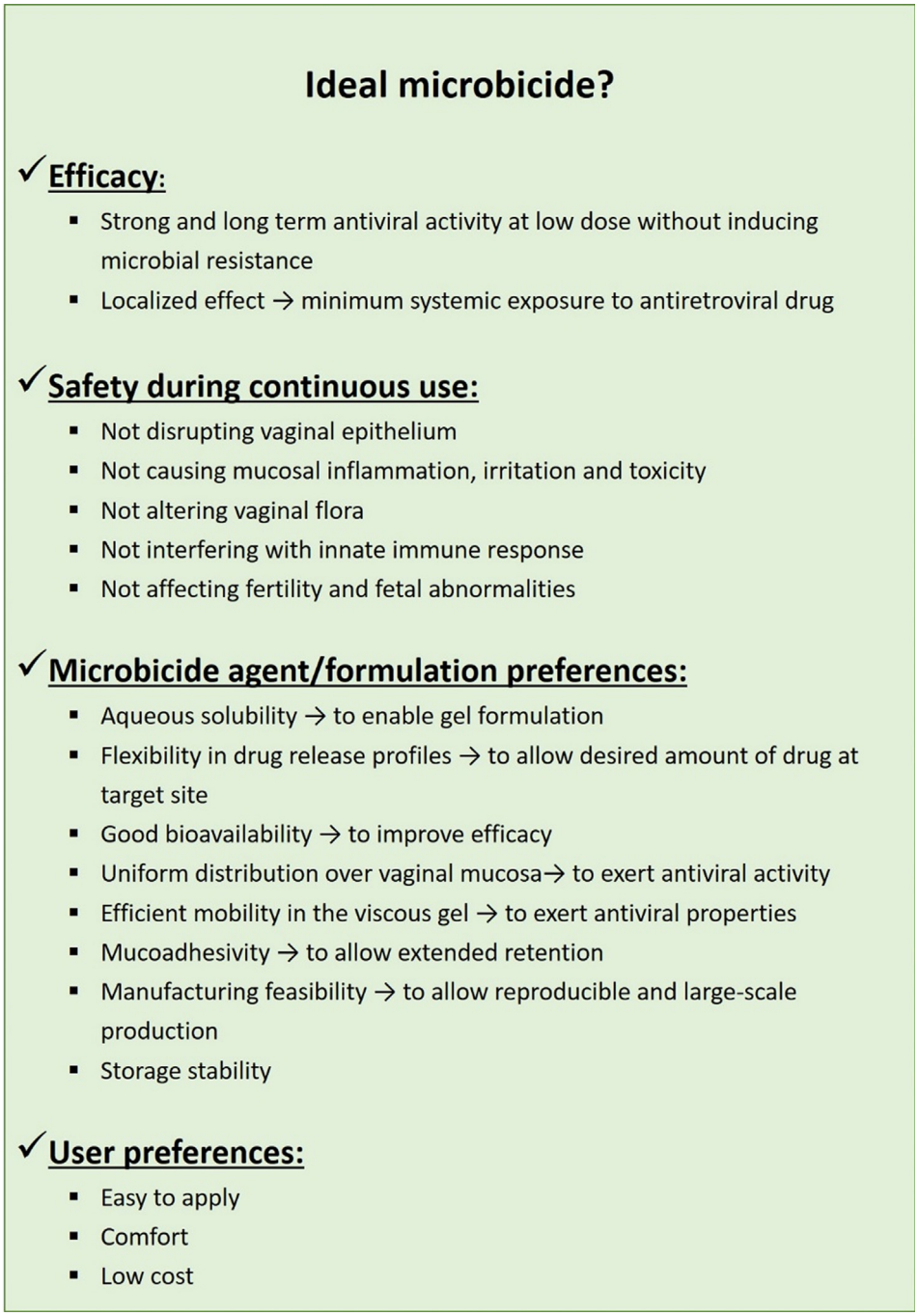


Fig. 8. Characteristics of optimal microbicide.

immunodeficiency syndrome (AIDS) in which the immune system weekens and diseases develop. The virus transmission occurs also from mother to child during pregnancy, labor and breastfeeding, and requires antiretroviral therapy of both HIV-positive mother and infant [220]. According to the WHO report for 2019, there were 38 million HIV positive people, living with HIV worldwide receiving lifelong oral antiretroviral therapy [219]. Moreover, growing drug resistance to HIV, due to the virus ability to mutate and replicate in the presence of antiretroviral drugs, significantly contribute to the therapy failure, particularly with the first-line drugs, such as efavirenz [221]. Very high levels of HIV drug resistance, up to 69%, were seen in infants born by HIV-infected mothers [222]. Unfortunately, to date, all attempts to develop anti-HIV vaccine were unsuccessful, and condoms and microbicides play a substantial role in controlling the spread of HIV.

Microbicides are substances designed as vaginal or rectal formulations to prevent or reduce the spread of STIs, especially HIV [223]. They can exhibit different mechanism of action; by increasing natural protection mechanism, formation of a physical barrier between cell and pathogens, blocking viral entrance into the cells, inactivating cell mechanisms or inhibiting viral replication [224]. Based on the mechanism of antiviral action, microbicides are classified as:

- HIV-nonspecific compounds, i.e. microbicides without antiretroviral drugs (acidifiers, surfactants, polyanions, gp120 neutralizing monoclonal antibodies), and

- HIV-specific compounds, i.e. microbicides containing antiretroviral drugs (entry inhibitors, inhibitors of viral replication or viral enzymes like maraviroc, tenofovir, dapivirine, raltegravir, sanquinavir, etc.) [223–224].

Microbicides display antiviral activity at the different sites in genital tract. For instance, viruses can be inactivated in vaginal environment by surfactants, polyanions, and monoclonal antibodies or at the mucosal level by maintenance the healthy vaginal pH with acidifiers. On the other hand, entry inhibitors can prevent internalization into CD4 + T cells when the virus crosses the vaginal epithelial layer (ibalizumab) or via blocking viral replication with viral enzyme inhibitors such as tenofovir, dapivirine, raltegravir, ritonavir, and sanquinavir [224]. The proposed characteristics of an optimal microbicide are summarized in Fig. 8.

Unfortunately, some of the perspective microbicides including nonoxynol-9 and SAVVY gel (surfactants) were withdrawn because they were ineffective in HIV prophylaxis, and contrary to expectation, enhanced the incidence of genital lesions increasing the risk for both HIV and other STIs [225–226]. Likewise, polyanion (PRO 2000 gel) and acidifier (BufferGel) exhibited little or no protective effect in preventing HIV infections although the acidifier was well tolerated by women [227]. Currently, most of the available microbicides are antiretroviral drugs; however, fully effective and safe microbicide is still not available [220].

4.7.3.2. Nanomicrobicides (microbicide-loaded nanomedicines). To overcome the limitations of conventional microbicides by improving bioavailability and biocompatibility and/or reducing their systemic exposure, research focus in last decades have been on developing nanomicrobicides. Nanotechnology allows protection of encapsulated sensitive drugs, modulation of drug release profile, enhanced intracellular/targeted drug delivery, and improved drug safety/toxicity issues, finally resulting in increased microbicide activity [228]. Particularly interesting are nanomedicines exhibiting intrinsic antiviral activity such as dendrimers and inorganic NPs, as discussed later in this subchapter.

A variety of polymeric NPs, including conventional, mucoadhesive, mucus-penetrating or stimuli responsive NPs, were studied as carriers for antiviral agents (tenofovir, dapivirine, raltegravir, griffithsin, etc), as well as nanofibers and liposomes. For instance, Meng et al. [82] prepared mucoadhesive, chitosan-based NPs to enable controlled release of tenofovir and maximize its contact with vaginal mucosa. Low encapsulation of tenofovir (6%), a limitation for effective drug delivery, has been significantly increased (20%) by addition of ethanol as a chitosan co-solvent modified the release profile and mucoadhesiveness. Continuing the research with chitosan–thioglycolic acid-conjugated NPs containing tenofovir, 5-fold higher mucoadhesiveness was obtained as compared to CNPs [229].

The ability of nanomedicines to provide stimuli responsive release is considered beneficial. Since HIV's transmission occurs via seminal fluid, which significantly changes the vaginal conditions from acidic to neutral/slightly alkaline [230], neutralization was used as a trigger in tenofovir and tenofovir disoproxil fumarate loaded pH-sensitive NPs. PLGA/Eudragit S-100 NPs facilitated bioresponsive release of encapsulated drug in the presence of semen fluid simulant (SFS, pH 7.6,) up to 70%, while about 20% of the drug was released in VFS (pH 4.2). Approximately, 50% of the drug was internalized by vaginal cells in 24 h by caveolin-mediated endocytosis. The microbicide-loaded NPs were well tolerated in human vaginal and endocervical epithelial cells, and *Lactobacillus crispatus* [231].

Stimuli responsive release of the encapsulated tenofovir was also achieved via enzyme-triggered release by hyaluronic acid-based NPs. Namely, presence of hyaluronidase in human semen was used as a target allowing triggered release of tenofovir. Almost 90% of the microbicide was released after 24 h in the presence of enzyme (pH 7.1), in comparison to 39% achieved in the absence of hyaluronidase, while *in vitro* cytotoxicity studies demonstrated biocompatibility with vaginal epithelial cells [232].

Continuing the research with tenofovir, Agrahari et al. [233] evaluated microbicidal potential of bioresponsive, mucoadhesive nanofibers. A thiolated hyaluronic acid, used to prepare tenofovir-loaded electrospun nanofibers, triggered rapid release of tenofovir within 1 h (87%) in the presence of hyaluronidase. Such release profile was considered beneficial in comparison to slow release of tenofovir from hyaluronic NPs [232]. Besides, *in vitro* anti-HIV studies proved that tenofovir nanofibers inhibited the replication of pseudo typed HIV virus and that drug activity was preserved after the electrospinning process. Following vaginal administration in mice, improved drug bioavailability in vaginal tissue was attained without significant CD45 immune cell-infiltration [233].

Despite of great promises of tenofovir-loaded nanomedicines, their main obstacle remains limited association of the drug with both hydrophobic and hydrophilic polymeric nanomedicines resulting in lower encapsulation efficiency in comparison to other antiretroviral drugs, such as dapivirine [234]. However, inclusion of hydrophobic excipients in polymeric NPs, such as stearylamine, significantly increased loading of tenofovir. To prolong the retention of the drug at mucosal tissue, the tenofovir-loaded PLGA/stearylamine NPs were incorporated into mucoadhesive film. The release of the drug occurred by biphasic pattern with 30% of tenofovir released in 30 min followed by slower release (up to 24 h). Although the cytotoxicity of the formulation was enhanced by incorporation of stearylamine, it has still remained at the levels acceptable for vaginal delivery. Following once-daily administration for 14 days (mice), the formulation confirmed its safety [235].

Dapivirine, a non-nucleoside reverse transcriptase inhibitor, is a highly potent microbicide that has been clinically investigated in various dosage forms including gel, ring and film [236]. Very recently, European Medicines Agency (EMA) approved dapivirine ring for HIV prevention in women with high HIV burden settings [237]. Since the drug is characterized by poor water solubility and low selectivity index [236], research efforts have also been directed to engineering nanomedicines that could potentiate dapivirine anti-viral activity and simultaneously reduce its cytotoxicity. Poloxamer 338 NF (PEO), sodium lauryl sulfate (SLS) and cetyltrimethylammonium bromide (CTAB) surface-modified poly (ε-caprolactone) (PCL) NPs containing dapivirine were prepared by nanoprecipitation method allowing nearly 100% association of dapivirine. All prepared NPs were in the size range 180–200 nm and permitted favorable fast release of the microbicide within 1–2 h of incubation in buffer, pH 7.4 and VFS, pH 4.2, followed by slower release rate [78]. Surface modification of the PCL NPs affected their stability during storage. Thus, negatively charged PEO-PCL and SLS-PCL NPs maintained their initial size and dapivirine content up to one-year storage at 5–40 °C. On the other hand, positively charged CTAB-PCLs aggregated in a period of 30–90 days, depending on the storage conditions. Such instability of the cationic nanoparticles has reflected to *in vitro* release profile, where approximately only 40% of dapivirine was released after one-year storage, while PEO-PCLs and SLS-PCLs release profiles remained unchanged [238]. Regardless of the surface charge, all NPs improved cellular uptake of dapivirine by several epithelial and immune cell lines, used for microbicide assessment. PEO-PCL NPs revealed enhanced antiviral activity and reduced cytotoxicity. SLS-PCL NPs demonstrated similar *in vitro* toxicity profile as free dapivirine, while CTAB-PCL NPs exhibited high cytotoxicity [78]. Further research on the interaction of the NPs with VFS containing mucin, demonstrated impact of the surface charge on the transport

through the mimicked cervicovaginal mucus. Overall, the negatively charged PEO-PCL and SLS-PCL NPs displayed subdiffusive transport, while positively charged CTAB-PCL dapivirine NPs interacted with the negatively charged mucin causing their inability to cross the mucus layer. Changing the pH of VFS from 4.2 to pH 7 reflected to NPs' diffusivity. Negatively charged NPs needed up to 1.7 h to cross the mucus layer, compared to 7 h required for CTAB-PCL NPs [239]. Surface modification of NPs also affected their permeability/retention in the tested cell monolayers (CaSki and Caco-2 cells) and pig's vaginal/rectal mucosa. Thus, PEO-PCL NPs reduced penetration of the drug through the monolayers and mucosal tissues in comparison to increased permeation attained with CTAB-PCL NPs, while SLS-PCL NPs did not affect drug permeability. Interestingly, all NPs improved accumulation of the drug in the mucosal tissues and the cell monolayers as compared to the free drug [240]. Since PEO-PCL NPs were the most promising dapivirine carrier, they were evaluated *in vivo* in a female mouse to determine their cervicovaginal distribution, pharmacokinetics and safety. Although high proportion of the NPs (70%) was rapidly eliminated due to the liquid nature of the nanoformulation, the retained NPs were distributed throughout vagina and lower uterus, crossed the mucus and penetrated the epithelial cells. Moreover, pharmacokinetic evaluation confirmed localization of dapivirine in vaginal lavages, vaginal and lower uterine tissues, while the systemic exposure decreased as compared to the free drug. Finally, during once-daily application for 14 consecutive days, dapivirine-loaded PEO-PCL NPs were found to be safe [241].

The aforementioned studies clearly indicate that the size and surface properties have an important role in nanomicrobicides delivery. Generally, the NPs ranging between 100 and 500 nm allow deeper penetration into the mucus to reach the epithelial cells. They should be insensitive to dilution and pH rising upon ejaculation [47], and finally, be made of biocompatible materials assuring safe use over longer period.

Following this approach, das Neves et al. [242] evaluated dapivirine-loaded PLGA NPs. Compared to the previously reported PCL NPs [78], lower dapivirine encapsulation was obtained in 170 nm-sized PLGA NPs (up to 80%) because of the lesser hydrophobicity of PLGA, restricting complete association of the drug with the polymer. Subsequently burst dapivirine release occurred in 4 h [242] in comparison to the release from PCL NPs in 1–2 h [78]. Presence of mucin significantly reduced the transport of dapivirine across HEC-1-A and CaSki cell monolayers both as NPs and free dapivirine, but the drug retention in the monolayers was enhanced for NPs [242].

In addition to single drug encapsulation, nanoformulations allow loading of two or more microbicidal compounds in the same nanoformulation. For instance, Yang et al. [243] designed PLGA NPs containing two antiretroviral agents, griffithsin and dapivirine, with the aim of providing sufficient and long-term HIV prophylaxis via targeting the fusion with CD4 + T cells (griffithsin) and transcription phase of HIV replication (dapivirine). Both microbicides were efficiently encapsulated in PLGA NPs (180–200 nm), either individually or simultaneously (40–45% griffithsin, 70% dapivirine), and were biocompatible with the *in vitro* model of cervical cells maintaining anti-HIV activity. The application of the microbicides at ratio 1:1 of IC_{50} values resulted in strong synergistic activity for both free and PLGA-loaded microbicides. Opposite to the findings by das Neves' group, where dapivirine was released in a few hours, Yang et al. [243] demonstrated slow release of both microbicides over 7 days (in buffers pH 7.4 and 4.5), suggesting reduced dosing frequency to a weekly-based regimen. However, no studies have been performed in the presence of the simulated mucus layer, known to significantly affect the delivery of both free drug and NPs-based drug.

Co-administration of several antiretroviral drugs offers virus targeting via different mechanism of action. Moreover, nanomicrobicides offer reduction of the required dose, improved localized effect, safety and possibly, reduced dosage frequency.

In a very recent study, Minooei et al. [244] evaluated synergistic activity of oxidation-resistant variant of griffithsin (Q-GRFT) with several antiretroviral drugs (tenofovir, raltegravir and dapivirine), differing from each other in solubility and mechanism of antiviral activity. Each of the drug was sufficiently encapsulated in PLGA NPs (ranging from 15% for tenofovir up to 100% for raltegravir), which facilitated slow release of the individual drugs in VFS (pH 4.5) during 14 days. All drug-loaded NPs were well tolerated by VK2, Ect1 and Ed1/E6E7l cells up to 72 h, with exception of dapivirine NPs (48 h), probably due to the faster release of the drug contributing its cytotoxicity. Co-administration of free Q-GRFT with each free antiretroviral drug led to strong synergistic interactions, relative to each agent alone. Moreover, when the different drug-loaded PLGA NPs were co-administered with the Q-GRFT-loaded NPs, synergy across all nanoformulations was achieved, with the most potent interactions between nanoparticle-loaded Q-GRFT and dapivirine. Embedding of the Q-GRFT-loaded NPs and dapivirine-loaded NPs in PEO-PCL-multilayered nanofibers resulted in burst release of dapivirine that can provide rapid HIV protection, followed by more sustained release of Q-GRFT for long-term prophylaxis and treatment.

There are several other studies exploring potential use of nanomicrobicides based on the combination of antiviral agents (Table 1).

Cunha-Reis and collaborators [245] prepared tenofovir- and efavirenz-loaded PLGA NPs embedded into fast dissolving film facilitating prolonged retention of the nanomicrobicides at vaginal lavages and tissue *in vivo*. Although local concentrations for both drugs rapidly declined during time, association between the NPs and film improved the pharmacokinetic profile of efavirenz especially in the first 2 h after formulation administration. In another study, co-administration of efavirenz with raltegravir via PLGA NPs resulted in lower 90% effective concentration (EC_{90}) values than solution of both drugs; however, the difference between the drugs-in-NPs and solution was not significant. The NPs were not cytotoxic to HeLa cells during 14 days, and the intracellular concentrations of efavirenz were above EC_{90} for 14 days, while raltegravir intracellular concentrations were eliminated in 6 days. Incorporation into thermosensitive gel facilitated retention of the NPs at the cervicovaginal tissue, permitting transport of the NPs in the HeLa cells [81].

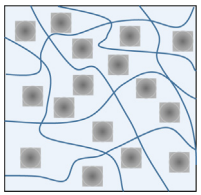
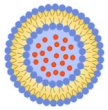
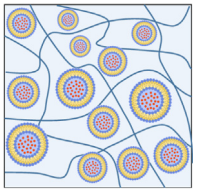
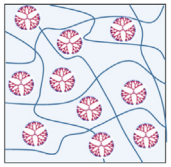
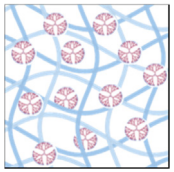
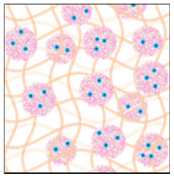
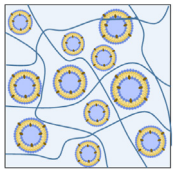
Contrary to all above reported studies on the drug-loaded conventional NPs embedded in a mucoadhesive vehicle, Krogstad et al. [246] designed mucus-penetrating NPs incorporated in mucoadhesive nanofibers. Such complex mucus-penetrating/mucoadhesive delivery system enabled enhanced retention of the nanomicrobicide in the genital tract by nanofibers and the fast diffusion of the efavirenz-loaded PEGylated PLGA NPs through the mucus layer. The nanofibers have significantly enhanced retention of the drug-loaded NPs *in vivo* to at least 3 days. In addition, pharmacokinetic showed extremely high efavirenz concentrations in cervicovaginal lavage (7 days) and 2-fold higher drug concentrations in vaginal tissue in comparison to suspension of the NPs, while systemic exposure to efavirenz was very low.

Development of siRNA-based vaginal microbicides presents a perspective approach for the HIV prophylaxis. To achieve improved delivery of siRNA across the cervicovaginal mucus, Gu et al. [247] engineered mucus-penetrating PLGA-PEG NPs and polyethylenimine (PEI)/siRNA complexes, functionalized with anti-HLA-DR antibody (siRNA-NP-Ab). The NPs were embedded in intravaginal film to allow retention at the cervicovaginal mucosa. Importantly,

Table 1
Overview of the representative nanomedicine-based formulations for the prevention or treatment of STDs.

Targeted microorganism	Nanomedicine (primary vehicle)	Formulation (secondary vehicle)	Active ingredient(s)	Outcome	Reference
HIV	*Polymeric nanoparticles*	Film	*Tenofovir*	Retention of the nanoparticles at vaginal mucosa; suitable release profile for HIV prophylaxis; confirmed safety (*in vivo*).	Machado et al., 2016 [235]
			Tenofovir, *efavirenz*	Enhanced retention of the nanomicrobicides in vaginal lavages and tissue *in vivo*; improved pharmacokinetic for efavirenz.	Cunha-Reis et al., 2016 [245]
		Nanofibers (multilayered)	*Dapivirine*, *Q-GRFT*	Rapid initial release of Q-GRFT and dapivirine, followed by slow release of Q-GRFT; retention of the microbicides in vaginal cavity; reduced cytotoxicity *in vitro*.	Minooei et al., 2021 [244]
		Gel (thermosensitive)	*Raltegravir*, *efavirenz*	Improved retention and localization of the nanoparticles at cervicovaginal tissue; sustained release and intracellular delivery of the drugs.	Date et al., 2012 [81]
	MP – polymeric nanoparticles	Nanofibers (mucoadhesive)	*Etavirine*	Significantly prolonged retention time for nanomicrobicide *in vivo* (at least 3 days); rapid transport of the nanoparticles over mucosa; long-lasting antiviral effect (up to 7 days).	Krogstad et al., 2017 [246]
	MP – polymeric nanoparticles (active targeting)	Film	*siRNA*	Rapid release of the siRNA-loaded nanoparticles, diffusion through the epithelial layer and uptake by KG-1-cells; improved targeting activity and significantly increased knockdown of SNAP-23 mRNA and protein compared with the nanoparticles without antibody conjugation.	Gu et al., (2015) [247]

Table 1 (*continued*)

Targeted microorganism	Nanomedicine (primary vehicle)	Formulation (secondary vehicle)	Active ingredient(s)	Outcome	Reference
	Silver nanoparticles 	Gel (mucoadhesive) 	-	Retention of the nanoparticles at cervicovaginal mucosa; rapid onset of anti-HIV activity; confirmed *in vitro* biocompatibility.	Lara et al., 2010 [261]
	Liposomes 		*MC-1220* ● *Tenofovir disoproxil fumarate, emtricitabine*	Improved retention of the microbicide at cevicovaginal tissue; incomplete HIV prophylaxis; confirmed safety (*in vivo*). Suitable retention and release of the microbicides at vaginal mucosa; enhanced permeation of the microbicides and reduced cytotoxicity *in vitro*.	Mourtas et al., 2011 [248]; Caron et al., 2010 [249] Faria et al., 2019 [251]
	G2-S16 Dendrimer 		-	Superior anti-HIV activity in the presence of semen; confirmed biocompatibility (mouse model).	Ceña-Diez et al., 2017 [259]
HIV, HSV-2	*SPL7013 dendrimer* 	Gel (mucoadhesive) 	-	Suitable vaginal retention and release of the dendrimers; strong antiviral activity; safety in several animal models; passed Phase 1 (HIV) and Phase 2 (HSV-2) clinical trials; unsafe for continuous use in women (rejected).	Rupp et al., 2007 [253]; Notario-Pérez et al., 2017 [224]
	Polymeric nanoparticles 	Gel (thermosensitive) 	● *Acyclovir*	Increased retention and tissue distribution; improved local drug bioavailability (*in vivo*).	Ramyadevi et al., 2016 [200]
HSV-2	*Liposomes* 	Gel (mucoadhesive) 	● *Acyclovir*	Sustained drug release, affected by the surface properties of the liposomes; increased *in vitro* stability and vaginal retention.	Pavelić et al., 2005 [65]

(*continued on next page*)

Table 1 (*continued*)

Targeted microorganism	Nanomedicine (primary vehicle)	Formulation (secondary vehicle)	Active ingredient(s)	Outcome	Reference
C. trachomatis	*G2-S16 dendrimer*		-	Retention and release of the dendrimer in vaginal cavity; superior anti-HSV-2 activity.	Ceña-Diez et al., 2016 [208]
	Liposomes	Gel (mucoadhesive)	● *Resveratrol*	Chitosan gel potentiated anti-chlamydial activity of liposomal resveratrol and anti-inflammatory activity (*in vitro*).	Jøraholmen et al., 2020 [126]
	SPL7013 dendrimer		-	Not effective at preventing infections *in vivo* (primate model).	Patton et al. 2006 [140]

MP, mucus-penetrating; Q-GRFT, oxidation-resistant variant of griffithsin; siRNA, small interfering RNA; SNAP-23, synaptose-associated 23-kDa protein.

siRNA-NPs were rapidly released from the film and able to penetrate the epithelial layer to be taken up by differentiated KG-1 cells. The formulation facilitated significant knock-down of SNAP-23 gene/protein expression by active targeting strategy, without significant impact on the cell viability.

Liposomes were also examined as carriers for HIV prophylaxis. However, there are only a few studies reporting on liposomes as nanomicrobicides. In one of the first studies, hydrophobic MC-1220 was encapsulated in conventional liposomes and incorporated in mucoadhesive gel; MC-1220 was better absorbed, and well tolerated in the rabbit model [248]. Assessment of the anti-HIV efficacy in rhesus macaques, confirmed partial protection (50–60%) against HIV with lower and medium MC-1220 concentrations in liposomal gels, while the formulation with the highest microbicide concentration failed to enhance the protection level, possibly due to the low microbicide solubility affected by pH [249].

In a study by Malavia et al. [250] a challenging approach based on the incorporation of synthetic lipids in liposomes, such as cardiolipin, demonstrated that by the balancing of synthetic and natural lipids enhanced anti-HIV activity can be obtained.

Strategy of vaginal co-delivery of two microbicides in one formulation, reported for polymeric NPs, was also applied for liposomal microbicides. Thus, tenofovir disoproxil fumarate-loaded liposomes were incorporated into Carbomer gel embedding emtricitabine. [251]. Such complex formulation enabled sustained drug release profile with 60% of both drugs released within 3–6 h, where the fast initial release was obtained with emtricitabine, while tenofovir disopoxil fumarate release was slower. The drug-loaded liposomes were non-cytotoxic to HEC-1-A and CaSki cells and able to facilitate the drug permeation *in vitro*.

Alukda et al. [252] proposed utilizing polylysine–heparin functionalized SLNs with tenofovir as HIV nanomicrobicide. Despite of the low encapsulation of tenofovir in functionalized SLNs, the nanoformulation was able to enhance the cellular uptake of tenofovir.

Although many of the nanomicrobicides reported above, particularly polymeric NPs, have shown a promise in preventing HIV infections, none of them have yet reached the clinical trial. The only exceptions are dendrimers, more precisely SPL7013 dendrimer (16,581 Da; Starpharma Pty Ltd, Melbourne, Australia), which was built from a divalent core, the bezhyldrylamine amide of L-lysine [83]. SPL7013 was confirmed very effective in prophylaxis of both HIV and HSV infections [253], and very lately, against severe SARS-CoV-2 virus in a form of a nasal spray [254]. The mechanism of its intrinsic antiviral activity lies in the polyanionic surface enabling attachment of SPL7013 to gp 120 proteins, thus obstructing viral fitting to CD4 receptors of the human cells. SPL7013 was formulated at a concentration of 3% (w/w) in Carbopol gel (VivaGel™) to facilitate the dendrimers retention at cervicovaginal mucus. However, despite the great success in preclinical animal and clinical studies, summarized by Rupp et al. [253]; the adverse effects associated with the formulation led to its withdrawal from further clinical investigations [224,255,256].

The inherent antiviral properties of dendrimers are particularly valuable in terms of the emergence of resistance to conventional antiretroviral therapy. Among alternative dendrimer-based nanomicrobicides are polyanionic carbosilane G1-S4 or G2-S16 dendrimers, acting as entry inhibitors similarly as SPL7013. Moreover, G2-S16 exhibited a dual action, namely the inhibition is made on viral attachment, protecting the cell against viral infection and acting directly against the virus. In that sense, polyanionic carbosilane dendrimers, G1-S4 and G2-S16, have demonstrated the best results against HIV-1 infections [257]. However, investigation on the possible HIV resistance, demonstrated that G1-S4 treatment caused significant mutations in HIV-$1_{NL4.3}$. On the contrary, G2-S16 did not result with resistance-associated mutation, proposing

Table 2
Advantages and limitations of nanomedicines for vaginal drug delivery.

Advantages	Limitations
Ability to incorporate a variety of drugs/active compounds differing in Mw and lipophilicity	Lower loading of hydrophilic drugs
Protection of sensitive drug from vaginal environment	Storage stability
Modulation of drug release	Possible leakage of loaded drug
Improved drug pharmacokinetics/ bioavailability	Limited loading capacity for high dosing drugs
Overcoming mucus barrier	Long-term safety for human use (disruption of the vaginal epithelium, inflammations, irritations, toxicity, etc.)
Ability to deliver high drug load at the site of action	Scale up
Potential for active drug targeting	Cost
Enhanced intracellular delivery	
Reduction of drug dose required for an efficient therapeutic outcome	
Increased antimicrobial activity (reduced MIC values)	
Decreased ability of resistance development	
Compatibility and efficient mobility within the secondary vehicle	
Long-lasting drug effect	
Reduced dosing frequency	
Reduced cytotoxicity/improved biocompatibility	
Improved localized effect	

MIC, minimum inhibitory concentration.

that G2-S16 is safe as a HIV-entry inhibitor [258]. Noteworthy, G2-S16 dendrimer did not cause any irritation or inflammation in the vaginal epithelium, proving that this dendrimer is a safe vaginal nanomicrobicide [259].

Inorganic NPs have also shown promising microbicidal potential. For instance, PEGylated gold NPs inhibited the viral entry by binding with gp120 thus preventing CD4 attachment. Interestingly, their activity was even higher than activity of UC-781 (antiretroviral drug) in the fusion assay [260]. Similarly, polyvinylpyrrolidone coated AgNPs exerted anti-HIV activity by the same mechanism of action as the gold nanoparticles. When incorporated into hydrogel they enabled effective prevention of the HIV transmission. Moreover, the NPs were not cytotoxic to the explant for 48 h, and importantly; only 1 min of the NPs pretreatment was required to prevent HIV-1 transmission. This effect was lasting for 2 days, offering safe and long-lasting protection of the cervical tissue from infection [261].

We tried to provide an overview of numerous potentials nanomedicine offers for the prevention and efficient topical treatment of the vaginal, as well as genital infections. However, the challenging limitations slowing nanomedicine's path to translation, summarized in Table 2 require focused attention. It is expected that better understanding of the interplay between nanoformulation and targeted site for drug/active ingredient action might help in design of optimal nanoformulation.

5. Addressing toxicity

With the increased attention on marketed nanoformulations destined for various administration sites, the issue of potential toxicity, especially related to repeated administration, become more relevant [262]. As a mucosal site, vagina can be potentially affected by the applied formulations. Considering the localized treatment of vaginal infections, relying on the administration of antimicrobial drugs and active ingredients, it is very important to consider the restoration of normal vaginal microflora, pH, as well as avoidance of any tissue toxicity. Available literature mostly reports concerns related to nanocarriers as drug delivery systems for parenteral, pulmonary and rather recently, skin routes. Toxicity of nanoformulations repeatedly applied to vaginal site deserves more attention than currently given. Although many of the nanocarriers discussed in the review have rather impressive safety profile, it is important to address the potential safety issues, especially for formulations that are at preclinical developmental stages. The toxicity issues can range from rather mild concerns such as the buffer strength of nanoformulation to be applied vaginally that needs to be carefully addressed according to the WHO Note on osmolality. The pH deviations from the normal vaginal pH (acidic) are considered toxic. More data are available on the safety of vaginal semi-solid and solid formulations rather than nanoformulations [6,263], probably since most nanoformulations, with the exception of dendrimers, have not reached the market. More comprehensive systemic studies are needed, extending beyond classical cytotoxicity testing [6,263].

Another highly relevant issue is the concentration of nanosystems at the vaginal site. Smaller volumes of nanocarriers exposed to the cervicovaginal mucus can be safely eliminated by the natural self-cleansing mechanisms of the genital tract relying on the mucus turnover and discharge. A higher concentration of NPs might interfere with the organized mucus causing a collapse of mucin fibers [47]. Moreover, possible genital irritation and systemic toxicity related to the delivery system especially in biofilm treatment should be followed [8]. Although dendrimers are one of the most successful nanoformulation destined for vaginal site, there are concerns regarding the disruptive effect of charged dendrimers toward bio-membranes, leading to the cytotoxicity caused by the aggregation processes involved into cellular membranes [264].

Finally, serious issues such as potential immune response, need to be urgently considered; it is know that upon the exposure to vaginal products (even the conventional ones), the vaginal epithelium may respond by secreting immune mediators further enhancing the susceptibility to STIs. Most of the studies determining the potential toxicity so far relied heavily on the cell toxicity testing; even when animal studies were performed, the results are often rather difficult to directly correlate to human data [265,266]. The limited choice of reliable models able to predict the safety of nanopharmaceuticals in a robust manner remains to be considered a major drawback in the development of microbicides for HIV prevention [267].

Highly relevant for localized therapy of vaginal infections is assurance that antimicrobials applied locally do not penetrate into systemic circulation. Chen and Yang [268] demonstrated that the vaginal administration of nanosilver particles lead to absorption into blood circulation; vaginal administration caused ultrastructural changes to the vaginal mucosa, urethra and rectum, with accumulation of particles in all tissues as evidenced by ultrastructural pathological changes promoting cytoxicity. The TEM examination of the hepatic portal vein blood revealed evidences of migration for the first time. The findings are highly relevant for localized antimicrobial therapy of pregnant patients where unwanted systemic exposure could lead to toxicity both to mother as well as fetal toxicity. Moreover, systemic exposure would be contraindicated in topical therapy.

6. Conclusions and perspectives

The importance of treating vaginal infections more successfully brought novel approaches, challenged certain dogmas, as well as raised public awareness, although still not to satisfactory level. Comparing to other fields, localized treatment of vaginal infections is still explored to a limited depth. The urgency to respond to the increasing treat of AMR summarized in Fig. 9, highlights the need for novel therapy approaches.

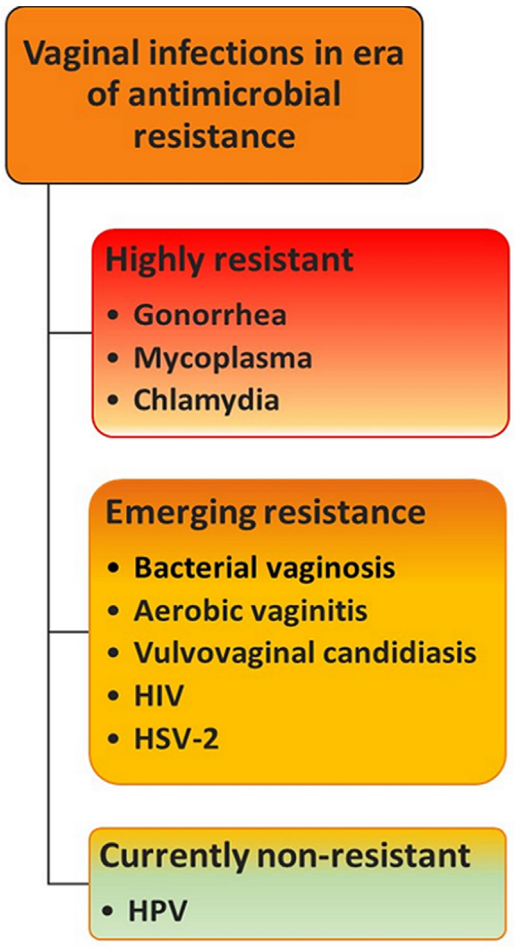


Fig. 9. Vaginal infections in the era of antimicrobial resistance.

New delivery systems presented in this review range in the origin of their building blocks, size, surface modifications and their interaction with the vaginal mucosa. It is rather challenging to summarize their features as we tried in Table 2, or even more to propose the superior nanocarrier for localized therapy of vaginal infections.

We would rather suggest that a deeper insight on the interplay between:

- Nanoparticle size
- Surface modification (mucoadhesiveness/penetration)
- Load of drug/active ingredient, alone or in combination
- Release profile of the drug/s
- Interaction/synergy with secondary vehicle used to improve system's retention at vaginal site
- Safety profile,

needs to be consider when tuning the properties of nanoformulation.

However, the optimization should also address the particular challenges the particular infection bears; some of the infections would require deeper penetration in the epithelial layer, while for others rather superficial contact between nanocarrier and microorganism will be sufficient.

From pharmaceutical perspective, the manufacturing of the nanoformulation, such as simplicity, cost and attractiveness, needs to be considered.

To optimize the formulation, there is a need for better models able to predict both qualitative and quantitative profiles of drugs delivered to vaginal site; animal infection models are very limited; mechanisms of action of some of the promising active molecules are not fully elucidated, therefore, in parallel to efforts invested in optimizing the novel nanoformulation, the development of better, more specific and robust *in silico*, *in vitro* and animal models is urgently needed.

In the era of AMR, and emerging threats of STIs resistance to current first lines of treatment, it is last moment to start seriously discussing the alternative ways to treat STIs (Fig. 9). There are different pathways which could potentially offer the "way out" of raising limitation of available antibiotics:

i) Choice of antimicrobial: novel chemical entities, natural origin, biomimetics, siRNA-based, "recycling" of antimicrobials currently not administered vaginally
ii) Choice of nanoformulations: more focus on synergy between drug and pharmaceutical ingredients with intrinsic antimicrobial properties
iii) Addressing treatment of pregnant patients
iv) Focus on user-friendliness of formulation, often neglected when designing and optimizing the formulations for vaginal site. This would enhance the patient compliance.

Finally, addressing the road map to translation, why are there no more products in clinical trials, reaching the market? What should be done? The barriers to translation are multiple and rather hard to address. They range from limited understanding of the infection targets, not yet smart enough approaches in designing the targeted delivery systems able to bring the molecules to pathogen in an optimal mode, availability of *in vitro*, *in vivo* models to optimize formulation, better correlation between limited animal and human models, more specific toxicity studies, especially regarding pregnant patients. However, the societal challenges such as awareness of increasing prevalence of STIs both in developing and developed countries need to be address. Finally, addressing a rather limited focus of health care providers regarding woman health as well as seeking more attention from pharmaceutical companies, are the barriers we need to focus on in parallel to scientific efforts.

Declaration of Competing Interest

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