Sveučilište u Zagrebu

Farmaceutsko-biokemijski fakultet

Sabina Keser

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DOKTORSKI RAD

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Mentor: prof. dr. sc. Željka Vanić

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VESICULAR PHOSPHOLIPID GELS FOR LOCALIZED SKIN DRUG DELIVERY

DOCTORAL DISSERTATION

Supervisor: Prof. Željka Vanić, Ph.D.

Zagreb, 2025

Doktorski rad je predan na ocjenu Fakultetskom vijeću Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta radi stjecanja akademskog stupnja doktora znanosti iz područja biomedicine i zdravstva, polje farmacija, grana farmacija.

Rad je izrađen u Zavodu za farmaceutsku tehnologiju Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta pod mentorstvom prof. dr. sc. Željke Vanić. Dio eksperimenata proveden je u Zavodu za biokemiju i molekularnu biologiju Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

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SAŽETAK

Učinkovito lokalno liječenje bolesti i ozljeda kože, koje su nerijetko praćene infekcijama, od velikog je medicinskog značaja. Svrha ovog rada bila je razviti biokompatibilan i učinkovit terapijski nanosustav za dermalnu primjenu, baziran na fosfolipidima, koži-sličnim tvarima. Riječ je o visokokoncentriranoj liposomskoj disperziji - vezikularnom fosfolipidnom gelu (VFG), koji bi zbog svoje polučvrste konzistencije omogućio dostavu visoke koncentracije lijeka na oboljelo mjesto, a integriranjem koži-sličnih sastojaka formulacije u kožu ili onih koji potiču zacjeljivanje, omogućila bi se njezina brža regeneracija. Istraživanja su obuhvatila razvoj i optimizaciju industrijski prikladne metode priprave VFG-ova s hidrofilnim modelnim lijekom (ciprofloksacinklorid, CPF), karakterizaciju CPF-VFG-ova u pogledu fizičko-kemijskih i reoloških svojstava, simulirane *in vivo* i fizičke stabilnosti, bioadhezivnosti, biokompatibilnosti, *in vitro* antimikrobne aktivnosti i *in vitro* učinka zacjeljivanja ozlijeđene kože. Rezultati provedenih ispitivanja su pokazali utjecaj fluidnosti (fosfo)lipidnih dvoslojeva i viskoznosti CPF-VFG-ova na profil oslobađanja CPF-a i njegovu permeabilnost u kožu. Neovisno o sastavu, svi CPF-VFG-ovi su bili kompatibilni sa stanicama keratinocita te su učinkovito inhibirali rast patogenih bakterija, čestih uzročnika infekcija kože i mekih tkiva. Antibakterijska aktivnost svih CPF-VFG-ova u prevenciji razvoja biofilmova *Pseudomonas aeruginosa* i meticilin-rezistentnog *Staphylococcus aureus* (MRSA) kliničkog izolata je bila dva puta veća od aktivnosti CPF otopine. CPF-VFG-ovi s hidrogeniranim fosfolipidima i kitozanom su se pokazali optimalnim nanoformulacijama zbog snažnog proliferativnog učinka na keratinocite, brzog zacjeljivanja ozlijeđenog epidermisa *in vitro* i lokalizacije lijeka na/u koži. Pritom bi CPF-VFG s kitozanom bio prikladan za liječenje površinskih infekcija s kompromitiranim epidermisom, dok bi CPF-VFG s hidrogeniranim fosfolipidima bio poželjan za liječenje dubljih infekcija kože. Njihovo dobro zadržavanje na površini koži te produljeno oslobađanje CPF-a tijekom duljeg vremena u koncentracijama koje značajno premašuju minimalne biofilm inhibitorne koncentracije CPF-a na patogene bakterije na mjestu infekcije, rezultiralo bi učinkovitom lokalnom terapijom i prevencijom razvoja kroničnih infekcija kože. Time bi se značajno unaprijedila postojeća lokalna antimikrobna terapija, a dodatno bi se smanjila učestalost doziranja, što je posebno važno kod tretiranja bolnih područja ozlijeđene kože.

Ključne riječi: vezikularni fosfolipidni gel, liposomi, ciprofloksacinklorid, kitozan, dermalna primjena lijeka, infekcija, biokompatibilnost, zacjeljivanje rana

SUMMARY

Introduction: Dermal drug delivery is a simple, non-invasive, well-tolerated and patient-acceptable route of drug administration that allows its action on a localized area of the skin. However, conventional dermal formulations, such as crems, ointments and hydrogels, are not always effective in assuring sufficient drug concentration at the diseased site in the skin. These could be caused by unfavorable physicochemical properties of drug as well as vehicle, resulting in inappropriate drug release and/or limited drug localization within the skin. Moreover, the nature of skin disease, characterized by thickened or damaged superficial skin layers, frequently associated with infections, also contributes to the therapeutic outcome. When the drug fails to penetrate the *stratum corneum*, it cannot accumulate in a sufficient amount within the skin, at the site of action. This will result in a weak or no therapeutic effect, while in the case of compromised epidermis, unfavorable systemic absorption may occur. The situation is even worse in the case of bacterial infections where ineffective topical antimicrobial therapy would promote the growth of biofilms, the treatment of which require oral administration of high doses of antibiotics, thus enhancing the risk of antimicrobial resistance. Therefore, the importance of developing therapeutically effective dermal drug delivery systems that could enable controlled drug delivery to the targeted skin area is highly appreciated. Various drug nanoformulations have been studied for advanced topical dermatotherapy. Due to their phospholipid origin and similarity with skin structures, liposomes are particularly attractive. Namely, specific structure of liposomes enables encapsulation of different drugs, which can be released in a sustained and controllable manner, while membrane fluidity regulates their skin penetration potential. The efficacy of liposomes in dermal drug delivery can be hampered by low encapsulation of hydrophilic drugs and liquid nature of nanoformulation causing leakage of liposomes from the site of application. Incorporation of liposomes into a hydrogel is a common approach for increasing their viscosity and skin retention. However, this strategy could be a limitation for administration of drugs that need to be applied in a larger dose. Another challenge is the preparation of liposomes, which is restricted by phospholipid concentration (< 40 mg/mL). This could be a problem especially for encapsulation of hydrophilic drugs, known for being poorly encapsulated in liposomes. These deficiencies can be overcome by vesicular phospholipid gel (VPG), a highly concentrated phospholipid dispersion in water of semisolid consistency. VPG is composed of tightly packed liposomes with very low portion of outer water phase between the neighboring liposomes, resulting in high and stable drug load. Up to now, VPGs have not been studied *via* cutaneous route of drug administration.

Hence, the aim of this thesis was to develop and evaluate the potential of using VPG for improved dermal drug delivery. It was hypothesized that VPG would facilitate the delivery of a high concentration of the drug to the affected skin area, whereby the integration of skin-like ingredients of the VPG into the skin, or those that promote healing effect, would lead to faster skin regeneration. Ciprofloxacin hydrochloride (CPX) was selected as a model hydrophilic drug for encapsulation into VPGs. It has not yet been examined in VPGs, nor has it been explored in the form of liposomes for dermal application.

Several CPX-VPGs varying in composition were prepared and evaluated in terms of their physicochemical and rheological properties, *in vitro* drug release profile and permeation into the skin, physical and simulated *in vivo* stabilities, bioadhesiveness, *in vitro* antibacterial activity, biocompatibility and wound healing potential.

Methods: CPX-VPGs, which differed from each other in (phospho)lipid composition, presence of chitosan and propylene glycol, were prepared by high-pressure homogenization method. They were converted to CPX-VPG liposomes and characterized for morphology, size (average diameter, polydispersity index), zeta potential, bilayer fluidity and entrapment of CPX. Semisolid CPX-VPGs were subjected to rheological testing (viscosity and oscillatory measurements), assessment of bioadhesiveness on porcine skin (*ex vivo*) and *in vitro* release in two media simulating the conditions of healthy and infected skin. Stability of CPX-VPG liposomes under conditions simulating *in vivo* biological environment, was examined by determining the size, surface charge and content of the originally encapsulated CPX after their mixing and incubation in simulated wound fluid. Moreover, all CPX-VPGs were subjected to storage stability evaluation. For that purpose, oscillatory frequency rheometric studies were performed immediately after preparation of CPX-VPGs and after 2 months of storage at 4 °C. In addition, physical stability assessment of CPX-VPGs involved measurements of average diameters, polydispersity indexes and zeta potentials of the corresponding liposomes for 6-months storage period of CPX-VPGs at 4 °C. Antibacterial potential of all CPX-VPGs was explored against three different bacterial strains typical for skin and soft tissue infections: *Staphylococcus aureus*, *Pseudomonas aeruginosa* and methicillin-resistant *S. aureus* (MRSA). The CPX-VPG liposomes were challenged against both planktonic and biofilm-forming strains. Biocompatibility of all the nanoformulations was assessed *in vitro* on a human skin keratinocytes monolayer (HaCaT), while *in vitro* wound healing potential of the selected CPX-VPG liposomes was determined using the scratch test. Finally, the most perspective CPX-VPGs

were evaluated for their skin permeability on a full thickness porcine ear skin model employing automated Franz diffusion cells system.

Results: CPX-VPGs were successfully prepared using the high-pressure homogenization method in one cycle at 500 bars. Hydrophilic model drug (CPX) was entirely incorporated into all VPGs, which differed from each other in (phospho)lipid composition, polymer or co-solvent. All CPX-VPGs were of suitable viscosity for dermal use and consisted of unilamellar liposomes with mean diameters less than 200 nm. The manufacturing process was completely environmentally friendly and suitable for industrial production due to its simplicity, robustness and reproducibility. The composition of CPX-VPGs had a significant impact on the physicochemical properties of CPX-VPG liposomes (size, surface charge, bilayer fluidity), encapsulation efficiency, stability under simulated *in vivo* application conditions, storage stability, *in vitro* drug release profile and skin permeability, bioadhesiveness, biocompatibility, and *in vitro* wound healing effect. The fluidity of the CPX-VPGs' bilayers affected the size of liposomes and the amount of liposomally-encapsulated CPX. Bilayers' rigidity was reflected in the increase of liposomal diameters and hence, CPX encapsulation. The viscosity of CPX-VPGs was primarily determined by the concentration of the main ingredient, soybean phospholipids (SPC). All CPX-VPGs were characterized by pseudoplastic properties, a linear viscoelastic region, and a viscoelastic solid structure that remained unchanged even after a 2-months storage period. The composition of CPX-VPGs had a significant effect on their bioadhesiveness, while viscosity had no effect. The highest detachment force from the skin surface and the work of adhesion were obtained with the low-viscous SPC/P90H/CPX VPG containing hydrogenated phospholipids, while the viscous CPX-VPG incorporating chitosan (SPC/CHIT/CPX VPG) was slightly less bioadhesive. The prolonged and controlled release of CPX was achieved from all the CPX-VPGs and fit the Korsmeyer-Peppas model. Viscosity and bilayers' fluidity (rigidity/elasticity) of CPX-VPGs had a predominant influence on the release rate. The least viscous CPX-VPG (SPC/P90H/CPX VPG) enabled an initially faster drug release, followed by a slow CPX release from the corresponding liposomes characterized by extremely rigid bilayers. Such a release profile is suitable for the rapid onset of the drug action and maintenance of the therapeutic drug concentrations over an extended period. Composition of CPX-VPGs influenced physical stability of CPX-VPG liposomes during storage. The presence of chitosan or propylene glycol within the CPX-VPGs had a favorable effect on preserving the original size of liposomes for 3 months. Under simulated *in vivo* conditions mean diameters of all CPX-VPG liposomes significantly enlarged; however, they were still suitable for topical dermal delivery

(< 300 nm). Interestingly, the interaction of CPX-VPG liposomes with albumin from simulated wound fluid did not cause the release of the originally encapsulated drug.

Encapsulation of CPX into VPGs did not reduce the antibacterial effect of CPX. Moreover, the activity of the drug was even increased against planktonic *P. aeruginosa*, while against *S. aureus* and MRSA clinical isolate, all CPX-VPG liposomes retained the same antibacterial effect as CPX solution. The effectiveness of CPX-VPGs has also been confirmed in the inhibition of biofilm formation. Namely, all CPX-VPGs were twice as effective as CPX solution in preventing growth of *P. aeruginosa* and MRSA biofilms. The performed *in vitro* biocompatibility studies proved that CPX-VPGs did not exhibit cytotoxic effects on the HaCaT cells. On the contrary, they were completely biocompatible with the keratinocytes, where those embedding hydrogenated phospholipids and chitosan demonstrated a strong proliferative effect. The superiority of SPC/CHIT/CPX- and SPC/P90H/CPX-VPG liposomes was confirmed by an *in vitro* keratinocyte migration test (scratch test), with both CPX nanoformulations showing an exceptionally strong epithelialization activity.

SPC/CPX-, SPC/CHIT/CPX- and SPC/P90H/CPX-VPGs significantly reduced penetration of CPX through the skin compared to the CPX solution, thus permitting the drug localization on/within the skin. Viscosity, bioadhesiveness and rigidity of CPX-VPGs' bilayers were responsible for controlling CPX penetration and localization within the skin. Thus, the application of the least viscous and extremely bioadhesive SPC/P90H/CPX VPG with the most rigid bilayers resulted in the highest level of the accumulated drug within the skin, while the opposite effect was achieved with the viscous and bioadhesive SPC/CHIT/CPX VPG of moderately rigid bilayers. The most viscous and less bioadhesive SPC/CPX VPG with the least rigid bilayers allowed better accumulation of CPX within the skin than SPC/CHIT/CPX VPG, but higher retention of CPX on the skin surface than SPC/P90H/CPX VPG.

Conclusions: The research conducted within this doctoral thesis represents the first study in which VPGs were proposed and successfully developed for improved dermal drug delivery. CPX-VPGs with hydrogenated phospholipids and chitosan have proved to be optimal nanoformulations due to their biocompatibility, superior proliferative effects on keratinocytes, rapid healing of injured epidermis (*in vitro*) and localization of the drug on/within the skin. Their good retention on the skin surface and prolonged CPX release over a longer period in concentrations significantly exceeding the minimum biofilm inhibitory concentration of CPX against pathogenic bacteria at the site of infection, would result in effective local treatment of skin infections and prevention the development of chronic infections. This would significantly

improve topical dermal therapy, and further reduce the frequency of dosing, which is especially important when treating painful areas of injured skin. CPX-VPG with chitosan would be suitable for the treatment of superficial infections with compromised epidermis, while the CPX-VPG with hydrogenated phospholipids would be preferable for the treatment of deeper skin infections. However, further studies on an *in vivo* animal model are needed to prove these assumptions.

Keywords: vesicular phospholipid gel, liposomes, ciprofloxacin hydrochloride, chitosan, dermal drug delivery, infection, biocompatibility, wound healing

SADRŽAJ

POPIS KRATICA I OZNAKA

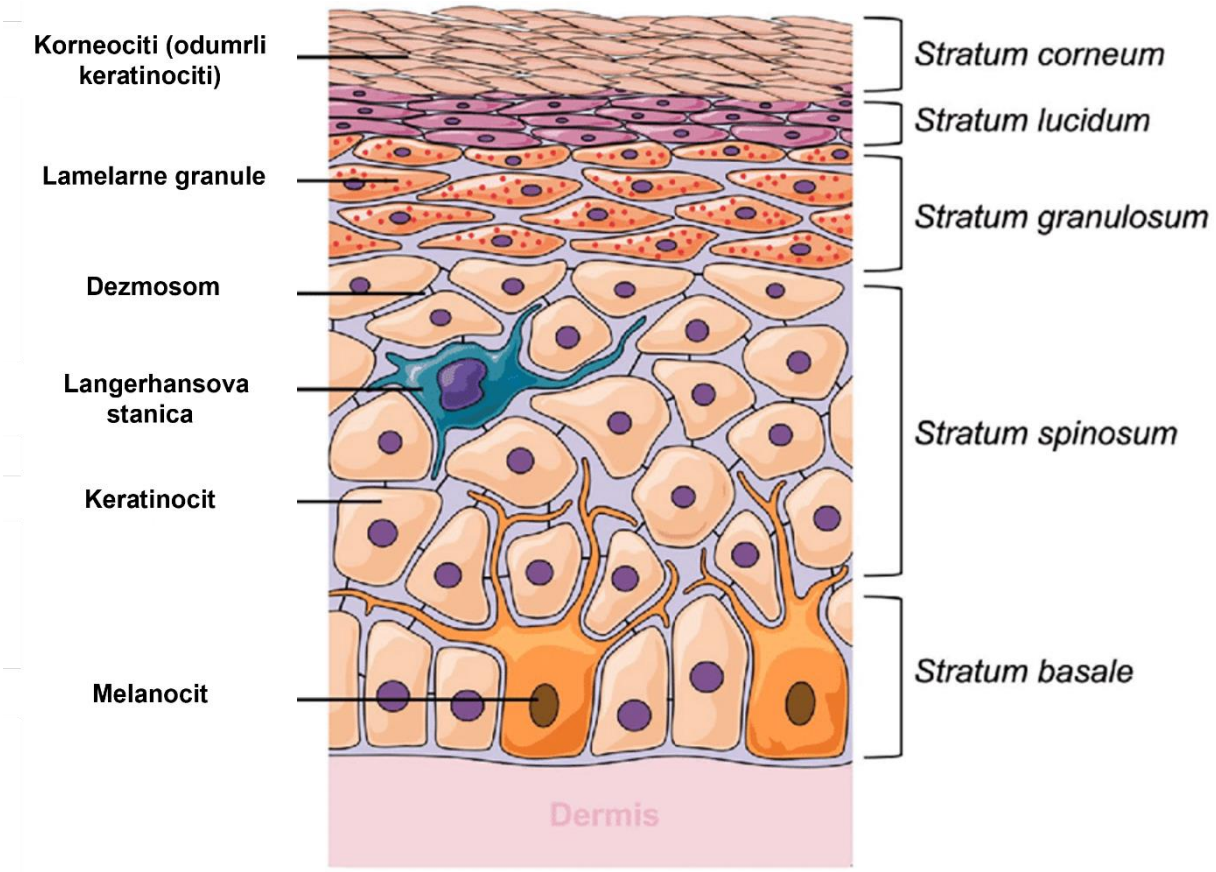
AIDS	sindrom stečene imunodeficijencije (engl. *Aquired Immunodeficiency Syndrome*)
ANOVA	analiza varijance
CHIT	kitozan
CHOL	kolesterol
CPF	ciprofloksacinklorid hidrat
DMEM	Dulbekov hranidbeni medij s visokim sadržajem glukoze
DNK	deoksiribonukleinska kiselina
E	stupanj membranske elastičnosti
EDTA	etilendiamintetraoctena kiselina
EPC	fosfatidilkolin iz jaja
EPC80	lecitin iz jaja s 80 % fosfatidilkolina
EPS	izvanstanične polimerne tvari (engl. *extracellular polymeric substances*)
G'	modul pohrane (engl. *storage modulus*)
G"	modul gubitka (engl. *loss modulus*)
G-CSF	stimulirajući faktor rasta granulocita
HaCaT	stanična linija humanih keratinocita
HEPC	hidrogenirani fosfatidilkolin iz jaja
HPLC	tekućinska kromatografija visoke djelotvornosti (engl. *high-performance liquid chromatography*)
HSPC	hidrogenirani fosfatidilkolin iz soje
J	masa liposomske disperzije nakon ekstruzije kroz odgovarajuću membranu r_p
LB	Luria-Bertani medij
LUV	veliki unilamelarni liposomi
MBEK	minimalna biofilm-eliminirajuća koncentracija
MBIK	minimalna biofilm-inhibirajuća koncentracija
MBK	minimalna baktericidna koncentracija
MHA	Müller-Hinton agar
MHB	Müller-Hinton bujon
MIK	minimalna inhibitorna koncentracija

MLV	multilamelarni liposomi
MRSA	meticilin-rezistentni *Staphylococcus aureus*
MTT	3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid
MVV	multivezikularni liposomi
NMF	prirodni faktor vlažnosti kože
okr/min	okretaji u minuti
P90H	Phospholipon 90H (hidrogenirani fosfolipid, > 90 % hidrogeniranog fosfatidilkolina)
PBS	fiziološka otopina puferirana fosfatnim puferom (engl. *phosphate-buffered saline*)
PDI	indeks polidisperznosti
PES	polietersulfon
PG	propilenglikol
Ph. Eur.	Europska farmakopeja
QS	*'quorum sensing'*
r_p	promjer pora membrane kod ispitivanja elastičnosti
r_v	srednji promjer liposoma nakon ekstruzije kroz membranu r_p
SAFit2	selektivni antagonist FKBP51 proteina (engl. *selective antagonist of FKBP51 by induced fit*)
SFR	simulirani fluid rane
SLPC 80	sojin monoacil fosfatidilkolin
SPC	fosfatidilkolin iz soje
SPC 100	sojin lecitin (> 94 % fosfatidilkolina)
SUV	mali unilamelarni liposomi
TEM	transmisijska elektronska mikroskopija
TRP 2	protein srodan tirozinazi 2
TSB	triptik soja bujon
TSBGlc	tekući TSB obogaćen s 0,25 % D-(+) glukoze
VFG	vezikularni fosfolipidni gel
VISA	*S. aureus* s djelomičnom otpornosti na vankomicin (engl. *Vancomycin intermediate S. aureus*)

1. UVOD

1.1. STRUKTURA I FUNKCIJA KOŽE

Koža predstavlja najveći tjelesni organ s površinom od otprilike 1,7 m^2 i masom koja iznosi oko 15 % ukupne tjelesne mase prosječne osobe. Ima značajnu ulogu u održavanju homeostaze te služi kao zaštitna barijera od štetnih fizičkih i kemijskih agensa, sprječava ulazak stranih patogena, štiti od UV zračenja i gubitka vode. Osim ovih primarnih funkcija, koža je uključena u brojne procese: regulaciju tjelesne temperature, percepciju osjeta, apsorpciju tvari, imunosne reakcije te sintezu melanina i vitamina D (Benson i Watkinson, 2011; Gilaberte i sur., 2016).



Slika 1. Struktura epidermisa. Preuzeto i prilagođeno iz Ramadon i sur. (2022), uz dozvolu *Springer Naturea.*

Kožu čine tri sloja: epidermis, dermis i hipodermis, uz pripadajuće kožne privjeske (nokti, dlake, žlijezde lojnice i znojnice).

Epidermis (Slika 1), vanjski sloj kože, slojeviti je skvamozni epitel sastavljen prvenstveno od keratinocita, a manji dio čine melanociti, Merkelove i Langerhansove stanice. Ovaj se sloj kontinuirano obnavlja, a keratinociti, jedine stanice unutar epidermisa koje

podliježu staničnoj diobi, prolaze kroz diferencijaciju od bazalnog sloja (lat. *stratum basale*), preko trnastog (lat. *stratum spinosum*), zrnatog (lat. *stratum granulosum*), svijetlog (lat. *stratum lucidum*), do najgornjeg, rožnatog sloja (lat. *stratum corneum*). U zrnatom sloju keratinociti su još vijabilni, a daljnjom diferencijacijom nastaju plosnate stanice bez jezgre, tzv. korneociti. Keratinociti, melanociti (proizvodnja melanina), Merkelove stanice (senzoričko opažanje) i Langerhansove stanice (imunosna funkcija) imaju važnu ulogu u funkcioniranju živog epidermisa (Benson i Watkinson, 2011; Celleno i Tamburi, 2009; Gilaberte i sur., 2016; Gupta i sur., 2012).

Stratum corneum, formiran od korneocita međusobno povezanih dezmosomima, djeluje kao primarna barijera koja sprječava gubitak vode i održava optimalni pH kože (4,1 - 5,8) (Proksch, 2018). Intercelularni lipidi, određene aminokiseline, soli iz znoja i lojnih izlučevina te razgradni produkti proteina, zajedno s lipidima, doprinose ukupnom učinku barijere. Koža posjeduje mnogo enzima koji sudjeluju u procesima sazrijevanja i ljuštenja keratinocita, stvaranju prirodnog faktora vlažnosti kože (NMF) te općenito u homeostazi. Treba imati na umu da su ti enzimi također sposobni metabolizirati lokalno primijenjene djelatne tvari (Benson i Watkinson, 2011).

Dermis se nalazi ispod epidermisa i debljine je otprilike 2 do 5 mm. Sastoji se od mukopolisaharidnog gela kojeg zajedno drže kolagena i elastična vlakna. Kolagena vlakna čine 70 % dermisa, dajući mu snagu i čvrstoću, elastin održava normalnu elastičnost i fleksibilnost, a proteoglikani osiguravaju viskoznost i hidrataciju. Fibroblasti, najbrojnije stanice dermisa, doprinose strukturnim funkcijama, dok su manje brojni mastociti, limfociti i histiociti zaslužni za imunosnu funkciju dermisa. Krvne žile, živčani završeci, folikuli dlake i žlijezde lojnice i znojnice, također se nalaze unutar dermisa. Ovaj sloj predstavlja malu prepreku penetraciji većine lijekova, ali može smanjiti prodiranje jako lipofilnih lijekova dublje u tkivo (Benson i Watkinson, 2011; Celleno i Tamburi, 2009).

Hipodermis ili potkožno tkivo sastoji se od sloja masnih stanica s međusobno povezanim vlaknima kolagena i elastina. Njegova funkcija je toplinska izolacija tijela, zaštita od mehaničke traume te skladištenje energije. Hipodermis pridonosi cjelokupnoj strukturnoj i nutritivnoj potpori kože, te je veza između gornjh slojeva kože i mišićnog tkiva (Benson i Watkinson, 2011; Kinnunen i Mrsny, 2014).

Poznavanje anatomije i fiziologije kože, pogotovo njezine barijerne funkcije, važno je prilikom dizajniranja terapijski učinkovitih topikalnih i transdermalnih farmaceutskih oblika lijekova (Benson i Watkinson, 2011; Gilaberte i sur., 2016).

1.2. LOKALNA PRIMJENA LIJEKOVA NA KOŽU

Lokalna primjena lijekova na kožu, poznata i kao topikalna ili dermalna primjena, jednostavan je i neinvazivan put primjene lijeka koji omogućuje njegovo djelovanje na ograničenom, oboljelom, mjestu na/u koži. U odnosu na oralnu primjenu lijekova, ovim putem primjene se izbjegava metabolizam prvog prolaza lijeka kroz jetru, te osigurava lokalizirana dostava djelatne tvari (lijeka) na oboljelo mjesto uz primjenu značajno nižih doza u usporedbi sa sistemskom primjenom (Antimisiaris i sur., 2021; Hagen i Baker, 2017). Na taj način se smanjuje izloženost organizma visokim dozama lijeka i rizik od sistemskih nuspojava (Leppert i sur., 2018). Za razliku od topikalne/dermalne primjene lijekova, gdje su ciljana mjesta djelovanja epidermis i dermis, kod transdermalne primjene lijekovi prolaze kroz sve slojeve kože te se apsorbiraju u sistemsku cirkulaciju (Brown i sur., 2006; Flynn, 1993).

Kako bi se osigurala učinkovita lokalna terapija, lijek (djelatna tvar) mora dostići ciljano (oboljelo) mjesto te se na njemu zadržati dovoljno dugo u koncentraciji prikladnoj za postizanje terapijskog učinka. Na ovaj proces utječe niz čimbenika, uključujući fizičko-kemijska svojstva djelatne tvari (lijeka), fiziološke parametre (dob, barijera kože, mjesto primjene na koži) te značajke samog farmaceutskog oblika tj. formulacije lijeka (Gupta i sur., 2012; Hagen i Baker, 2017).

Konvencionalni topikalni oblici za dermalnu primjenu poput masti i krema nisu uvijek dostatno učinkoviti. Razlozi su mnogobrojni, uključujući i nesuradljivost pacijenata zbog primjerice ljepljivosti masne podloge, neugodnog mirisa pripravka, ali i pojave iritacija i alergijskih reakcija uslijed visokih koncentracija djelatne tvari u podlozi ili sastojaka podloge, nedostatne permeabilnosti djelatne tvari u kožu ili pak hlapljenja aktivnog sastojka iz pripravka. Permeabilnost djelatne tvari može biti ograničena bilo zbog svojstava same polučvrste podloge (formulacije), ograničene topljivosti djelatne tvari u podlozi i/ili njezinog slabog oslobađanja iz podloge (Gupta i sur., 2012; Richard i sur., 2020). Dodatni čimbenik je priroda oboljenja, koja može biti karakterizirana zadebljalim ili pak oštećenim površinskim slojem kože te je često praćena bakterijskim infekcijama (Cui i sur., 2021; Las Heras i sur., 2020; Stefanov i sur., 2021; Vastarella i sur., 2020). Ukoliko lokalno primijenjeni lijek ne penetrira kroz rožnati sloj kože i ne nakuplja se na ciljanom mjestu djelovanja u koži u odgovarajućoj koncentraciji, terapijski učinak je slab ili izostaje. Nasuprot tome, zbog oštećenosti rožnatoga sloja i narušenog integriteta kožne barijere, moguća je nekontrolirana sistemska apsorpcija, pogotovo dobro permeabilnih lijekova (Aulton i Taylor, 2017; Gupta i sur., 2012). Stoga je za učinkovito lokalno liječenje oboljenja kože važno primjenjivati terapijski učinkovite i fiziološki

prihvatljive farmaceutske oblike koji omogućuju kontroliranu i lokaliziranu dostavu djelatne tvari na ciljano (oboljelo) mjesto u koži.

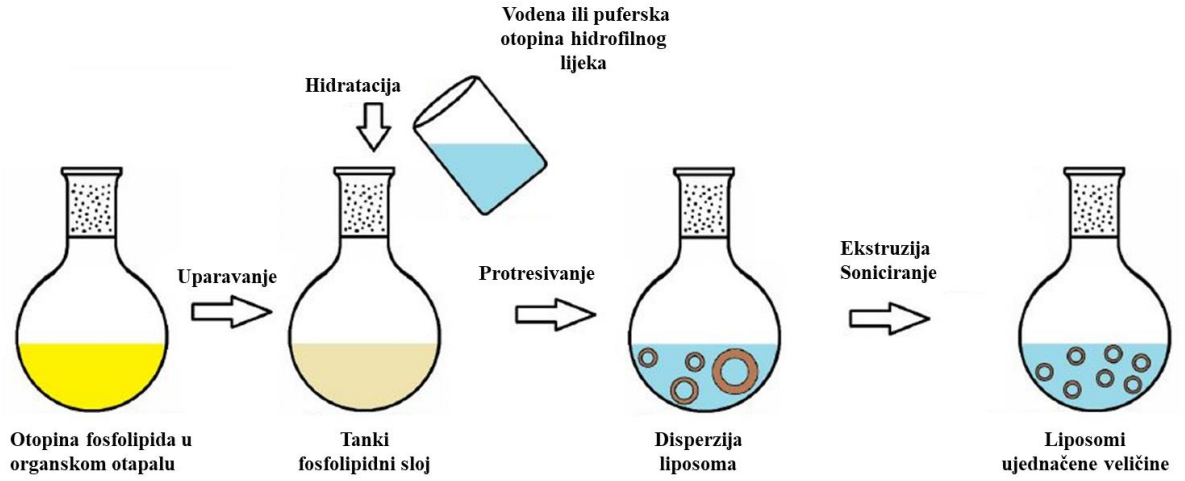
1.3. FOSFOLIPIDNI TERAPIJSKI NANOSUSTAVI

Među brojnim istraživanim lipidnim i polimernim nanosustavima za dermalnu primjenu (lipidne nanočestice, nanostrukturirani lipidni nosači, nano- i mikro-emulzije, polimerne micele, niosomi, polimerne nanočestice), fosfolipidni vezikularni nanosustavi zauzimaju istaknuto mjesto (Cui i sur., 2021; Stefanov i sur., 2021). Jedna od njihovih najvećih prednosti je ta što se sastoje od fosfolipida, koji su biokompatibilni, biorazgradivi i netoksični jer dijele sličnosti sa stanicama i strukturama površinskog sloja kože, te se mogu proizvesti u velikim količinama, a da su pritom zadovoljavajuće farmaceutske kakvoće (van Hoogevest i sur., 2021; Vanić i sur., 2015). Zbog svih tih pozitivnih svojstava fosfolipida, fosfolipidni terapijski nanosustavi su formulacije od posebnog značaja, a obuhvaćaju različite vrste liposoma i vezikularne fosfolipidne gelove.

1.3.1. Liposomi

Liposomi su sferične fosfolipidne vezikule sastavljene od jednog ili više koncentrično položenih fosfolipidnih dvoslojeva koji okružuju vodenu jezgru. Osim fosfolipida, dvosloj mogu sačinjavati i drugi lipidi, dok se površina vezikula može modificirati sterički stabilizirajućim polimerima i/ili ligandima. Strukturna svojstava liposoma omogućuju uklapanje djelatnih tvari različite lipofilnosti (hidrofilne, hidrofobne i amfipatske). Optimiranjem (fosfo)lipidnog sastava i metode priprave moguće je pripraviti liposome željenih fizičko-kemijskih svojstava (veličina, lamelarnost, naboj na površini, fluidnost dvosloja), postići kontrolirano i/ili produljeno oslobađanje i isporuku djelatne tvari u oboljela područja na/u koži (Rahnfeld i Luciani, 2020; Vanić i sur., 2015).

Postoji više metoda za pripremu liposoma, no najčešće se koristi metoda hidratacije tankog fosfolipidnog sloja (Slika 2). Postupak izrade liposoma ovom metodom obuhvaća: (i) uparavanje otopine fosfolipida i hidrofobnog lijeka (ukoliko se uklapa u liposome) u organskom otapalu, (ii) hidrataciju osušenog tankog fosfolipidnog sloja (filma) u vodenom mediju koji može sadržavati otopljeni hidrofilni lijek i (iii) homogenizaciju nastalih liposoma postupcima soniciranja ili ekstruzije (Dymek i Sikora, 2022; Richard i sur., 2020).



Slika 2. Shematski prikaz priprave liposoma metodom hidratacije tankog fosfolipidnog sloja (tzv. film metoda). Preuzeto i prilagođeno iz Dymek i Sikora (2022), uz dozvolu *Elseviera.*

Liposomi se mogu kategorizirati prema veličini, broju fosfolipidnih dvoslojeva i sastavu. Veličina liposoma može varirati od 20-ak nm do 10-ak μm pri čemu veći liposomi (> 500 nm) obično sadrže više koncentrično položenih dvoslojeva. Metoda izrade značajno utječe na morfologiju i veličinu liposoma. Ovisno o korištenoj metodi, moguće je pripraviti multilamelarne liposome (MLV), male ili velike unilamelarne liposome (SUV ili LUV), pa čak i multivezikularne liposome (MVV) koji sadrže mnogo nekoncentrično položenih fosfolipidnih dvoslojeva (Dymek i Sikora, 2022; Rahman i sur., 2019; Richard i sur., 2020).

U proizvodnji liposoma se najčešće koristi fosfatidilkolin te čini osnovu dvosloja. Ovisno o (fosfo)lipidnom sastavu, liposomi za (trans)dermalnu primjenu se kategoriziraju u: konvencionalne, deformabilne i propilenglikol liposome, etosome i invasome. Kod konvencionalnih liposoma, fosfatidilkolin se najčešće kombinira s dodatkom kolesterola, koji sam po sebi ne tvori dvosloj, već se interkalira između molekula fosfolipida i na taj način čini membranu čvršćom (rigidnijom), što smanjuje njezinu propusnost za uklopljene hidrofilne lijekove (Richard i sur., 2020). Takve rigidne vezikule zadržavaju se većinom na površini kože i površinskim slojevima epidermisa, što ih čini neprikladnim kada je lijek potrebno dostaviti dublje u kožu ili transdermalno. Stoga su razvijeni liposomi fluidnijih (deformabilnih, elastičnih) membrana. Etosomi, fosfolipidne vezikule koji sadrže 20 - 50 % etanola, pokazali su značajno poboljšanje dermalne i transdermalne dostave lijeka u odnosu na konvencionalne liposome. Deformabilni liposomi, poznati i kao elastični, fleksibilni, ultra-deformabilni ili Transfersomi®, su fosfolipidne vezikule koje dodatno sadrže jednolančani surfaktant (rubni

aktivator) u koncentraciji do 25 %. Najčešće korišten rubni aktivator je natrijev kolat, ali slična svojstva pokazuju deoksikolat, parcijalni esteri sorbitana i masnih kiselina te esteri masnih kiselina i polioksietilensorbitana. Zbog drugačije geometrije od molekula fosfatidilkolina, rubni aktivatori narušavaju pravilnu organizaciju liposomskog dvosloja, čineći membranu liposoma elastičnom, zbog čega deformabilni liposomi, kada su primijenjeni u neokluzivnim uvjetima, mogu penetrirati kroz *stratum corneum* (Dymek i Sikora, 2022; Palac i sur., 2015; Richard i sur., 2020).

Dosadašnja istraživanja su potvrdila učinkovitost liposoma u dostavi antimikrobnih lijekova i prevenciji razvoja biofilmova, što je od velikog značaja u sprječavanju razvoja kroničnih infekcija (Dymek i Sikora, 2022; Rukavina i sur., 2018; Rukavina i Vanić, 2016). Važno je istaknuti da i 'prazni' liposomi (bez uklopljene djelatne tvari), povoljno djeluju na hidrataciju kože i obnavljanje narušenog integriteta površinskog sloja kože zbog toga što sadrže koži-slične tvari (fosfolipidi, kolesterol) (Vanić i sur., 2015). Unatoč brojnim prednostima, mala sposobnost uklapanja hidrofilnih djelatnih tvari u liposome te tekuća konzistencija liposomske suspenzije mogu predstavljati ograničenja za njihovu dermalnu primjenu (Rukavina i sur., 2023).

Umješavanje liposoma u hidrogel uobičajeni je način povećanja viskoznosti liposomskih suspenzija. Polučvrsta konzistencija liposomskih gelova prikladna je za primjenu na kožu, a uz to omogućuje i produljeno oslobađanje uklopljene djelatne tvari (Palac i sur., 2015). S druge strane, udio liposoma s uklopljenim lijekom u hidrogelu većinom iznosi 10 – 30 % (*m/m*) (Vanić i sur., 2014, 2015), što može predstavljati ograničenje u liječenju određenih kožnih bolesti koje zahtijevaju primjenu većih doza lijeka. Poseban izazov u industrijskoj proizvodnji liposoma su metode priprave koje zahtijevaju specifične proizvodne postupke i opremu te korištenje ekološki neprihvatljivih otapala koji se iz pripravka moraju u potpunosti ukloniti. Gotovo svi postupci izrade liposoma limitirani su količinom (fosfo)lipida u nanoformulaciji koja obično iznosi 10 - 30 mg/mL, što može predstavljati ograničenje u pogledu kapaciteta uklapanja djelatne tvari i količine nanoformulacije koja se može primijeniti (Filipczak i sur., 2020; Worsham i sur., 2019). Navedene nedostatke moguće je prevladati razvojem vezikularnih fosfolipidnih gelova.

1.3.2. Vezikularni fosfolipidni gelovi

Vezikularni fosfolipidni gelovi (VFG) predstavljaju visoko-koncentrirane, polučvrste disperzije fosfolipida u vodi ili puferu (Brandl 2007). Sastavljeni su od gusto pakiranih fosfolipidnih vezikula (liposoma) s iznimno malim udjelom vanjske vodene faze. Nasuprot klasičnih liposomskih suspenzija, VFG-ove karakterizira izrazito visoko uklapanje hidrofilnih lijekova u liposome te su stabilni tijekom skladištenja jer je onemogućeno oslobađanje lijeka iz unutarnje u vanjsku vodenu fazu VFG-a (Brandl i sur., 1997).

Osim što su VFG-ovi korisni kao depo sustavi s produljenim oslobađanjem, mogu se primijeniti i u razrijeđenom obliku budući da miješanjem s vodenim medijem stvaraju homogene disperzije većinom unilamelarnih liposoma (Brandl i sur., 1997; Tardi i sur., 1998). Variranjem (fosfo)lipidnog sastava, koncentracije (fosfo)lipida i suotapala, moguće je pripraviti VFG-ove različite fluidnosti/rigidnosti fosfolipidnih dvoslojeva, čime se može modificirati, odnosno kontrolirati oslobađanje uklopljene djelatne tvari (Tardi i sur., 1998). VFG-ovi su posebno prikladni za uklapanje hidrofilnih i ioniziranih lijekova (Brandl, 2007; Brandl i sur., 1997; Tardi i sur., 1998). Primarno su razvijani i istraživani za parenteralnu primjenu citostatika poput 5-fluorouracila (Kaiser i sur., 2003), gemcitabina (Moog i sur., 2002), cetroreliksa (Grohganz i sur., 2005) i proteinskih lijekova (Tian i sur., 2010), a tek je nedavno predložena njihova oftalmička primjena (Fang i sur., 2021) (Tablica 1).

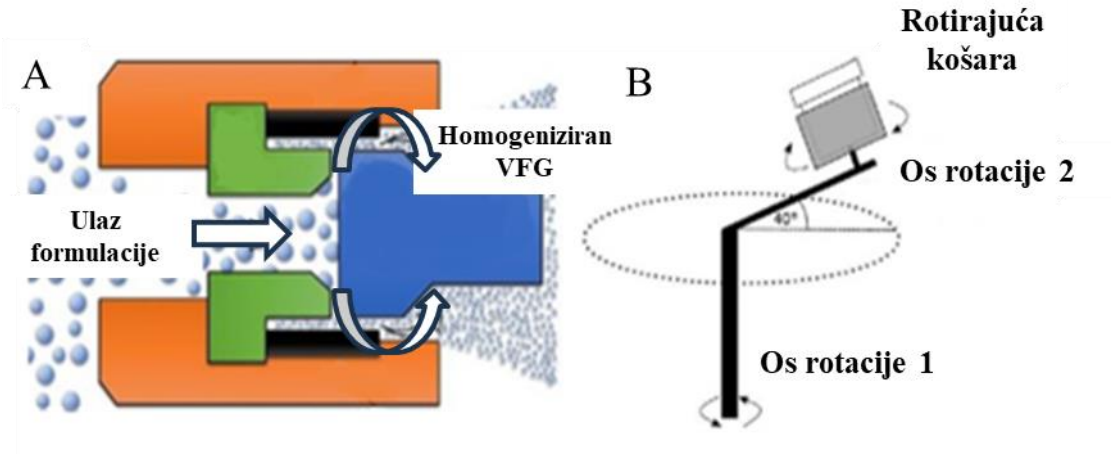
Tablica 1. Sažeti pregled dosadašnjih istraživanja VFG-ova kao nosača djelatnih tvari.

Djelatna tvar	Metoda izrade	Primjena/oblik	Indikacija	Lipidna i vodena faza	Rezultati	Literatura
Vinkristin	Visokotlačna homogenizacija	Intravenski* (injekcija)	Antitumorsko liječenje	HEPC:kolesterol (molarni omjer 1:1); 40% (fosfo)lipida; citratni pufer, pH 5,6	Bolji antitumorski učinak u odnosu na otopinu lijeka zbog pasivnog usmjeravanja lijeka u tumorsko tkivo (*in vivo*).	Güthlein i sur., 2002
Gemcitabin	Visokotlačna homogenizacija	Intravenski* (injekcija)	Antitumorsko liječenje	HEPC:kolesterol (molarni omjer 1:1); 40% (fosfo)lipida; fosfatni pufer, pH 7,3	4 x veća razina lijeka u tumorskom tkivu; bolja farmakodinamička i farmakokinetička svojstva u odnosu na vodenu otopinu gemcitabina.	Moog i sur., 2002
5-Fluorouracil	Visokotlačna homogenizacija	Intravenski* Depo oblik (*in vitro*)	Antitumorsko liječenje	HSPC/kolesterol (molarni omjeri 25/75–45/55); 30% (fosfo)lipida; fosfatni puferi pH 7,4 i pH 8,0	Bolje uklapanje lijeka u VFG-ove s manjim udjelom kolesterola (40%); rigidnost membrane kontrolira oslobađanje lijeka.	Kaiser i sur., 2003
Cetroreliks	Visokotlačna homogenizacija	Depo oblik (*in vitro*)	Zaustavljanje preuranjene ovulacije	EPC80 (30-50%); voda	Oslobađanje terapijskih doza cetotreliksa u periodu do 6 tjedana.	Grohganz i sur., 2005
Eritropoetin (modelni lijek)	Dualno asimetrično centrifugiranje	Depo (*in vitro*)	-	EPC80 (30-55%); PBS pH 7,2	Stabilnost lijeka nije narušena procesom izrade; produženo oslobađanje lijeka tijekom 2 tjedna; brzina oslobađanja raste smanjenjem udjela fosofolipida.	Tian i sur., 2010
Citarabin	Visokotlačna homogenizacija, dualno asimetrično centrifugiranje	Depo (implantacija tijekom operacije)	Antitumorski učinak	EPC80, natrij-kolesteril sulfat, oktadecilamin, ε-polilizin, γ-poliglutamska kiselina; 40% (fosfo)lipida; PBS, pH 7,4	Produženo oslobađanje (16 dana, *in vitro*); terapijske koncentracije održane tijekom 28 dana u radijusu do 5 mm od mjesta implantacije (*in vivo*, miševi).	Qi i sur., 2012, 2014
Timopentin	Homogenizacija na magnetskoj mješalici	Depo (supkutano)	Stimulacija imunosnog odgovora	EPC80 (30-40%); acetatni pufer, pH 6,8	Terapijski učinak VFG-a (1x tjedno) usporediv s otopinom timopentina (1x dnevno, 7 dana)	Zhong i sur., 2013

G-CSF	Dualno asimetrično centrifugiranje	Depo (implantacija tijekom operacije)	Pomoć pri zacjeljivanju tetiva	EPC80 (40-49%); acetatni pufer (pH 3,9), Tween (0,004%) i sorbitol (5%)	Oslobađanje G-CSF-a kinetikom nultog reda (*in vitro*); dobra podnošljivost i poboljšanje imunohistoloških i biomehaničkih svojstava primjenom niske doze G-CSF VFG-a (*in vivo*, štakori).	Buchmann i sur., 2015
Interferon beta-1b	Dualno asimetrično centrifugiranje	Depo oblik (*in vitro*)	-	EPC80 (35%), fosfatni pufer, pH 7,2	Izostanak oslobađanja interferona *(in vitro)* zbog jake interakcije proteina s fosfolipidima.	Neuhofer, 2015
TRP 2	Dualno asimetrično centrifugiranje	Depo oblik (*in vitro*)	-	SPC (30%); PBS, pH 7,4	Sporo i nepotpuno oslobađanje TRP 2 zbog interakcije peptida s fosfolipidima.	Even, 2015
Eksenatid	Homogenizacija na magnetskoj mješalici	Depo oblik (supkutano)	Tip 2 dijabetesa	SPC (20–40%); acetatni pufer, pH 4,0	Produženo oslobađanje eksenatida (3 tjedna, *in vitro*); hipoglikemijski učinak injekcije VFG-a (1 u 10 dana) usporediv s injekcijama otopine lijeka (2 x dnevno, 10 dana) (*in vivo)*	Zhang i sur., 2016
SAFit2 ligand	Dualno asimetrično centrifugiranje	Depo oblik (supkutano)	Tolerancija na glukozu	EPC80 (50%); PBS, pH 7,4	Učinak lijeka na metaboličke parametre prisutan do 8 dana nakon primjene VFG-a (*in vivo*, miševi).	Balsevich i sur., 2017
			Upalna stanja (neuropatska bol)		Smanjena mehanička preosjetljivost najmanje 16 dana nakon primjene VFG-a, u odnosu na otopinu SAFit2 liganda (24 sata).	Maiaru i sur., 2018
Flurbiprofen	Visokotlačna homogenizacija	Oftalmička primjena	Prednji uveitis	EPC (30–60%); PBS, pH 7,4	Produljeno zadržavanje lijeka na rožnici; poboljšana transkornealna dostava lijeka (*ex vivo*); poboljšana bioraspoloživost; snažan protuupalni učinak i izbjegavanje iritacija (*in vivo*, kunići).	Fang i sur., 2021

EPC, fosfatidilkolin iz jaja; EPC80, lecitin iz jaja s 80 % fosfatidilkolina; G-CSF, stimulirajući faktor rasta granulocita; HEPC, hidrogenirani fosfatidilkolin iz jaja; HSPC, hidrogenirani fosfatidilkolin iz soje; PBS, fiziološka otopina puferirana fosfatnim puferom; SAFit2 ligand, selektivni antagonist FKBP51 proteina; SPC, fosfatidilkolin iz soje; TRP 2, protein srodan tirozinazi 2; *Neposredno prije primjene VFG-ovi su prevedeni u liposome.

Elektronskom mikroskopijom je potvrđeno da se VFG-ovi sastoje od gusto pakiranih vezikula unilamelarne ili oligolamelarne strukture ovisno o udjelu i tipu korištenih fosfolipida (Brandl i sur., 1998; Breitsamer i Winter, 2019). Biorazgradivi su i biokompatibilni te se mogu pripraviti bez upotrebe organskih otapala. Za veličinu i distribuciju veličina liposoma unutar VFG-a zaslužna je tehnika izrade. Primjerice, klasična homogenizacija fosfolipida na magnetskoj mješalici rezultira vezikulama velikog promjera i široke distribucije veličina (Zhong i sur., 2013). Nasuprot tome, kompleksnijim metodama priprave poput visokotlačne homogenizacije i dualnog asimetričnog centrifugiranja (Slika 3) nastaju VFG-ovi s liposomima uske distribucije veličina. Naime, optimizacijom tlaka tijekom visokotlačne homogenizacije, odnosno brzine centrifugiranja u dualnoj asimetričnoj centrifugi, dobivaju se unilamelarni liposomi malog srednjeg promjera i niskog indeksa polidisperznosti (Breitsamer i Winter, 2019).



Slika 3. Različite metode pripreme VFG-ova; A, visokotlačna homogenizacija (Yong i sur., 2021) i B, dualno asimetrično centrifugiranje (Breitsamer i Winter, 2019). Preuzeto i prilagođeno uz dozvolu *Frontiersa* (A) i *Elseviera* (B).

Znakovita prednost VFG-a u odnosu na tekuće liposomske disperzije je da je lijek u potpunosti uklopljen u VFG te je zbog odsutnosti koncentracijskog gradijenta onemogućeno oslobađanje liposomski-uklopljenog lijeka u vanjsku vodenu fazu tijekom skladištenja VFG-a (Brandl, 2007; Tardi i sur., 1998).

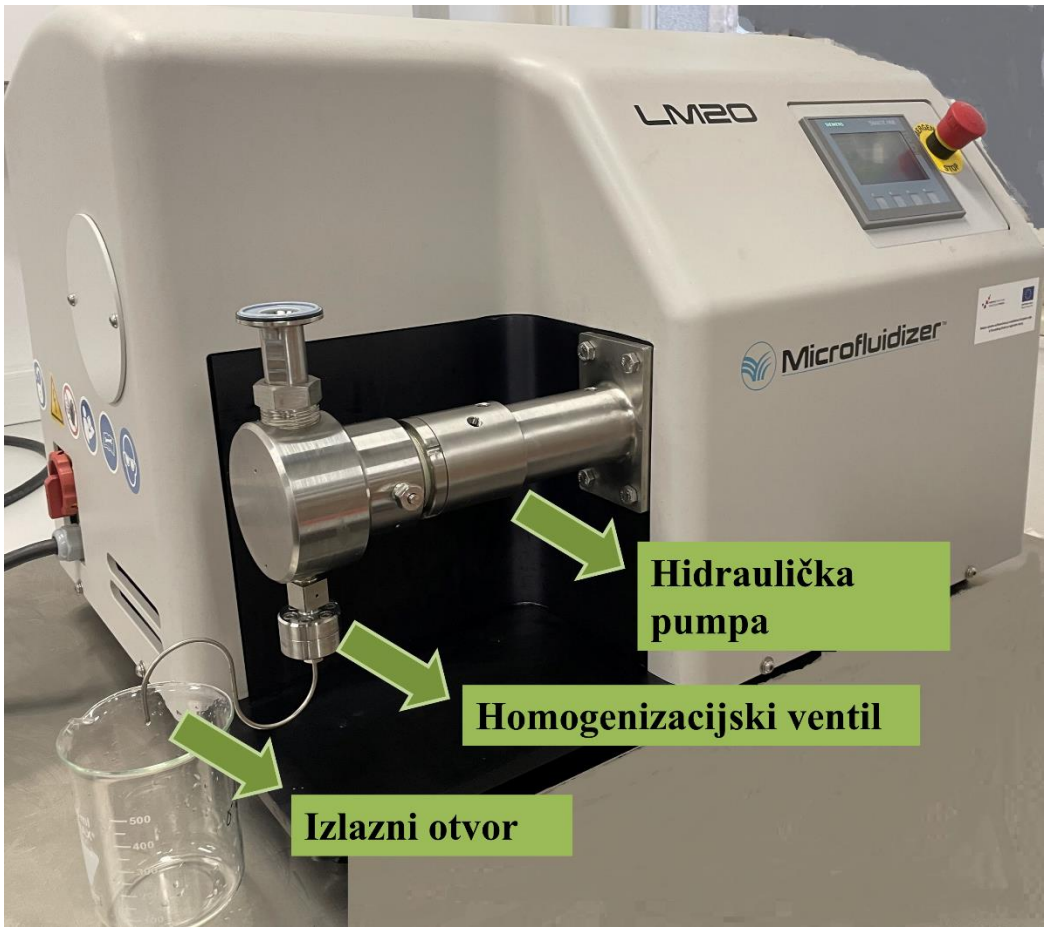
VFG-ovi omogućuju produljeno oslobađanje uklopljene djelatne tvari, a ovisno o udjelu i tipu korištenih fosfolipida, djelatna tvar se oslobađa u vremenu od nekoliko sati do nekoliko dana (Breitsamer i Winter, 2019). Na profil oslobađanja značajno utječe koncentracija djelatne tvari unutar VFG-a, ali i unutar liposoma VFG-a. Tardi i sur. (1998) su pokazali da se uklopljeni

hidrofilni marker (kalcein) oslobađa iz VFG-a preko više mehanizama. To su erozija fosfolipidnog matriksa i difuzija hidrofilnog markera iz vanjske vodene faze VFG-a, procesi koji se odvijaju istovremeno. Dodatni proces koji se događa je difuzija hidrofilnog markera iz unutarnje vodene faze liposoma. Upravo je taj mehanizam odgovoran za produljeno oslobađanje djelatne tvari iz VFG-a (Breitsamer i Winter, 2019; Grohganz i sur., 2005; Tardi i sur., 1998).

1.4. VISOKOTLAČNA HOMOGENIZACIJA

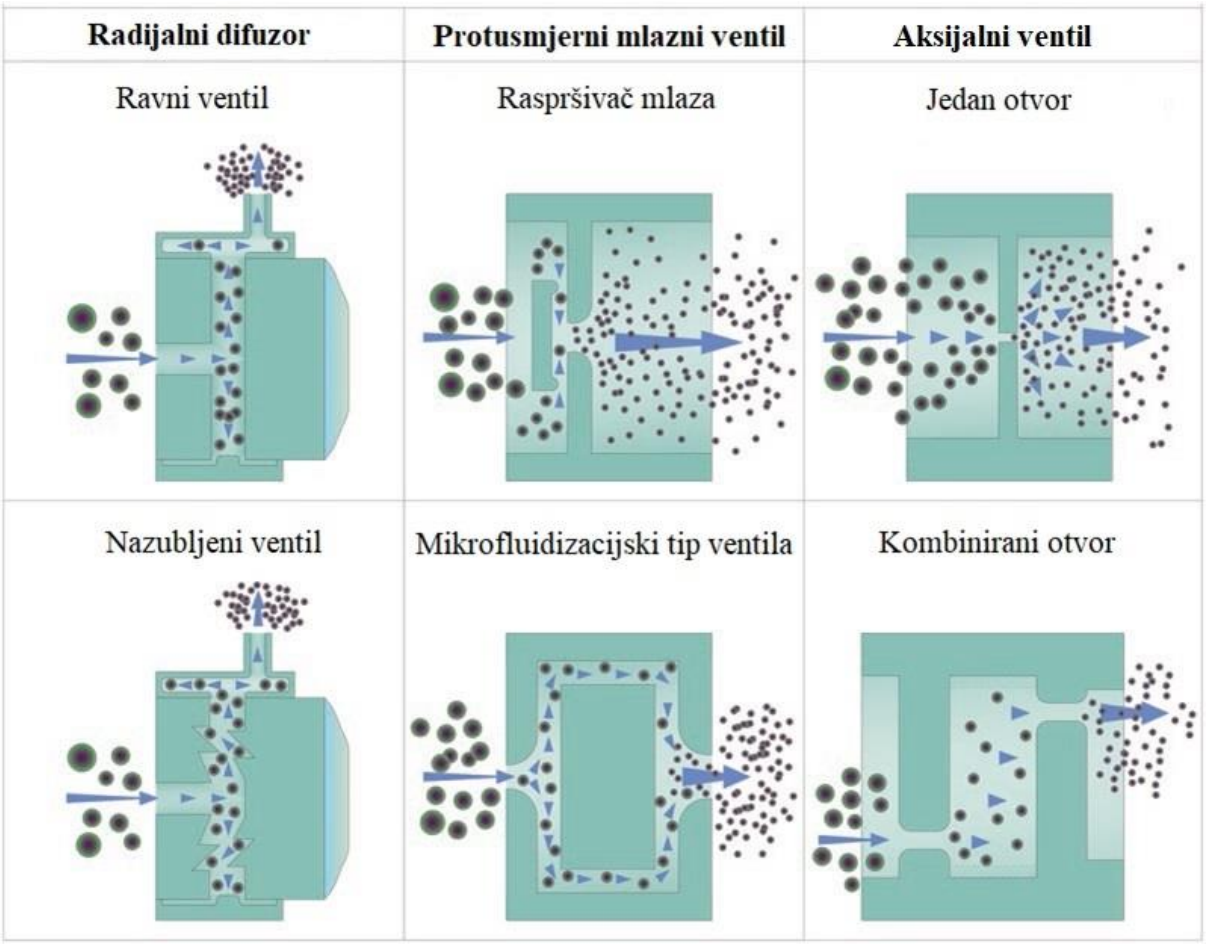
Visokotlačnu homogenizaciju je uvelike popularizirao Auguste Gaulin početkom 20. stoljeća za obradu mlijeka radi poboljšane stabilnosti proizvoda. Otada se razvila u ključnu tehnološku operaciju u raznim industrijama (prehrambena, farmaceutska), obradi otpadnih voda, proizvodnji materijala i biotehnologiji (Inguva i sur., 2024). Mayhew i suradnici su još 1983. godine koristili visokotlačnu homogenizaciju kako bi smanjili i ujednačili veličinu liposoma te su kao prednost ove metode homogenizacije naveli mogućnost procesiranja visokih koncentracija lipida u formulaciji. Naime, visoka koncentracija lipida u sustavu je značajna jer omogućuje uklapanje veće količine djelatne tvari (Mayhew i sur., 1984).

Postoje dvije glavne kategorije tehnologija homogenizacije; standardna homogenizacija, koja koristi tlakove do 50 MPa i visokotlačna homogenizacija koju karakteriziraju tlakovi do 300 MPa (Osorio-Arias i sur., 2020). Visokotlačna homogenizacija je mehanički proces koji uključuje protiskivanje tekuće disperzije kroz uski otvor ili ventil pri ekstremno visokim tlakovima. Visokotlačni homogenizatori mogu biti različitih izvedbi, no svi sadrže tri osnovna dijela: hidrauličku pumpu, homogenizacijski ventil te izlazni otvor (Slika 4). Pumpa potiskuje tekući uzorak u ventil, u kojem uslijed protiskivanja uzorka kroz mali otvor dolazi do smanjivanja i ujednačavanja veličine čestica tekuće disperzije (Diels i Michiels, 2006; Vinchhi i sur., 2021). Homogenizator djeluje kao 'usko grlo' u kojem je uzorak izložen turbulentnim uvjetima protoka i fenomenima kavitacije uslijed velike brzine strujanja i pada tlaka nakon prolaska uzorka kroz ventil. Ova tehnologija ima veliku primjenu u oblikovanju tekućih i polučvrstih farmaceutskih oblika: omogućuje smanjenje veličine čestica disperzije kao preduvjet bolje stabilnosti pripravka te potpomaže procese otapanja, miješanja, dispergiranja i uklapanja djelatne tvari u višefaznim sustavima (Inguva i sur., 2024; Kluge i sur., 2012; Osorio-Arias i sur., 2020; Vinchhi i sur., 2021).



Slika 4. Visokotlačni homogenizator (Microfluidizer ML 20, Microfluidics, Sjedinjene Američke Države).

Na tržištu su dostupni različiti tipovi visokotlačnih homogenizatora, ovisno o načinu postizanja tlaka. Odgovorajući tlak se može postići povećanjem protoka tekućine pri jednakoj veličini otvora ventila ili smanjenjem otvora ventila, dok je protok tekućine konstantan (Inguva i sur., 2024; Kluge i sur., 2012; Osorio-Arias i sur., 2020; Patrignani i Lanciotti, 2016). Ventili mogu biti različitih izvedbi uključujući protusmjerni mlazni ventil, radijalni difuzor i ventil s aksijalnim protokom (Slika 5). Protusmjerni mlazni ventil (zastupljen u uređaju Microfluidizer LM20, Microfluidics) nema pokretnih dijelova te se oslanja na sudar dvaju turbulentnih tokova. Tlak se u sustavu postiže povećanjem protoka uzorka koji se usmjerava kroz mikrokanale ventila (Osorio-Arias i sur., 2020; Schultz i sur., 2004; Vinchhi i sur., 2021).



Slika 5. Shematski prikaz različitih vrsta ventila u visokotlačnom homogenizatoru. Preuzeto i prilagođeno iz Vinchhi i sur. (2021), uz dozvolu *Springer Naturea*. Protusmjerni mlazni ventil (mikrofluidizacijski tip ventila) je korišten za pripremu uzoraka u ovoj doktorskoj disertaciji.

Visokotlačna homogenizacija je napredna i sveobuhvatna tehnologija koja, oslanjajući se na složenu dinamiku fluida unutar homogenizacijskog ventila, pronalazi primjenu u različitim industrijama (prehrambena, farmaceutska, kozmetička). Napredak u dizajnu ventila, konfiguracijama sustava i radnim parametrima otvara nove mogućnosti primjene ove važne homogenizacijske tehnike.

1.5. INFEKCIJE KOŽE

1.5.1. Bakterijske infekcije kože i mekih tkiva

Bakterijske infekcije kože i mekih tkiva su jedne od najučestalijih infekcija diljem svijeta. U mnogim slučajevima izvori su kronične rane, čija se prevalencija povećava s dobi pacijenta, dok druge infekcije potječu iz malih površinskih lezija, akutnih traumatskih ozljeda ili kirurških rana. Kliničke manifestacije mogu varirati od blagih površinskih lezija do po život opasnih nekrotizirajućih i sistemskih infekcija. Točna i na vrijeme dijagnosticirana infekcija kože i mekih tkiva je ključna kako bi se omogućilo pravovremeno liječenje odgovarajućim antibioticima (Russo i sur., 2016; Sunderkötter i Becker, 2015). Uobičajene infekcije kože i mekih tkiva mogu se karakterizirati na temelju kliničke prezentacije i morfološkog izgleda, koji pružaju naznake o mogućem bakterijskom uzročniku. Početno liječenje uključuje empirijsku terapiju koja je usmjerena na pokrivanje patogena za koje se pretpostavlja da su prisutni u patološkom području. Ta početna terapija također može biti vođena saznanjima o samom pacijentu, poput oslabljenog imunosnog sustava, postojećih bolesti, kontaktu sa životinjama, nedavnim putovanjima, povijesti prethodnih trauma, hobija (npr. vrtlarstvo, planinarenje, kampiranje), alergijama i trenutnoj farmakoterapiji. Terapija usmjerena na specifične mikroorganizme treba se primijeniti neposredno po primitku nalaza o izoliranom patogenu i njegovoj osjetljivosti na antibiotike (Kahn i Goldstein, 1993; Lee i sur., 2021).

Težina infekcije ovisi o različitim čimbenicima, a klinički spektar obuhvaća blage oblike do po život opasnih varijanti. U tretiranju složenih infekcija kože i mekih tkiva bolesnicima je često potrebna kompleksna terapija, uključujući kiruršku obradu patološkog područja i liječenje antibioticima, no infekcije kože i mekih tkiva mogu obuhvatiti i one bolesnike koji imaju pridružene komorbiditete poput dijabetesa, sistemske imunosupresije ili neuroloških bolesti. Ova druga kategorija obuhvaća i zdrave pojedince s teškim infekcijama te pacijente s komorbiditetima i relativno blagim infekcijama. Postupak liječenja se prilagođava težini i mjestu infekcije, uzimajući pritom u obzir komorbiditete bolesnika (Russo i sur., 2016). Infekcije kože i mekih tkiva klasificiraju se na:

- površinske infekcije (impetigo, folikulitis, erizipel),
- duboke infekcije (celulitis, nekrotizirajući fascitis) i
- ostale infekcije (apscesi, inficirane rane, dijabetičke infekcije stopala).

Površinske nekomplicirane infekcije se mogu liječiti lokalnim antimikrobnim lijekovima, dok kožni apscesi dobro reagiraju na drenažu i općenito se preporučuje propisivanje sistemskih

antibiotika. Bolesnici s nekrotizirajućim infekcijama zahtijevaju kirurško čišćenje rane i empirijski polimikrobni antibiotski tretman (Bessa 2023).

Racionalno korištenje antibiotika važno je zbog rastuće mikrobne rezistencije. Opće pravilo za upotrebu antibiotika je da njihov spektar djelovanja treba biti širi da pokrije potencijalnog uzročnika infekcije, ali ne nepotrebno preširok (Sunderkötter i Becker, 2015).

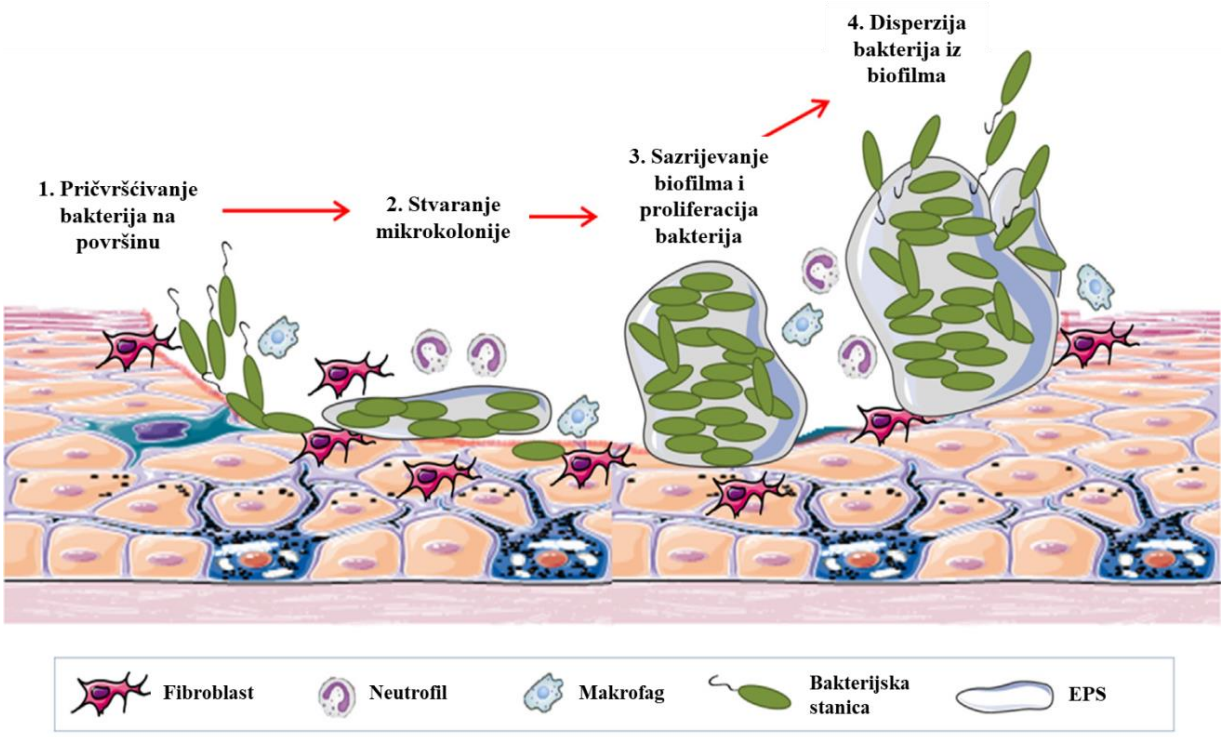
Sistemsko liječenje infekcija kože i mekih tkiva suočava se s izazovima poput nedovoljne koncentracije lijeka na ciljanom mjestu djelovanja, interakcija s drugim lijekovima i sistemskim nuspojavama. Upotreba sistemskih antibiotika za liječenje određenih vrsta kroničnih rana s prisutnim biofilmovima, poput dijabetičkih ulkusa, nije poželjna jer nema dovoljno dokaza o učinkovitosti. Lokalnoj antibiotskoj terapiji daje se prednost nad sistemskom terapijom jer omogućuje postizanje veće lokalne koncentracije lijeka i smanjuje potrebu za visokim sistemskim dozama antibiotika. Međutim, prisutnost biofilma u kroničnim ranama ograničava učinkovitost lokalno primjenjenih antibiotika zbog nedostatne penetracije antibiotika u biofilm. Stoga topikalna dostava djelatnih tvari koji imaju antibiofilm učinak, samostalno ili u kombinaciji s antibioticima, može imati značajnu terapijsku vrijednost u liječenju kroničnih rana. Naime, tvari s antibiofilm učinkom mogu razgraditi ili narušiti integritet biofilma i time omogućiti penetraciju antibiotika u biofilm (Razdan i sur., 2022). Osim toga, da bi bilo učinkovito, liječenje mora biti pravovremeno, odgovarajućeg trajanja i uz dovoljno visoku dozu topikalno primjenjenog anitmikrobnog lijeka/sredstava (Sunderkötter i Becker, 2015).

1.5.2. Biofilmovi

Biofilmovi, ili kako ih neki nazivaju 'gradovi mikroba', su organizirane zajednice mikroorganizama, najčešće bakterija, koje mogu rasti na inertnim površinama (npr. medicinska pomagala koja se ugrađuju u tijelo) i na živim tkivima. Prvi put ih spominje davne 1684. godine Antonie Van Leeuwenhoek, koji je primijetio da su 'životinjice' (bakterije) unutar plaka na zubima bile otpornije na djelovanje octa nego bakterije izvan plaka, koje su bile uništene. Danas je poznato da je jedno od glavnih obilježja biofilma ekstremna tolerancija na antimikrobna sredstva (Bjarnsholt, 2013; Razdan i sur., 2022). Takve mikrobne zajednice se razvijaju unutar izvanstaničnog matriksa kojeg čine izvanstanične polimerne tvari (engl. *extracellular polymeric substances,* EPS) poput polisaharida, proteina i DNK koje izlučuju sami mikroorganizmi. EPS čini 90 % biomase biofilma, pružajući zaštićeno okruženje koje omogućuje mikroorganizmima preživljavanje u neprijateljskim uvjetima, nesmetano razmnožavanje te širenje van biofilma (Cavallo i sur., 2024; Vyas i sur., 2021). EPS igra ključnu

ulogu u antimikrobnoj rezistenciji, otpornosti na imunosne odgovore, razmjeni gena i komunikaciji bakterija unutar biofilma koja je poznata pod nazivom *'quorum sensing*' (QS). Pojam 'quorum' odnosi se na minimalni broj bakterija okupljenih unutar određenog volumena koji je potreban za donošenje 'odluke' o aktiviranju ekspresije gena kontroliranih QS-om te regulira nekoliko procesa ključnih za razvoj biofilma, otpornost na antibiotike i virulenciju (Bjarnsholt, 2013; Vyas i sur., 2021).

Formiranje biofilma je višestupanjski dinamički proces koji se sastoji od četiri faze prikazane na Slici 6: i) pričvršćivanje bakterija na površinu, ii) formiranje mikrokolonija, iii) sazrijevanje biofilma i iv) odvajanje (disperzija) pojedinačnih bakterija iz zrelog biofilma (makrokolonije). Biofilmovi mogu sadržavati bakterije s istim genskim sastavom, ali različitom genskom ekspresijom. Također je zanimljivo da više vrsta mikroorganizama može formirati zajedničku koloniju, a u samom biofilmu postoji funkcionalna heterogenost, što znači da različite skupine bakterija obavljaju specijalizirane funkcije. Među različitim mikrobnim biofilmovima, bakterijski biofilmovi su najviše proučavani, a neki od njih, primjerice biofilmovi bakterije *Pseudomonas aeruginosa* često se koriste kao model u istraživanjima tvari s antibiofilm učinkom (Cavallo i sur., 2024; Razdan i sur., 2022).



Slika 6. Proces stvaranja i sazrijevanja biofilma: 1 - pričvršćivanje bakterija na površinu, 2 - stvaranje mikrokolonija, 3 - proliferacija bakterija i sazrijavanje biofilma, 4 - disperzija (širenje) bakterija iz makrokolonije (zrelog biofilma). EPS, izvanstanične polimerne tvari. Preuzeto i prilagođeno iz Razdan i sur. (2022), uz dozvolu *Elseviera*.

Kod oboljenja ili ozljeda kože koje su praćene sekundarnim infekcijama (primjerice opekline i rane), neučinkovita lokalna terapija može pogodovati razvoju biofilma i nastanku teško-zarastajućih, kroničnih rana (Las Heras i sur., 2020). Rane koje ne uspiju zacijeliti i povratiti anatomske i funkcionalne značajke unutar mjesec dana smatraju se kroničnim ranama, a uključuju dijabetičke ulkuse, dekubituse i ulkuse uzrokovane venskom stazom. Kronične rane pogoduju razmnožavanju bakterija zbog prisutnosti nekrotičnog tkiva, smanjene razine kisika, specifičnog mikrookoliša i oslabljenog imunosnog odgovora. Formiranje biofilma ima ključnu ulogu u neuspjehu liječenja kroničnih rana (Versey i sur., 2021; Vyas i sur., 2021). Infekcije povezane s biofilmom posebno su problematične jer bakterije unutar biofilma mogu biti do 1000 puta otpornije na antibiotike od svojih planktonskih oblika (Bjarnsholt, 2013; Razdan i sur., 2022).

Zbog svoje otpornosti na antibiotike i imunosni sustav, biofilmovi predstavljaju veliki klinički i javnozdravstveni izazov. Doze antibiotika temelje se na minimalnoj inhibitornoj koncentraciji (MIK) i minimalnoj baktericidnoj koncentraciji (MBK), koje su potrebne za inhibiciju rasta ili eradikaciju planktonskih bakterija uzgajanih na hranjivim podlogama. Međutim, zbog značajno povećane otpornosti bakterija u biofilmu, postoji potreba za određivanjem minimalne koncentracije antibiotika za eradikaciju biofilma (MBEK). Nažalost, istraživanja su otkrila da MBEK mnogih antibiotika premašuje maksimalne propisane doze tih lijekova. Kao rezultat toga, standardne oralne doze antibiotika, koje učinkovito uništavaju planktonske bakterije, nemaju ili imaju mali antimikrobni učinak na bakterije u biofilmu *in vivo* (Ceri i sur., 2001; Phillips i sur., 2008; Simsekli i sur., 2023; Tillander i sur., 2022).

Važno je naglasiti da se tolerancija biofilma na antibiotike treba razlikovati od antibiotske rezistencije. Iako bakterije unutar biofilma često preživljavaju antibiotsko liječenje, postaju osjetljive na terapiju nakon što se biofilm razgradi (Bjarnsholt 2013). Konvencionalni oblici lijekova za dermalnu primjenu poput krema i gelova pokazuju slabu penetraciju kroz barijeru biofilma. Razumijevanje formiranja, strukture i funkcije biofilma stoga je ključno za razvoj učinkovitih topikalnih farmaceutskih oblika lijekova (Versey i sur., 2021; Vyas i sur., 2021).

1.5.3. *Pseudomonas aeruginosa*

P. aeruginosa je sveprisutna Gram-negativna bakterija koja se može pronaći u različitim okolišima i domaćinima. Njezin uspjeh u prilagodbi različitim staništima temelji se na izuzetnoj metaboličkoj fleksibilnosti i sposobnosti preživljavanja u nepovoljnim uvjetima. Jedan je od najznačajnijih patogena u bolničkim uvjetima, gdje uzrokuje infekcije povezane s visokom stopom smrtnosti, osobito kod imunokompromitiranih pacijenata, osoba s kroničnim stanjima poput cistične fibroze, raka, sindroma stečene imunodeficijencije (AIDS), bronhiektazija, kroničnih rana i urinarnih infekcija (Krell i Matilla, 2024; Poole, 2011; Russo i sur., 2016; Wu i sur., 2015). Infekcije uzrokovane bakterijom *P. aeruginosa* osobito su učestale kod pacijenata na odjelima intenzivne njege. Također, poznata je i kao jedan od najčešćih patogena izoliranih iz opeklina. Infekcije povezane s opeklinama često su ozbiljne zbog teškog stanja pacijenata i povećane otpornosti na antibiotike. Ova bakterija često uzrokuje traumatske i postoperacijske infekcije te kronične rane, što dodatno povećava rizik od komplikacija (Bassetti i sur., 2018). Osim bolničkih infekcija, *P. aeruginosa* je odgovorna za folikulitis i sindrome poput sindroma zelenog nokta, koji se može pojaviti u osoba nakon rekreacijskog izlaganja mikrobiološki neispravnoj vodi u bazenima i hidromasažnim kadama. Također 'plivačko uho' ili vanjski otitis je blaga vanjska infekcija uha do koje može doći ako voda zaražena bakterijama uđe u uho. U teškim slučajevima, posebno kod dijabetičara ili imunokompromitiranih osoba, može napredovati u životno opasni maligni vanjski otitis. Infekcije dišnih puteva uzrokovane ovom bakterijom češće su kod osoba s postojećim plućnim bolestima, poput bronhiektazija ili cistične fibroze (Kerr i Snelling, 2009).

Velik problem u zdravstvu predstavlja izuzetna intrinzična otpornost *P. aeruginosa* na glavne skupine antibiotika kao što su β-laktami, aminoglikozidi, kinoloni i polimiksini, zbog niske propusnosti stanične membrane, prisutnosti višestrukih efluksnih pumpi i enzima za modifikaciju antibiotika, kao i sposobnosti stvaranja biofilma. Osim toga, ova bakterija može razviti otpornost na antibiotike mutacijskim promjenama i horizontalnim prijenosom gena za otpornost. Sve to rezultira sojevima bakterija koji su postali rezistentni na više vrsta lijekova istovremeno, što dovodi do dulje hospitalizacije bolesnika, porasta zdravstvenih troškova, ali i povećanog morbiditeta/mortaliteta (Bassetti i sur., 2018; Krell i Matilla, 2024; Poole, 2011; Wu i sur., 2015).

Jedan od najvažnijih izazova u kliničkom liječenju *P. aeruginosa* infekcija je stvaranje biofilma. Zbog ograničene razine kisika i hranjivih tvari, bakterije unutar biofilma su manje metabolički aktivne što pridonosi njihovoj otpornosti na antibiotike (Poole, 2011; Wu i sur.,

2015). Sve veća otpornost bakterija *P. aeruginosa* na više skupina lijekova istodobno i povećana pojavnost infekcija ukazuju na hitnu potrebu za pronalaženjem efikasnijih strategija prevencije i liječenja.

1.5.4. *Staphylococcus aureus*

S. aureus, poznat i pod nazivom 'zlatni stafilokok', pripada skupini Gram-pozitivnih bakterija te je jedan od najpoznatijih i najraširenijih uzročnika infekcija u svijetu. Odgovoran je za različita infektivna stanja, poput nekompliciranih infekcija kože do ozbiljnijih, invazivnih bolesti, uključujući upalu pluća, infekcije kirurških rana, zglobnih proteza, kardiovaskularnih infekcija kao i bolničku bakterijemiju (Howden i sur., 2023; Idrees i sur., 2021). Prema podacima iz 2012. godine, incidencija bakterijemije uzrokovane bakterijom *S. aureus* iznosila je 20 – 50 slučajeva na 100.000 ljudi godišnje, s 10 – 30 % smrtnosti među zaraženima. Smatra se da je uzrok većeg broja smrtnih slučajeva nego što ih uzrokuju AIDS, tuberkuloza i virusni hepatitis zajedno (Cheung i sur., 2021; Nandhini i sur., 2022).

Osim bakterijemije, *S. aureus* uzrokuje razne infekcije kože, poput čireva, apscesa i rana, koje iako uobičajeno nisu životno ugrožavajuće, mogu uzrokovati značajnu bol i morbiditet. S obzirom na učestalost, s milijunima slučajeva godišnje samo u Sjedinjenim Američkim Državama, ove infekcije predstavljaju ozbiljan javnozdravstveni problem (Cheung i sur., 2021). *S. aureus* je često prisutan na ljudskoj koži, u nosnicama, pazusima i drugim područjima bez izazivanja simptoma bolesti. Međutim, pod određenim uvjetima, može uzrokovati infekcije. Na primjer, osobe s atopijskim dermatitisom su sklonije kolonizaciji bakterijom *S. aureus* te je poznato da pridonosi razvoju i komplikacijama atopijskog dermatitisa (Howden i sur., 2023; Idrees i sur., 2021).

Jedan od glavnih izazova u liječenju infekcija uzrokovanih bakterijom *S. aureus* je njezina otpornost na antibiotike, posebno kod sojeva otpornih na meticilin (meticilin-rezistentni *S. aureus*, MRSA). Učestalost meticilinske otpornosti značajno varira među državama; u Skandinaviji je niska, dok u zemljama poput Sjedinjenih Američkih Država i Kine prelazi 50 % te se smatra glavnim patogenom u svim regijama Sjeverne Amerike, Latinske Amerike i Europe (Cheung i sur., 2021; Russo i sur., 2016). Dok je u razvijenim zemljama zabilježen pad broja bolničkih MRSA infekcija zahvaljujući poboljšanim higijenskim mjerama, u siromašnijim regijama, poput Afrike, broj infekcija nastavlja rasti. Osim na meticilin, *S. aureus* pokazuje otpornost na gotovo sve dostupne antibiotike. Vankmicin ostaje lijek 'posljednje linije

obrane' za MRSA infekcije, iako postoje sojevi s djelomičnom otpornosti na ovaj antibiotik (engl. *Vancomycin intermediate Staphylococcus aureus*, VISA) (Cheung i sur., 2021).

Biofilm kojeg proizvodi *S. aureus* je jedan od glavnih uzroka pojave antimikrobne rezistencije jer stvara barijeru koja ograničava djelovanje antibiotika i imunosnog sustava. Otpornost bakterija unutar biofilma izrazito je visoka u odnosu na planktonski oblik, što značajno otežava liječenje (Idrees i sur., 2021). Biofilmovi *S. aureus* na medicinskim pomagalima, poput katetera i implantata, predstavljaju veliki problem u bolničkom okruženju. Bakterija ima izuzetnu sposobnost prianjanja na površinu pomagala ubrzo nakon njegova umetanja. Ako nisu adekvatno sterilizirani, mogu uzrokovati infekcije koje zahtijevaju uklanjanje i zamjenu pomagala, čime se povećava rizik od komplikacija i troškova liječenja (Cheung i sur., 2021; Howden i sur., 2023). Biofilmovi se također formiraju na površinama u prehrambenoj industriji, gdje predstavljaju rizik za kontaminaciju hrane i prijenos bolesti (Idrees i sur., 2021). Osim ključne uloge bakterijskih biofilmova u otpornosti na lijekove, i drugi čimbenici doprinose razvoju i širenju antimikrobne rezistencije. To uključuje sposobnost izbjegavanja imunosnog sustava pacijenta, plastičnost genoma bakterije i prilagodljivost kroz evoluciju gena i razmjenu genetskog materijala (Idrees i sur., 2021). Širina mehanizama kojima *S. aureus* izbjegava imunosni sustav odražava dugotrajnu interakciju ovog patogena s ljudima, obilježenu trajnom kolonizacijom i povremenim invazivnim infekcijama. *S. aureu*s proizvodi velik broj čimbenika virulencije i mehanizama za izbjegavanje imunosnog sustava, koji ometaju obrambene reakcije ljudskog organizma. Neutrofili, kao ključna linija obrane protiv stafilokoknih infekcija, često su meta ovih mehanizama, kao i ometanje adaptivnih imunosnih odgovora B i T limfocita, čime slabi zaštitni imunitet. Također, značajnu ulogu imaju mehanizmi koji zaobilaze urođeni imunosni odgovor, poput inhibicije kemotaksije i uništavanja neutrofila, sprječavanja aktivacije komplementa i fagocitoze, ubijanja stanica domaćina te stvaranja bakterijskih agregata. Kao rezultat prisutnosti čimbenika koji inhibiraju adaptivne imunosne reakcije, infekcija uzrokovana bakterijom *S. aureus* ne izaziva zaštitni imunosni odgovor, što dovodi do čestih recidiva tijekom života (Howden i sur., 2023; Nandhini i sur., 2022).

Glikopeptidi predstavljaju antibiotike izbora za invazivne MRSA infekcije, no smanjena osjetljivost uzrokovana prilagodbom putem mutacija dodatno je otežala liječenje. To je dovelo do primjene drugih antimikrobnih lijekova 'posljednje linije obrane' i kombinirane terapije više skupina antibiotika (Howden i sur., 2023).

Budući koraci u borbi protiv infekcija uzrokovanih bakterijom *S. aureus* uključuju istraživanje i bolje razumijevanje sastava i funkcije njegovog biofilma, te razvoj novih pristupa za eradikaciju biofilmova i sprječavanje razvoja otpornosti na antibiotike.

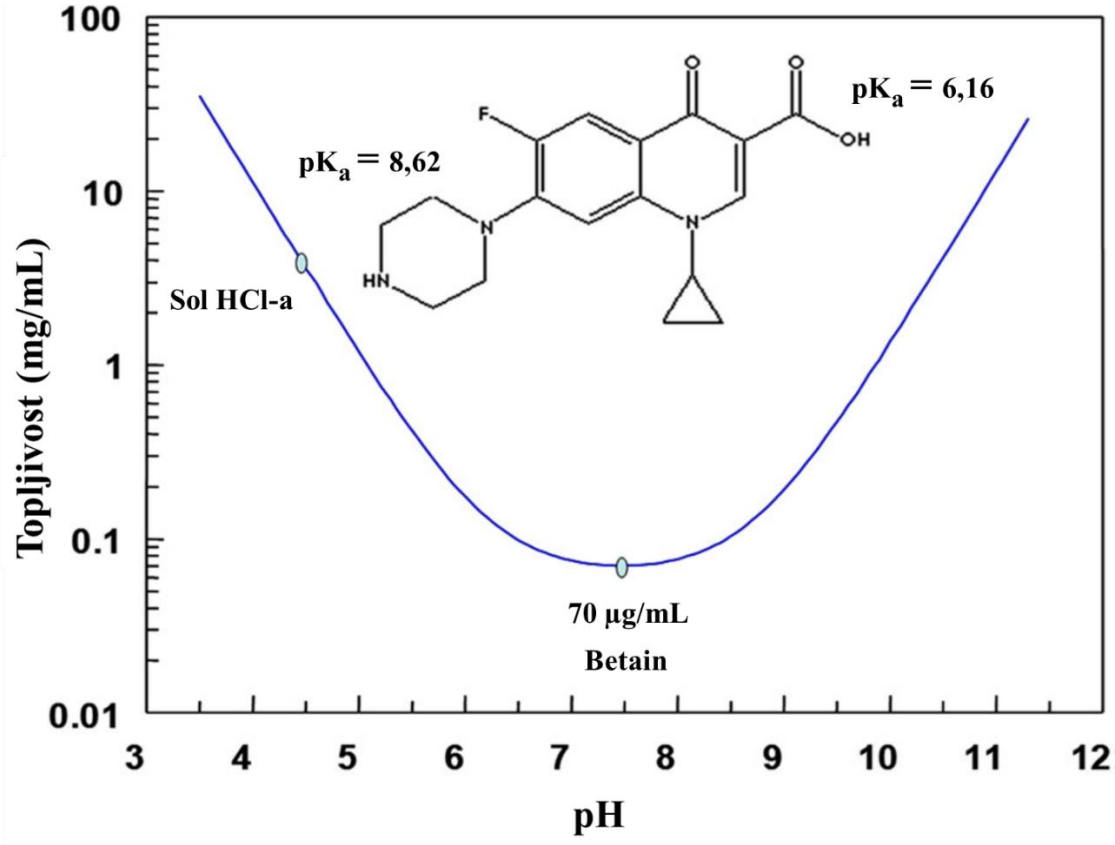
1.6. CIPROFLOKSACIN

Fluorokinolonski antibiotik ciprofloksacin predstavlja ključno sredstvo u borbi protiv širokog spektra bakterijskih infekcija. Postoji u obliku monohidroklorid monohidratne soli 1-ciklopropil-6-fluor-1,4-dihidro-4-okso-7-(1-piperazinil)-3-kinolin karboksilne kiseline, molekulske težine 385,82 g/mol, koja izgleda kao blijedo do svijetlo žuti kristalni prah (Ciloxan SPC; Torniainen i sur., 1996). Ciprofloksacin je stekao široku upotrebu od sredine 80.-ih godina prošlog stoljeća (Torniainen i sur., 1996). Od ostalih kinolona se razlikuje jedinstvenom molekularnom strukturom, koja uključuje atom fluora na položaju 6, piperazinski prsten na položaju 7 i ciklopropilni prsten na položaju 1 (Ciloxan SPC). Sinergističko međudjelovanje komponenti fluora i piperazina je zaslužno za izvanrednu *in vitro* aktivnost protiv širokog spektra bakterija, uključujući Gram-negativne organizme kao što je *P. aeruginosa* i Gram-pozitivne organizme poput *S. aureusa* (uključujući sojeve otporne na meticilin), česte uzročnike bakterijskih infekcija kože (Las Heras i sur., 2020). Snažno baktericidno djelovanje proizlazi iz njegove interferencije s enzimom DNK girazom i topoizomerazom IV, što rezultira inhibicijom replikacije DNK i transkripcije (Ciloxan SPC; Sharma i sur., 2010; Torniainen i sur., 1996; Weers, 2019).

Profil nuspojava ciprofloksacina uključuje gastrointestinalne smetnje, osip, glavobolju i nemir. Također su zabilježene rijetke alergijske reakcije poput osipa i anafilaktičkog šoka, te ozbiljne nuspojave kao što su psihoza uzrokovana lijekovima, imunosna reakcija preosjetljivosti, periferna neuropatija, povišen intrakranijalni tlak, tendinitis, traumatsko ili netraumatsko pucanje tetive. Također je opisano akutno zatajenje bubrega, uglavnom u slučajevima povezanim s predoziranjem (Ciloxan SPC; Olivera i sur., 2011; Thai i sur., 2023).

Ciprofloksacin (Slika 7) sadrži dvije skupine koje se mogu ionizirati: karboksilnu skupinu s pKa od 6,2 i sekundarni amin s pKa od 8,6. Pri neutralnom pH, karboksilna skupina je u velikoj mjeri deprotonirana, a aminska skupina protonirana, što rezultira neutralnim *zwitterionskim* oblikom koji se naziva ciprofloksacin betain. Ciprofloksacin betain je praktički netopljiv u vodi s topljivošću od 70 μg/mL. Pri niskom pH, skupina karboksilne kiseline je protonirana, a molekula postaje pozitivno nabijena. Stvaranje hidrokloridne soli s pozitivno

nabijenom amino skupinom povećava topljivost lijeka u vodi za više od 250 puta (20 mg/mL pri pH 3,2) (Varanda i sur., 2006; Weers, 2019).



Slika 7. Struktura ciprofloksacina i topljivost ovisna o pH. Preuzeto i prilagođeno iz Weers (2019), uz dozvolu *Springer Naturea*.

Ciprofloksacin je registriran za sistemsku primjenu, a lokalno se u obliku otopine primjenjuje jedino za liječenje infekcija oka i uha (Orange Book; Thai i sur., 2023).

Širok spektar djelovanja ciprofloksacina i dobar sigurnosni profil čine ga izuzetno vrijednim lijekom, često rezerviranim za liječenje infekcija otpornih na antibiotike. Njegova specifična molekularna struktura i mehanizmi djelovanja podupiru njegovu učinkovitost protiv različitih bakterijskih patogena, pridonoseći njegovoj nezamjenjivoj ulozi u kliničkoj praksi.

2. OBRAZLOŽENJE TEME

Učinkovito lokalno liječenje bolesti i ozljeda kože, koje su često praćene infekcijama, od velikog je medicinskog značaja. Time se izbjegava sistemska primjena lijekova, neželjene nuspojave te smanjuje rizik razvoja rezistencije mikroorganizama na antimikrobne lijekove. Primjenom topikalnih formulacija koje osiguravaju kontroliranu isporuku antibiotika na ciljanom mjestu djelovanja u dostatnoj koncentraciji i trajanju, značajno bi se unaprijedila lokalna terapija bakterijskih infekcija kože te smanjila potreba za sistemskom primjenom antibiotika.

Istraživanja koja su provedena u sklopu ovog rada imala su za cilj razviti terapijski učinkovit, siguran i stabilan polučvrsti pripravak za dermalnu primjenu, baziran na fosfolipidima, spojevima koji su strukturni elementi bioloških membrana i kože. Riječ je o VFG-u, fosfolipidnoj nanoformulaciji koja za razliku od klasičnih liposomskih suspenzija omogućuje uklapanje visoke koncentracije hidrofilne djelatne tvari, pri čemu je onemogućeno njezino spontano oslobađanje tijekom uskladištenja formulacije. Polučvrsta konzistencija VFG-a pridonosi lokaliziranoj dostavi lijeka na oboljelom mjestu u koži, dok fosfolipidi pogoduju fiziološkoj podnošljivosti pripravaka. Osim toga, integriranje fosfolipida u oštećene površinske slojeve kože doprinijelo bi njezinoj bržoj regeneraciji i zacjeljivanju.

Pretraživanjem literature je utvrđeno da trenutno nisu provedena istraživanja koja su se bavila ispitivanjem i razvojem VFG-ova namijenjenih dermalnoj primjeni, niti su provedena ispitivanja VFG-ova s antimikrobnim lijekovima (neovisno o putu primjene).

Hipoteza ovog rada je da se optimiranjem sastava i koncentracije (fosfo)lipida, polimera i suotapala mogu pripraviti VFG-ovi različite fluidnosti/rigidnosti fosfolipidnih dvoslojeva čime se može modificirati, odnosno kontrolirati oslobađanje i permeabilnost djelatne tvari u kožu, a integriranjem koži-sličnih tvari iz VFG-a u kožu povoljno utjecati na proces zacjeljivanja. Nadalje, razvojem metode priprave VFG-a bez uporabe organskih otapala korištenjem visokotlačnog homogenizatora osigurali bi se preduvjeti za ekološki prihvatljivu proizvodnju u industrijskim mjerilima.

Ciprofloksacinklorid, fluorokinolonski antibiotik učinkovitog djelovanja protiv *P. aeruginosa* i *S. aureus*, uobičajenih uzročnika bakterijskih infekcija kože, do sada nije istraživan za lokalnu primjenu na kožu, stoga je odabran kao modelni lijek. Zbog svojih hidrofilnih svojstava predstavlja izazov za uklapanje u liposome.

Specifični ciljevi ovog rada su uklopiti ciprofloksacinklorid u nekoliko različitih nanoformulacija VFG-ova namjenjenih dermalnoj primjeni koje se razlikuju po udjelu i tipu korištenog (fosfo)lipida, polimera i suotapala. Pritom će se optimirati procesni i formulacijski

parametri s obzirom na ciljana svojstva pripravka (veličinu liposoma, naboj, fluidnost dvosloja, profil oslobađanja djelatne tvari te reološka i teksturna svojstva). Provest će se detaljna karakterizacija VFG-ova s obzirom na fizičko-kemijska i reološka svojstva, *in vitro* antimikrobnu aktivnost na tri bakterijska soja karakteristična za inficirane rane (uključujući rezistentne sojeve) te *in vitro* biokompatibilnost koja će se ispitati na HaCaT staničnoj liniji humanih keratinocita. U konačnosti, odabrane nanoformulacije podvrgnut će se *ex vivo* ispitivanjima permeabilnosti kako bi se utvrdila mogućnost lokalizacije lijeka na/u koži, a njihov epitelizacijski učinak procijenit će se pomoću *in vitro* testa migracije keratinocita.

3. MATERIJALI I METODE

3.1 MATERIJALI

Za izradu VFG-ova korišteni su: sojin lecitin s više od 94 % fosfatidilkolina (Lipoid SPC 100, u daljnjem tekstu SPC), monoacil fosfatidilkolin iz soje (SLPC 80) i Phospholipon 90H (s najmanje 90 % hidrogeniranih fosfolipida, u daljnjem tekstu P90H) proizvođača Lipoid GmbH (Njemačka), kitozan niske molekulske mase (50,000 - 190,000 Da, 75 - 85 % deacetiliran) i kolesterol (Sigma-Aldrich, Njemačka) te propilenglikol (T.T.T., d.o.o., Hrvatska). Djelatna tvar, ciprofloksacin u obliku hidroklorid monohidrata (CPF), je kupljena od proizvođača Biosynth (Švicarska). Voda koja se koristila u izradi VFG-ova, pripremi pufera, medija i ispitivanjima bila je visoko pročišćena voda (u daljnjem tekstu voda).

Fosfatni pufer, pH 7,4, izrađen je otapanjem 2,98 g dinatrijevog hidrogenfosfat dihidrata (Fluka Chemika, Njemačka), 0,19 g kalijevog dihidrogenfosfata (Gram-mol, Hrvatska) i 8,0 g natrijevog klorida (Biosynth, Švicarska) u vodi, u odmjernoj tikvici od 1000 mL.

Simulirani fluid rane (SFR) izrađen je dodatkom 2 % goveđeg serumskog albumina (Sigma-Aldrich, Njemačka) u fosfatni pufer, pH 7,4 (Boateng i sur., 2013).

Za izradu minikolona u postupcima odjeljivanja liposoma s CPF-om korišten je Sephadex G-50 (Sigma-Aldrich, Njemačka).

Za uzgoj humanih staničnih linija keratinocita kože HaCaT (Cell Line Services, Njemačka) korišteni su sljedeći materijali: posude za uzgoj stanica (Corning, Costar te Falcon, Sjedinjene Američke Države), Dulbekov hranidbeni medij s visokom dozom glukoze (DMEM) (Gibco, Invitrogen, Paisley, Ujedinjeno Kraljevstvo) uz dodatak 10 % fetalnog goveđeg seruma (Biosera, Francuska), smjesa antibiotika penicilina, streptomicina i amfotericina B (Lonza, Švicarska), etilendiamintetraoctena kiselina (EDTA) (Lonza, Švicarska), tripsin 2,5 %-tni (Lonza, Švicarska), fiziološka otopina puferirana fosfatnim puferom (PBS; pH 7,3 - 7,5) bez Ca i Mg (Lonza, Švicarska). Za utvrđivanje vijabilnosti tretiranih stanica u kulturi korišten je 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid (MTT; Sigma-Aldrich, Njemačka) te izopropanol i klorovodična kiselina (Kemig, Hrvatska).

Antimikrobno djelovanje VFG-ova testirano je na tri bakterijska soja: *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 6538 i MRSA MFBF 10679 iz zbirke mikroorganizama Zavoda za mikrobiologiju Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Korištene kemikalije i materijali u antimikrobnim ispitivanjima bili su:

- za uzgoj *P. aeruginosa* ATCC 27853: Luria-Bertani agar (Liofilchem, Italy), Luria-Bertani (LB) medij (BD Difco, Sjedinjene Američke Države), M63 minimalni medij koji se sastoji od kalijevog dihidrogen fosfata (13,6 g/L) (Sigma-Aldrich, Njemačka), željezovog (II)

sulfat heptahidrata (0,5 mg/L) (Sigma-Aldrich, Njemačka) i amonijevog sulfata (2 g/L) (Sigma-Aldrich, Njemačka), uz dodatak magnezij sulfata (1 mM) (Sigma-Aldrich, Njemačka), glukoze (0,2 %) (Fluka, Sjedinjene Američke Države) i L-arginina (0,4 %) (Merck, Njemačka);

- za uzgoj *S. aureus* ATCC 6538 i MRSA MFBF 10679: Müller-Hinton agar (MHA) (Merck, Njemačka), Müller-Hinton bujon (MHB) (Merck, Njemačka), triptik soja bujon (TSB) (Difco, Sjedinjene Američke Države);
- MTT (Sigma-Aldrich, Njemačka) i kristal violet (Kemika, Hrvatska).

Korištena su dva tipa sterilnih mikrotitarskih pločica s 96 jažica: pločice s ravnim dnom (Greiner Bio-One, Njemačka) te s 'U' oblikom dna (Ratiolab, Njemačka). Mikrobiološki materijali, pribor te hranidbeni mediji korišteni u mikrobiološkim ispitivanjima su nabavljeni kao sterilni materijali ili su naknadno sterilizirani autoklaviranjem pri 121 °C/1 atmosfera tijekom 20 minuta.

Kemikalije korištene u svim ispitivanjima bile su analitičkog stupnja čistoće. Kao otapalo za (fosfo)lipide korišten je metanol (VWR International S.A.S., Francuska), a za kitozan je korištena octena kiselina (Lach-Ner, Češka). Otapala korištena za tekućinsku kromatografiju visoke djelotvornosti (HPLC) su zadovoljavala HPLC stupanj čistoće.

3.2. METODE

3.2.1. Priprava VFG-ova

Pripremljeno je nekoliko različitih nanoformulacija VFG-a (Tablica 2), koristeći SPC kao osnovni fosfolipid, bez ili s dodatkom monoacil fosfatidilkolina (SLPC80), hidrogeniranih fosfolipida (P90H), kolesterola, kitozana i propilenglikola. Koncentracija CPF-a je u svim nanoformulacijama bila jednaka i iznosila je 2 % (*m/m*).

Tablica 2. Sastav različitih nanoformulacija VFG-ova s CPF-om. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

VFG	SPC (g)	CHIT (g)	P90H (g)	CHOL (g)	SLPC80 (g)	PG (g)	CPF (g)	Voda (g)
SPC/CPF	34						2	64
SPC/CHIT/CPF	30	1					2	67
SPC/P90H/CPF*	16,5		8,5				2	73
SPC/CHOL/CPF*	28,3			3,3			2	66,4
SPC/SLPC80/CPF	29,8				5,3		2	62,9
SPC/PG/CPF	35					18,9	2	44,1
SPC/SLPC80/PG/CPF	22,4				4	5,2	2	66,4

CHIT, kitozan korišten kao 1,5 %-tna otopina u 0,5 %-tnoj octenoj kiselini; CHOL, kolesterol; CPF, ciprofloksacinklorid monohidrat; PG, propilenglikol; P90H, Phospholipon 90H (> 90 % hidrogeniranih fosfolipida); SLPC 80, sojin monoacil fosfatidilkolin; SPC, sojin lecitin s minimalno 94 % fosfatidilkolina. Masa pojedinog CPF-VFG-a je iznosila 100 g. *Hidratacija i homogenizacija SPC/CHOL/CPF i SPC/P90H/CPF VFG-ova provedena je na 60 °C.

VFG-ovi su pripravljeni u dvije faze. Prva je uključivala hidrataciju smjese (fosfo)lipida s otopinom lijeka u vodi (ili smjesi vode i propilenglikola) pomoću magnetske miješalice (600 okretaja u minuti, 90 minuta) na sobnoj temperaturi. Nakon toga je uslijedila homogenizacija (druga faza) na visokotlačnom homogenizatoru (Microfluidizer LM20, Microfluidics, Sjedinjene Američke Države), jedan ciklus pri tlaku od 500 bara. Izrada VFG-ova koji su sadržavali kolesterol i P90H provedena je na 60 °C.

Istom metodom, pod jednakim uvjetima, pripravljeni su 'prazni' VFG-ovi (bez CPF-a). Korišteni su kao kontrole u *in vitro* ispitivanjima antibakterijske aktivnosti i biokompatibilnosti.

pH vrijednosti svih VPG-ova određene su pH metrom (Mettler-Toledo, Greifensee, Švicarska) primjenom elektrode za polučvrste pripravke. Mjerenja su provedena na 25 °C u triplikatu nakon kalibracije uređaja trima standardnim otopinama (Čačić i sur., 2023).

Sve formulacije VFG-ova pohranjene su u hladnjak na 4 °C, a prije ispitivanja su temperirane na sobnu temperaturu.

Prije određivanja veličine (srednjeg promjera i indeksa polidisperznosti), morfologije, zeta potencijala, fluidnosti dvoslojeva, uspješnosti uklapanja CPF-a u liposome (unutarnju vodenu fazu VFG-a), *in vitro* stabilnosti VFG-a u SFR-u, *in vitro* antimikrobne aktivnosti, biokompatibilnosti i epitelizacijskog učinka, VFG-ovi su prevedeni u tekuće liposomske disperzije. Postupak je baziran na homogenom dispergiranju VFG-a (1 g) u vodi (9 g) na magnetskoj mješalici (600 okr/min) tijekom 30 minuta, uz postepen dodatak vode. Rekonstitucija VFG-ova s vodom je provedena na sobnoj temperaturi, osim nanoformulacija koje su sadržavale kolesterol i P90H gdje je postupak proveden na 60 °C.

3.2.2. Određivanje morfologije CPF-VFG-ova i CPF-VFG liposoma

Morfološke karakteristike odabranih hidratiziranih smjesa fosfolipida u otopini CPF-a i pripadajućih CPF-VFG-ova evaluirane su svjetlosnim mikroskopom Olympus BX51 (Olympus, Japan), opremljenim kamerom QICAM 1394 (QImaging, Canada). Mjerenja su provedena pripremom nativnog preparata koji je vizualiziran pri povećanju od 200 puta.

Strukturna obilježja SPC/CPF VFG-a te CPF-VFG liposoma nastalih konverzijom iz SPC/CPF i SPC/P90H/CPF VFG-ova vizualizirana su transmisijskim elektronskim mikroskopom (TEM) Morgagni 268D (FEI Company, Sjedinjene Američke Države). U tu svrhu CPF-VFG liposomi su prvo inkubirani na Formvar®/ugljik/bakrenoj mrežici (Sigma Aldrich, Sjedinjene Američke Države) 5 minuta te isprani jednom u ultračistoj vodi (Milli-Q, Merck Millipore, Sjedinjene Američke Države). Mrežica s uzorkom je zatim inkubirana u kapljici UranyLess™ kontrastne otopine (Uranyless, Francuska) 5 minuta te je nakon bojenja isprana tri puta u ultračistoj vodi. Nakon sušenja, uzorci su vizualizirani pri 70 kV.

3.2.3. Određivanje srednjeg promjera, indeksa polidisperznosti i zeta potencijala

Srednji promjeri, indeksi polidisperznosti i zeta potencijali liposoma unutar različitih VFG-ova su izmjereni fotonskom korelacijskom spektroskopijom koristeći Zetasizer Ultra (Malvern Panalytical Ltd, Ujedinjeno Kraljevstvo). Liposomske disperzije su netom prije mjerenja razrjeđivane s vodom, prethodno filtriranom kroz Minisart filtere (Sigma Aldrich, Njemačka) veličine pora 200 nm. Mjerenja su provedena pri temperaturi od 25 °C te kutu raspršenja od 90 ° (srednji promjer i indeks polidisperznosti). Za određivanje zeta potencijala, elektroforetskim raspršenjem svjetlosti na 25 °C, korištena je kapilarna kiveta. Mjerenja veličine i zeta potencijala također su provedena u SFR-u na 32 °C. Svi uzorci su mjereni u triplikatima.

3.2.4. Određivanje fluidnosti fosfolipidnih dvoslojeva CPF-VFG-ova

Fluidnost (elastičnost/rigidnost) fosfolipidnih dvoslojeva liposoma unutar VFG-a je određena pomoću uređaja vlastoručne izrade (Vanić i sur. 2014) nakon konverzije VFG-a u liposomsku suspenziju (CPF-VFG liposomi). CPF-VFG liposomi su protisnuti kroz polikarbonatne membrane promjera pora 100 nm (r_p), pod konstantim tlakom od 2,5 bara, tijekom 5 minuta. Određena je masa (g) tekuće disperzije liposoma koja je prošla kroz membranu (J) te srednji promjer (nm) liposoma nakon ekstruzije (r_v). Fluidnost fosfolipidnih dvoslojeva iskazana je kao stupanj membranske elastičnosti (E) prema izrazu:

$$E = J \cdot (r_v/r_p)^2.$$

SPC/CHOL/CPF- i SPC/P90H/CPF-VFG liposomi su prije ispitivanja zagrijani na 60 °C. Ispitivanja su provedena u triplikatu.

3.2.5. Određivanje uspješnosti uklapanja CPF-a u liposome VFG-a

Određivanju sadržaja liposomski-ukopljenog CPF-a, prethodilo je odijeljivanje neukoplјene frakcije CPF-a (vanjska vodena faza VFG-a) od liposoma s uklopljenim lijekom. Za odjeljivanje su korištene dvije metode: metoda centrifugiranja minikolona i postupak ultracentrifugiranja.

3.2.5.1. Metoda centrifugiranja minikolona

U vodi izbubren Sephadex G-50 stavljen je u minikolone volumena 2 mL te je nakon centrifugiranja (Heraeus Megafuge 1.0, Sepatech, Ujedinjeno Kraljevstvo) pri 2000 okr/min tijekom 3 min dobiven suhi stupac gela (minikolona) visine 1,8 cm. Na tako pripremljenu minikolonu pažljivo je naneseno 400 μL liposomske disperzije. Nakon centrifugiranja (3 min, 2000 okr/min), eluat liposoma s uklopljenim lijekom sakupljen je u kiveti za centrifugiranje. U sljedećem koraku na kolonu je stavljeno 500 μL vode te je ponovljen postupak centrifugiranja (eluat je pripadao liposomskoj frakciji). Potom su minikolone ispirane s 200 μL vode, uz centrifugiranje pod istim uvjetima. Eluati su sadržavali liposomski-neuklopljeni (slobodan) CPF-a. Postupak ispiranja minikolona s vodom je ponavljan dok slobodni (neuklopljeni) CPF nije u potpunosti uklonjen iz minikolone (Vanić i sur., 2014).

Prva 2 eluata koja su sadržavala liposomski-uklopljen CPF tretirana su metanolom, kako bi se otopili (fosfo)lipidi liposoma i odredio sadržaj lijeka u liposomima. Sve ostale frakcije su razrijeđivane s vodom i služile su za određivanje sadržaja neuklopljenog CPF-a (vanjska vodena faza VFG-a).

3.2.5.2. Metoda ultracentrifugiranja

Liposomska disperzija (1 ml), nastala razrjeđenjem CPF-VFG-a s vodom (3.2.1.), je stavljena u kivetu za ultracentrifugiranje i dodatno razrijeđena s vodom (6 mL). Postupak ultracentrifugiranja je proveden na uređaju Beckman Optima LE-80 K Ultracentrifuge (Beckman Coulter Inc., Fullerton, Sjedinjene Američke Države) tijekom 60 minuta na 120.000 x g pri temperaturi od 25 °C. Supernatant, koji je sadržavao neuklopljeni lijek, je odvojen, a pelet (liposomi s uklopljenim CPF-om) je resuspendiran u 1 mL vode. Kako bi se kvantificirao sadržaj uklopljenog lijeka, CPF-VFG liposomi su tretirani metanolom (otapanje fosfolipidnog dvosloja) (Rukavina i sur., 2018).

Sadržaj CPF-a iz svih frakcija je kvantificiran spektrofotometrijski pri valnoj duljini od 278 nm, na način opisan u potpoglavlju 3.2.6.

Za svaku formulaciju su izračunati relativna uspješnost uklapanja i analitički prinos prema izrazima:

$$Uspješnost\ uklapanja(\%) = \frac{m_{\text{uklopljeni lijek}}}{m_{\text{uklopljeni lijek}} + m_{\text{slobodni lijek}}} \times 100$$

$$Analitički\ prinos\ (\%) = \frac{m_{\text{uklopljeni lijek}} + m_{\text{slobodni lijek}}}{m_{\text{ukupni lijek}}} \times 100$$

3.2.6. Određivanje sadržaja CPF-a

Sadržaj CPF-a u svim nanoformulacijama je određen dvjema metodama: UV/Vis spektrofotometrijskom metodom te HPLC-om. HPLC metoda je korištena samo u ispitivanjima permeabilnosti.

3.2.6.1. UV/Vis spektrofotometrija

Za CPF analizu je korišten UV-Vis spektrofotometar Cary 50 Probe (Varian, Palo Alto, Sjedinjene Američke Države). Kalibracijske krivulje su napravljene za vodu (R^2 = 0,9978) i metanol (R^2 = 0,9989), korištenjem osam CPF koncentracija u rasponu od 1 - 15 μg/mL. Svaki uzorak mjeren je tri puta na valnoj duljini od 278 nm te je za izradu kalibracijskog pravca uzimana prosječna vrijednost apsorbacije.

3.2.6.2. HPLC metoda

Kromatografski sustav sastojao se od: uređaja za automatsko uzorkovanje, upravljačke jedinice, pumpe, degazera, termostatirane pećnice, UV-VIS detektora, svi iz serije 1260 Infinity II (Agilent, Santa Clara, Sjedinjene Američke Države). Za obradu podataka korišten je program *OpenLab*. Uzorci su prije analize filtrirani kroz 0,22 μm polietersulfonske (PES) filtere (Nantong FilterBio Membrane Co., Ltd. Nantong, Kina). HPLC metoda za kvantifikaciju CPF-a je prilagođena iz Europske farmakopeje (Ph. Eur. 11.0, 01/2023:0888). Odjeljivanje je provedno na Kinetex C18 koloni (250 × 4,6 mm, veličina čestica 5 μm) (Phenomenex, Torrance, Sjedinjene Američke Države) s pripadajućom zaštitnom pretkolonom (Phenomenex, Torrance, Sjedinjene Američke Države). Mobilnu fazu činila je mješavina 13 volumena acetonitrila i 87 volumena 2,45 g/L otopine fosforne kiseline, prethodno podešene na pH 3,0 s trietilaminom. Temperatura pećnice je bila postavljena na 40 °C, a brzina protoka je iznosila 1,5 mL/min. Analiza pojedinačnog uzorka je trajala 10 minuta, s retencijskim vremenom CPF-a od 6,5 minuta. Volumen injektiranja je bio 20 μL, a valna duljina detekcije 278 nm. Otopine CPF-a u metanolu u koncentracijskom rasponu 1 – 15 μg/mL poslužile su za izradu kalibracijske krivulje (R^2 = 0,9980). Sva mjerenja su provedena u triplikatima.

3.2.7. Ispitivanje reoloških svojstava CPF-VFG-ova

3.2.7.1. Mjerenje viskoznosti

Viskoznost VFG-ova određena je modularnim kompaktnim reometrom MCR 102 (Anton Paar GmbH, Austrija) koristeći mjerni stožac CP25, promjera 25 mm i nagibnog kuta od 1°. Uzorci su nakon nanošenja na mjerno tijelo ostavljeni da stoje 10 minuta prije početka samog mjerenja kako bi se osiguralo da nema razlike u mjerenjima zbog manipulacije uzorkom, te kako bi se temperirali na postavljenu temperaturu. Rotacijska ispitivanja provedena su u rasponu brzina smicanja od 0,01 do 1000 s^{-1}, u 30 točaka, pri temperaturama od 25 i 32 °C.

3.2.7.2. Test promjene amplitude

Test promjene amplitude proveden je pomoću modularnog kompaktnog reometra MCR 102 (Anton Paar GmbH, Austrija) opremljenog mjernim sustavom s paralelnom pločom PP25 (promjer 25 mm), s razmakom postavljenim na 1 mm. Uzorci su nakon nanošenja na mjerno tijelo ostavljeni da stoje na 32 °C tijekom 10 minuta. Ispitivanja su provedena s angularnom frekvencijom od 10 s^{-1}, u rasponu smičnog naprezanja od 0,1 – 1000 Pa.

3.2.8. Ispitivanje bioadhezivnosti CPF-VFG-ova

Bioadhezivnost CPF-VFG-ova procijenjena je na svinjskoj koži korištenjem analizatora teksture (TA.XT Plus Texture Analyzer, Stable Micro Systems, Ujedinjeno Kraljevstvo) opremljenog s nastavkom za ispitivanje mukoadhezivnosti. Svinjska koža s područja leđa kupljena je u lokalnoj mesnici te je pomoću Nouvag Dermatoma s TCL3000BL motornim sustavom (Nouvag GmbH, Švicarska) izrezana na 1,0 mm debljine i pohranjena na -20 °C do uporabe. Neposredno prije ispitivanja je odmrznuta na sobnu temperaturu, isprana s fiziološkom otopinom i lagano posušena staničevinom (gornja strana kože). Izrezana je na diskove promjera 10 mm i pričvršćena na gornju sondu analizatora teksture cijanoakrilatnim ljepilom. Uzorci CPF-VFG-a (0,10 - 0,15 g) u obliku diska su postavljeni na donju platformu nastavka za ispitivanje bioadhezije.

Postavke mjerenja bile su: brzina spuštanja sonde do početne pozicije za ispitivanje 0,5 mm/s, brzina spuštanja sonde tijekom ispitivanja do kontakta s uzorkom 0,1 mm/s, vrijeme kontakta 60 s korištenjem konstantne sile od 0,5 N i brzina povratka sonde na početnu poziciju nakon ispitivanja 0,1 mm/s.

Bioadhezivnost je procijenjena na temelju maksimalne sile odvajanja (F_{max}) sonde s kožom od donje platforme s uzorkom CPF-VFG-a i rada adhezije (W_{ad}), izračunatog kao površina ispod krivulje udaljenosti i sile. U tu svrhu korišten je program *Exponent Connect Version 7.0.6.0* (Stable Micro Systems, Ujedinjeno Kraljevstvo). Kao kontrola je upotrijebljena otopina CPF-a u vodi (20 mg/mL). Sva ispitivanja su provedena u pentaplikatima.

3.2.9. *In vitro* oslobađanje CPF-a iz VFG-ova

Ispitivanje oslobođanja CPF-a iz pripravljenih VFG-ova je provedeno u dva medija, vodi i fosfatnom puferu, pH 7,4. Voda je odabrana kako bi se bolje simulirali nepuferirani uvjeti koji bi se mogli naći u koži. Također, zbog kiselih svojstava CPF-a, rezultirajući pH medija bio je između 4,70 i 5,55, što odgovara pH vrijednosti rožnatog sloja kože (4,1-5,8) (Proksch 2018). Kako bi se oponašali lužnatiji uvjeti prisutni u inficiranim ranama, eksperimenti su provedeni i u fosfatnom puferu, pH 7,4.

Ispitivanja su provedena tzv. dijalizacijskom metodom. Ukratko, uzorak CPF-VFG-a, čija masa odgovara 15 mg CPF-a, stavljen je u dijalizacijsku vrećicu (Medicell Membranes, Ujedinjeno Kraljevstvo, Mw cut-off 14 000 Da) i dijaliziran u 200 mL vode pri temperaturi od 32 °C uz kontinuirano miješanje (300 okr/min). U određenim vremenskim intervalima (1, 2, 3, 4, 5, 6, 24, 27 i 30 sata nakon postavljanja pokusa) uzimano je po 2 mL dijalizacijskog medija uz istovremeno nadomiještanje uzorkovanog volumena svježim, termostatiranim, medijem (Čačić i sur., 2023). Količina oslobođenog CPF-a u uzorkovanim volumenima dijalizacijskog medija određena je spektrofotometrijski (3.2.6.).

Na isti način ispitivanje je provedeno i u fosfatnom puferu, pH 7, 4, s tim da je volumen dijalizacijskog medija iznosio 100 mL, a masa CPF-VFG-a odgovarala 1 mg CPF-a (postizanje uvjeta osigurane topljivosti CPF-a).

3.2.10. Ispitivanje stabilnosti CPF-VFG-ova u SFR-u

Kako bi se bolje simulirali uvjeti kojima bi CPF-VFG-ovi bili izloženi nakon primjene na ozlijeđenu kožu, provedeno je ispitivanje njihove stabilnosti u SFR-u. Prije testiranja, CPF-VPG-ovi su prevedeni u CPF-VFG liposome, a neuklopljena frakcija lijeka je odijeljena od liposomski uklopljenog CPF-a ultracentrifugiranjem (3.2.5.). Utjecaj albumina iz SFR-a na stabilnost liposoma procijenjena je tako da je 0,5 mL liposoma s uklopljenim CPF-om pomiješano je s 1,5 mL SFR-a ili pufera, pH 7,4. Smjese su inkubirane na 32 °C i nakon 24 sata

određen je sadržaj liposomski uklopljenog CPF-a. Količina oslobođenog CPF-a iz liposoma detektirana je spektrofotometrijski (3.2.6.) nakon odjeljivanja oslobođenog lijeka iz liposoma pomoću centrifugalnog koncentratora Vivaspin 6 (Sartorius, Ulm, Njemačka). Centrifugalni koncentrator sadrži PES membranu koja zadržava molekule molekulske mase veće od 30.000. Uzorci naneseni na koncentrator su centrifugirani (Heraeus Megafuge 1.0, Sepatech, Ujedinjeno Kraljevstvo) 10 minuta na 2.000 okr/min kako bi se uklonili albumin ili lipidi koji bi mogli ometati spektrofotometrijsko mjerenje.

Stabilnost CPF-VPG-ova u SFR-u također je evaluirana određivanjem srednjih promjera, indeksa polidisperznosti i zeta potencijala CPF-VFG liposoma prema postupku opisanom u 3.2.3. Međutim, umjesto vode kao medija za dispergiranje liposoma upotrijebljen je SFR, a mjerenja su provedena na 32 °C.

3.2.11. Ispitivanje *in vitro* antibakterijske aktivnosti CPF-VFG-ova

Antimikrobni potencijal različitih nanoformulacija CPF-VPG-ova testiran je na tri bakterijska soja: *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 6538 i MRSA MFBF 10679. Planktonske bakterije su se koristile za određivanje MIK-ova, a njihovi biofilmovi za određivanje MBIK-ova i MBEK-ova. Ispitivanja su provedena s CPF-VFG liposomima nastalim konverzijom iz CPF-VFG-ova (3.2.1.). Kako bi se utvrdio mogući ujecaj sastavnica liposoma VFG-a na antibakterijsku aktivnost, ispitivanja su obuhvatila i 'prazne' liposome (bez CPF-a), nastale razrjeđenjem 'praznih' VFG-ova u vodi (3.2.1.). Provedena su po 3 testiranja za svaki bakterijski soj, a svako testiranje je izvedeno u tetraplikatu.

3.2.11.1. Priprema inokuluma i uzoraka VFG-ova za testiranje

Bakterijske kulture su uzgajane na 37 °C aerobno na odgovarajućim agar pločama. Za pripremu inokuluma korištene su 24-satne bakterijske kulture uzgojene u mediju prikladnom za svaki bakterijski soj (navedeno u poglavlju 3.1.). Bakterije su suspendirane u tom istom mediju do gustoće od 0,5 McFarlanda (denzitometar DEN-1, Grant Instruments, Ujedinjeno Kraljevstvo), što odgovara koncentraciji bakterija od $1,5 \times 10^8$ CFU/mL. Prije nasađivanja na ploče, bakterije su dodatno razrijeđene 100 puta u odgovarajućem svježem mediju kako bi se dobila koncentracija od oko 10^6 bakterijskih stanica.

Početne koncentracije CPF-VFG liposoma za testiranje na bakterijskim sojevima su dobivene razrjeđivanjem s hranidbenim medijima, ovisno o testiranom bakterijskom soju i tipu

ispitivanja koji je rađen (MIK/MBIK/MBEK). Na jednak način su pripremljeni i uzorci 'praznih' liposoma (bez CPF-a), nastali razrjeđivanjem VFG-ova bez CPF-a u vodi (3.2.1.).

3.2.11.2. Određivanje MIK-ova

Određivanje MIK-ova CPF-VFG liposoma na različitim planktonskim bakterijskim sojevima provedeno je metodom dvostruke mikrodilucije prema smjernicama Nacionalnog odbora za kliničke laboratorijske standarde (*National Committee for Clinical Laboratory Standards* (NCCLS, 2000). Kako bi se dobila početna koncentracija prikladna za testiranje, svi CPF-VFG liposomi su razrijeđeni do početne koncentracije od 16 µg/mL ekvivalenta CPF-a.

U mikrotitarsku pločicu ravnog dna s 96 jažica dodano je po 100 µL medija u sve jažice, osim u prvi stupac. Zatim je u prvi stupac dodano 200 µL CPF-VFG liposoma s početnom koncentracijom od 16 µg/mL CPF-a. Dvostrukim serijskim razrjeđenjem dobiven je raspon koncentracija ispitivanih uzoraka između 8 i 0,25 µg/mL CPF-a. Nakon toga je u sve jažice dodano po 100 µL razrijeđenog bakterijskog inokuluma te su pločice s uzorcima inkubirane 24 sata pri 37 °C. Planktonske bakterije u mediju su korištene kao pozitivna kontrola (kontrola rasta), a čisti medij, bez dodatka bakterija, kao negativna kontrola.

Osim CPF-VFG liposoma testirana je i otopina CPF-a u istom rasponu koncentracija kao i ispitivane nanoformulacije kako bi se procijenio utjecaj liposoma na antimikrobni učinak. Iz istog razloga su pod jednakim uvjetima ispitani i 'prazni' liposomi (bez CPF-a).

Nakon 24 sata inkubacije na 37 °C, MIK-ovi su procijenjeni mjerenjem apsorbancije na 570 nm (Wallac 1420 Victor 2, PerkinElmer, Sjedinjene Američke Države). MIK je definiran kao najniža koncentracija antimikrobnog sredstva koja ograničava rast bakterija (Babić i sur., 2010; Rukavina i sur., 2018).

3.2.11.3. Određivanje MBIK-ova i MBEK-ova

Određivanje MBIK-ova i MBEK-ova je provedeno prema modificiranoj metodi koju je izvorno opisao O'Toole (2011). Za ispitivanje inhibicije stvaranja biofilma, bakterije koje su suspendirane u hranidbenom mediju do gustoće od 0,5 McFarland-a dodatno su razrijeđene u omjeru 1 : 100 s minimalnim medijem M63 uz dodatak magnezijevog sulfata, glukoze i arginina u slučaju *P. aureginosa* ATCC 27853, odnosno u tekućem TSB-u obogaćenom s 0,25 % D-(+) glukoze (TSBGlc) za *S. aureus* ATCC 6538 i MRSA MFBF 10679 sojeve. Po 50 µL medija je dodano u sve jažice 96-mikrotitarske pločice s 'U' dnom, osim u prvi stupac. U prvi stupac

jažica je dodano po 100 µL ispitivane CPF nanoformulacije, razrijeđene odgovarajućim medijima ovisno o ispitivanom bakterijskom soju do koncentracije od 16 µg/mL CPF-a, te je metodom dvostruke mikrodilucije ispitan raspon koncentracija od 8 - 0,25 µg/mL CPF-a. U kontrolne jažice je dodano 100 µL čistog medija (negativna kontrola), odnosno 50 µL čistog medija pomiješanog s 50 µL bakterijske kulture kao pozitivna kontrola rasta. U sve ostale jažice je dodano po 50 µL prethodno pripremljenih bakterijskih suspenzija. Ploče su aerobno inkubirane na 37 °C tijekom 24 sata. Planktonske stanice su uklonjene, a biofilm je bojan sa 125 µL 0,1%-tnog kristal violeta tijekom 15 minuta. Mikrotitarska ploča isprana je tri puta vodom, osušena, te je dodano 150 µL 30 %-tne octene kiseline i miješano dok se svi kristali ne otope. Količina od 125 µL solubiliziranog kristal violeta prenesena je na novu mikrotitarsku ploču s ravnim dnom i izmjerena je apsorbancija na 540 nm (Wallac 1420 Victor 2, Perkin Elmer) (Perković i sur., 2023). MBIK je određen kao najmanja koncentracija CPF-VFG liposoma koja je inhibirala vidljivi bakterijski rast i formiranje biofilma nakon 24 sata inkubacije.

Za ispitivanje eradikacije biofilma, bakterije koje su suspendirane u hranidbenom mediju do gustoće od 0,5 McFarland-a dodatno su razrijeđene u omjeru 1 : 100 s minimalnim medijem M63 uz dodatak magnezijevog sulfata, glukoze i arginina u slučaju *P. aureginosa* ATCC 27853 soja, odnosno u tekućem TSB-u obogaćenom s 0,25 % D-(+) glukoze (TSBGlc) u slučaju *S. aureus* ATCC 6538 i MRSA MFBF 10679 sojeva. Po 100 µL bakterijskih suspenzija je dodano u sve jažice 96-mikrotitarske pločice, osim u posljednji stupac koji je ostavljen kao negativna kontrola rasta i u njega je dodano 100 µL čistog medija, te je ploča inkubirana 24 sata pri 37 °C kako bi se u jažicama formirao biofilm. Nakon 24-satne inkubacije i formiranja biofilma, uklonjen je medij iz jažica, potom su jažice tri puta isprane vodom. Po 50 µL svježeg medija je dodano u sve jažice 96-mikrotitarske pločice osim u prvi stupac. U prvi stupac jažica je dodano po 100 µL ispitivane CPF nanoformulacije, razrijeđene odgovarajućim medijima, ovisno o ispitivanom bakterijskom soju, do koncentracije od 1 mg/mL, te je metodom dvostruke mikrodilucije ispitan raspon koncentracija od 1 mg/mL do 2,0 µg/mL CPF-a. Predzadnji stupac pločice je ostavljen kao pozitivna kontrola te se u njemu nalazio samo hranidbeni medij s bakterijama.

Ploče su zatim ponovno inkubirane tijekom 24 sata pri 37 °C, nakon čega je uklonjen medij s ispitivanim nanoformulacijama. Za vizualizaciju biofilma provedeno je bojenje sa 125 µL 0,1 %-tnog kristal violeta tijekom 15 minuta. Mikrotitarska ploča isprana je tri puta vodom, osušena, te je dodano 150 µL 30 %-tne octene kiseline i miješano dok se svi kristali ne otope.

Količina od 125 µL solubiliziranog kristal violeta prenesena je na novu mikrotitarsku ploču s ravnim dnom i izmjerena je apsorbancija na 540 nm (Wallac 1420 Victor 2, Perkin Elmer) (Perković i sur., 2023). MBEK je određen kao najmanja koncentracija ispitivanih nanoformulacija za koju nije detektirana vijabilnost bakterija u biofilmu nakon 24 sata inkubacije.

3.2.12. Ispitivanje *in vitro* biokompatibilnosti CPF-VFG-ova

3.2.12.1. Uzgoj HaCaT stanične linije

Biokompatibilnost CPF-VFG-ova prevedenih u CPF-VFG liposome ispitana je pomoću stanične linije humanih keratinocita HaCaT (Cell Line Services, Njemačka). Za uzgoj je korišten DMEM hranidbeni medij s dodatkom 10 % fetalnog goveđeg seruma i mješavinom penicilina, streptomicina i amfotericina B. Stanične kulture uzgajane su u inkubatoru (Sanyo CO2, Nagasaki, Japan) na 37 °C, 5 % CO_2. Hranidbeni medij je mijenjan svaka 2 - 3 dana, rast stanica je praćen invertnim mikroskopom (Olympus CKX41, Japan), a stanice su presađivane kada je postignuto 80 – 90 % konfluentnosti. Prilikom presađivanja, prvo je hranidbeni medij odsisan i stanice su isprane PBS-om (pH 7,4), koji je potom uklonjen. Zatim su stanice inkubirane 3 minute u inkubatoru u otopini EDTA, nakon čega je slijedila tripsinizacija s 0,125 %-tnom (*m/V*) otopinom tripsina dok nije bilo vidljivo odvajanje stanica od podloge. Učinak tripsina inhibiran je dodatkom hranidbenog medija u volumnom omjeru 3 : 2 (hranidbeni medij : tripsin). Sadržaj je aspiriran iz tikvice za uzgoj, prebačen u epruvetu za centrifugiranje te centrifugiran 3 minute pri 800 okr/min (Heraeus Megafuge 1.0, Sepatech, Ujedinjeno Kraljevstvo). Medij iznad peleta stanica je uklonjen, a stanice su resuspendirane u svježem hranidbenom mediju te ponovno nasađene u novu tikvicu za ugoj ili ploču za testiranje.

3.2.12.2. Ispitivanje in vitro biokompatibilnosti CPF-VFG-ova MTT-testom

Postupak testiranja je modificiran prema izvornom postupku opisanom u Rukavina i sur. (2018). HaCaT stanice su nasađene na ploče s 96 jažica pri gustoći od 10^4 stanice/jažici te su ostavljene 48 sati kako bi se postigla konfluentnost. Prije dodatka nanoformulacija za testiranje, hranidbeni medij je uklonjen, a stanice su isprane PBS-om. CPF-VFG liposomi (prevedeni iz CPF-VFG-ova na način opisan u potpoglavlju 3.2.1.), CPF otopina i 'prazni' liposomi (nastali konverzijom iz 'praznih' VFG-ova) su razrjeđeni dodatkom hranidbenog medija bez dodatka suplemenata. Stanice su tretirane tijekom 24 sata, dodatkom 100 µL

uzoraka koncentracija: 1, 8, 16 i 32 μg/mL CPF-a. Stanice inkubirane u DMEM-u bez suplemenata su korištene kao negativna kontrola. Nakon 24 sata inkubacije, uzorci su pažljivo odsisani i stanice su isprane dva puta s PBS-om te je dodan kompletan hranidbeni medij. Nakon 24 sata stanicama je ispitana vijabilnost kolorimetrijskim MTT testom. U tu svrhu, u sve jažice je dodano 20 μL otopine MTT u DMEM-u bez suplemenata (5 μg/mL), a stanice su inkubirane 1 sat na 37 ºC. Princip određivanja stanične aktivnosti je baziran na činjenici da se žuto obojeni MTT u živim stanicama reducira te prelazi u ljubičasti, u vodi netopljiv, formazan. Nakon inkubacije, MTT otopina je uklonjena, dodano je 100 μL izopropanola u svaku jažicu kako bi se formazan otopio i količina formazana je kvantificirana spektrofotometrijski na 570 nm (Victor, Perkin-Elmer, Sjedinjene Američke Države). Izmjerena apsorbancija je izravno proporcionalna koncentraciji formazana te predstavlja mjeru vijabilnosti stanica.

3.2.13. Ispitivanje *in vitro* učinka CPF-VFG-ova na zacjeljivanje testom migracije keratinocita (tzv. *scratch* test)

Procjena *in vitro* učinka CPF-VFG-ova na zacjeljivanje ozlijeđene kože je provedeno prema metodi koju su razvili Liang i sur. (2007). Ispitivanje se bazira na stvaranju ogrebotine na monosloju stanica, snimanju monosloja na početku i zatim u redovitim intervalima tijekom dijeljenja stanica kako bi se mogle usporediti snimke zatvaranja ogrebotine te na taj način odrediti brzina migracije stanica (učinak zacjeljivanja).

Ispitivanje je provedeno na HaCaT staničnoj liniji. Stanice su nasađene na ploče s 24 jažice pri gustoći od 10^4 stanica/jažici i ostavljene tijekom jednog dana u hranidbenom mediju (DMEM s dodanim suplementima) kako bi postigle konfluentnost. Svaka je jažica označena s donje strane povlačenjem vodoravne linije, kako bi se osigurala točnost praćenje dijela jažice s ogrebotinom. Sljedeći dan je hranidbeni medij uklonjen i dodan je svježi DMEM (bez dodanih suplemenata). Stanice su ponovno ostavljene 24 sata, nakon čega su bile spremne za analizu. Napravljena je ravna okomita ogrebotina (pukotina) vrhom pipete od 200 μL na staničnom monosloju te je isti temeljito ispran s PBS-om (pH 7,4) kako bi se uklonile odvojene stanice. Ogrebotine su 24 sata bile izložene otopini CPF-a i CPF-VFG liposomima (nastalim iz odgovarajućih VFG-ova): SPC/CPF-, SPC/CHIT/CPF-, SPC/P90H/CPF-, SPC/CHOL/CPF- i SPC/SLPC80/PG/CPF-VFG liposomima te DMEM-u bez suplemenata koji je predstavljao kontrolu. Ispitivanja su provedena u koncentraciji CPF-a od 16 μg/mL, koja je utvrđena na temelju MIK i MBIK vrijednosti te rezultata ispitivanja *in vitro* biokompatibilnosti. Zatvaranje

ogrebotina uslijed migracije keratinocita (cijeljenje 'rane') praćeno je tijekom 24 sata pomoću invertnog mikroskopa Olympus CKX41 (Japan) pri povećanju od 200 puta. Brzina zacjeljivanja određena je na temelju razlika između površine 'rane' (ogrebotine) u trenutku njezinog nastanka (nulto vrijeme) i nakon 24 sata. Površina ogrebotine izračunata je korištenjem programa *ImageJ* (National Institutes of Health, Sjedinjene Američke Države). Učinkovitost zacjeljivanja (zarastanja) 'rane' (ZR) izražena je kao postotak zatvaranja ogrebotine nakon 24 sata u usporedbi s početnim stanjem, prema izrazu:

$$ZR\ (\%) = \frac{A(0) - A(24)}{A(0)}\ x\ 100$$

gdje je A(0) površina 'rane' u vremenu 0 (početno stanje), i A(24) je površina nakon 24 sata.

Ispitivanja su provedena u triplikatu.

3.2.14. Ispitivanje *ex vivo* permeabilnosti CPF-VFG-ova u kožu

Permeabilnost odabranih CPF-VFG-ova u kožu (*ex vivo*) određena je korištenjem sustava Phoenix™ Dry Heat (Teledyne Hanson, Sjedinjene Američke Države). Sustav je opremljen Peltierovim grijaćim blokom i automatiziranom platformom za uzorkovanje i sakupljanje sa 6 Franz-difuzijskih ćelija (nominalni volumen od 15 mL) (Phoenix RDS). U ispitivanjima je korištena koža svinjskog uha dobivena iz lokalne klaonice. U periodu od samo nekoliko sati od pogubljenja životinje, uške su obrađene na način da je prvo uklonjen višak dlake brijanjem dorzalnog dijela kože. Potom je kirurškim priborom odijeljena koža (dermis s epidermisom) od potkožnog vezivnog tkiva, isprana s fiziološkom otopinom, lagano posušena staničevinom, umotana u aluminijsku foliju te pohranjena u zamrzivač na temperaturu od -20 °C do ispitivanja.

Neposredno prije ispitivanja, uzorci kože su odmrznuti pri sobnoj temperaturi, isprani fosfatnim puferom, pH 7,4 te učvršćeni između donorskog i receptorskog odjeljka Franz-difuzijske ćelije (površina 1,77 cm^2), s rožnatim slojem okrenutim prema donorskom odjeljku. Receptorski odjeljak (15 mL) je napunjen otopinom fosfatnog pufera, pH 7,4, a sustav je termostatiran na 37 °C i kontinuirano miješan brzinom od 400 okretaja/min.

Uzorci CPF-VFG-ova ili kontrole (otopina CPF-a) u količini koja je odgovarala 1 mg CPF-a stavljena je u donorski odjeljak koji je potom zatvoren. Alikvoti od 200 µL izvučeni su iz receptorskog odjeljka nakon 1, 2, 3, 4, 5, 6, 7, 8, 16 i 24 sata te su zamijenjeni svježim

fosfatnim puferom (pH 7,4) istog volumena. Nakon 24 sata ostaci zaostale formulacije s površine kože su prenijeti u tikvicu od 10 mL, a površina kože pažljivo je isprana metanolom (3 mL). Oba su uzorka zatim sjedinjena i dopunjena metanolom do 10 ml. Taj sjedinjeni uzorak poslužio je za određivanje CPF-a koji nije penetrirao u kožu. Koža je zatim izrezana na male komadiće koji su podvrgnuti ekstrakciji u metanolu tijekom 16 sati (Rukavina i sur., 2018). Sadržaj CPF-a u svim uzorcima je kvantificiran HPLC metodom (3.2.6.). Ispitivanja su provedena u triplikatu.

3.2.15. Ispitivanja fizičke stabilnosti CPF-VFG-ova

Fizička stabilnost CPF-VFG-ova procijenjena je na temelju promjena njihovih srednjih promjera, indeksa polidisperznosti i zeta potencijala tijekom 6 mjeseci skladištenja na 4 °C. Mjerenja su provedena na način opisan u potpoglavlju 3.2.3., a uzorci CPF-VFG-ova su neposredno prije mjerenja prevedeni u tekuće liposomske disperzije (3.2.1.).

Kako bi se utvrdile moguće promjene u mehaničkim svojstvima CPF-VFG-ova tijekom pohrane pripravaka, nanoformulacije su podvrgnute reološkim ispitivanjima. U tu svrhu korišten je oscilacijski frekvencijski test koji je proveden neposredno nakon priprave CPF-VFG-ova te nakon 2 mjeseca njihova skladištenja na 4 °C. Mjerenja su provedena pomoću modularnog kompaktnog reometra MCR 102 (Anton Paar GmbH, Austrija) opremljenog mjernim tijelom s paralelnim pločama (promjer 25 mm, PP25). Razmak je postavljen na 1 mm, kutna frekvencija je bila u rasponu od 1 - 100 rad/s, smična deformacija 0,01 %, te je ispitivanje provedeno pri 32 °C. Za svaki uzorak dodano je vrijeme 'čekanja' od 10 minuta, nakon nanošenja na mjernu ploču i pritiska gornje ploče na uzorak, kako bi se osiguralo da nema razlike u mjerenjima zbog manipulacije uzorkom.

Ispitivanja su provedena u triplikatu.

3.2.16. Statistička analiza podataka

Statistička obrada podataka je provedena primjenom dvosmjerne analize varijance (engl. *two-way ANOVA*), uz *post-hoc* multiparametrijski *Tukey* test, za tri ili više grupa podataka. Srednje vrijednosti su se smatrale značajno različitima kada je razina značajnosti $p < 0,05$. Za obradu podataka je korišten program *GraphPad 8 Prism* (GraphPad Software, Sjedinjene Američke Države).

4. REZULTATI I RASPRAVA

4.1. Fizičko-kemijska karakterizacija CPF-VFG-liposoma

Fizičko-kemijska karakterizacija ima ključnu ulogu u dobivanju relevantnih podataka tijekom razvoja i optimizacije farmaceutskog oblika lijeka (terapijskog sustava, nanoformulacije). Naime, fizičko-kemijska svojstva nanoformulacije utječu na farmakokinetička svojstva uklopljenog lijeka, njegovu permeabilnost kroz biološke barijere, stabilnost nanoformulacije tijekom uskladištenja te njezino ponašanje u organizmu, što se posredno reflektira na terapijski učinak (Bhattacharjee, 2016; Vanić i sur., 2019). Kada je riječ o VFG-u, odabir odgovarajućih (fosfo)lipida, otapala i suotapala, ali i njihove koncentracije u sustavu, definiraju značajke VFG-a, odnosno liposoma kao njihovih konstitutivnih elemenata (Brandl i sur., 1998). Podešavanjem količine, tipa i omjera navedenih sastavnica VFG-ova može se utjecati na viskoznost nanoformulacije, fluidnost fosfolipidnih dvoslojeva, uspješnost uklapanja lijeka u liposome, veličinu i naboj na površini liposoma te oslobađanje lijeka iz VFG-a. Osim toga, uvjeti pri kojima se provodi homogenizacija također imaju značajan utjecaj na fizičko-kemijska svojstva VFG-a, prvenstveno na veličinu liposoma, što je potvrđeno i u prvim studijama koje su se bavile razvojem VFG-ova (Brandl i sur., 1998, 1997).

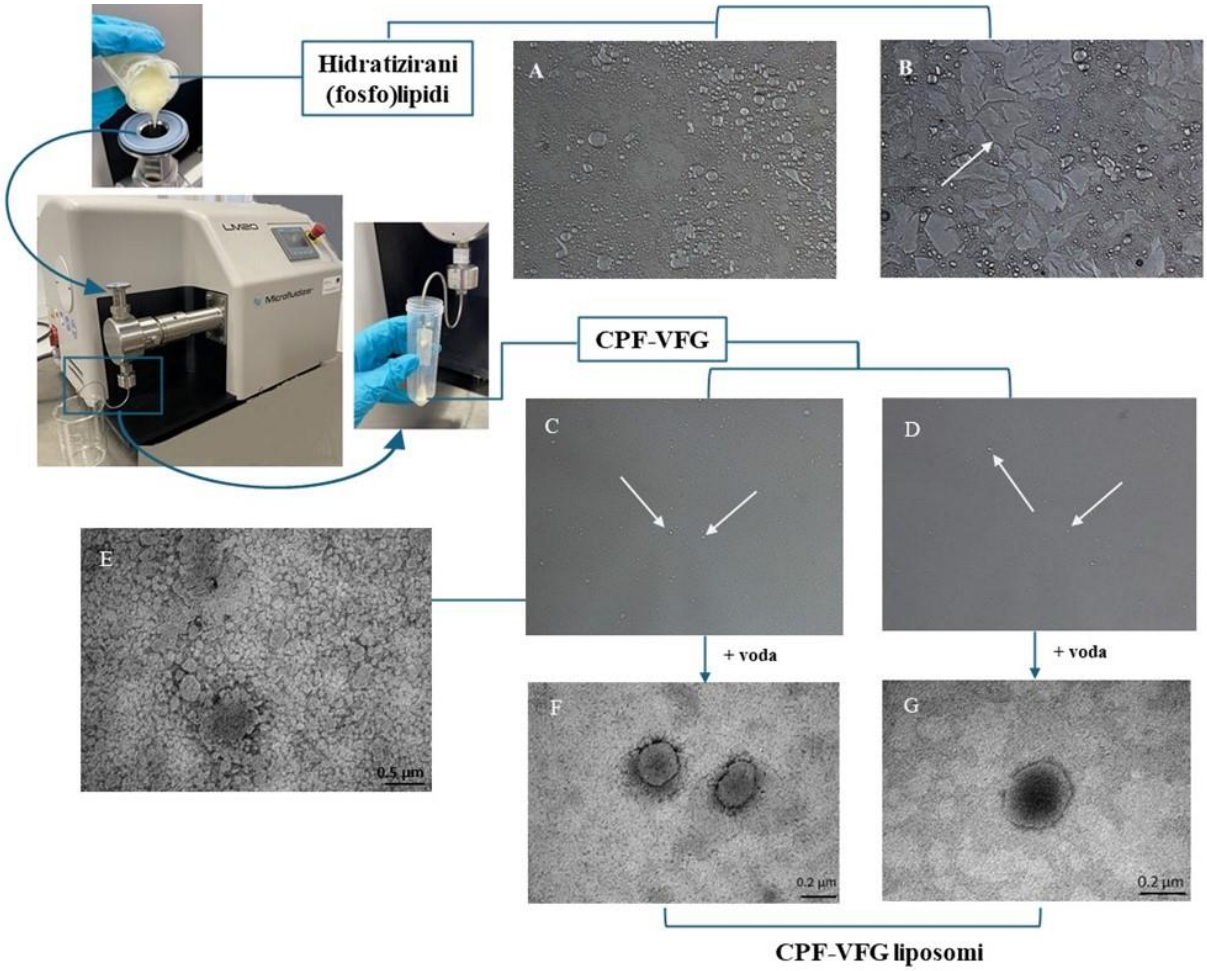
4.1.1. Veličina, naboj na površini i fluidnost CPF-VFG liposoma

Postupak i uvjeti izrade VFG-a utječu na svojstva konačne formulacije. Pokazalo se da su za formiranje polučvrste konzistencije VFG-a važni koncentracija i vrsta korištenih fosfolipida te primjenjen tlak i broj provedenih ciklusa visokotlačne homogenizacije (Brandl, 2007).

CPF-VFG-ovi su izrađeni visokotlačnom homogenizacijom hidratiziranih smjesa (fosfo)lipida u vodenoj otopini CPF-a u samo jednom ciklusu pri tlaku od 500 bara (Slika 8). Neposredno prije provođenja visokotlačne homogenizacije, SPC/CPF i SPC/P90H/CPF hidratizirane smjese fosfolipida su evaluirane mikroskopski.

Analiza uzoraka provedena korištenjem Olympus BX51 mikroskopa (Olympus, Japan) spregnutog s QICAM 1394 kamerom (QImaging, Kanada) upućuje na heterogenu smjesu hidratiziranog SPC-a (Slika 8A), a pogotovo smjese SPC-a i P90H u otopini lijeka, kod koje su uočene pločaste strukture hidrogeniranih fosfolipida (Slika 8B). Visokotlačnom homogenizacijom hidratiziranih (fosfo)lipida u otopini CPF-a nastale su viskozne homogene disperzije CPF-VFG-ova (Slike 8C i 8D) sastavljene od gusto pakiranih unilamelarnih liposoma, što potvrđuju TEM slike SPC/CPF VFG-a (Slika 8E) i SPC/CPF- i SPC/P90H/CPF-

VFG liposoma, nastalih konverzijom SPC/CPF- i SPC/P90H/CPF-VFG-ova u vodi (Slike 8F i 8G).



Slika 8. Homogenizacija hidratizirane smjese (fosfo)lipida u vodenoj otopini CPF-a na visokotlačnom homogenizatoru (500 bara) i formiranje CPF-VFG-a. Hidratizirani SPC/CPF (A) i SPC/P90H/CPF (B) u vodi te homogene disperzije SPC/CPF VFG-a (C) i SPC/P90H/CPF VFG-a (D), promatrani Olympus BX51 mikroskopom spregnutim s QICAM 1394 kamerom (povećanje od 200 x). Bijele strelice označavaju svijetlu pločastu strukturu hidrogeniranih fosfolipida (B) te sitne vezikule (liposome) (C i D). TEM prikaz homogene strukture SPC/CPF VFG-a (E), uz mjerilo od 0,5 µm. Konverzijom CPF-VFG-a u vodi (3.2.1.) nastali su unilamelarni liposomi (F, G). TEM prikazi SPC/CPF- (F) i SPC/P90H/CPF-VFG liposoma (G); mjerilo pokazuje 0,2 µm.

Mjerenja veličine liposoma svih pripravljenih CPF-VFG-ova provedena su metodom dinamičkog raspršenja svjetlosti (3.2.3.) određivanjem srednjeg promjera i indeksa

polidisperznosti, pokazatelja homogenosti/heterogenosti uzorka liposomske disperzije (Danaei i sur., 2018). Rezultati provednih ispitivanja, prikazani Tablicom 3, pokazuju da su se srednji promjeri svih CPF-VFG liposoma kretali između 126 i 193 nm, što je potvrđeno TEM analizom (Slike 8E-G) te je u skladu s rezultatima mjerenja veličine liposoma VFG-ova koja su proveli Brandl i sur. (1997). U tim studijama *cryo*-TEM metodom je određen promjer liposoma u rasponu od 100 do 200 nm (Brandl i sur., 1998, 1997).

Tablica 3. Srednji promjeri i indeksi polidisperznosti CPF-VFG-ova prevedenih u CPF-VFG liposome. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

CPF-VFG liposomi	Srednji promjer (nm)	Indeks polidisperznosti
SPC/CPF	130,0 ± 2,6	0,40 ± 0,00
SPC/CHIT/CPF	179,8 ± 4,4*	0,32 ± 0,04
SPC/P90H/CPF	193,2 ± 2,5*	0,69 ± 0,07*
SPC/CHOL/CPF	144,8 ± 2,2	0,49 ± 0,09
SPC/SLPC80/CPF	129,2 ± 2,0	0,45 ± 0,02
SPC/PG/CPF	166,2 ± 1,9*	0,27 ± 0,01*
SPC/SLPC80/PG/CPF	126,8 ± 1,4	0,43 ± 0,02

Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 3). Statistički značajna razlika u odnosu na SPC/CPF VFG liposome ($p < 0,05$).

Indeksi polidisperznosti CPF-VFG liposoma su se kretali od 0,27 do 0,69 (Tablica 3) što upućuje na blage razlike u ujednačenosti veličine liposoma unutar VFG-ova koja je posljedica njihovog različitog sastava. Najveći liposomi (193 nm, indeks poliderznosti 0,69) su bili oni koji su sadržavali hidrogenirane fosfolipide (SPC/P90H/CPF). Potom su slijedili SPC/CHIT/CPF- i SPC/PG/CPF-VFG liposomi. Najsitniji su bili SPC/SLPC80/PG/CPF VFG liposomi što je posljedica utjecaja jednolančanog fosfolipida (SLPC80) koji povećava deformabilnost dvosloja i time smanjuje veličinu liposoma (Vanić i sur., 2019). Naime, jednolančani surfaktanti djeluju kao rubni aktivatori te povećavaju elastičnost ovojnice liposoma, čineći je deformabilnijom (fleksibilnijom). Sličnu ulogu ima i propilenglikol (Palac i sur., 2014; Rukavina i sur., 2018).

Naboj na površini liposoma ima značajnu ulogu u stabilnosti liposomskih disperzija tijekom njihovog uskladištenja, ali može utjecati i na uklapanje djelatne tvari u vezikule uslijed elektrostatskih interakcija fosfolipida s molekulama lijeka. Osim toga, on znatno utječe i na

interakcije sa stanicama i bakterijama u biološkom okruženju (Qi i sur., 2020; Rukavina i sur., 2018).

Određivanje zeta potencijala, kao mjere površinskog naboja liposoma, provedeno je s CPF-VFG liposomima nastalim dispergiranjem CPF-VFG-ova u vodi (Tablica 4). Međutim, takve tekuće disperzije liposoma su uz liposomski uklopljeni CPF sadržavale i neuklopljeni CPF u vanjskoj vodenoj fazi disperzije. Budući da je CPF blago kiselih svojstava, njegovo prisustvo u vanjskoj vodenoj fazi liposomskih disperzija može rezultirati prekrivanjem izvornog naboja CPF-VPG liposoma. Stoga je, da bi se utvrdio izvorni naboj CPF-VFG liposoma, ultracentrifuginanjem odijeljen liposomski-uklopljen od neuklopljenog (slobodnog) CPF-a (3.2.5.2.).

Tablica 4. Zeta potencijali CPF-VFG liposoma. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

CPF-VFG liposomi	**Zeta potencijal (mV)**	
	Liposomi s uklopljenim CPF-om	Liposomi s uklopljenim i slobodnim CPF-om
SPC/CPF	-6,01 ± 0,49	+23,75 ± 0,51**
SPC/CHIT/CPF	+26,88 ± 1,43*	+26,82 ± 1,55
SPC/P90H/CPF	-4,22 ± 2,46	+18,53 ± 0,93**
SPC/CHOL/CPF	-1,58 ± 0,24*	+27,06 ± 0,99**
SPC/SLPC80/CPF	-8,50 ± 0,30*	+32,12 ± 0,70**
SPC/PG/CPF	+8,22 ± 0,84*	+29,85 ± 1,58**
SPC/SLPC80/PG/CPF	+5,26 ± 1,13*	+25,16 ± 0,91**

Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 3). * Statistički značajna razlika u odnosu na SPC/CPF VFG liposome ($p < 0,05$). ** Statistički značajna razlika u odnosu na CPF-VFG liposome koji sadrže samo uklopljeni CPF ($p < 0,05$).

Na vrijednosti zeta potencijala CPF-VFG liposoma značajno su utjecali sastojci VFG-a: fosfolipidi, kolesterol, propilenglikol i kitozan. Liposomi građeni od SPC-a, bez ili s dodatkom P90H ili SLPC80, imali su blago negativne vrijednosti zeta potencijala (-4 do -9 mV). Prisustvo kolesterola je rezultiralo blagim smanjenjem zeta potencijala SPC/CHOL/CPF VFG liposoma (-1,5 mV). S druge strane, prisutnost propilenglikola u SPC/PG/CPF- i SPC/SLPC80/PG/CPF-VFG liposomima dovela je do daljnjeg porasta zeta potencijala (+8 mV). Takvi blago negativni/pozitivni zeta potencijali liposoma mogli bi pridonijeti smanjenoj penetraciji uklopljenog lijeka u kožu (Ibaraki i sur., 2019), potičući time lokalizaciju CPF-a na oboljelom mjestu u koži. Najveći zeta potencijal (+25 mV; $p < 0,05$) imali su liposomi s kitozanom

(SPC/CHIT/CPF VFG liposomi), budući da kitozan pokazuje polikationsku strukturu u kiselom mediju (pKa ≈ 6,5) (Hu i Luo, 2021). Takav pozitivan naboj na površini liposoma bi mogao imati povoljan učinak na antimikrobnu aktivnost liposoma, što je pokazano u ispitivanjima antimikrobne aktivnosti liposoma s azitromicinom (Rukavina i sur., 2023). Zanimljivo je da su u prisutnosti CPF-a, koji je ostao u vanjskoj vodenoj fazi liposoma nakon rekonstitucije CPF-VFG-a s vodom, svi CPF-VFG liposomi imali pozitivan zeta potencijal (18 do 32 mV) koji je posljedica prekrivanja izvornih zeta potencijala CPF-VFG liposoma pozitivnim nabojem CPF-a (Tablica 4).

Termodinamičko stanje dvosloja liposoma značajno pridonosi permeabilnosti lijeka i liposoma u kožu, profilu oslobađanja uklopljenog lijeka te stabilnosti liposoma tijekom njhovog uskladištenja (Rukavina i sur., 2018.). Na fluidnost (čvrstoću/elastičnost) CPF-VFG-ova utjecali su svi konstitutivni sastojci membrane, uključujući propilenglikol i kitozan (Tablica 5). Elastičnost je rasla u prisustvu SLPC80, a pogotovo propilenglikola, što je u suglasnosti s prethodnim ispitivanjima u kojima su ispitivani deformabilni i propilenglikol liposomi (Rukavina i sur., 2018; Vanić i sur., 2019). Tako je stupanj membranske elastičnosti (E) iznosio 3,71 za SPC/PG/CPF VFG liposome i 3,81 za SPC/SLPC80/PG/CPF VFG liposome, u usporedbi sa SPC/CPF VFG liposomima kod kojih je E bio 1,59. S druge strane, prisutnost kitozana se odrazila na laganom povećanju čvrstoće dvoslojeva SPC/CHIT/CPF VFG liposoma (0,96), najvjerojatnije kao posljedica interkaliranja polimernih lanaca kitozana unutar membrana liposoma (Efimova i sur., 2021; Tan i sur., 2013). Kolesterol, a pogotovo hidrogenirani fosfolipidi (P90H) značajno su povećali čvrstoću fosfolipidnih dvoslojeva CPF-VPG-ova ($p < 0,0001$), što je u skladu s dobro poznatim spoznajama o utjecaju kolesterola (Santhosh i sur. 2020) i hidrogeniranih fosfolipida (Song i sur., 2022; Tai i sur., 2020) na termodinamičko stanje liposomskog dvosloja.

Slično kao u prethodnim ispitivanjima membranske elastičnosti (Palac i sur., 2014; Rukavina i sur., 2018; Vanić i sur., 2014, 2019) i u ovom istraživanju je stupanj membranske elastičnosti (E) najvećim dijelom bio ovisan o masi ekstrudiranih liposoma (J), a manjim dijelom o veličini liposoma nakon protiskivanja kroz 100 nm-sku membranu (Talica 5).

Tablica 5. Fluidnost dvoslojeva CPF-VFG liposoma. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

CPF-VFG liposomi	$(r_v/r_p)^2$	J (g)	E
SPC/CPF	28,93 ± 0,02	1,12 ± 0,08	1,59 ± 0,12
SPC/CHIT/CPF	40,58 ± 0,23	0,34 ± 0,17	0,96 ± 0,49
SPC/P90H/CPF	38,28 ± 0,46	0,07 ± 0,02	0,18 ± 0,09*
SPC/CHOL/CPF	37,65 ± 0,66	0,21 ± 0,03	0,47 ± 0,07*
SPC/SLPC80/CPF	31,61 ± 0,09	1,28 ± 0,34	2,13 ± 0,47*
SPC/PG/CPF	43,42 ± 0,37	1,21 ± 0,27	3,71 ± 0,50*
SPC/SLPC80/PG/CPF	30,41 ± 0,11	2,50 ± 0,62	3,81 ± 1,13*

E, stupanj membranske elastičnosti; J, masa liposoma nakon ekstruzije kroz membranu od 100 nm; rv, srednji promjer liposoma nakon ekstruzije; rp, veličina pora membrane (100 nm). Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 3). *Statistički značajna razlika u odnosu na SPC/CPF VFG liposome ($p < 0.05$).

4.1.2. Uklapanje CPF-a u liposome VFG-a

Učinkovita nanoformulacija za dermalnu primjenu bi trebala omogućiti visoko uklapanje djelatne tvari kako bi se osigurala dostatna koncentracija lijeka na ciljanom mjestu djelovanja u koži. Velika prednost VFG-a u odnosu na liposome je da je lijek u cijelosti uklopljen u polučvrstu nanoformulaciju (Brandl, 2007). To je pogotovo važno za hidrofilne lijekove koje karakterizira nisko uklapanje u liposome. S obzirom na činjenicu da su se *in vitro* antimikrobna i ispitivanja citotoksičnosti (biokompatibilnosti) provodila s nanoformulacijama u tekućem obliku, provedena je konverzija CPF-VFG-ova u liposome. Pritom je slobodni CPF (iz vanjske vodene faze VFG-a) uklonjen kako bi se simulirali uvjeti primjene CPF-VFG-a u kojem je sav lijek uklopljen unutar VFG-a.

Udio (%) liposomski uklopljenog CPF-a u liposome VFG-a određen je nakon odjeljivanja liposomski-neuklopljenog CPF-a dvjema metodama; metodom centrifugiranja minikolona i metodom ultracentrifugiranja (Tablica 6).

Metodom centrifugiranja minikolona dobivene su nešto više vrijednosti liposomski uklopljenog CPF-a (24,7 - 49,6 %) u odnosu na one dobivene metodom ultracentrifugiranja (18 - 33 %). Niže vrijednosti liposomski-uklopljenog CPF-a, kvantificirane nakon provedenog postupka ultracentrifugiranja, vjerojatno su posljedica primjene izrazito jake sile (120.000 x g, 60 minuta) kako bi se liposomi odijelili od neuklopljenog (slobodnog) lijeka iz vanjske vodene

faze CPF-VFG-a. Zbog velikog stresa kojem su liposomi bili izloženi tijekom postupka ultracentrifugiranja, moguće je da je došlo do gubitka dijela liposomski-uklopljenog CPF-a.

Analitički prinos za metodu centrifugiranja minikolona bio je u rasponu od 76 do 102 %, dok je za metodu ultracentrifugiranja bio značajno uži i iznosio je 94 - 102 %. Zbog jednostavnosti metode i boljeg analitičkog prinosa, ultracentrifugiranje je odabrano kao metoda odjeljivanja liposoma za daljnja ispitivanja.

Sastav VFG-a imao je velikog utjecaja na uklapanje CPF-a u liposome. U prisustvu kitozana, hidrogeniranih fosfolipida i propilenglikola udio liposomski-uklopljenog CPF-a je bio značajno viši ($p < 0,05$) u odnosu na osnovnu nanoformulaciju (SPC/CPF VFG) (Tablica 6). Takvi rezultati posljedica su većeg promjera SPC/CHIT/CPF-, SPC/P90H/CPF- i SPC/PG/CPF-VFG liposoma (Tablica 3) te time boljeg uklapanja hidrofilnog lijeka u liposome. Niže vrijednosti uklopljenog CPF-a u VFG liposome koji su sadržavali SLPC80 (Tablica 6) u sprezi je s manjom veličinom liposoma (Tablica 3) te je u skladu s rezultatima istraživanja provedenih s deformabilnim liposomima, koje karakterizira manji promjer i nešto slabije uklapanje lijeka u usporedbi s konvencionalnim liposomima (Palac i sur, 2014; Vanić i sur., 2014, 2019).

Tablica 6. Uspješnost uklapanja CPF-a u liposome VFG-a. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

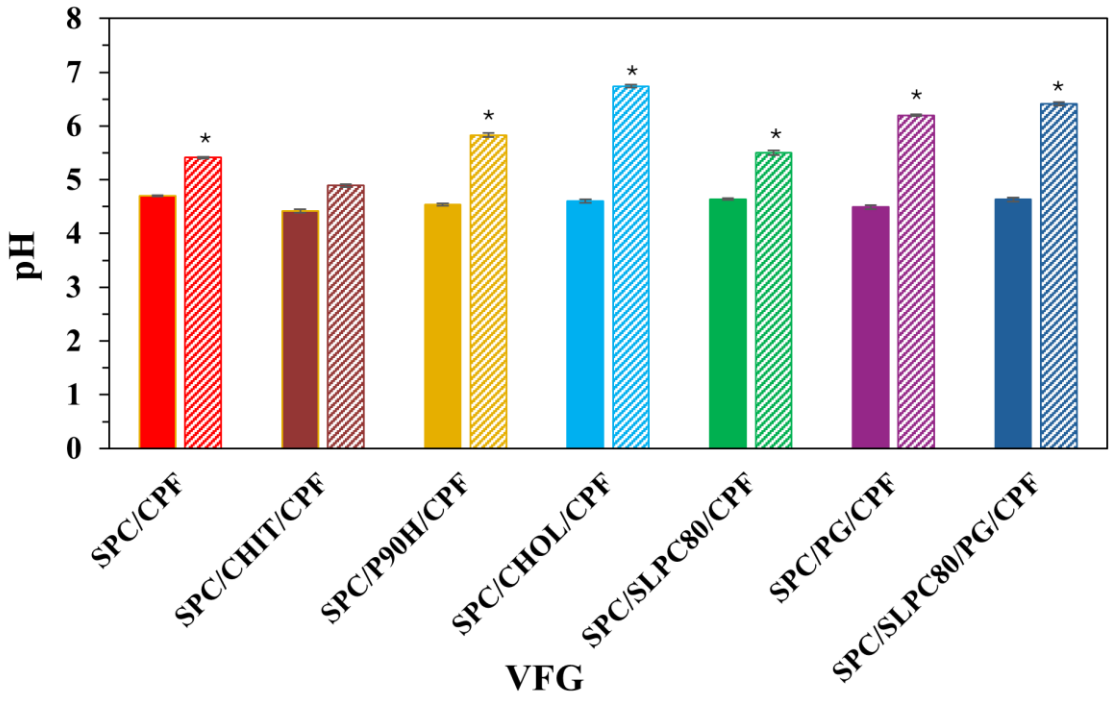
CPF-VFG	**Udio (%) uklopljenog CPF-a u liposome VFG-a**	
	Centrifugiranje minikolona	**Ultracentrifugiranje**
SPC/CPF	29,5 ± 2,1	18,0 ± 2,0*
SPC/CHIT/CPF	26,8 ± 0,6	28,5 ± 0,7
SPC/P90H/CPF	49,6 ± 3,4	26,3 ± 1,0*
SPC/CHOL/CPF	33,5 ± 0,7	22,7 ± 2,1*
SPC/SLPC80/CPF	27,0 ± 0	18,0 ± 2,0
SPC/PG/CPF	44,5 ± 0,7	33,0 ± 1,0*
SPC/SLPC80/PG/CPF	24,7 ± 2,0	25,0 ± 1,4

Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 3). *Statistički značajna razlika u odnosu na metodu centrifugiranja minikolona ($p < 0,05$).

4.2. Karakterizacija CPF-VFG-ova

Nakon što bi se nanoformulacija CPF-VPG-a primijenila na kožu, trebala bi se ravnomjerno raspodijeliti po njezinoj površini i ostati na mjestu primjene dovoljno dugo da omogući oslobađanje uklopljenog lijeka i njegov terapijski učinak. Takve karakteristike su strogo određene sastavom VFG-a i njegovim svojstvima te su reološka evaluacija, ispitivanja bioadhezivnosti i profila *in vitro* oslobađanja lijeka bitni za procjenu karakteristika pripremljenih CPF-VFG-ova. Osim toga, primjenjena nanoformulacija ne bi smjela narušavati barijernu funkciju i fiziološke značajke kože. Primjerice, pH površine zdrave kože se kreće između 4,1 i 5,8 te se povećava u infekciji do neutralnog ili čak alkalnog pH (Proksch, 2018; Sim i sur., 2022). Stoga bi primjena pripravaka s blago kiselim pH bila poželjna u lokalnoj terapiji. U tom smislu je jedan od prvih ciljeva tijekom razvoja CPF-VFG-a bio postići odgovarajući pH koji je usklađen s pH zdrave kože.

Uistinu, pH vrijednosti ispitivanih CPF-VFG-ova (Slika 9) kretale su se u rasponu pH 4,4 do 4,7 te su bile usuglašene s pH vrijednostima zdrave kože. Blago kiseli pH svih CPF-VFG-ova posljedica je prisutnog CPF-a te je povoljan u smislu očuvanja stabilnosti lijeka (Rodríguez-López i sur., 2021) i osiguravanja zaštitne barijere kože (Proksch, 2018). Nasuprot tome, pH 'praznih' VFG-ova bio je nešto viši i kretao se između pH 5,4 i 6,4, osim SPC/CHIT VFG-a čiji je pH iznosio 4,9 ($p < 0,0001$). Na takav, značajno niži, pH kitozanskog VFG-a, utjecala je octena kiselina korištena za otapanje kitozana.



Slika 9. pH vrijednosti VFG-ova s uklopljenim CPF-om (puni stupci) te 'praznih' VFG-ova (stupci s uzorkom). *Statistički značajna razlika u odnosu na istu formulaciju s uklopljenim CPF-om ($p < 0,05$). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

4.2.1. Reološka ispitivanja

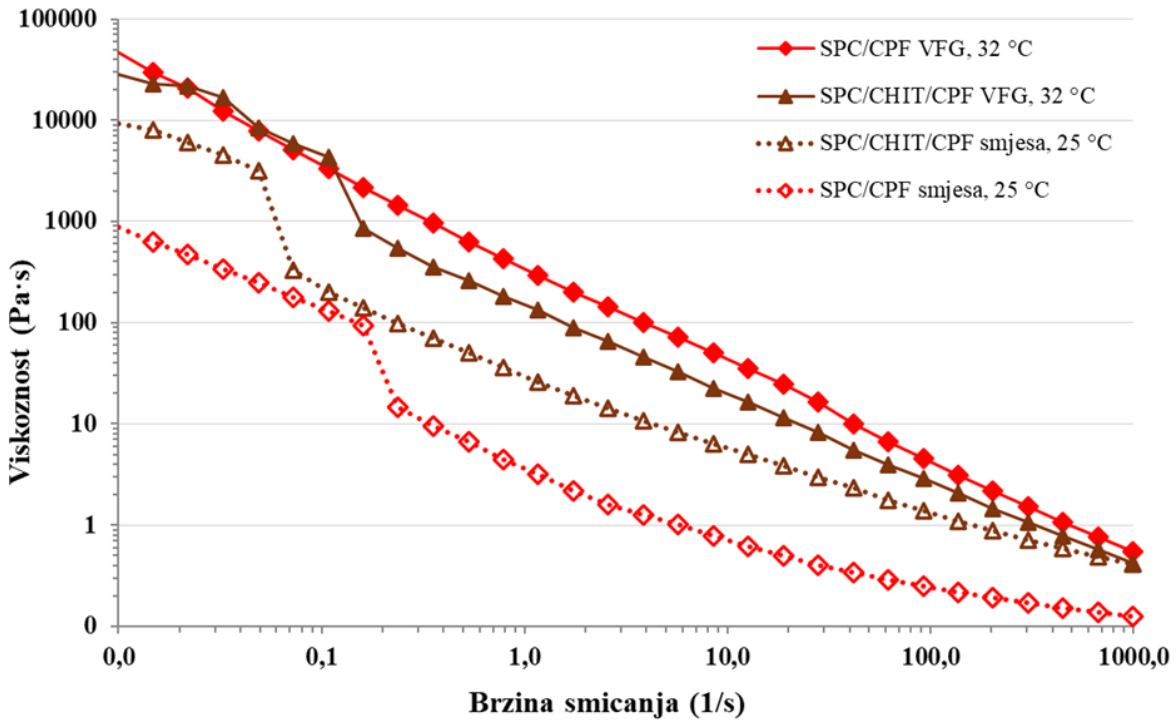
Reologija značajno utječe na karakteristike formulacije kao što su izgled, razmazivost, zadržavanje na mjestu primjene, stabilnost i učinak (Simões i sur., 2020). Razumijevanje reološkog ponašanja formulacije ključno je za objašnjenje složenog odnosa unutar njezine strukture, kemijskih interakcija sastavnica i rezultirajućih svojstava formulacije (Stojkov i sur., 2021). Iz tih je razloga bilo važno reološki okarakterizirati pripravljene CPF-VFG-ove. U tu svrhu su provedena rotacijska i oscilatorna mjerenja. Rotacijskim testovima se željelo utvrditi kako visokotlačna homogenizacija utječe na svojstva tečenja različitih VFG-ova te kako pojedini konstitutivni sastojci CPF-VFG-ova pridonose njegovoj viskoznosti, dok su oscilacijski amplitudni testovi provedeni kako bi se ispitala njihova viskoelastičnost.

4.2.1.1. Viskoznost CPF-VFG-ova

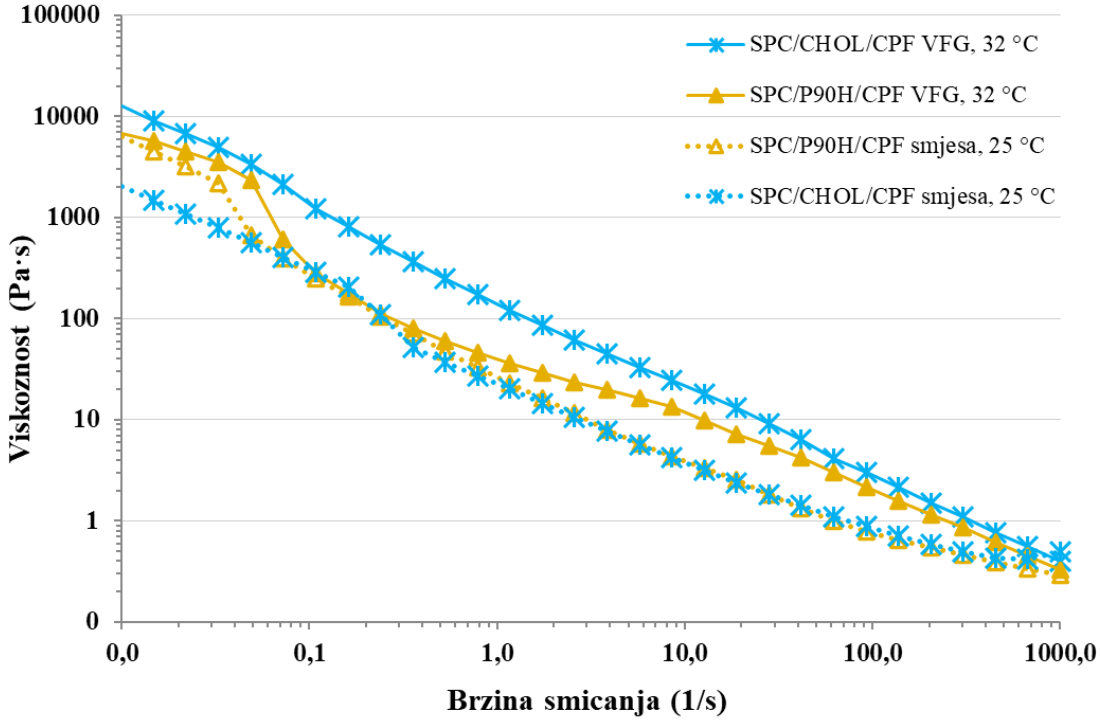
Mjerenjem viskoznosti procjenjuje se otpornost polučvrstog pripravka na razaranje njegove strukture (Simões i sur., 2020). Ispitivanjima viskoznosti podvrgnute su hidratizirane smjese fosfolipida u otopini CPF-a (prije visokotlačne homogenizacije) te pripravljeni CPF-

VPG-ovi. Hidratizirane smjese fosfolipida su testirane na 25 °C, dok su CPF-VFG-ovi ispitivani na 32 °C (temperatura površine kože).

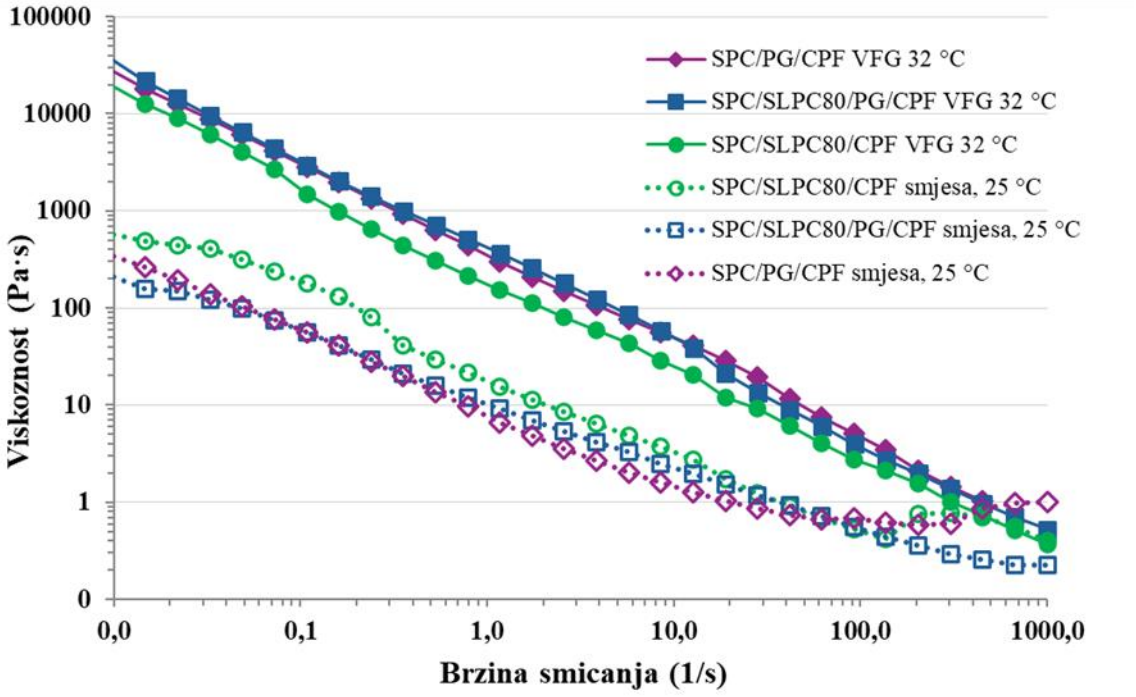
Primjenom visokotlačne homogenizacije, viskoznost svake CPF-fosfolipidne smjese povećala se za jedan red veličine (Slike 10 - 12) uslijed formiranja brojnih, gusto zbijenih, malih unilamelarnih liposoma unutar VFG-a (Slika 8E). Međuvezikularni, vodeni, prostori su pritom smanjeni što je dovelo do steričkih interakcija između susjednih liposoma unutar VFG-a i posljedično formiranja konzistencije nalik na gel (Elnaggar i sur., 2014).



Slika 10. Profili viskoznosti SPC/CPF i SPC/CHIT/CPF hidratiziranih fosfolipidnih smjesa (25 °C) i VFG-ova (32 °C), prije i nakon visokotlačne homogenizacije. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.



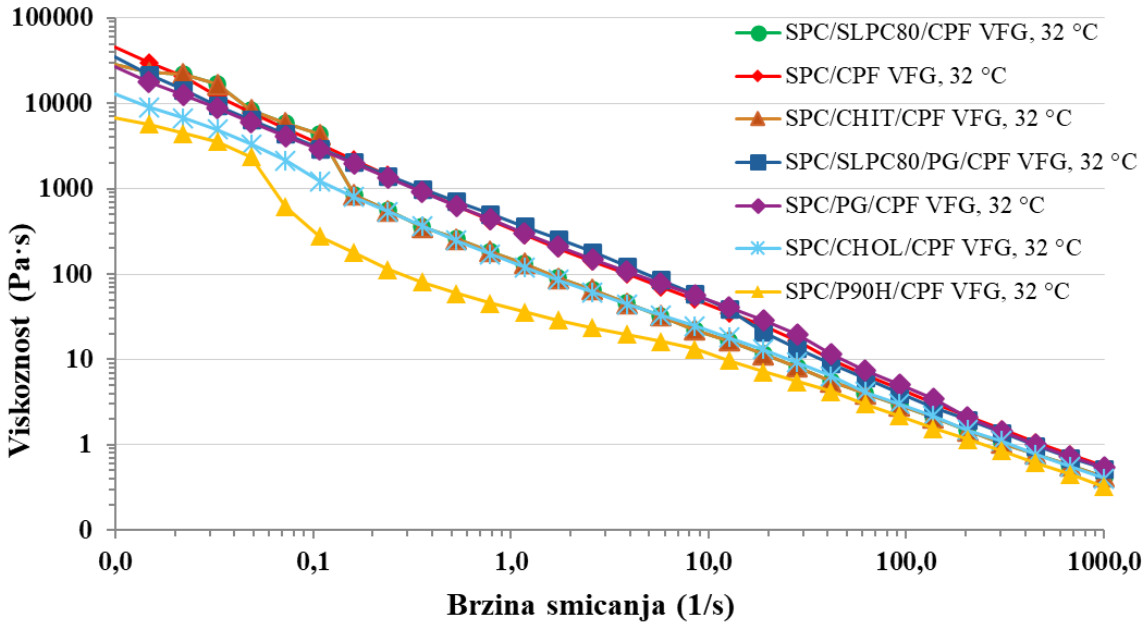
Slika 11. Profili viskoznosti SPC/P90H/CPF i SPC/CHOL/CPF hidratiziranih fosfolipidnih smjesa (25 °C) i VFG-ova (32 °C), prije i nakon visokotlačne homogenizacije. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.



Slika 12. Profili viskoznosti SPC/SLPC80/CPF, SPC/PG/CPF, SPC/SLPC80/PG/CPF hidratiziranih fosfolipidnih smjesa (25 °C) i VFG-ova (32°C), prije i nakon visokotlačne homogenizacije. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Sastavnice VFG-a odgovorne za čvrstoću (P90H, kolesterol) i elastičnost (propilenglikol, SLPC80) fosfolipidnog dvosloja nisu utjecali na viskoznost CPF-VFG-ova budući da koncentracija SPC-a nije bila konstantna za sve nanoformulacije. Naime, sastav CPF-VFG-ova je optimiziran na način kako bi se osiguralo da sve hidratizirane (fosfo)lipidne smjese imaju tekuću konzistenciju kako bi se mogle unijeti u visokotlačni homogenizator tijekom proizvodnje VPG-ova. Viskoznost je bila prvenstveno određena koncentracijom SPC-a (preliminarna istraživanja), što je u suglasnosti s prethodnim istraživanjima VFG-ova (Tardi i sur., 1998; Elnaggar i sur., 2014; Tian i sur., 2010). Sukladno tome, konačna viskoznost CPF-VFG-ova optimirana je prilagodbom količine SPC-a u svakoj nanoformulaciji.

Sumarni prikaz profila viskoznosti svih pripravljenih CPF-VFG-ova na 32 °C prikazan je Slikom 13. Svi VFG-ovi su pokazali ne-*Newtonovsko* ponašanje, karakteristično za pseudoplastične sustave, pri čemu se povećanjem brzine smicanja smanjivala viskoznost svih nanoformulacija (Slike 10-13).



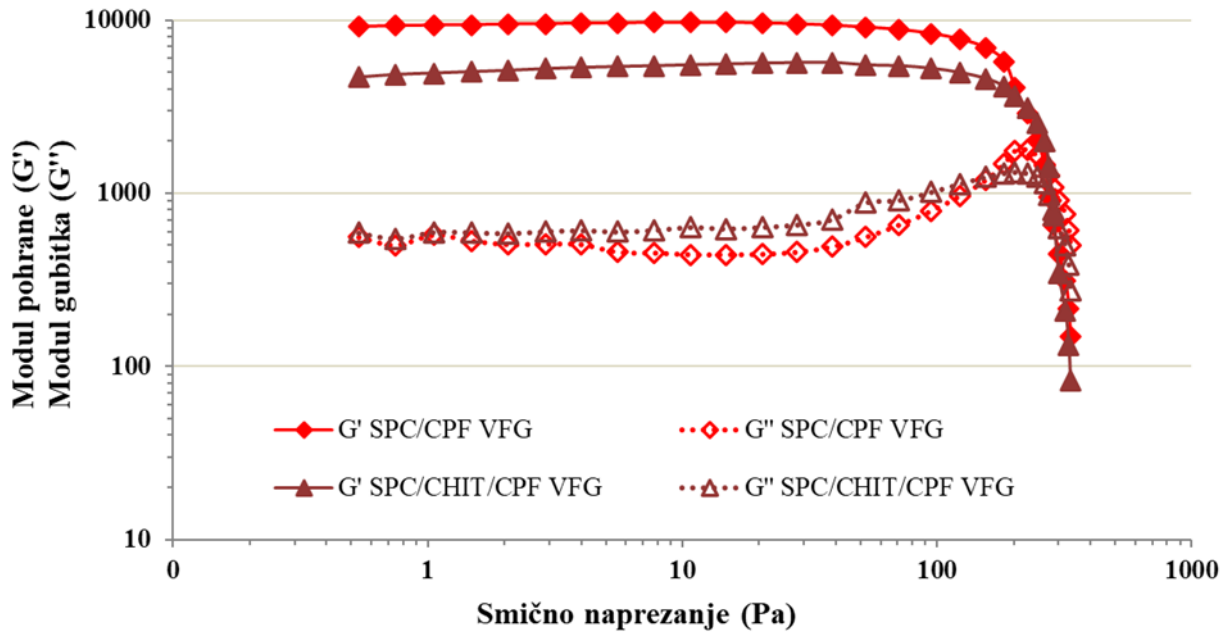
Slika 13. Profili viskoznosti pripravljenih CPF-VFG-ova na 32 °C.

4.2.1.2. Test promjene amplitude

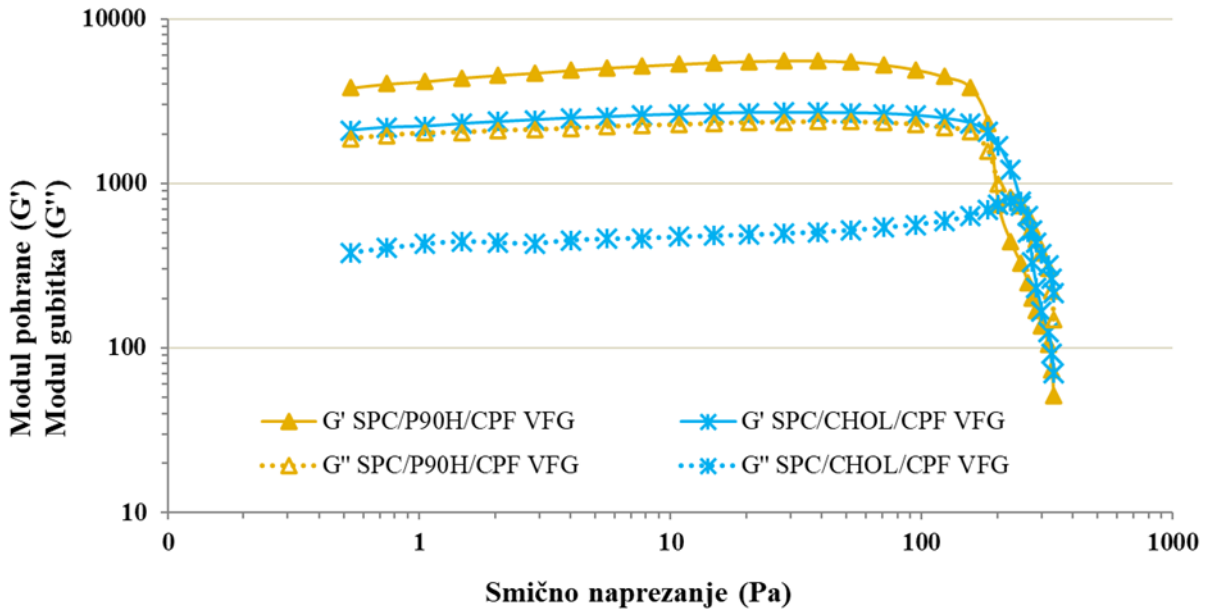
Oscilacijski testovi preferiraju se u reološkoj karakterizaciji polučvrstih formulacija jer omogućuju procjenu utjecaja sastava formulacije na viskoelastičnu strukturu gelova (Yu i sur.

2011). Provedeni su na 32 °C kako bi se procijenila svojstva tečenja CPF-VFG-ova u uvjetima dermalne primjene (rasprostiranja formulacije na koži).

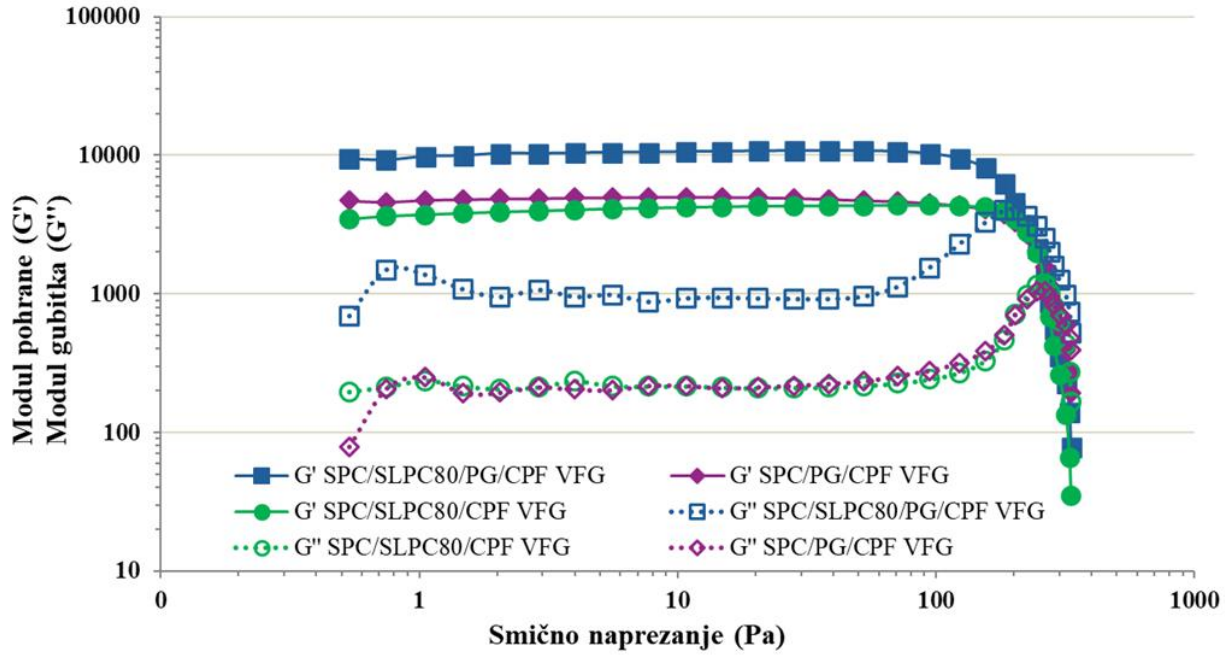
Svi CPF-VFG-ovi pokazali su linearno viskoelastično područje, tj. konstantni plato gdje su vrijednosti modula pohrane (G') i modula gubitka (G'') neovisne o smičnom naprezanju i koreliraju samo s molekularnom strukturom (Simoes i sur., 2020). Za sve testirane CPF-VFG-ove G' je bio viši od G'' (Slike 14 - 16), što ukazuje da CPF-VFG-ovi posjeduju viskoelastičnu čvrstu strukturu. Ovo zapažanje je u skladu s istraživanjem Qija i suradnika (2020.) gdje je modul pohrane također bio veći od modula gubitka za sve testirane VFG-ove koji su sadržavali citarabin.



Slika 14. Test promjene amplitude za SPC/CPF i SPC/CHIT/CPF VFG-ove pri temperaturi od 32 °C. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.



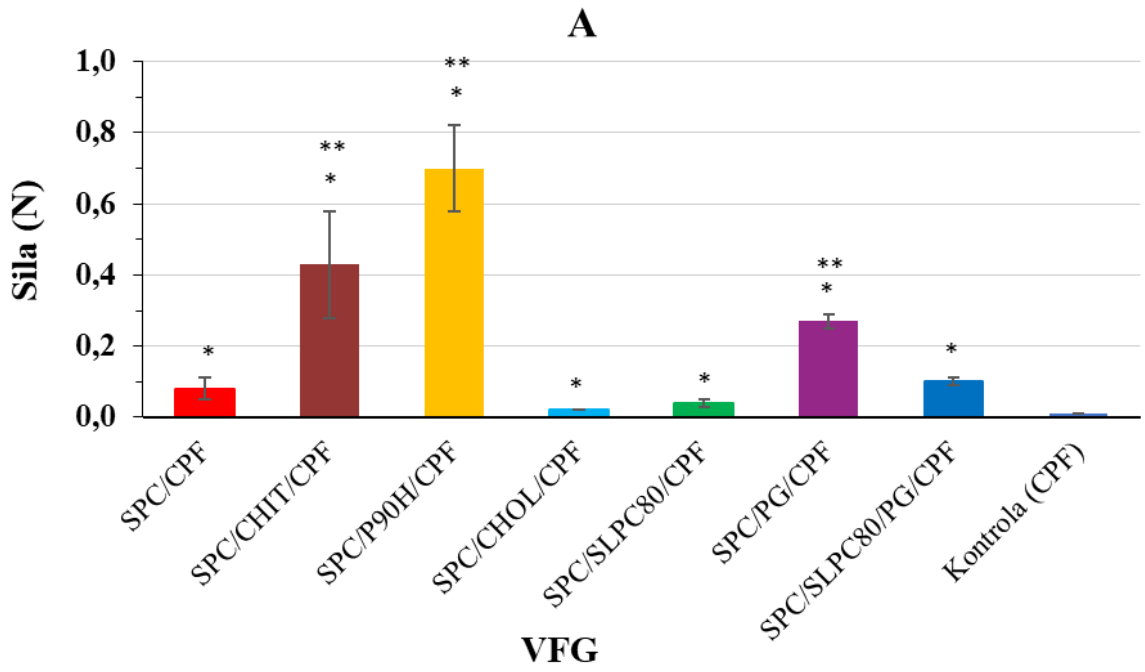
Slika 15. Test promjene amplitude za SPC/P90H/CPF i SPC/CHOL/CPF VFG-ove pri temperaturi od 32 °C. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

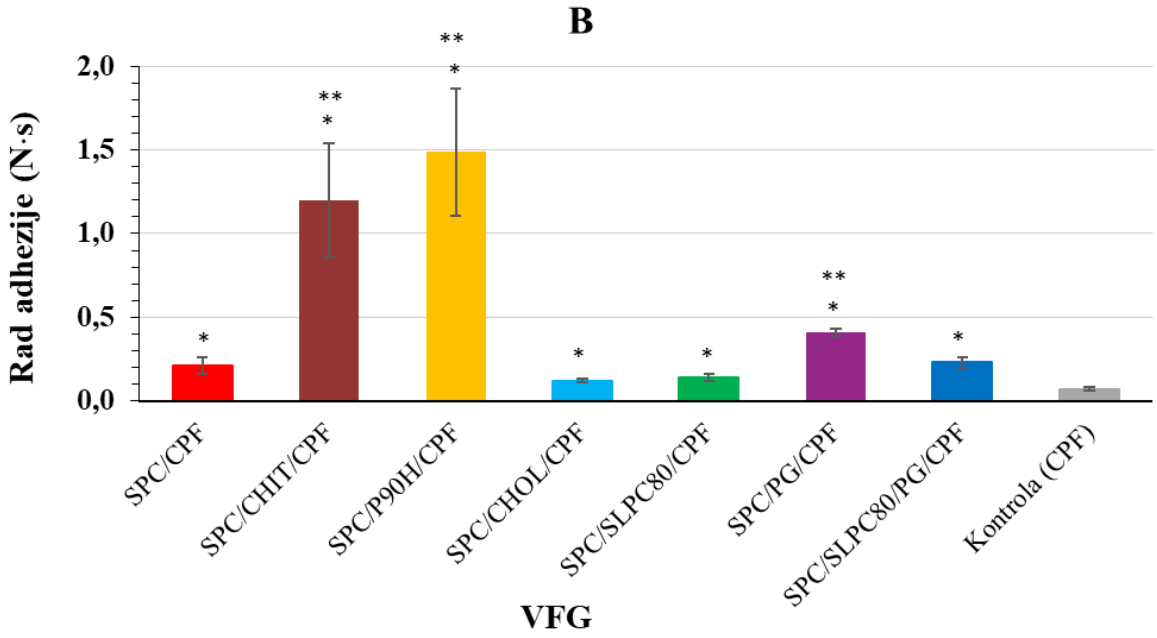


Slika 16. Test promjene amplitude za SPC/SLPC80/CPF, SPC/PG/CPF i SPC/SLPC80/PG/CPF VFG-ove pri temperaturi od 32 °C. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

4.2.2. Bioadhezivnost

Dostatno zadržavanje formulacije lijeka na mjestu primjene preduvjet je njezine terapijske učinkovitosti (Čačić i sur., 2023). Stoga je u sljedećoj fazi karakterizacije CPF-VFG-ova evaluirana njihova bioadhezivnost. Mjerenja su provedena vlačnim testom korištenjem analizatora teksture (3.2.8.), pri čemu je određivana sila odvajanja (Slika 17A) i rad potreban za odvajanje CPF-VFG-a od kože (Slika 17B). Ispitivanja su provedena na svinjskoj koži koja je među različitim animalnim modelima (Flaten i sur., 2015) najprikladnija zbog velike sličnosti s humanom kožom u pogledu strukture, debljine, folikula dlaka, pigmentacije, prisutnih kolagena i lipida (Oesch i sur., 2018; Summerfield i sur., 2015).





Slika 17. Bioadhezivnost različitih CPF-VFG-ova: sila odvajanja (A) i rad adhezije (B). Kontrolu je predstavljala otopina CPF-a. Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 5). *Statistički značajna razlika ($p < 0,0001$) u usporedbi s kontrolom. **Statistički značajna razlika ($p < 0,0001$) u usporedbi sa SPC/CPF VFG-om. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Sastav CPF-VFG-ova značajno je utjecao na njihovu bioadhezivnost pri čemu fluidnost (elastičnost/rigidnost) fosfolipidnih dvoslojeva CPF-VFG-ova nije imala utjecaja. Najveća sila potrebna za odvajanje uzorka s površine kože i rad adhezije ($p < 0,001$) zabilježeni su s CPF-VFG-om koji sadrži hidrogenirane fosfolipide (P90H), a potom slijedi CPF-VFG s kitozanom (Slika 17). Oba VFG-a su bila karakterizirana čvrstim membranama (Tablica 5). Nasuprot tome, SLPC80 i propilenglikol, ekscipijensi koji remete 'uređenost' fosfolipidnog dvosloja i povećavaju njegovu elastičnost (Vanić i sur., 2019), negativno su utjecali na bioadhezivnost CPF-VFG-ova. Sličan negativan utjecaj na bioadhezivnost zapažen je i kod VFG-a s kolesterolom (Slika 17), karakteriziranog rigidnim (čvrstim) membranama (Tablica 5).

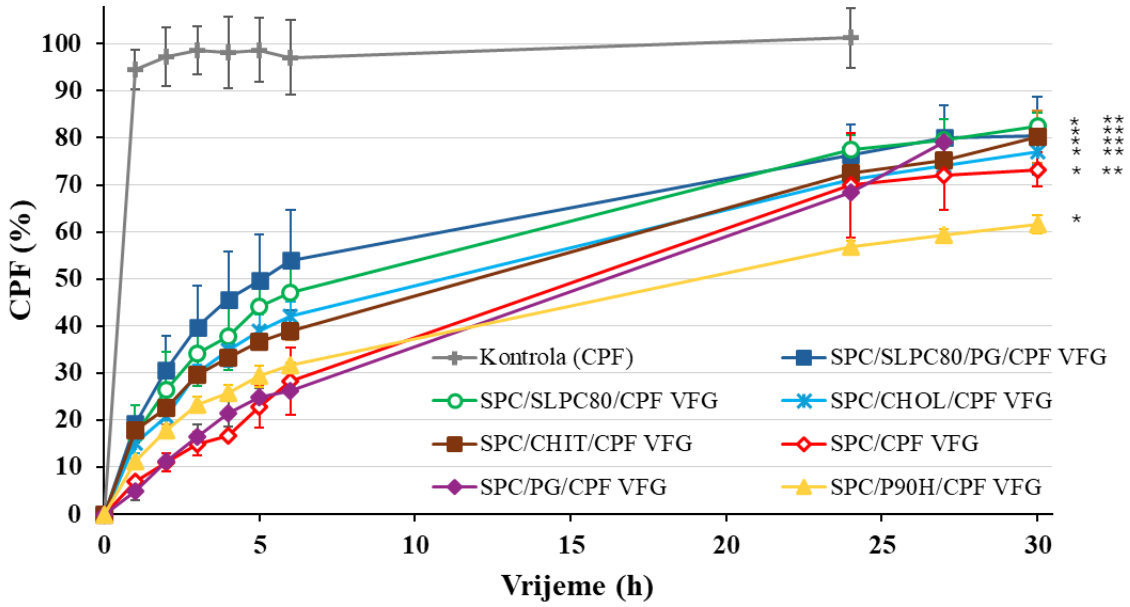
Zanimljivo je napomenuti da na bioadhezivnost CPF-VFG-ova nije utjecala njihova viskoznost, odnosno koncentracija SPC-a u nanoformulaciji. Primjerice, bioadhezivnost jako viskoznog SPC/CPF VFG-a (Slika 13) je bila gotovo 6 puta manja u usporedbi s podjednako viskoznim SPC/CHIT/CPF VFG-om i čak više od 7 puta niža od najmanje viskoznog SPC/P90H/CPF VFG-a (Slika 13). Dobra bioadhezivnost kitozanskog VFG-a je na određeni način očekivana budući je kitozan poznat po svojoj mukoadhezivnosti i dobrom prianjanju na

sluznice (Wang i sur., 2024; Čačić i sur., 2023). Osim sluznica, kitozanski hidrogelovi su, ovisno o koncentraciji kitozana u hidrogelu, pokazali veću ili podjednaku bioadhezivnost na koži od 0,5%-tnog Carbopol® Ultrez 10 hidrogela (Hurler i Škalko-Basnet, 2012). Najveća bioadhezivnost CPF-VFG-ova, zabilježena sa SPC/P90H/CPF VFG-om (Slika 17), posljedica je prisutnih hidrogeniranih fosfolipida, za koje je potvrđeno da povećavaju zadržavanje formulacije na površini kože i održavaju netaknutom zaštitnu barijeru kože (Lipoid, 2022). Naime, zbog visoke temperature faznog prijelaza, hidrogenirani fosfolipidi su u gel-stanju kada se nanesu na kožu. Osim toga, svojom strukturom oponašaju lamelarnu strukturu izvanstaničnog lipidnog matriksa kože, jačajući integritet zaštitne barijere (van Hoogevest i Fahr, 2019). Točan mehanizam kojim P90H stupa u interaciju s kožom doprinoseći superiornoj bioahezivnosti SPC/P90H/CPF VFG ostaje nerazjašnjen. Pretpostavlja se da se P90H integrira u izvanstanični lipidni matriks te povećava zadržavanje VFG-a na površini kože, no potrebno je provesti dodatna ispitivanja kako bi se ta pretpostavka potvrdila.

4.3. *In vitro* oslobađanje CPF-a iz CPF-VFG-ova

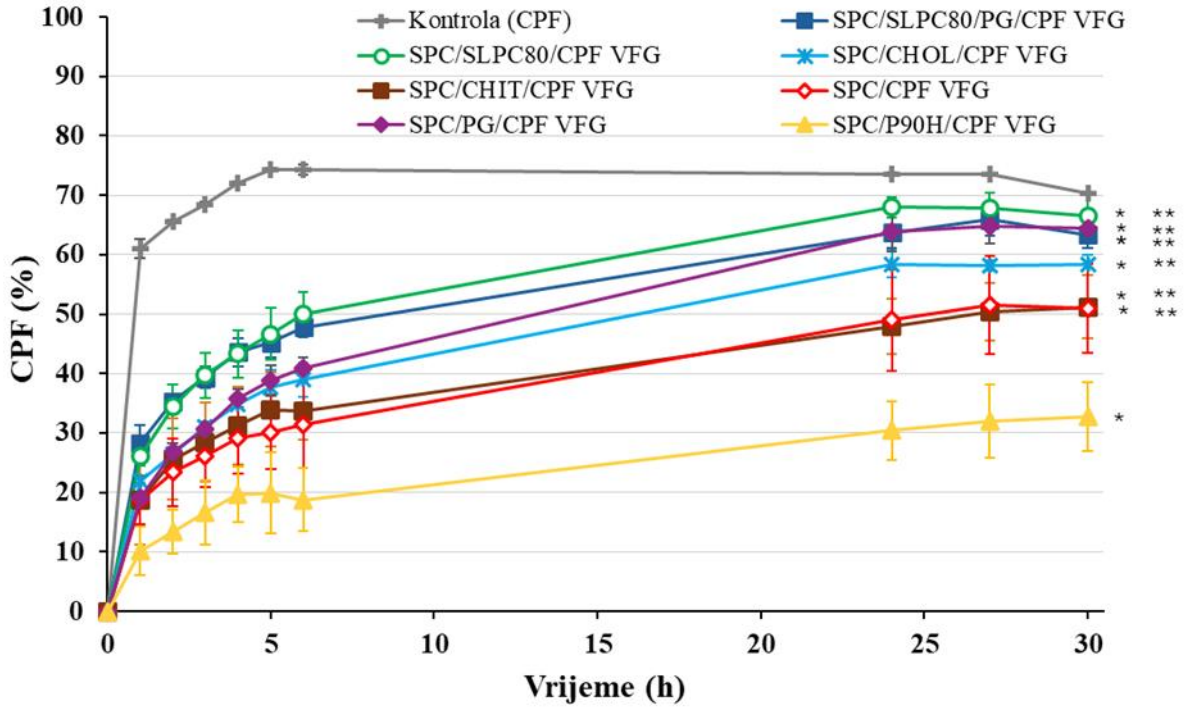
Kontrolirano oslobađanje CPF-a iz CPF-VFG-ova u mediju koji simulira uvjete *in vivo* primjene relevantno je za njihovu dermalnu primjenu. Naime, podaci dobiveni iz tih ispitivanja mogli bi poslužiti za određivanje početka djelovanja lijeka, trajanje učinka i učestalosti doziranja (Bruschi, 2015).

In vitro ispitivanje oslobađanja CPF-a iz različitih VFG-ova provedena su dijalizacijskom metodom u dva različita medija; vodi (Slika 18) i fosfatnom puferu, pH 7,4 (Slika 19). Voda je odabrana kako bi se bolje simulirali nepuferirani uvjeti koji se mogu naći u koži. Zbog kiselog pH CPF-a, rezultirajući pH medija (nakon 30. sata ispitivanja) bio je između 4,70 i 5,55, što odgovara pH vrijednosti površinskog sloja kože koji iznosi 4,1 - 5,8 (Proksch, 2018). Fosfatni pufer, pH 7,4, je odabran kako bi se oponašali lužnatiji uvjeti prisutni u otvorenim, inficiranim ranama (Sim i sur., 2022).



Slika 18. Kumulativni prikaz *in vitro* oslobađanja CPF-a iz CPF-VFG-ova u vodi. Kontrolu je predstavljala otopina CPF-a u vodi u istoj koncentraciji kao u VFG-u. Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 3). *Statistički značajna razlika ($p < 0,0001$) u usporedbi s kontrolom (1 – 24 h). ** Statistički značajna razlika ($p < 0,05$) u odnosu na SPC/P90H/CPF-VFG (nakon 24 h). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Rezultati prikazani Slikom 18 pokazuju produljeno oslobađanje CPF-a iz svih CPF-VPG-ova u usporedbi s kontrolom (CPF otopina), u kojoj je sav lijek oslobođen već unutar prvog sata ispitivanja. Među različitim CPF-VFG-ovima, oni koji sadrže SLPC80, karakterizirani elastičnim dvoslojevima (Tablica 5), omogućili su najbrže oslobađanje CPF-a (83 % tijekom 30 sati). Nasuprot tome, najsporije oslobađanje lijeka bilo je iz SPC/P90H/CPF VFG-a karakteriziranog rigidnim dvoslojevima (61 %, 30 sati). Sličan trend oslobađanja CPF-a iz različitih CPF-VFG-ova dobiven je kada su pokusi provedeni u puferu, pH 7,4 (simulacija uvjeta inficirane kože), ali je udio oslobođenog CPF-a bio niži (Slika 19) nego kad su ispitivanja provedena u vodi (Slika 18).



Slika 19. Kumulativni prikaz *in vitro* oslobađanja CPF-a iz CPF-VFG-ova u fosfatnom puferu, pH 7,4. Kontrolu je predstavljala otopina CPF-a u vodi u istoj koncentraciji kao u VFG-u. Prikazane vrijednosti predstavljaju srednju vrijednost ± S.D. (n = 3). *Statistički značajna razlika ($p < 0,0001$) u usporedbi s kontrolom (1 - 6 h). ** Statistički značajna razlika ($p < 0,05$) u odnosu na SPC/P90H/CPF-VFG (nakon 24 h). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Neovisno o ispitivanom mediju, na razlike u oslobađanju CPF-a iz različitih CPF-VFG-ova utjecala je fluidnost dvoslojeva VFG-ova i koncentracija fosfolipida. Tako je iz SPC/PG/CPF VFG-a s izraženom elastičnosti fosfolipidnih dvoslojeva (Tablica 5), oslobađanje CPF-a u prvih 6 sati bilo značajno niže ($p < 0,001$) nego kod VFG-a koji je sadržavao SLPC80 (slične elastičnosti kao VFG s propilenglikolom) budući da je koncentracija lipida bila viša u SPC/PG/CPF VFG-u (Slika 18). To je u suglasnosti s rezultatima istraživanjima Tardi i suradnika (1998) koji su pokazali značajan utjecaj koncentracije lipida VFG-a na obrazac oslobađanja lijeka; povećanjem koncentracije lipida postignuto je sporije oslobađanje lijeka i obrnuto. Zanimljivo je spomenuti da se oslobađanje CPF-a iz SPC/PG/CPF VFG-a povećalo u razdoblju od 6. do 24. sata, a razina lijeka oslobođenog nakon 24 sata bila je jednaka onoj dobivenoj iz VFG-ova koji sadrže SLPC80. Takav profil oslobađanja vjerojatno je rezultat

sporije erozije polučvrstog matriksa SPC/PG/CPF VFG-a u usporedbi s VFG-ovima koji sadrže nižu koncentraciju lipida, uključujući VFG-ove s elastičnim i čvrstim dvoslojevima. Erozija polučvrstog matriksa rezultirala je oslobađanjem CPF-a zarobljenog između liposoma VFG-a (vanjska vodena faza VFG-a), nakon čega je uslijedilo oslobađanje lijeka uklopljenog u liposome. Fluidnost membrana liposoma imala je dominantan utjecaj na oslobađanje CPF-a iz liposoma. Najsporije oslobađanje je bilo iz SPC/P90H/CPF VFG-a, rigidnih membrana (Tablica 5) iako je ukupna koncentracija lipida bila najniža u ovoj formulaciji (Slike 18 i 19).

Produljeno oslobađanje lijeka, zabilježeno kod svih VFG-ova tijekom razdoblja od 30 sati, poželjno je za osiguranje lokalnog učinka lijeka, sprječavanje ponovnog rasta bakterija te smanjenje učestalosti primjene pripravka, što je posebno značajno kod liječenja bolnih, upalnih, područja kože, uključujući rane i opekline.

Provedena ispitivanja oslobađanja CPF-a iz CPF-VFG-ova poslužila su i za procjenu kinetike oslobađanja lijeka u dva različita medija, vodi (Tablica 7) i fosfatnom puferu, pH 7,4 (Tablica 8).

Tablica 7. Koeficijenti determinacije (R^2) CPF/VFG-ova za različite kinetičke modele: ispitivanja provedena u vodi.

Uzorak	**Kinetički model**				
	Nulti red	Prvi red	Higuchi	Korsmeyer-Peppas	
				R^2	**n**
SPC/CPF VFG	0,98	1	0,99	0,99	0,72
SPC/CHIT/CPF VFG	0,96	0,99	0,99	1	0,44
SPC/P90H/CPF VFG	0,96	0,99	0,99	0,99	0,47
SPC/CHOL/CPF VFG	0,94	0,99	0,98	0,98	0,47
SPC/SLPC80/CPF VFG	0,93	0,99	0,98	0,98	0,44
SPC/PG/CPF VFG	0,99	0,99	1	0,98	0,78
SPC/SLPC80/PG/CPF VFG	0,87	0,96	0,94	0,94	0,38

Idealan kinetički model procijenjen je preko koeficijenata determinacije (R^2) za različite kinetičke modele: nulti red, prvi red, Higuchijev i Korsmeyer-Peppasov model, prema sljedećim jednadžbama:

Nulti red: $Q = Q_0 + k * t$,

Prvi red: $Q / Q_0 = 1 - e^{(-k * t)}$,

Higuchijev model: $Q = k * t^{1/2}$,

Korsmeyer-Peppasov model: $Q / Q_0 = k * t^n$.

Q predstavlja količinu lijeka koja je oslobođena u vremenu t; Q_0 je početna količina lijeka; k je konstanta oslobađanja; n je eksponent oslobađanja koji ukazuje na mehanizam oslobađanja. Za sustave s $n < 0,45$, dominantan je Fickov mehanizam difuzije, dok n vrijednosti u rasponu $0,45 < n < 0,89$ označavaju ne-Fickovu difuziju (Bruschi, 2015).

U slučaju ispitivanja oslobađanja CPF-a u vodi, konstante determinacije bile su najviše za prvi red za većinu CPF-VFG-ova. Izuzetak su bili SPC/CHIT/CPF VFG, gdje je Korsmeyer-Peppasov model najbolje odgovarao i SPC/PG/CPF VFG kod kojeg je Higuchijev model bio dominantan. Vrijednosti Korsmeyer-Peppasovih eksponenta difuzije su bile u rasponu od 0,38 do 0,78 (Tablica 7), ovisno o formulaciji CPF-VFG-a. Formulacije s eksponentima između $0,1 < n < 0,45$ pokazuju da difuzija CPF-a iz CPF-VFG-ova u medij za oslobađanje (voda) slijedi Fickov mehanizam difuzije. SPC/CPF VFG i SPC/PG/CPF VFG imali su vrijednosti Korsmeyer-Peppasovih eksponenta difuzije u rasponu između $0,45 < n < 0,89$, što ukazuje na ne-Fickovu difuziju (Bruschi, 2015; Romić i sur., 2016). Ove razlike u kinetici oslobađanja lijeka i mehanizmima transporta mogle bi biti posljedica upotrebe vode kao medija za oslobađanje. S obzirom na činjenicu da voda nije puferirana, oslobađanje CPF-a iz nanoformulacija izravno je utjecalo na pH medija što bi moglo biti uzrokom navedenih razlika.

Tablica 8. Koeficijenti determinacije (R^2) za različite CPF-VFG za različite kinetičke modele: ispitivanja provedena u fosfatnom puferu, pH 7,4.

Uzorak	**Kinetički model**				
	Nulti red	Prvi red	Higuchi	Korsmeyer-Peppas	
				R^2	**n**
SPC/CPF VFG	0,96	0,98	0,99	1	0,3
SPC/CHIT/CPF VFG	0,91	0,95	0,97	0,98	0,28
SPC/P90H/CPF VFG	0,92	0,94	0,97	0,97	0,33
SPC/CHOL/CPF VFG	0,92	0,95	0,97	0,99	0,29
SPC/SLPC80/CPF VFG	0,88	0,96	0,95	0,97	0,27
SPC/PG/CPF VFG	0,92	0,96	0,98	0,99	0,35
SPC/SLPC80/PG/CPF VFG	0,88	0,92	0,95	0,98	0,24

Kada su ispitivanja oslobađanja CPF-a iz CPF-VFG-ova provedena u puferu, pH 7,4, konstante determinacije bile su najveće za Korsmeyer-Peppasov model. To je u skladu s istraživanjima koje su proveli Wu i sur. (2019) prema kojem je oslobađanje lijeka iz unilamelarnih liposoma slijedilo Korsmeyer-Peppasov model.

Vrijednosti Korsmeyer-Peppasovih eksponenata difuzije su bile u rasponu od 0,24 do 0,35 (Tablica 8), što ukazuje da je difuzija CPF-a iz CPF-VFG-ova u medij za oslobađanje (fosfatni pufer, pH 7,4) slijedila Fickov mehanizam difuzije. Takav rezultat je očekivan s obzirom na to da je CPF hidrofilni lijek. Naime, kada je hidrofilni lijek uklopljen u nosač (matricu), oslobađanje se odvija putem mehanizma difuzije (Bruschi, 2015).

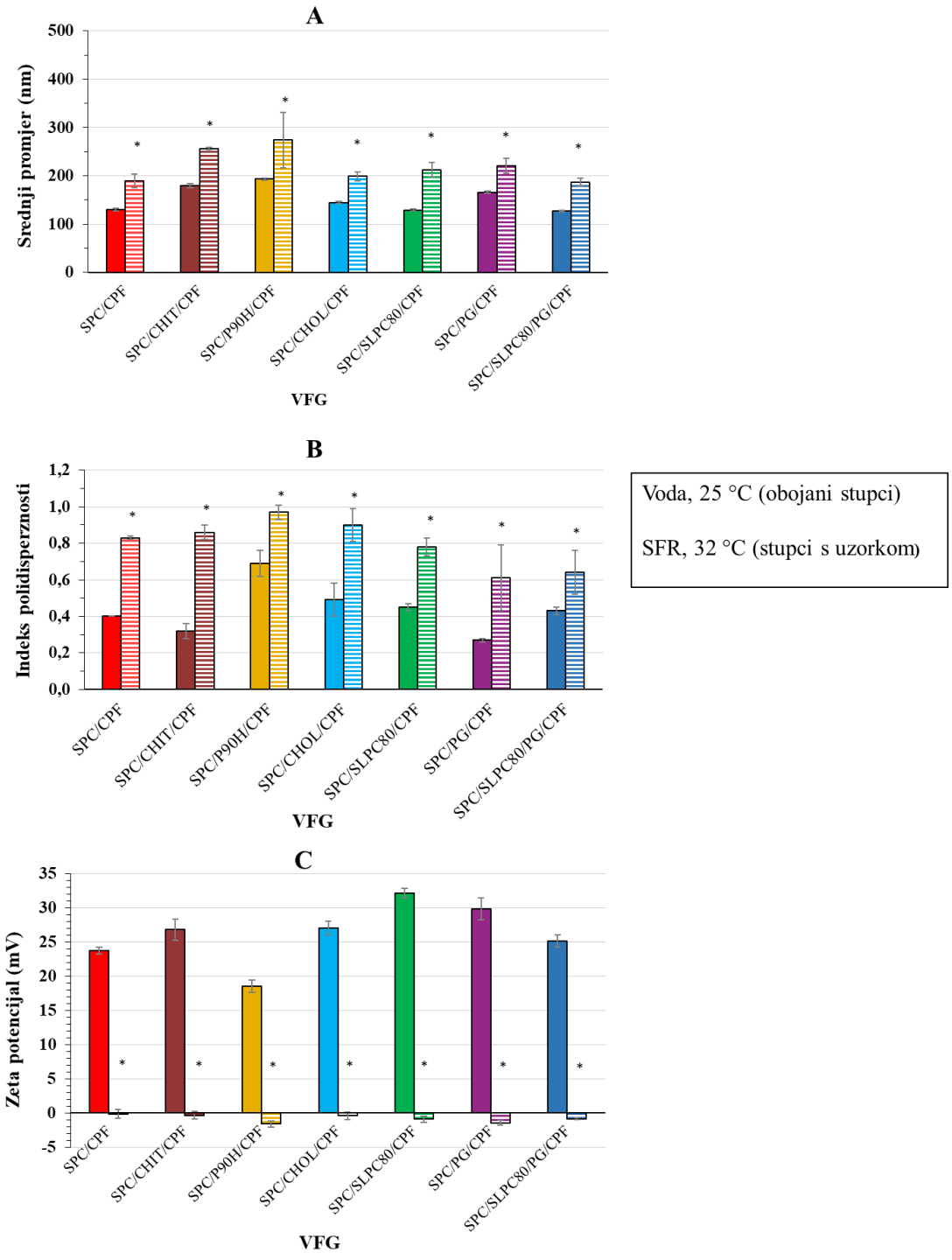
4.4. Stabilnost CPF-VFG liposoma u simuliranim *iv vivo* uvjetima

Primjena klasičnih formulacija lijekova za kožu, posebice onih za liječenje ozlijeđene i inficirane kože, može rezultirati neadekvatnim terapijskim učinkom ili čak izostankom učinka uslijed nedostatne koncentacije lijeka na mjestu djelovanja (Rukavina i sur., 2018). Razlog tome može biti neodgovarajuće oslobađanje lijeka i/ili njegova nestabilnost na mjestu primjene (biološkom okruženju). Kako bi se osigurala visoka lokalna koncentracija lijeka na mjestu infekcije, uz odgovarajući profil oslobađanja, farmaceutski oblik bi također trebao zaštititi uklopljeni lijek od nepovoljnog utjecaja biološkog miliea (Aulton i Taylor, 2017).

Nakon što bi se CPF-VFG aplicirao na kožu, uslijedila bi erozija matriksa VFG-a pri čemu bi se prvo oslobodio CPF zarobljen između vezikula VFG-a, a CPF-VFG liposomi (odgovorni za sporije oslobađanje lijeka) bili bi izloženi okolini ozlijeđene i inficirane kože koja podrazumijeva okruženje s pH vrijednosti višom od 7,0 te prisutnost proteina, kao što je albumin (Boateng i sur., 2013; Metcalf i sur., 2019). Budući da bi takvo okruženje moglo utjecati na fizička svojstva CPF-VFG liposoma (veličina, površinski naboj) te uzrokovati destabilizaciju liposomskog dvosloja i oslobađanje uklopljenog CPF-a, bilo je potrebno provesti ispitivanja stabilnosti liposoma u simuliranim uvjetima *in vivo* primjene. Fizičke karakteristike CPF-VFG liposoma ispitane su neposredno nakon njihove priprave dispergiranjem uzoraka liposoma u vodi, mediju u kojem su CPF-VFG-ovi izrađeni (Tablice 3 i 4), čime se ujedno oponašaju uvjeti njhove primjene na neozlijeđenu kožu koja nije zahvaćena infekcijom, te SFR-u koji simulira patološko, inficirano područje (Slike 20A-C). Osim toga, provedena su i ispitivanja u kojima je testiran učinak neutralnog pH i prisutnog albumina iz SFR-a na stabilnost CPF-VFG liposoma u smislu oslobađanja izvorno uklopljenog CPF-a iz liposoma (Slika 21).

Rezultati provedenih ispitivanja pokazuju da se izvorna veličina svih CPF-VFG liposoma značajno povećala ($p < 0,001$) nakon izlaganja simuliranim patološkim uvjetima (Slike 20A i 20 B) u usporedbi s rezultatima dobivenim mjerenjima srednjeg promjera i indeksa

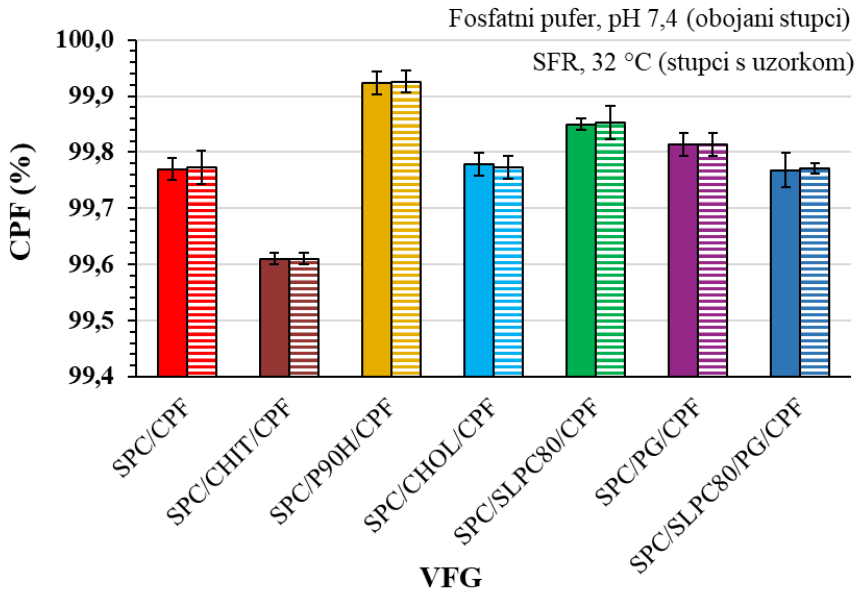
polidisperznosti u vodi. Zeta potencijali CPF-VFG liposoma također su se značajno promijenili ($p < 0,001$) te ukazuju na neutralan površinski naboj svih uzoraka (Slika 20 C).



Slika 20. Srednji promjeri (A), indeksi polidisperznosti (B) i zeta potencijali (C) CPF-VFG liposoma. CPF-VFG liposomi su dispergirani u vodi (25 °C) ili SFR-u (32 °C). Rezultati su izraženi kao srednja vrijednost ± S.D. (n = 3). *Statistički značajna razlika ($p < 0,0001$) u usporedbi s CPF-VFG liposomima (mjerenja u vodi, 25 °C). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Promjene u veličini i naboju na površini CPF-VFG liposoma u prisustvu SFR-a (32 °C) (Slika 20) vjerojatno su posljedica elektrostatske interakcije površine liposoma s pozitivno nabijenim albuminom te formiranja sloja proteina na površini liposoma (Taguchi i sur., 2021). Međutim, ta interakcija nije rezultirala oslobađanjem izvorno uklopljenog CPF-a iz liposoma, čak niti nakon 24 sata njihove inkubacije u SFR-u na 32 °C (Slika 21). Štoviše, sadržaj izvorno uklopljenog CPF-a ostao je nepromijenjen, jednak kontroli koju su predstavljali CPF-VFG liposomi inkubirani u fosfatnom puferu, pH 7,4.

Usporedbom rezultata provedenih ispitivanja utvrđeno je da je, unatoč značajnom povećanju veličine CPF-VFG liposoma ($p < 0,001$) u simuliranim *in vivo* uvjetima, njihov srednji promjer ostao manji od 300 nm (Slika 20 A) što se smatra prihvatljivim za lokalnu primjenu (Hemmingsen i sur., 2021). Naime, liposomi većeg promjera osiguravaju zadržavanje lijeka u površinskim slojevima eipidermisa ili na samoj površini kože (El Maghraby i sur., 2006), što pogoduje lokaliziranom učinku dobro permeabilnih lijekova.



Slika 21. Stabilnost CPF-VFG liposoma u simuliranim *in vivo* uvjetima, iskazana kao sadržaj CPF-a zadržan u liposomima nakon 24 sata inkubacije u SFR-u (pH 7,4) na 32 °C. Kontrolu su predstavljali CPF-VFG liposomi inkubirani u fosfatnom puferu, pH 7,4 na 32 °C. Rezultati su izraženi kao srednja vrijednost ± S.D. (n = 3). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

4.5. Fizička stabilnost CPF-VFG-ova tijekom skladištenja

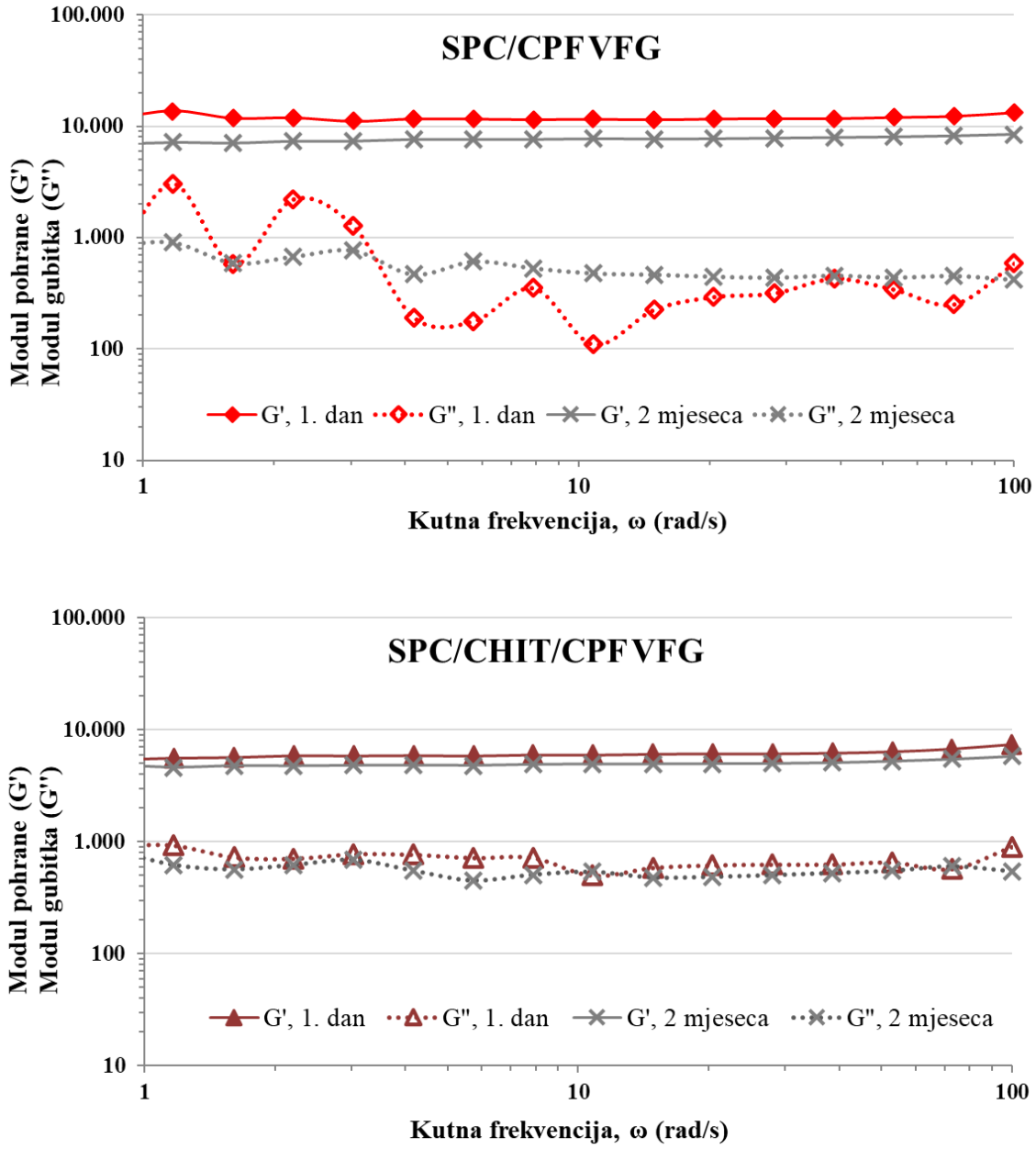
Fizička stabilnost topikalnih formulacija lijekova je iznimno važna kako pripravak ne bi izgubio na svojoj učinkovitosti i sigurnosti tijekom uskladištenja (Aulton i Taylor, 2017). Stabilnost CPF-VFG-ova tijekom njihove pohrane na 4 °C procijenjena je na temelju reoloških karakteristika (Slike 22 - 25) i promjena u veličini i površinskom naboju CPF-VFG liposoma tijekom skladištenja CPF-VPG-ova na 4 °C (Slika 25).

Poznavanje reoloških karakteristika polučvrste topikalne formulacije relevantno je ne samo zbog utjecaja na izgled formulacije, njezinu razmazivost po koži i zadržavanje na mjestu primjene, već i zbog uvida u moguću fizičku nestabilnost formulacije tijekom skladištenja. Detaljna reološka karakterizacija pruža uvid u promjene koje bi mogle dovesti do nestabilnosti, poput razdvajanja faza pripravka tijekom predviđenog roka valjanosti. Nadalje, pomoću reoloških testova se može odrediti postoji li značajan utjecaj primjene sile na mikrostrukturu formulacija kada ih se primjerice dozira iz tube/spremnika za doziranje. Pored toga, biofarmaceutska svojstva, uključujući oslobađanje uklopljenog lijeka, permeabilnost, bioadhezivnost i razmazivost, su također definirani reološkim profilom formulacije, pri čemu svaka promjena izvornih reoloških karakteristika može dovesti do promjena biofarmaceutskih značajki formulacije (Simões i sur., 2020; Sivaraman i sur., 2017). Zbog svega navedenog procjena fizičke stabilnosti CPF-VFG-ova je obuhvatila ispitivanje njihovog reološkog profila (svojstva tečenja) pri čemu je korišten test promjene frekvencije (Adeyeye i sur., 2002; Batheja i sur., 2011; Namjoshi i sur., 2020; Simões i sur., 2020; Sivaraman i sur., 2017).

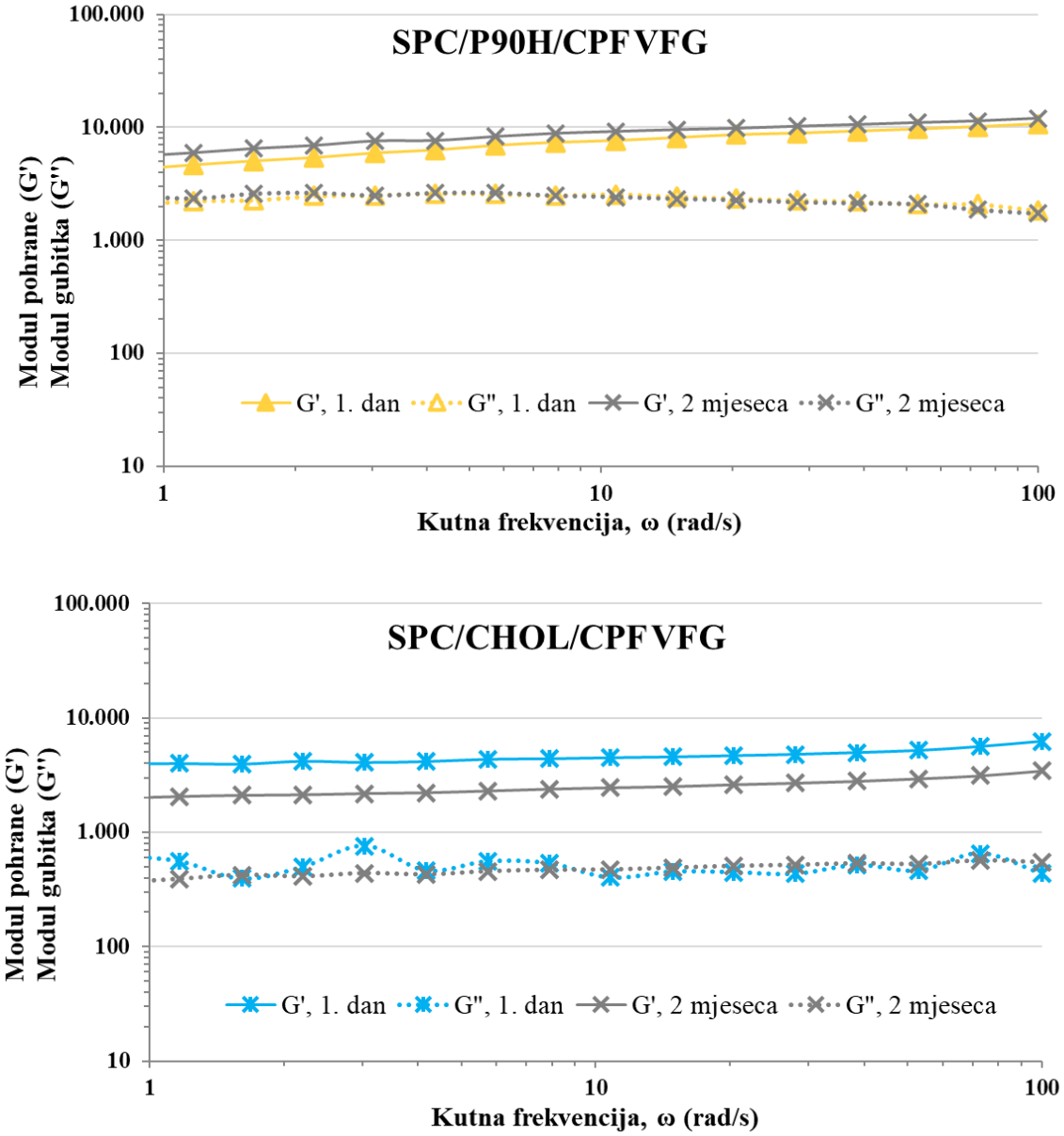
Test promjene frekvencije je dinamički reološki test u kojem je uzorak izložen postupnom povećanju frekvencije uz primjenu konstantne vrijednosti smične deformacije (Korhonen i sur., 2000). Smična deformacija je određena primjenom testa promjene amplitude (3.2.7.2.), te je utvrđeno da je optimalna vrijednost iznosila 0,01 % (4.2.1.2.).

Rezultati provedenog testa promjene frekvencije (Slike 22 - 25) pokazuju dominaciju modula pohrane (G') nad modulom gubitka (G") kod svih CPF-VFG-ova, neposredno nakon izrade, te nakon 2 mjeseca skladištenja na 4 °C. Stoga proizlazi da se svi CPF-VFG-ovi mogu smatrati viskoelastičnim sustavima tijekom ispitivanog vremenskog perioda, budući da u njihovoj mikrostrukturi dominiraju gusto zbijeni liposomi koji tvore oblik fosfolipidnog gela. Kada se za neki mjereni uzorak utvrdi da je $G' > G''$, to znači da prevladavaju elastična svojstva materijala, što podrazumijeva da će se materijal nakon uklanjanja deformirajuće sile vratiti u prvobitno stanje. Drugim riječima, tijekom procesa deformiranja, prevalencija elastičnih

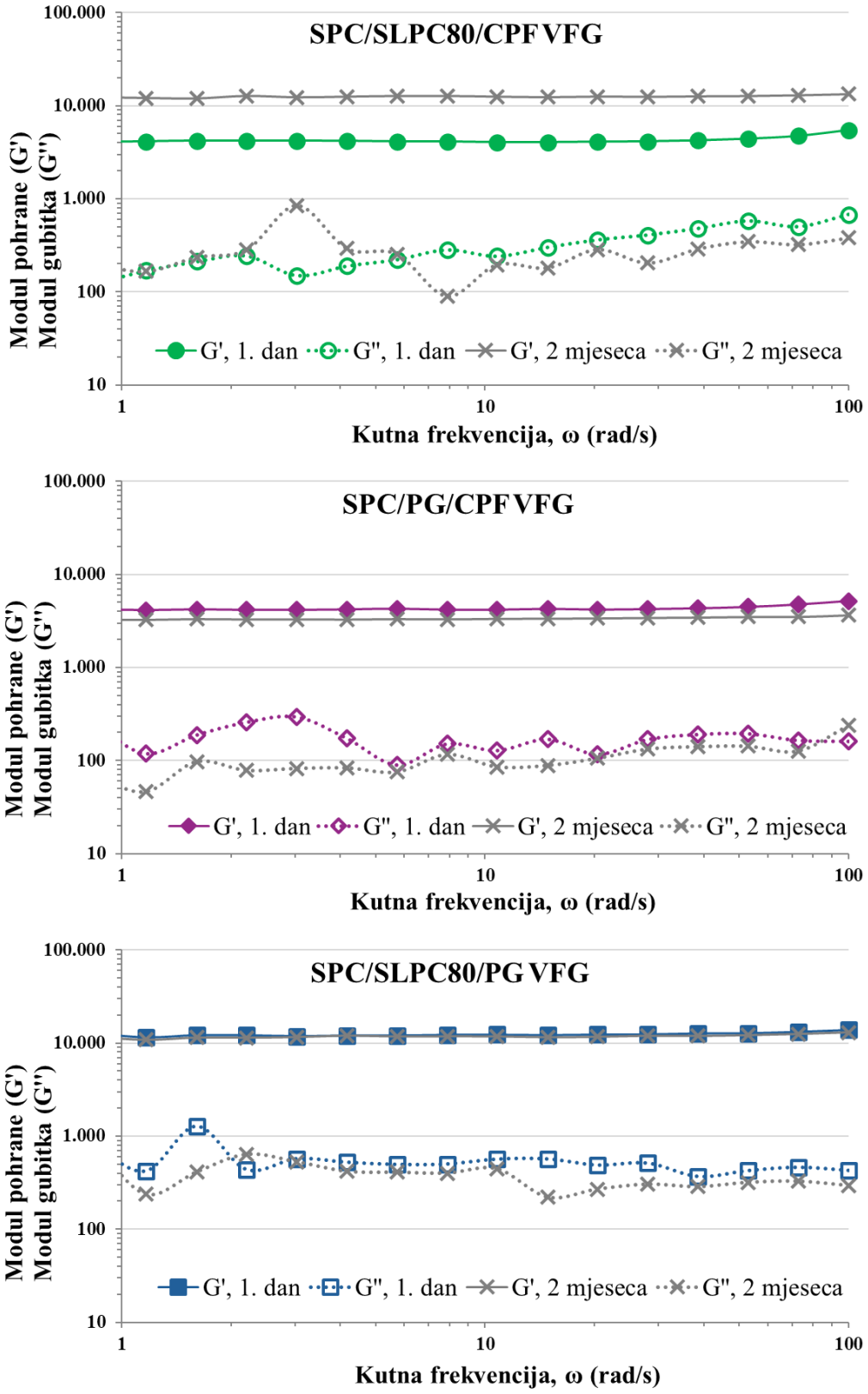
svojstava također određuje stabilniju mikrostrukturu, budući da reverzibilne deformacije (G') nadvladaju ireverzibilne (G") (Adeyeye i sur., 2002; Simões i sur., 2020).



Slika 22. Test promjene frekvencije za SPC/CPF VFG i SPC/CHIT/CPF VFG neposredno nakon izrade te nakon 2 mjeseca pohrane na 4 °C. Uzorci su mjereni na 32 °C uz smično naprezanje od 0,01 %. Rezultati su izraženi kao srednja vrijednost 2 mjerenja. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.



Slika 23. Test promjene frekvencije za SPC/P90H/CPF VFG i SPC/CHOL/CPF VFG neposredno nakon izrade te nakon 2 mjeseca pohrane na 4 °C. Uzorci su mjereni na 32 °C uz smično naprezanje od 0,01 %. Rezultati su izraženi kao srednja vrijednost 2 mjerenja. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.



Slika 24. Test promjene frekvencije za SPC/SLPC80/CPF VFG, SPC/PG/CPF VFG i SPC/SLPC80/PG/CPF VPG neposredno nakon izrade te nakon 2 mjeseca pohrane na 4 °C. Uzorci su mjereni na 32 °C uz smično naprezanje od 0,01 %. Rezultati su izraženi kao srednja vrijednost 2 mjerenja. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Fizička stabilnost CPF-VFG-ova ključna je za dugotrajno očuvanje integriteta nanoformulacija. Time se osigurava zadržavanje izvornih fizičko-kemijskih svojstva CPF-VFG liposoma, što se u konačnosti reflektira na njihovu učinkovitost u lokalnoj terapiji.

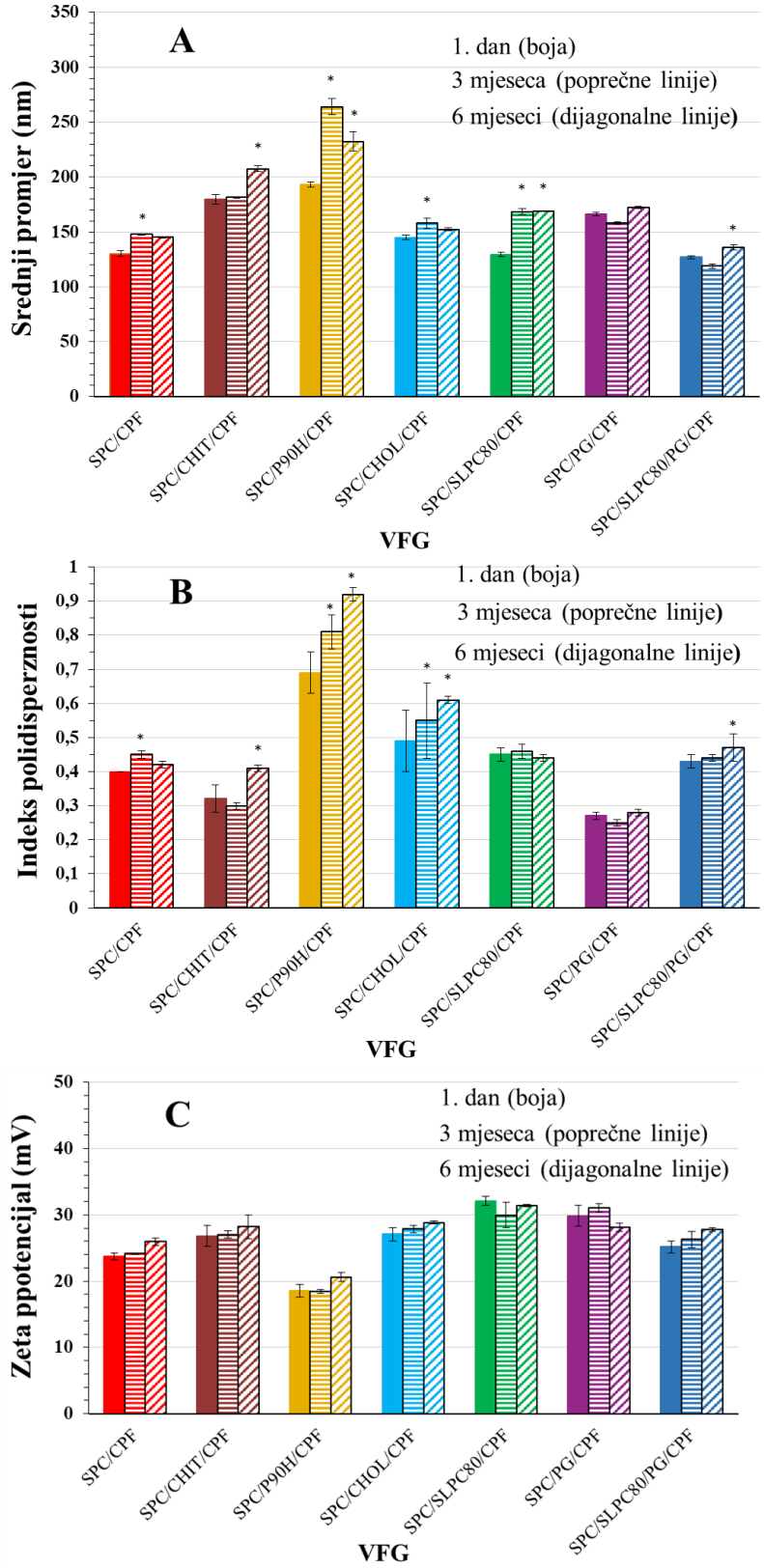
Osim reološkim ispitivanjem, fizička stabilnost CPF-VFG-ova procijenjena je mjerenjem srednjeg promjera, indeksa polidisperznosti i zeta potencijala CPF-VFG liposoma (nastalih konverzijom iz odgovarajućih VFG-ova) nakon 3 i 6 mjeseci pohrane CPF-VFG-ova na 4 °C. Pritom su izmjerene vrijednosti uspoređene s onima dobivenim neposredno nakon priprave CPF-VFG-ova.

Sastav CPF-VFG-ova značajno je utjecao na stabilnost CPF-VFG liposoma (Slika 25). Prisutnost kitozana ili propilenglikola unutar CPF-VFG-a povoljno je utjecao na očuvanje izvorne veličine liposoma tijekom 3 mjeseca. Njihovi srednji promjeri ostali su gotovo nepromijenjeni ($p > 0,05$) u odnosu na izvorne vrijednosti (Slike 25A i 25B). Dok je za uzorak s propilenglikolom veličina ostala konstantna čak nakon 6 mjeseci, VFG s kitozanom bilježi značajan porast srednjeg promjera i indeksa polidisperznosti (Slike 25A i 25B).

Veličina SPC/CPF-, SPC/CHOL/CPF- i SPC/SLPC80/CPF-VFG liposoma je nakon 3 mjeseca pohrane uzoraka na 4 °C blago porasla, ali je ostala nepromijenjena tijekom naredna 3 mjeseca skladištenja (Slike 25A i 25B).

Najveća promjena izvorne veličine CPF-VFG liposoma utvrđena je kod nanoformulacije s hidrogeniranim fosfolipidima (SPC/P90H/CPF VFG, 264 nm). Ta je nanoformulacija bila najmanje viskozna (Slika 13) s liposomima najvećeg srednjeg promjera (Tablica 3) među ispitivanim CPF-VFG-ovima. Pretpostavlja se da bi niska viskoznost mogla biti uzrokom lošije fizičke stabilnosti SPC/P90H/CPF VFG-a. Zanimljivo je spomenuti da je unatoč signifikantnom porastu srednjeg promjera ($p < 0,001$) nakon 3 mjeseca pohrane, tijekom naredna 3 mjeseca, veličina SPC/P90H/CPF VFG liposoma ostala konstantna (232 nm) (Slika 25A).

Površinski naboj svih CPF-VFG liposoma (Slika 25C) ostao je nepromijenjen tijekom ispitivanog razdoblja. Pozitivni zeta potencijali liposoma mogu se pripisati prisutnosti pozitivno-nabijenog CPF-a u svim CPF-VFG-ovima te kitozanu u SPC/CHIT/CPF VFG-u. Nazočnost CPF-a u vanjskoj vodenoj fazi CPF-VFG-ova utjecao je na zeta potencijale svih CPF-VFG liposoma nastalih nakon dispergiranja CPF-VFG-ova u vodi (Slika 25C). Pretpostavlja se da takav pozitivan površinski naboj uzrokuje elektrostatske interakcije između susjednih liposoma CPF-VFG-ova, što doprinosi stabilnosti formulacija.

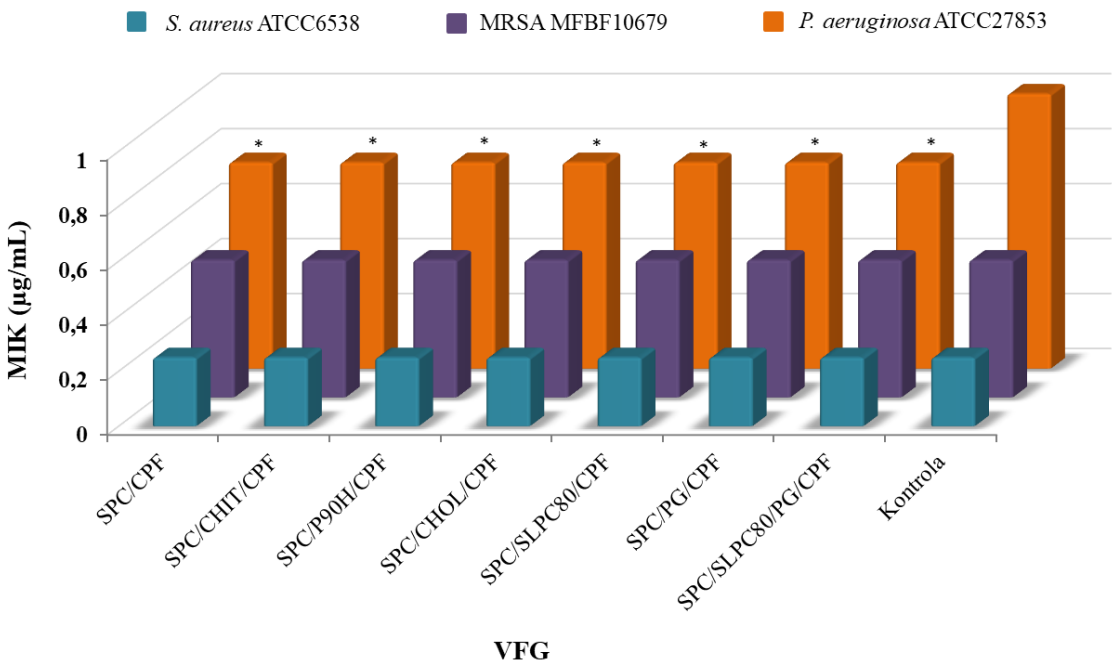


Slika 25. Fizička stabilnost CPF-VFG liposoma tijekom 6 mjeseci pohrane na 4 °C: srednji promjeri (A), indeksi polidisperznosti (B) i zeta potencijali (C). Rezultati su izraženi kao srednja vrijednost ± S.D. (n = 3). *Statistički značajna razlika u odnosu na izvorne vrijednosti ($p < 0,0001$). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

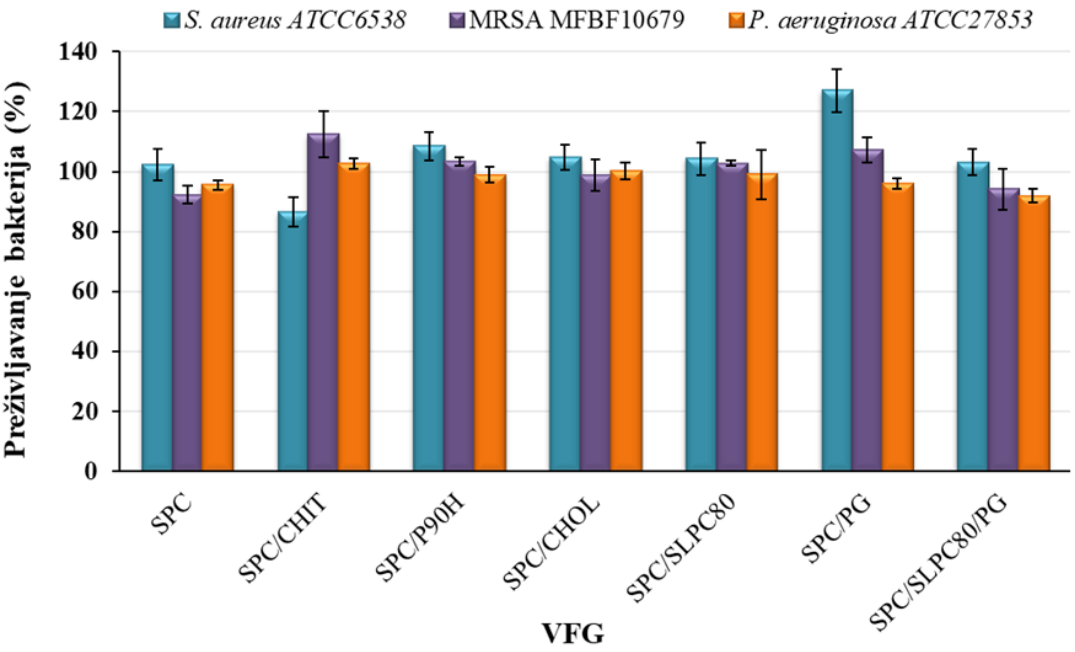
4.6. *In vitro* antibakterijska aktivnost

Učinkovito lokalno liječenje infekcija kože i mekih tkiva trebalo bi omogućiti potpunu eliminaciju patogenih bakterija i sprječiti njihov ponovni rast. Time bi se smanjila potreba za sistemskom primjenom visokih doza antibiotika i umanjio rizik od rastuće antimikrobne rezistencije (Sunderkötter i Becker, 2015). CPF je potentan antibiotik (Shariati i sur., 2022) koji se zasad topikalno koristi samo za liječenje infekcija oka i uha (Thai i sur., 2023). Uklapanje CPF-a, koji je odabran kao modelni hidrofilni lijek, u VFG, može se odraziti na njegov antibakterijski učinak. Kako bi se utvrdilo narušava li uklapanje CPF-a u VFG njegovu antibakterijsku aktivnost, svi CPF-VFG liposomi su podvrgnuti testiranju antibakterijskog učinka na 3 najčešća uzročnika bakterijskih infekcija kože i mekih tkiva: *S. aureus*, *P. aeruginosa* i MRSA. Istovremeno su ispitivanja provedena i s 'praznim' VFG-liposomima u istoj lipidnoj koncentraciji kao kod testiranja CPF-VFG liposoma. Antibakterijski učinak svih uzoraka ispitan je na sojevima planktonskih bakterija (Slike 26 i 27) i bakterijskih biofilmova (Slike 28 i 29).

Ispitivanja provedena na planktonskim bakterijama (Slika 26) pokazuju da uklapanje CPF-a u VFG-ove različitog sastava, nije ometalo antibakterijski učinak CPF-a. Naprotiv, aktivnost lijeka je čak povećana kada su provedena testiranja na *P. aeruginosa* ATCC 27853, dok su na *S. aureus* ATCC 6538 i MRSA MFBF 10679 kliničkom izolatu svi CPF-VFG liposomi imali isti antibakterijski učinak kao i otopina CPF-a (kontrola). Rezultati su tim više važniji ako se uzme u obzir da je *P. aeruginosa* ATCC 27853 pokazala najveću otpornost na CPF u odnosu na druge dvije testirane bakterijske vrste (MIK je iznosio 0,75 μg/mL). Među ispitivanim bakterijskim sojevima, *S. aureus* ATCC 6538 je bio najosjetljiviji na CPF-VFG s MIK vrijednosti od 0,2 μg/mL CPF-a. 'Prazni' liposomi, bez CPF-a (nastali konverzijom iz 'praznih' VFG-ova), također su testirani u istoj lipidnoj koncentracija kao CPF-VFG liposomi, kako bi se istražio mogući antibakterijski učinak samih sastavnica liposoma. Rezultati prikazani Slikom 27 pokazuju izostanak antimikrobnog učinka svih testiranih 'praznih' liposoma u koncentraciji fosfolipida koja korelira s CPF-VFG liposomima u MIK-u za ispitivani bakterijski soj.



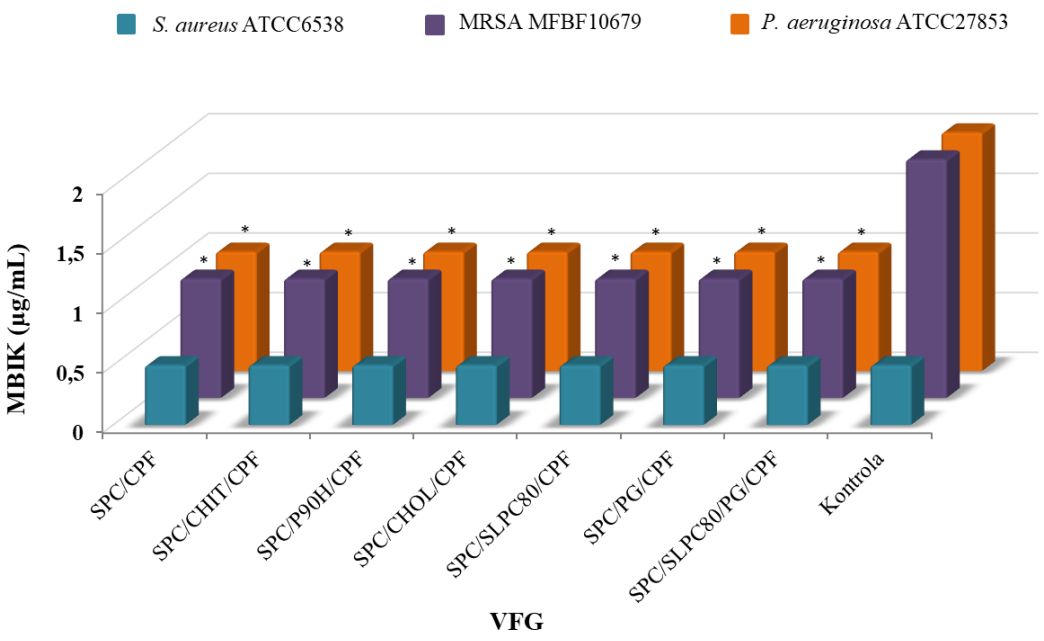
Slika 26. *In vitro* aktivnost CPF-VFG liposoma i CPF otopine (kontrola) na planktonske bakterije. Rezultati su izraženi kao srednja vrijednost ± S.D. (n = 3). *Statistički značajna razlika ($p < 0,0001$) u odnosu na kontrolu. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.



Slika 27. *In vitro* aktivnost 'praznih' liposoma (nastali konverzijom iz 'praznih' VFG-ova) na planktonske bakterije u koncentraciji fosfolipida koja korelira s CPF-VFG liposomima u MIK koncentraciji za pojedini bakterijski soj. Rezultati su prikazani kao prosječna vrijednost ± S.D. (n = 3). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Primjena nedovoljno učinkovitih topikalnih pripravaka antibiotika može dovesti do odgode u procesu cijeljenja ozlijeđene i inficirane kože, što rezultira razvojem biofilmova i nastankom kroničnih rana (Daeschlein, 2013). Kako bi se ispitao učinak CPF-VFG liposoma na prevenciju razvoja bakterijskih biofilmova, ispitivani bakterijski sojevi su tijekom stvaranja biofilma bili tretirani CPF-VFG liposomima u koncentracijama od 0,25 – 8 μg/mL CPF-a (3.2.11.3.).

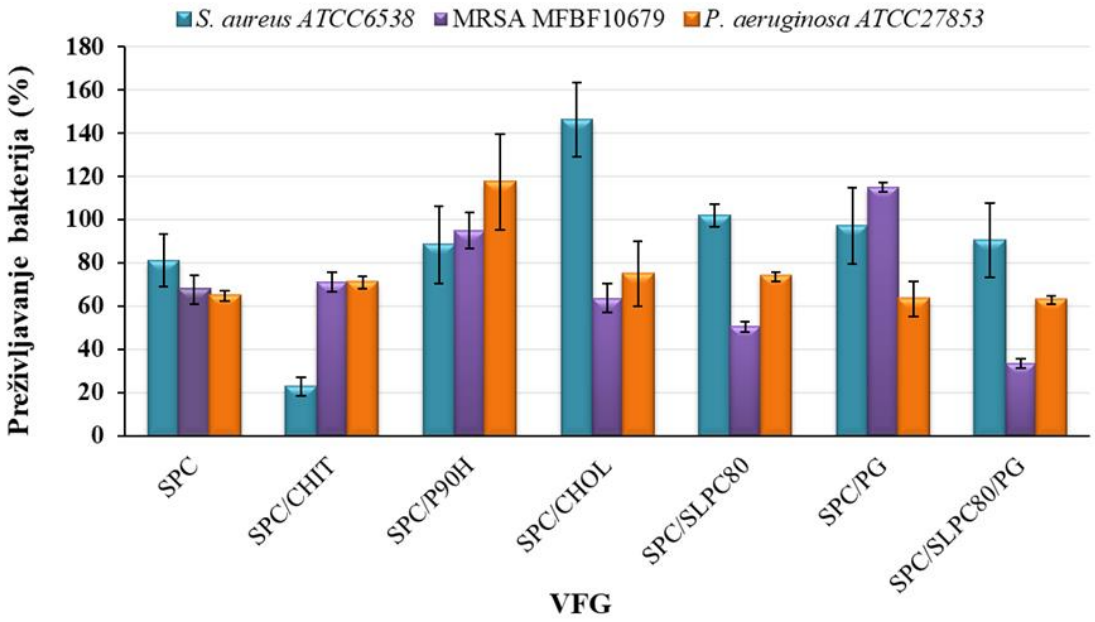
Rezultati testiranja ilustrirani Slikom 28 pokazuju veću učinkovitost svih CPF-VFG liposoma u sprječavanju nastanka *P. aeruginosa* ATCC 27853 i MRSA MFBF 10679 biofilmova. Vrijednosti MBIK-ova bile su čak 2 puta niže od onih za slobodni CPF (kontrola). Ekvivalentno rezultatima na planktonskim bakterijama (Slika 26), učinkovitost CPF-VFG liposoma u inhibiciji *S. aureus* ATCC 6538 biofilma bila je ista kao i s otopinom CPF-a (Slika 28).



Slika 28. *In vitro* aktivnost CPF-VFG liposoma i otopine CPF-a (kontrola) u prevenciji stvaranja bakterijskih biofilmova. Rezultati su izraženi kao srednja vrijednost ± S.D. (n = 3). *Statistički značajna razlika ($p < 0,0001$) u odnosu na kontrolu. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Zanimljivo je istaknuti da su 'prazni' VFG liposomi (bez CPF-a) pokazali određene učinke na sprječavanje nastaka biofilma (Slika 29), posebno SPC/CHIT- i SPC/SLPC80/PG-

VFG liposomi. Međutim, niti jedan od ispitivanih uzoraka 'praznih' liposoma nije mogao u potpunosti zaustaviti formiranje biofilma kao što su to učinili CPF-VFG liposomi (Slika 28). Pritom su uočene razlike u aktivnosti pojednih VFG liposoma na određene bakterijske sojeve. Tako su SPC/CHIT VFG liposomi bili najučinkovitiji na *S. aureus* ATCC6538 (preživljavanje bakterija 23 %), dok su SPC/SLPC80/PG VFG liposomi pokazali najveću aktivnost na MRSA MFBF10679 (preživljavanje bakterija 34 %) (Slika 29).



Slika 29. *In vitro* aktivnost 'praznih' liposoma (nastali konverzijom iz 'praznih' VFG-ova) u prevenciji stvaranja bakterijskih biofilmova u koncentraciji fosfolipida koja korelira s CPF-VFG liposomima u MBIK koncentraciji za pojedini bakterijski soj. Rezultati su prikazani kao prosječna vrijednost ± S.D. (n = 4). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Osim ispitivanja aktivnosti CPF-VFG liposoma u prevenciji formiranja biofilma, testirana je i njihova učinkovitost u eradikaciji formiranih biofilmova, no niti jedna nanoformulacija VFG-a nije bila efikasna. Takvi rezultati su donekle očekivani zbog izrazito velike otpornosti bakterijskih biofilmova na antibiotike (Forier i sur., 2014; Malone i sur., 2017).

4.7. *In vitro* biokompatibilnost

Sigurnost je jedan od osnovnih zahtjeva kojem treba udovoljiti svaki lijek, odnosno farmaceutski oblik lijeka (Aulton i Taylor, 2017). Naime, osim molekula djelatne tvari, svaki sastojak formulacije može ispoljiti toksične učinke kada se primjeni na žive stanice, pogotovo ako se nanosi na ozlijeđenu i upaljenu kožu. Stoga je u razvoju VFG-a namijenjenog lokalnoj primjeni na kožu bilo važno provesti ispitivanja kojima se procjenjuje njegova kompatibilnost, odnosno potencijalna toksičnost na stanice kože.

Biokompatibilnost različitih CPF-VFG-ova ispitana je *in vitro* na staničnoj liniji humanih keratinocita (HaCaT) primjenom MTT testa za procjenu metaboličke aktivnosti tretiranih stanica. Stanična linija je bila izložena djelovanju CPF-VFG liposoma tijekom 24 sata u koncentracijama CPF-a koje višestruko nadilaze MIK i MBIK vrijednosti CPF-VFG-ova (1 - 32 μg/mL). Kao kontrole, slobodni CPF i 'prazni' VFG liposomi testirani su u koncentracijama ekvivalentnim CPF-VFG liposomima (3.2.12.2.). Kriterij za netoksičnost ispitivanih uzoraka na staničnim linijama preuzet je iz smjernice za određivanje *in vitro* citotoksičnosti medicinskih proizvoda prema kojem se materijali smatraju netoksičnim ako je vijabilnost stanica nakon izlaganja ispitivanim materijalima ≥ 70 % (ISO 90113-1).

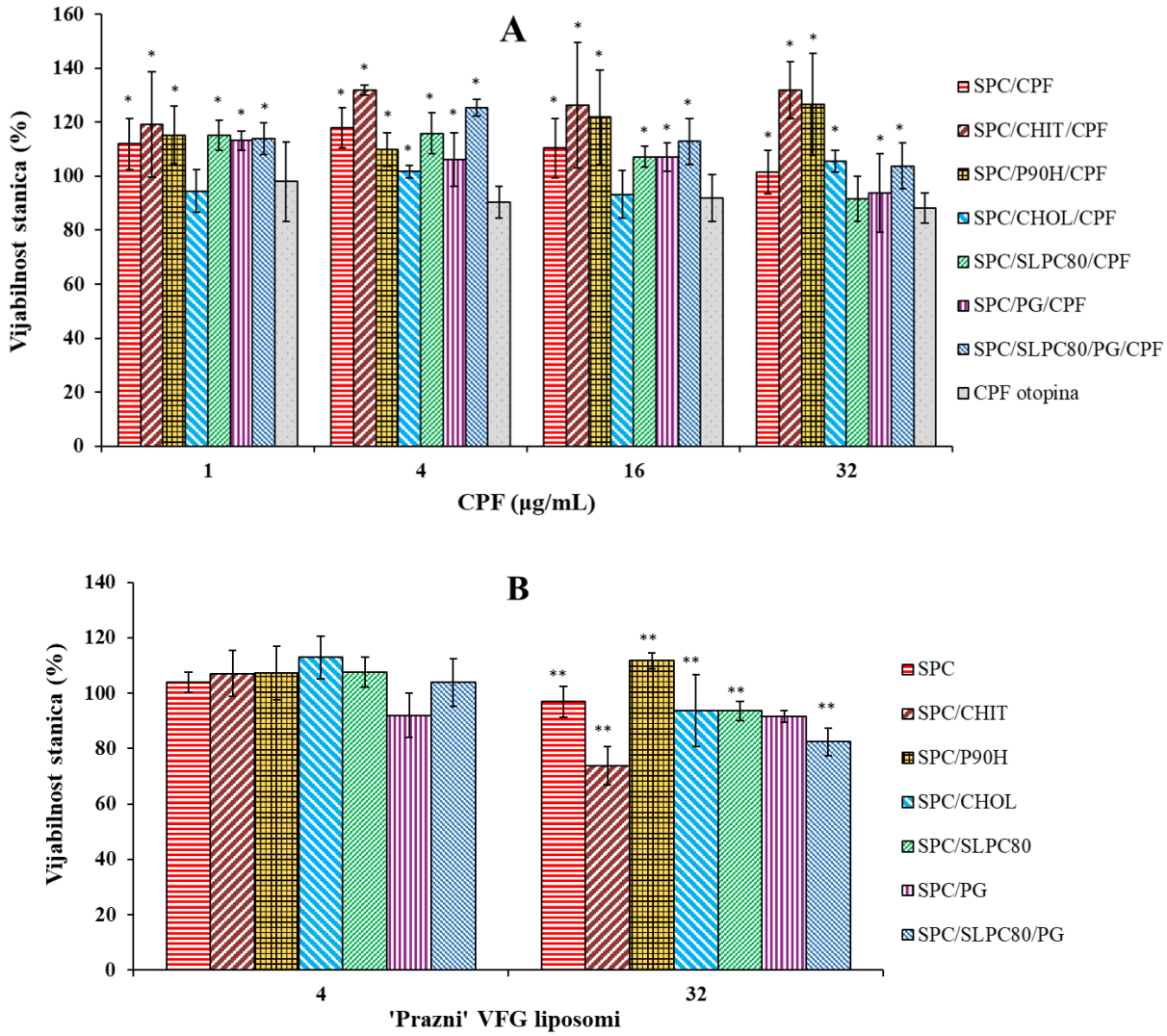
Rezultati provedenih ispitivanja pokazuju da su svi CPF-VFG liposomi bili biokompatibilni s keratinocitima. Stanice su bile vijabilne kod svih testiranih koncentracija pri čemu je, očekivano, najbolje preživljavanje bilo kod nižih koncentracija (Slika 30A). Zanimljivo je istaknuti da su određeni CPF-VFG liposomi pokazali proliferativni učinak na keratinocite, koji je pogotovo bio izražen pri višim koncentracijama CPF-a (16 i 32 μg/mL). Najjaču proliferativnu aktivnost imali su CPF-VFG liposomi koji sadrže hidrogenirane fosfolipide (P90H) ili kitozan, ekscipijense poznate po svojim blagotvornim učincima na koži i procesu zacjeljivanja rana (van Hoogevest i Fahr, 2019; Feng i sur., 2021). Iako je kolesterol prirodni sastojak svih bioloških membranama (Saxena i Das, 2016), nanoformulacija s kolesterolom bila je manje podnošljiva za keratinocite pri najnižoj testiranoj koncentraciji, dok je obrnuti učinak zapažen tijekom ispitivanja s najvišom koncentracijom CPF-a. Takvi rezultati vjerojatno su posljedica nižeg sadržaja osnovnog fosfolipida (SPC) u SPC/CHOL/CPF VPG-u, pri čemu dolazi do izražaja učinak slobodnog CPF-a. CPF-VFG liposomi koji sadrže veću količinu propilenglikola (SPC/PG/CPF) su pri najvišoj koncentraciji CPF-a (32 μg/mL), bili umjereno podnošljivi s HaCaT stanicama, slično kao i SPC/SLPC80/CPF VFG liposomi. Među elastičnim liposomima, oni koji su sadržavali manje monoacilfosfatidilkolina (SLPC80) i manje

propilenglikola u svom sastavu (SPC/SLPC80/PG/CPF VFG liposomi), bili su u potpunosti biokompatibilni te su u višim koncentracijama (4 - 32 μg/mL CPF-a) pospješivali rast keratinocita. Otopina CPF-a je imala najveći citotoksični učinak na keratinocite, što ukazuje na dobrobit uklapanja CPF-a u VFG, ne samo zbog kontroliranog i produljenog oslobađanja lijeka te pojačanog učinka na prevenciju stavaranja bakterijskih biofilmova, nego i zbog smanjenja toksičnog učinka lijeka na stanice kože.

Kako bi se provjerilo potencijalno toksično djelovanje sastavnica VFG-ova na vijabilnost keratinocita, ispitani su i 'prazni' VFG liposomi pri razrjeđenju koje odgovara koncentracijama CPF-VFG liposoma od 4 i 32 μg/mL CPF-a (Slika 30B). Pritom niti jedna od testiranih nanoformulacija 'praznih' VFG liposoma nije pokazala toksične učinke prema HaCaT stanicama, iako se vijabilnost neznatno smanjila za sve liposome pri najvišoj testiranoj koncentraciji liposoma, osim SPC/P90H VFG i SPC/CHIT VFG liposoma. SPC/P90H VFG liposomi su značajno poboljšali staničnu proliferaciju ($p < 0,001$), dok je održivost stanica tretiranih SPC/CHIT VFG liposomima smanjena na 73 %. Iako se radilo o značajnom smanjenju, vijabilnost stanica iznad 70 % i dalje se smatra netoksičnom (ISO 90113-1). Takav rezultat je posljedica neutralnog pH 'praznih' liposoma, gdje kitozan nije u aktivnom (ioniziranom) obliku (pKa 6,5). Naime, svi CPF-VFG liposomi su imali blago kiseli pH, zbog kisele prirode CPF-a koji je pogodovao kitozanskom CPF-VFG-u.

Proliferativni učinak liposoma izrazito je poželjan u liječenju bolesti kože s oštećenim integritetom epidermisa, a posebno u zacijeljivanju rana. Osim toga, hidrofilna priroda liposoma također doprinosi povećanju učinka hidratacije kože, što je posebno značajno u dermatologiji.

Na temelju rezultata postignutih u ovom ispitivanju, SPC/CPF-, SPC/CHIT/CPF-, SPC/P90H/CPF-, SPC/CHOL/CPF- i SPC/SLPC80/PG/CPF-VFG-ovi su odabrani za daljnja *in vitro* ispitivanja učinkovitosti na zacijeljivanje kože *in vitro* testom migracije keratinocita.



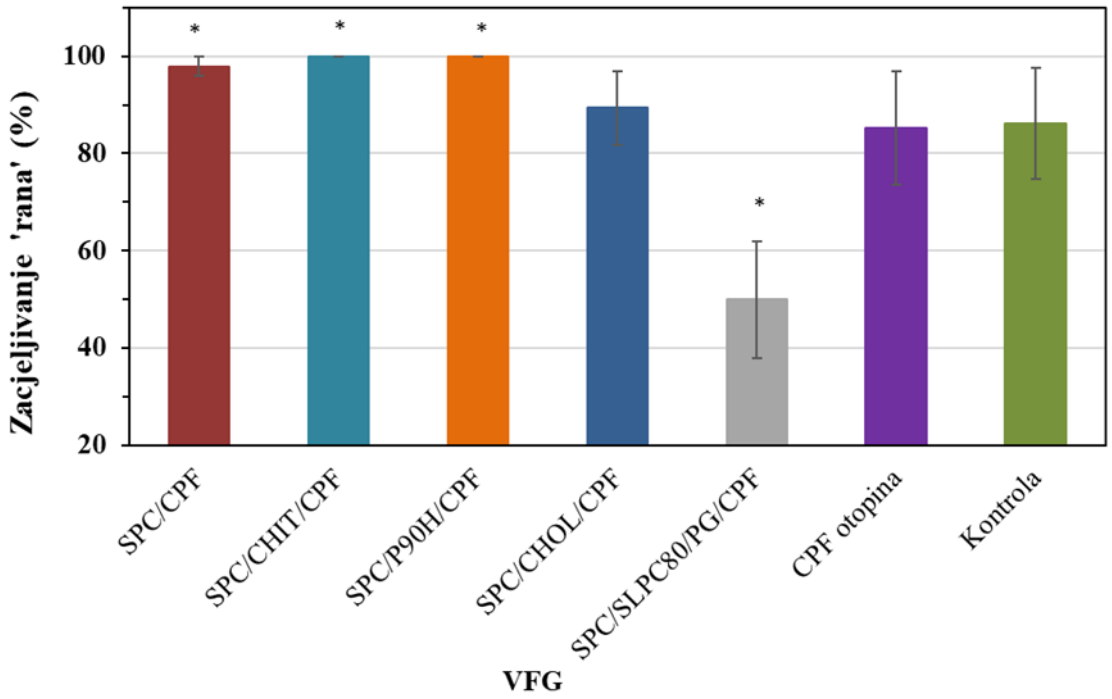
Slika 30. Vijabilnost HaCaT stanica (%) nakon 24 sata inkubacije na 37 °C s CPF-VFG liposomima i CPF otopinom (A) te 'praznim' VFG liposomima (B). Rezultati su izraženi kao srednja vrijednost ± S.D. (n = 4). *Statistički značajna razlika ($p < 0,0001$) u usporedbi sa slobodnim CPF-om (kontrola) pri ispitivanoj koncentraciji. **Statistički značajna razlika ($p < 0,0001$) u usporedbi s 'praznim' VFG liposomima testiranim pri nižoj koncentraciji. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

4.8. Učinak CPF-VFG-ova na zacjeljivanje kože *in vitro*

In vitro testom migracije keratinocita (tzv. *scratch* test) procijenjen je učinak odabranih nanoformulacija CPF-VFG-ova na zacjeljivanje, odnosno epitelizaciju ozlijeđene kože. Riječ je o jednostavnoj i ekonomičnoj metodi prikladnoj za brzi probir nanoformulacija s epitelizacijskim učinkom. Ispitivanje se bazira na stvaranju ogrebotine ('rane') na konfluentnom staničnom monosloju i praćenju njezinog zatvaranja uslijed migracije stanica s rubova ogrebotine prema sredini sve dok se ne uspostavi cjelovitost monosloja (Liang i sur., 2007).

Učinak zacjeljivanja odabranih CPF-VFG liposoma procijenjen je na monoslojevima humanih keratinocita (HaCaT). Među testiranim CPF-VFG liposomima, oni koji su sadržavali kitozan (SPC/CHIT/CPF VFG) ili hidrogenirani fosfolipid (SPC/P90H/CPF VFG) omogućili su potpuno zatvaranje ogrebotine nakon 24 sata (Slika 31), što je u suglasnosti s potvrđenim proliferativnim učinkom tih nanoformulacija na keratinocite (Slika 30A). Kitozan je dobro poznat po svojim blagotvornim učincima u procesu zacjeljivanja rana (Feng i sur., 2021) i poticanju migracije keratinocita (Blažević i sur., 2016; Mutlu i sur., 2022), dok hidrogenirani fosfatidilkolin povoljno djeluje na reepitelizaciju kože (van Hoogevest i Fahr, 2019; Lautenschlager, 2006).

Učinkovitost zacjeljivanja 'rane' bila je zanemarivo manja za SPC/CPF VFG liposome, dok je za SPC/CHOL/CPF VFG liposome iznosila 90 % (Slika 31). SPC/SLPC80/PG/CPF VFG liposomi nisu pokazali učinak zacjeljivanja u usporedbi s otopinom CPF-a i kontrolom (netretirane HaCaT stanice). Takav rezultat posljedica je citotoksičnog učinka monoacilfosfolipida na staničnu membranu (Tan i sur., 2020). Zanimljivo je spomenuti da u *in vitro* ispitivanju biokompatibilnosti (Slika 30A), SPC/SLPC80/PG/CPF VFG liposomi nisu djelovali citotoksično, vjerojatno zbog dominantnog učinka biokompatibilnog SPC-a kao glavne sastavnice VFG-a. S obzirom da je *scratch* test posebno prikladan za ispitivanje interakcija između stanica i izvanstaničnog matriksa, omogućio je precizniju procjenu utjecaja pojedinih komponenti CPF-VFG liposoma na stanice kože koje nisu bile jasno vidljive (izražene) ispitivanjem *in vitro* biokompatibilnosti.



Slika 31. Utjecaj CPF-VFG liposoma i otopine CPF-a na cijeljenje 'rana' *in vitro* (24 sata nakon tretmana). Učinkovitost zacjeljivanja 'rane' (%) predstavlja postotak zatvaranja ogrebotine u odnosu na početnu površinu ogrebotine. Koncentracija CPF-a u svim uzorcima bila je 16 µg/mL. Netretirane stanice služile su kao kontrola. Rezultati prikazuju srednju vrijednost ± S.D. (n = 3). *Statistički značajna razlika ($p < 0,05$) u odnosu na kontrolu. Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

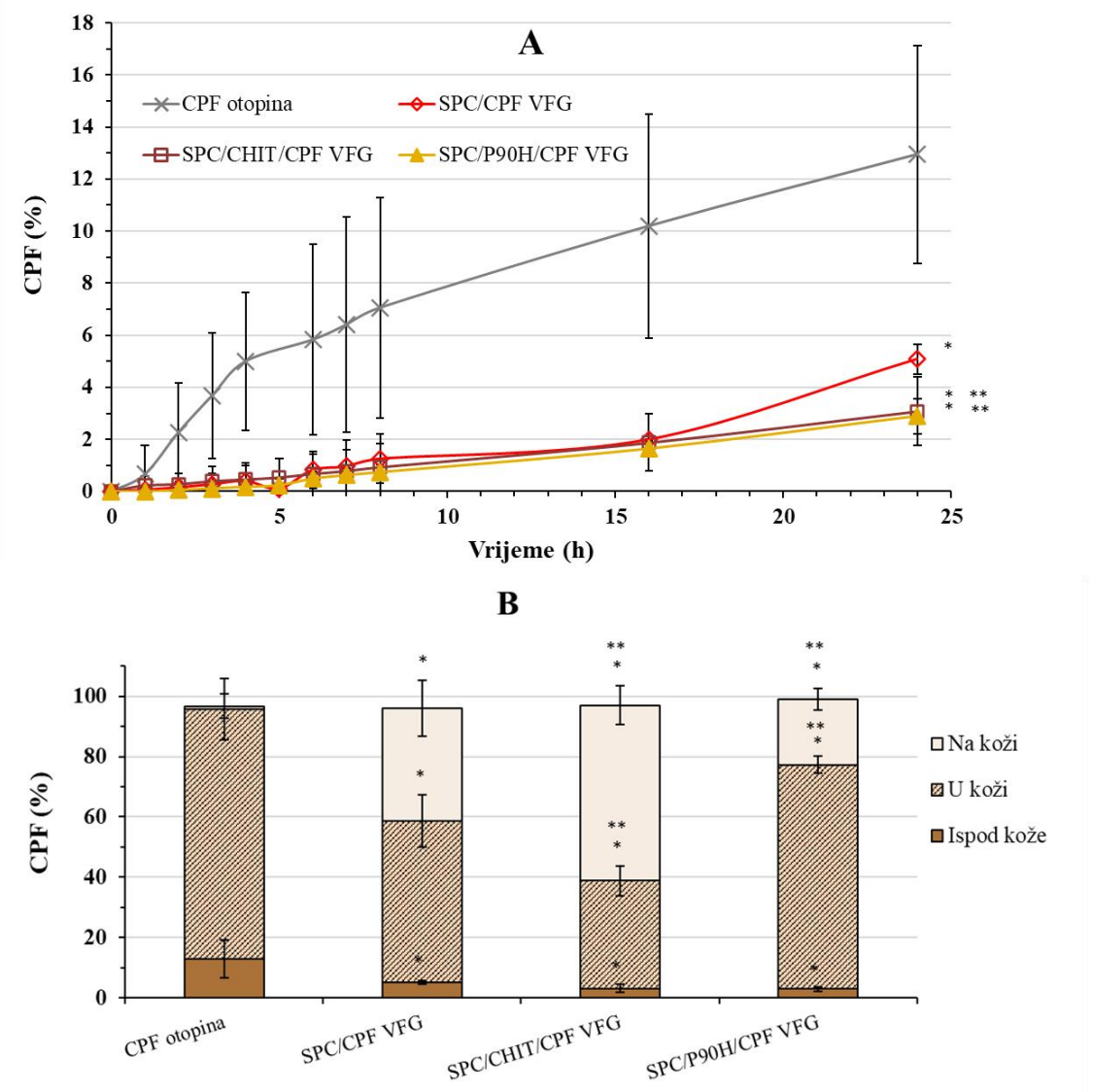
4.9. *Ex vivo* permeabilnost CPF-VFG-ova u kožu

Studije permeabilnosti neophodne su u razvoju svake formulacije lijeka za dermalnu primjenu budući da se na temelju dobivenih podataka može procijeniti njegova lokalizacija u koži, bolje predvidjeti lokalna bioraspoloživost lijeka te omogućiti odabir optimalne formulacije za daljnja *in vivo* istraživanja (Flaten i sur., 2015). Ispitivanja permeabilnosti CPF-a iz CPF-VFG-ova provedena su kako bi se utvrdila mogućnost lokalizacije lijeka na željenom mjestu djelovanja na/u koži. Testirani su CPF-VFG-ovi koji su pokazali superiorne rezultate u *in vitro* ispitivanju zacjeljivanja ozlijeđene kože pri čemu je korišten model kože svinjskog uha, koja je histološki i sastavom najsličnija humanoj koži (Flaten i sur., 2015).

CPF-VFG-ovi koji su podvrgnuti ispitivanju permeabilnosti razlikovali su se po fluidnosti (fosfo)lipidnog dvosloja, viskoznosti i bioadhezivnosti:

- SPC/CPF VFG (viskozna, osnovna nanoformulacija VFG-a, umjerene fluidnosti i bioadhezivnosti),
- SPC/CHIT/CPF VFG (viskozni VFG, umjerene rigidnosti dvoslojeva i veće bioadhezivnosti od SPC/CPF VFG-a), te
- SPC/P90H/CPF VFG (najmanje viskozan VFG, izrazito čvrstih dvoslojeva i najveće bioadhezivnosti).

Rezultati ispitivanja prikazani Slikom 32A potvrđuju da su svi testirani CPF-VFG-ovi značajno smanjili permeabilnost CPF-a kroz kožu (transdermalno) u odnosu na otopinu CPF-a (Slika 32A), čime se osigurava njegova lokalizacija na/u koži. Među ispitivanim CPF-VPG-ovima, najmanja permeabilnost CPF-a u kožu bila je s CPF-VFG-ovima čvrstih dvoslojeva, koji su sadržavali hidrogenirani fosfolipid ili kitozan. S druge strane, najveća količina CPF-a detektirana u receptorskom mediju nakon 24 sata ispitivanja postignuta je sa SPC/CPF VFG-om, karakteriziranim umjereno fluidnim dvoslojevima ($p < 0,0001$) (Sl. 32A). Takvi rezultati su na određeni način očekivani zbog učinka fluidnosti (rigidnosti/elastičnosti) membrana liposoma na permeabilnost (Vanić i sur., 2019). Primjerice, liposomi s azitromicinom elastičnih dvoslojeva su omogućili bolje nakupljanje azitromicina u koži, za razliku od konvencionalnih liposoma rigidnih dvoslojeva koji su se zadržavali na njezinoj površini (Rukavina i sur., 2018).



Slika 32. Permeabilnost CPF-a u kožu iz SPC/CPF VFG-a, SPC/CHIT/CPF VFG-a i SPC/P90H/CPF VFG-a. Otopina CPF-a u vodi predstavljala je kontrolu. Rezultati su prikazani kao kumulativna količina CPF-a koja je penetrirala u receptorski medij (A). Udio CPF-a (%) koji je zadržan na površini i unutar kože te koji je penetrirao kroz kožu (B). Prikazani rezultati su prosječne vrijednosti ± S.D. (n = 3). *Statistički značajna razlika u odnosu na CPF otopinu (kontrola) ($p < 0,05$). **Statistički značajna razlika u odnosu na SPC/CPF VFG ($p < 0,05$). Preuzeto i prilagođeno iz Keser i sur. (2024), uz dozvolu *Elseviera*.

Temeljem rezultata provedenih ispitivanja permeabilnosti proizlazi da su svi ispitivani CPF-VPG-ovi omogućili lokalizaciju CPF-a na površini i unutar kože (Slika 30B). Pritom su fluidnost dvoslojeva, viskoznost i bioadhezivnost CPF-VFG-ova, utjecali na mjesto lokalizacije CPF-a u koži. Tako je primjena najmanje viskoznog SPC/P90H/CPF VFG-a, kojeg

karakterizira izrazito visoka bioadhezivnost, rezultirala najvećom razinom lijeka akumuliranog unutar kože, dok se najmanje lijeka zadržalo na površini koži, u usporedbi s druge dvije formulacije. Obrnuti učinak primijećen je s viskoznim i nešto manje bioadhezivnim SPC/CHIT/CPF VFG-om, karakteriziranim umjereno čvrstim dvoslojevima, gdje je značajno veća količina lijeka bila na površini nego unutar kože ($p < 0,001$). S druge strane, viskozni SPC/CPF VFG, s umjereno fluidnim dvoslojevima (najmanje čvrstim u odnosu na druge dvije nanoformulacije), pokazao je bolje nakupljanje CPF-a unutar kože nego SPC/CHIT/CPF VFG, ali veće zadržavanje CPF-a na površini kože nego SPC/P90H/CPF VFG (Slika 32B).

Viskoznost nanoformulacija je značajno doprinijela lokalizaciji CPF-a u koži, ali nije imala nikakav utjecaj na njegovu permeabilnost kroz kožu. Ključnu ulogu u permabilnosti CPF-a imala je fuidnost (fosfo)lipidnih dvoslojeva CPF-VFG-ova. Tako je gotovo jednaka količina CPF-a određena u receptorskom mediju s jako viskoznim SPC/CHIT/CPF VFG-om i najmanje viskoznim SPC/P90H/CPF VPG-om (Slika 32A), pri čemu su oba bila karakterizirana čvrstim dvoslojevima (Tablica 5). Najviše CPF-a u receptorskom mediju nakon 24 sata postignuto s umjereno fluidnim i najviše viskoznom SPC/CPF VFG-om.

Permeabilnosti lijeka u/kroz kožu pridonosi i naboj na površini liposoma. Dok površinski negativno nabijeni liposomi povećavaju permeabilnost lijeka kroz kožu, liposomi neutralnog i pozitivnog naboja omogućuju njegovo zadržavanje na ili u površinskim slojevima kože (Ibaraki i sur., 2019; Ternullo i sur., 2019). Svi CPF-VFG liposomi (nastali dispergiranjem CPF-VFG-ova u vodi) imali su pozitivan površinski naboj zbog kationske prirode lijeka koji prekriva izvorni neutralni površinski naboj liposoma (Tablica 4). Stoga se pretpostavlja da bi efekt površinskog naboja CPF-VFG liposoma također mogao doprinosti učinku lokalizacije CPF-a unutar kože (Slika 32 B).

Na temelju rezultata svih provedenih istraživanja, proizlazi da su SPC/CHIT/CPF VFG i SPC/P90H/CPF VFG optimalne nanoformulacije koje omogućuju lokalizaciju lijeka u koži i smanjuju njegovu penetraciju u sistemsku cirkulaciju. Dobro prianjanje i zadržavanje VFG-a na površini koži zajedno s produljenim oslobađanjem CPF-a tijekom duljeg vremena u koncentracijama koje značajno premašuju MIK i MBIK vrijednosti CPF-a na patogene bakterije na mjestu infekcije, rezultiralo bi učinkovitim liječenjem infekcija kože i prevencijom razvoja kroničnih infekcija. Time bi se smanjila učestalost doziranja, što je posebno važno kod tretiranja bolnih, upalnih područja ozlijeđene kože. Obje formulacije bile su u potpunosti biokompatibilne te su imale snažan proliferativni učinak na keratinocite i omogućile brže

zacjeljivanje ozlijeđenog epidermisa *in vitro*. Sve to bi u konačnosti rezultiralo poboljšanom lokalnom terapijom infekcija kože. Budući da infekcije kože obuhvaćaju lezije koje zahvaćaju različite slojeve kože (od najgornjih slojeva epidermisa do potkožnog tkiva) (Augustin i sur., 2017), pretpostavlja se da bi SPC/CHIT/CPF VFG bio prikladan za liječenje površinskih infekcija s kompromitiranim epidermisom, dok bi SPC/P90H/CPF VFG bio prikladan za liječenje infekcija kože koje zahvaćuje dublje slojeve kože. Međutim, potrebno je provesti daljnja *in vivo* animalna ispitivanja kojima bi se te pretpostavke potvrdile.

5. ZAKLJUČCI

Istraživanja u okviru ovog doktorskog rada predstavljaju prvu studiju u kojoj su uspješno razvijeni VFG-ovi namijenjeni lokalnoj primjeni lijekova na kožu. Hidrofilni modelni lijek (CPF) u cijelosti je uklopljen metodom visokotlačne homogenizacije u nekoliko VFG-ova, koji su se međusobno razlikovali (fosfo)lipidnim sastavom, prisutnim polimerom ili suotapalom. Optimiranjem procesnih parametara i udjela fosfolipida izrađeni su različiti CPF-VFG-ovi primjerene viskoznosti za dermalnu primjenu s unilamelarnim liposomima srednjih promjera manjih od 200 nm. Proces izrade je bio u potpunosti ekološki prihvatljiv, a sama metoda prikladna za industrijsku proizvodnju zbog svoje jednostavnosti, reproducibilnosti i robusnosti.

Sastav CPF-VFG-ova značajno se odrazio na fizičko-kemijska svojstva CPF-VFG liposoma (veličinu, zeta potencijal, fluidnost fosfolipidnog dvosloja), uklapanje CPF-a u liposome VFG-a, stabilnost u simuliranim uvjetima *in vivo* primjene i tijekom skladištenja, *in vitro* profil oslobađanja lijeka i njegovu permeabilnost u kožu, bioadhezivnost, biokompatibilnost te *in vitro* epitelizacijski učinak (zacjeljivanje ozlijeđene kože).

Fluidnost fosfolipidnih dvoslojeva CPF-VFG-ova utjecala je na veličinu liposoma i sadržaj liposomski-uklopljenog lijeka. Povećanje čvrstoće membrane odrazilo se na porast veličine liposoma i posljedično veći udio uklopljenog CPF-a.

Viskoznost CPF-VFG-ova je bila prvenstveno određena koncentracijom SPC-a. Svi CPF-VFG-ovi su imali pseudoplastična svojstva, široko viskoleastično područje i viskoelastičnu čvrstu strukturu koja je ostala stabilna i nakon 2 mjeseca skladištenja uzoraka.

Sastav CPF-VFG-ova značajno se odrazio na njihovu bioadhezivnost, dok viskoznost nije imala utjecaja. Najveća sila odvajanja s površine kože i rad adhezije zabilježeni su s niskoviskoznim SPC/P90H/CPF VFG-om koji je sadržavao hidrogenirane fosfolipide, dok je viskozni CPF-VFG s kitozanom (SPC/CHIT/CPF VFG) bio neznatno manje bioadhezivan.

Produljeno i kontrolirano oslobađanje CPF-a, postignuto iz svih CPF-VFG-ova, slijedilo je Korsmeyer-Peppasov model, pri čemu su viskoznost i fluidnost membrana VFG-ova imali dominantan utjecaj na brzinu oslobađanja. Niskoviskozni CPF-VFG čvrstih membrana (SPC/P90H/CPF VFG) je omogućio početno brže oslobađanje lijeka, nakon čega je uslijedilo polagano oslobađanje CPF-a iz SPC/P90H/CPF VFG liposoma izrazito rigidnih membrana. Takav dvofazni profil oslobađanja pogodan je za brzi početak djelovanja i održavanje terapijske koncentracije lijeka kroz dulji vremenski period.

Sastav CPF-VFG-ova utjecao je na fizičku stabilnost CPF-VFG liposoma tijekom skladištenja. Prisutnost kitozana ili propilenglikola unutar CPF-VFG-a povoljno se odrazila na očuvanje izvorne veličine liposoma tijekom 3 mjeseca. U simuliranim uvjetima *in vivo* primjene veličina svih CPF-VFG liposoma značajno je porasla, no i dalje je ostala prikladna za lokalnu dermalnu primjenu (< 300 nm). Interakcija liposoma s albuminom iz SFR-a nije uzrokovala oslobađanje izvorno liposomski-uklopljenog CPF-a.

Uklapanje CPF-a u VFG-ove nije umanjilo antibakterijski učinak CPF-a. Štoviše, aktivnost lijeka je povećana na *P. aeruginosa*-u ATCC 27853, dok su na *S. aureus* ATCC 6538 i MRSA MFBF 10679 kliničkom izolatu svi CPF-VFG liposomi imali isti antibakterijski učinak kao i CPF-otopina. Djelotvornost CPF-VFG-ova potvrđena je i u inhibiciji stvaranja biofilmova. Svi CPF-VFG-ovi su bili dvostruko učinkovitiji od CPF-otopine u prevenciji nastanka *P. aeruginosa* ATCC 27853 i MRSA MFBF 10679 biofilmova.

CPF-VFG-ovi su bili u potpunosti biokompatibilni s keratinocitima *in vitro*, pri čemu su oni s hidrogeniranim fosfolipidima i kitozanom imali jaki proliferativni učinak. Superiornost SPC/CHIT/CPF- i SPC/P90H/CPF-VFG liposoma potvrđena je *in vitro* testom migracije keratinocita, pri čemu su obje nanoformulacije pokazale izrazito snažan epitelizacijski učinak.

SPC/CPF-, SPC/CHIT/CPF- i SPC/P90H/CPF-VFG-ovi su značajno smanjili permeabilnost CPF-a kroz kožu u odnosu na otopinu CPF-a i omogućili lokalizaciju lijeka u koži. Viskoznost, bioadhezivnost i fluidnost dvoslojeva CPF-VFG-ova su utjecali na mjesto lokalizacije lijeka u koži. Primjena najmanje viskoznog i izrazito bioadhezivnog SPC/P90H/CPF VFG čvrstih membrana, rezultirala je najvećom razinom akumuliranog lijeka u koži, dok je obrnuti učinak postignut s viskoznim i nešto manje bioadhezivnim SPC/CHIT/CPF VFG-om umjereno čvrstih dvoslojeva. Viskozni i manje bioadhezivan SPC/CPF VFG umjerene fluidnosti omogućio je bolje nakupljanje CPF-a unutar kože nego SPC/CHIT/CPF VFG, ali veće zadržavanje CPF-a na površini kože nego SPC/P90H/CPF VFG.

Uzimajući u obzir rezultate svih provedenih istraživanja proizlazi da su SPC/CHIT/CPF VFG i SPC/P90H/CPF VFG optimalne nanoformulacije s poželjnim biofarmaceutskim svojstvima. SPC/CHIT/CPF VFG bi bio prikladan za liječenje površinskih infekcija s kompromitiranim epidermisom, dok bi SPC/P90H/CPF VFG bio primjeren za liječenje infekcija kože koje zahvaćaju dublje slojeve kože. Međutim, potrebno je provesti daljnja, *in vivo* animalna, ispitivanja kojima bi se te pretpostavke potvrdile.

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7. ŽIVOTOPIS

Sabina Keser je rođena 27. ožujka 1988. godine u Zagrebu, gdje je završila IV. jezičnu gimnaziju. Godine 2006. upisala je integrirani preddiplomski i diplomski studij farmacije na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu. 2010. godine dodijeljena joj je Rektorova nagrada za studentski znanstveni rad pod naslovom „N-glikozilacija ukupnih serumskih proteina i transferina u ranom tijeku akutnog pankreatitisa". Diplomirala je 2011. godine te je nakon odrađene godine pripravničkog staža u Gradskoj ljekarni Zagreb dobila odobrenje za samostalan rad u ljekarničkoj djelatnosti.

Godine 2012. zaposlila se kao asistent-zamjena na Zavodu za farmakognoziju Farmaceutsko-biokemijskog fakulteta. Godine 2013. pridružila se odjelu Istraživanja i razvoja tvrtke Xellia d.o.o., gdje je radila kao istraživač na odjelu Formulacija, te se bavila razvojem parenteralnih dozirnih oblika. Godine 2015. zaposlila se kao asistentica na Zavodu za farmaceutsku tehnologiju Farmaceutsko-biokemijskog fakulteta. Poslijediplomski doktorski studij Farmaceutsko-biokemijske znanosti (grana Farmacija) na Farmaceutsko-biokemijskom fakultetu Sveučilišta u Zagrebu upisala je 2016. godine. Sudjelovala je u izvođenju nastave iz predmeta Oblikovanje lijekova, Magistralna receptura, Kozmetologija i Biofarmacija s farmakokinetikom. Do sada je u koautorstvu objavila šest znanstvenih radova zastupljenih u bazi *Web of Science Core Collection*, dva stručna rada te dva trenutno važeća patenta. Aktivno je sudjelovala na stranim i domaćim znanstvenim skupovima sa sedam posterskih priopćenja. Održala je nekoliko radionica na Festivalu znanosti i Danima otvorenih vrata Farmaceutsko-biokemijskog fakulteta. Godine 2023. se zapošljava kao viši istraživač-formulator u tvrtki Teva/Pliva d.o.o. u odjelu Istraživanja i razvoja, Formulacije.

POPIS RADOVA:

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VEZIKULARNI FOSFOLIPIDNI GELOVI ZA LOKALNU PRIMJENU LIJEKOVA NA KOŽU

Sabina Keser

SAŽETAK

Učinkovito lokalno liječenje bolesti i ozljeda kože, koje su nerijetko praćene infekcijama, od velikog je medicinskog značaja. Svrha ovog rada bila je razviti biokompatibilan i učinkovit terapijski nanosustav za dermalnu primjenu, baziran na fosfolipidima, koži-sličnim tvarima. Riječ je o visokokoncentriranoj liposomskoj disperziji - vezikularnom fosfolipidnom gelu (VFG), koji bi zbog svoje polučvrste konzistencije omogućio dostavu visoke koncentracije lijeka na oboljelo mjesto, a integriranjem koži-sličnih sastojaka formulacije u kožu ili onih koji potiču zacjeljivanje, omogućila bi se njezina brža regeneracija. Istraživanja su obuhvatila razvoj i optimizaciju industrijski prikladne metode priprave VFG-ova s hidrofilnim modelnim lijekom (ciprofloksacinklorid, CPF), karakterizaciju CPF-VFG-ova u pogledu fizičko-kemijskih i reoloških svojstava, simulirane *in vivo* i fizičke stabilnosti, bioadhezivnosti, biokompatibilnosti, *in vitro* antimikrobne aktivnosti i *in vitro* učinka zacjeljivanja ozlijeđene kože. Rezultati provedenih ispitivanja su pokazali utjecaj fluidnosti (fosfo)lipidnih dvoslojeva i viskoznosti CPF-VFG-ova na profil oslobađanja CPF-a i njegovu permeabilnost u kožu. Neovisno o sastavu, svi CPF-VFG-ovi su bili kompatibilni sa stanicama keratinocita te su učinkovito inhibirali rast patogenih bakterija, čestih uzročnika infekcija kože i mekih tkiva. Antibakterijska aktivnost svih CPF-VFG-ova u prevenciji razvoja biofilmova *Pseudomonas aeruginosa* i meticilin-rezistentnog *Staphylococcus aureus* (MRSA) kliničkog izolata je bila dva puta veća od aktivnosti CPF otopine. CPF-VFG-ovi s hidrogeniranim fosfolipidima i kitozanom su se pokazali optimalnim nanoformulacijama zbog snažnog proliferativnog učinka na keratinocite, brzog zacjeljivanja ozlijeđenog epidermisa *in vitro* i lokalizacije lijeka na/u koži. Pritom bi CPF-VFG s kitozanom bio prikladan za liječenje površinskih infekcija s kompromitiranim epidermisom, dok bi CPF-VFG s hidrogeniranim fosfolipidima bio poželjan za liječenje dubljih infekcija kože. Njihovo dobro zadržavanje na površini koži te produljeno oslobađanje CPF-a tijekom duljeg vremena u koncentracijama koje značajno premašuju minimalne biofilm inhibitorne koncentracije CPF-a na patogene bakterije na mjestu infekcije, rezultiralo bi učinkovitom lokalnom terapijom i prevencijom razvoja kroničnih infekcija kože. Time bi se značajno unaprijedila postojeća lokalna antimikrobna terapija, a dodatno bi se smanjila učestalost doziranja, što je posebno važno kod tretiranja bolnih područja ozlijeđene kože.

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VESICULAR PHOSPHOLIPID GELS FOR LOCALIZED SKIN DRUG DELIVERY

Sabina Keser

SUMMARY

The effective local treatment of skin diseases and injuries, which are often accompanied by infections, is of great medical importance. The purpose of this thesis was to develop a biocompatible and therapeutically effective dermal drug delivery nanosystem, based on phospholipids, skin-like substances. It was a vesicular phospholipid gel (VPG), a highly concentrated liposomal dispersion of semisolid consistency. VPG should allow the delivery of a high concentration of the drug to the affected area, whereby the integration of skin-like ingredients of the VPG into the skin, or those that promote healing effect, would lead to faster skin regeneration. The research involved the development and optimization of an industrially suitable method for the preparation of VPG containing a hydrophilic model drug (ciprofloxacin hydrochloride, CPX), and characterization of the several prepared CPX-VPGs in terms of their physicochemical and rheological properties, *in vitro* drug release profile, physical and *in vivo* simulated stabilities, bioadhesiveness, *in vitro* antibacterial activities, biocompatibility and wound healing potential. The results demonstrated the influence of the CPX-VPGs' bilayer fluidity and viscosity on the release profile of CPX and its permeability into the skin. Regardless of the VPG's composition, all CPX-VPGs were compatible with human keratinocytes *in vitro* and effectively inhibited the growth of pathogenic bacteria, typical for skin and soft tissue infections. The activity of all CPX-VPGs in preventing the growth of *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate biofilms was two-fold increased, compared to CPX solution. CPX-VPGs with hydrogenated phospholipids and chitosan have proved to be the optimal nanoformulations due to their potent proliferative effects on keratinocytes, rapid healing of injured epidermis *in vitro* and localization of the drug on/within the skin. CPX-VPGs with chitosan would be suitable for the treatment of superficial infections with compromised epidermis, while the CPX-VPG with hydrogenated phospholipids would be preferable for the treatment of deeper skin infections. Their good retention on the skin surface and prolonged CPX release over a longer period in concentrations significantly exceeding the minimum biofilm inhibitory concentration of CPX against pathogenic bacteria at the site of infection, would result in effective local therapy and prevention the development of chronic skin infections. This would significantly improve dermal therapy, and further reduce the frequency of dosing, which is especially important when treating painful areas of injured skin.

The thesis is deposited in the Central Library of University of Zagreb Faculty of Pharmacy and Biochemistry.
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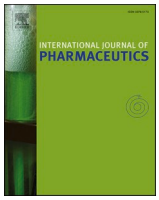

Vesicular phospholipid gels: A new strategy to improve topical antimicrobial dermatotherapy

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ABSTRACT

Therapeutically effective and biocompatible dermal formulations that can ensure localization of a high level of antimicrobial drug at the site of action for an appropriate duration, while at the same time providing intrinsic reepithelization properties, are of particular importance for the treatment of infected and injured skin. The current research aimed to explore the potentials of using vesicular phospholipid gels (VPGs), semisolid formulations consisting of tightly packed liposomes (100–200 nm), as innovative local depot drug vehicles for advanced topical dermatotherapy. Ciprofloxacin hydrocholoride (CPX) was selected as a model hydrophilic antibacterial drug and was loaded into several VPGs, differing in their composition. Various CPX-loaded VPGs (CPX-VPGs) were evaluated *in vitro* for the rheological and physicochemical characteristics, drug release profile, stability under *in vivo* mimicked conditions and during storage, skin permeability, biocompatibility with the epidermal cells, antibacterial efficacy and wound healing assay, to determine the optimal CPX-VPG for topical dermatotherapy. Viscosity and bilayers fluidity of VPGs affected the release of CPX from CPX-VPGs and its skin localization, limiting CPX percutaneous absorption. All CPX-VPGs exhibited even a 2-fold increase in anti-biofilm activity against both *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolate compared to the free drug, while showing no toxic effects on human keratinocytes *in vitro*. Based on the pronounced proliferative effects on keratinocytes, superior *in vitro* wound healing effect and drug localization on/inside the skin, CPX-VPGs containing chitosan and hydrogenated phospholipid proved to be the most promising for topical dermatotherapy. These findings, along with increased bioadhesiveness and the slow drug release, with CPX concentrations significantly above the minimum biofilm inhibitory concentrations for bacteria typical in infected wounds, would contribute not only to the improvement of the antimicrobial dermatotherapy, but also to reduction of the frequency of the drug administration.

1. Introduction

Effective topical therapy of skin diseases and injuries is of great importance in dermatology. It should ensure controlled and localized drug delivery to the diseased site in the skin with the use of significantly lower doses compared to the systemic administration of the drug (Antimisiaris et al., 2021). Despite relatively good tolerability and acceptance by patients, conventional dermal formulations have not always proven to be sufficiently effective. There are several reasons for their potential failure, such as unfavorable physicochemical properties

Abbreviations: BSA, bovine serum albumin; Chit, low molecular weight chitosan (≥ 75% deacetylation); Chol, cholesterol; CPX, ciprofloxacin hydrochloride; DMEM, Dulbecco's Modified Eagle's Medium; E, degree of bilayers' elasticity; EDTA, ethylenediamine tetra acetic acid; FBS, fetal bovine serum; G', storage modulus; G'', loss modulus; J, mass of extruded liposomes in elasticity measurements; LVR, linear viscoelastic region; MBIC, minimum biofilm inhibitory concentration; MD, mean diameter; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRSA, methicillin-resistant *Staphylococcus aureus*; PDI, polydispersity index; PG, propylene glycol; P90H, Phospholipon 90H (hydrogenated phosphatidylcholine ≥ 90.0 %); r_p, membrane pore size (100 nm); r_v, mean diameters of CPX-liposomes after extrusion through the membrane of 100 nm; SLPC80, soybean monoacyl phosphatidylcholine (approximately 80 % monoacyl phosphatidylcholine); SPC, soybean lecithin (≥ 94 % phosphatidylcholine); SWF, simulated wound fluid; VPG, vesicular phospholipid gel; CPX-VPG, vesicular phospholipid gel containing ciprofloxacin hydrochloride; ZP, zeta potential; WCR, wound closure rate.

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of the drug, limited solubility of the drug in the vehicle or its poor release and penetration into the skin (Richard et al., 2020). An additional parameter is the nature of skin disease, which can be characterized by thickened or compromised superficial skin layers often associated with bacterial infections (Cui et al., 2021; Las Heras et al., 2020; Stefanov and Andonova, 2021; Vastarella et al., 2020). If the topically applied drug does not penetrate through the *stratum corneum* of the skin and does not accumulate at the target site in the skin in an appropriate concentration, the therapeutic effect is weak or even absent, with the possible appearance of skin irritations. On the contrary, when the integrity of epidermal barrier is impaired and/or damaged, uncontrolled systemic absorption, especially of highly permeable drugs, is possible (Vanić et al., 2015; Williams, 2018). In the case of skin injuries accompanied by secondary infections (burns, wounds), ineffective topical dermatotherapy can favor the development of biofilms (Las Heras et al., 2020). Their treatment requires systemic administration of high doses of antibiotics, hence increasing the incidence of side-effects and the risk of growing antimicrobial resistance (Cui et al., 2021; Las Heras et al., 2020; Stefanov and Andonova, 2021; Vastarella et al., 2020). Therefore, the development of effective dermal formulations with controlled drug delivery to the targeted skin area is of high relevance.

Among various nanopharmaceuticals investigated for improved topical dermatotherapy (Cui et al., 2021; Las Heras et al., 2020), liposomes are attracting considerable attention. They are recognized as physiologically acceptable nanopharmaceuticals owing to their (phospho)lipid composition and similarity with cell membranes and the assemblies of the skin. Moreover, structural properties of liposomes enable incorporation of drugs of different lipophilicity as well as controlled release and skin penetration of the drug into the skin (Vanić et al., 2015).

Previous research has confirmed the effectiveness of liposomes in delivering antimicrobial drugs and preventing biofilm formation (Rukavina et al., 2018; Rukavina and Vanić, 2016; Vanić et al., 2019). However, low entrapment of hydrophilic drugs and the liquid consistency of liposomes can restrict their dermal application. Mixing liposomes into a hydrogel is a common approach to increase the viscosity of liposomal dispersions. The semi-solid consistency of liposomal gels is suitable for application to the skin, further extending release of the liposomal drug (Palac et al., 2015). On the other hand, the ratio of liposomes in the liposomal hydrogel is commonly 10–30 % (m/m) (Palac et al., 2015; Rukavina et al., 2023), which can be an obstacle to the effectiveness of treating certain skin diseases that require administration of larger doses of the drug. In addition, almost all preparation methods of liposomes are restricted by the amount of (phospho)lipids in the nanoformulation. Their concentration is typically 10–30 mg/mL, and consequently, may represent a limitation in terms of drug entrapment capacity (especially for hydrophilic drugs) and the amount of nanoformulation that needs to be applied to achieve an adequate drug dose (Filipczak et al., 2020; Worsham et al., 2019). These weaknesses can be overcome by vesicular phospholipid gel (VPG), a highly concentrated, semi-solid dispersion of phospholipid(s) in water prepared by high-pressure homogenization (Brandl et al., 1990; Brandl et al., 1997; Tardi et al., 1998). VPG consists of densely packed small unilamellar liposomes and an extremely low portion of the external aqueous phase surrounding liposomes (Brandl et al., 1997), resulting with stable drug load that can be released in a sustained and controllable manner (Brandl, 2007; Brandl et al., 1997; Tardi et al., 1998). In the presence of aqueous medium, VPG easily converts into liquid liposomal dispersion still allowing high drug loading (Brandl, 2007). So far, VPGs have been developed and investigated as depot and liquid nanoformulations for parenteral (Tardi et al., 1998; Güthlein et al., 2002; Kaiser et al., 2003; Breitsamer and Winter, 2019) and recently, for ophthalmic drug delivery (Fang et al., 2021).

To the best of our knowledge, VPGs have not been examined for skin administration of drugs so far. Therefore, in this study, for the first time, we evaluated their potential for dermal drug delivery. Ciprofloxacin hydrochloride (CPX) was selected as a model hydrophilic antibacterial drug, which has not yet been investigated in the form of liposomes for dermal drug delivery. It was incorporated into several VPGs varying in their composition. By optimizing the composition and concentration of (phospho)lipids, adding co-solvent or polymer, it is possible to prepare VPGs of appropriate bilayers' fluidity/rigidity, which can control the release and penetration of the incorporated drug into the skin. Semisolid consistency of VPG would facilitate dermal application and extend the retention of the drug at the site of action. Additionally, the phospholipids from VPG could be integrated into disturbed superficial skin layers, thus facilitating the effect of healing and skin regeneration.

To examine these assumptions, we prepared several CPX-VPGs and characterized them for the physical and rheological properties, *in vitro* CPX release and storage stability. The efficacy of CPX-VPGs in delivering the drug was tested *in vitro* against several biofilm-forming bacterial strains typical for infected wounds, i.e., *Staphylococcus aureus*, *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA). The optimized CPX-VPGs were explored for *ex vivo* skin permeability, biocompatibility with the human keratinocytes and *in vitro* healing effect.

2. Materials and methods

2.1. Materials

Soybean lecithin S100 (SPC, phosphatidylcholine ≥ 94.0 %), Phospholipon 90H (P90H, hydrogenated phosphatidylcholine ≥ 90.0 %) and soybean monoacyl phosphatidylcholine (SLPC80, monoacyl phosphatidylcholine content approx. 80 %) were obtained from Lipoid GmbH (Ludwigshafen, Germany). CPX in the form of hydrochloride monohydrate (Mw 385.82 g/mol) was purchased by Biosynth AG (Staad, Switzerland). Low molecular weight chitosan (≥75 % deacetylation), cholesterol, Sephadex G-50, bovine serum albumin (BSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), potassium dihydrogen phosphate, disodium hydrogen phosphate dihydrate, iron (II) sulfate heptahydrate, ammonium sulfate and magnesium sulfate were purchased from Sigma Aldrich (Taufkirchen, Germany). Acetonitrile, ethanol, and methanol were of HPLC grade and procured from BDH Prolabo (Lutterworth, UK). Dulbecco's Modified Eagle's Medium (DMEM) was obtained by Gibco (Invitrogen, UK), while fetal bovine serum (FBS) by Biosera (Cholete, France). Penicillin-streptomycin-amphotericin B mixture, ethylenediamine tetra acetic acid (EDTA), 2.5 % trypsin, and phosphate buffer (PBS, pH 7.4) without calcium and magnesium (used in *in vitro* antibacterial and biocompatibility studies), were purchased by Lonza (Basel, Switzerland). Glucose was obtained from Fluka Chemie GmbH (Buchs, Germany), while Müller-Hinton agar, Müller-Hinton broth, tryptic soy broth and L-arginine were procured by Merck (Darmstadt, Germany). Luria-Bertani agar and Luria-Bertani medium were from Liofilchem (Roseto degli Abruzzi, Italy) and BD Difco (Becton, USA), respectively. All other chemicals and solvents used in this research were of analytical grade and purchased from Kemika (Zagreb, Croatia) or Sigma Aldrich (Taufkirchen, Germany).

For cultivation of *P. aeruginosa* ATCC 27853, Luria-Bertani agar, Luria-Bertani medium and M63 minimal medium, consisting of potassium dihydrogen phosphate (13.6 g/l), iron (II) sulfate heptahydrate (0.5 mg/l), with the addition of magnesium sulfate (1 mM), glucose (0.2 %) and L-arginine (0.4 %), were used. Müller-Hinton agar, Müller-Hinton broth, and tryptic soy broth were used for the cultivation of *S. aureus* ATCC 6538 and MRSA clinical isolate MFBF 10679.

Phosphate buffer pH 7.4, used in *in vitro* release studies, was prepared by dissolving 2.98 g disodium hydrogen phosphate dihydrate, 0.19 g potassium dihydrogen phosphate and 8.0 g sodium chloride in distilled water, up to 1000 mL (Ph. Eur., 2023). A simulated wound fluid (SWF) was prepared by addition of 2 % BSA to the phosphate buffer, pH 7.4 (Boateng et al., 2013).

2.2. Methods

2.2.1. Preparation of VPGs

CPX-loaded VPGs were prepared by the modification of the method originally described by Brandl et al. (1997). In brief, the appropriate amounts of (phospho)lipids were hydrated in water solution of CPX, with or without addition of propylene glycol or chitosan (Table 1), using a magnetic stirrer (500 rpm, 2 h). The hydrated phospholipid mixture was then homogenized (500 bar, one cycle) by a high-pressure homogenizer (Microfluidizer LM20, Microfluidics) resulting with semisolid CPX-VPG. Quantitative composition of the different CPX-VPGs is shown in Table 1.

CPX-free VPGs were prepared under the same conditions, but without the addition of CPX. They were used as controls in *in vitro* cytotoxicity and antibacterial studies.

pH values of all the VPGs were determined by a pH meter (Mettler-Toledo, Greifensee, Switzerland) equipped with electrode for semisolid formulations. The measurements were performed at 25 °C in triplicates (Čačić et al., 2023).

All the VPGs were stored in a refrigerator at 4 °C. Prior to the measurement they were tempered at room temperature (30–40 min).

2.2.2. Physicochemical characterization of CPX-VPGs

Physicochemical evaluation of VPGs (size, zeta potential, bilayer elasticity and drug entrapment in internal phase of VPG) was performed after their conversion into liposomes by gradual dilution of VPG (1 g) with distilled water (9 g), during continuous magnetic stirring (600 rpm, 10 min). For VPGs containing cholesterol (SPC/Chol/CPX) and hydrogenated phosphatidylcholine (SPC/P90H/CPX), the reconstitution procedure was conducted at 60 °C.

Mean diameters, polydispersity indexes, and zeta potentials of the different CPX-VPGs were determined on a Zetasizer Ultra (Malvern Panalytical, Malvern, UK) at 25 °C. Liposomes, converted from VPGs, were diluted with distilled water to obtain semi-transparent dispersion appropriate for measurements. Multi-angle dynamic light scattering was applied for determination of mean diameters and polydispersity indexes. Zeta potential measurements were employed by electrophoretic light scattering using a disposable folded capillary cell. Size and zeta potential measurements were also performed in SWF at 32 °C. All the measurements were done in triplicate.

Bilayers' elasticity/rigidity of the different CPX-VPGs was determined by a homemade device allowing continuous extrusion (5 min) of liposomes under constant pressure (2.5 bar) through the membrane of 100 nm pore size (Vanić et al., 2014). The degree of bilayer's elasticity (E) was calculated according to formula:

$E = J \cdot (r_v/r_p)^2$,

where J corresponds to mass of extruded liposomes (g) in 5 min, r_v denotes the average diameter of liposomes after extrusion, while r_p is the membrane pore size.

Entrapment of CPX in liposomes of VPGs was determined after separation of liposomally-entrapped CPX by ultracentrifugation, according to the previously described procedure (Palac et al., 2015). Quantification of CPX, entrapped in liposomes (internal phase of VPG) and nonliposomal CPX (outer phase of VPG), was performed on UV/Vis spectrophotometer (Cary 50 Probe, Varian, Palo Alto, USA) at 278 nm. Solutions of CPX in water and methanol, ranging from 1 – 15 μg/mL, were used to construct standard calibration curves (R^2 = 0.9978, distilled water; R^2 = 0.9989, methanol).

Entrapment of CPX in liposomes of VPGs and the drug recovery were calculated according to the following equations:

$$\textit{Entrapment efficinecy}\ (\%) = \frac{\text{m liposomal CPX}}{\text{m liposomal CPX} + \text{m nonliposomal CPX}} \cdot 100$$

$$\textit{Recovery}(\%) = \frac{\text{m liposomal CPX} + \text{m nonliposomal CPX}}{\text{m total CPX}} \cdot 100$$

The content of liposomal CPX was determined after dissolving liposomes (previously separated from nonliposomal CPX) in methanol. The total CPX represented the amount of CPX in initial liposomal dispersion and was achieved after dissolving lipids of initial CPX-liposomes in methanol.

2.2.3. In vitro release studies

The release of CPX from the CPX-VPG was performed using a dialysis method in distilled water (simulation of healthy skin) and buffer, pH 7.4 (simulation of infected skin conditions). In brief, a sample of CPX-VPG, each corresponding to 15 mg CPX, was placed into a dialysis bag (Medicell Membranes, Mw cut-off 3500 Da) and dialyzed against 200 mL of distilled water during constant stirring (300 rpm) at 32 °C (corresponding to temperature of skin surface). At certain time intervals (1, 2, 3, 4, 5, 6, 24, 27 and 30 h), 2 mL of the dialysis medium containing the released drug was withdrawn and replaced with the fresh medium. The amount of the released CPX was determined spectrophotometrically.

In vitro release studies were also carried out in the buffer pH 7.4, since skin infections are characterized by an increase in pH value. The studies were conducted following the same protocol; however, to ensure sink conditions, the amount of CPX-VPG corresponded to 1 mg CPX, while the volume of buffer, pH 7.4 was 100 mL.

As a control, CPX solution in the same drug concentration as in the CPX-VPGs was used.

These studies were performed in three independent experiments.

2.2.4. Stability of CPX-VPGs in simulated wound fluid (SWF)

To be closer to *in vivo* conditions of infected skin, CPX-VPGs were examined for their stability in SWF. Prior to the testing, CPX-VPGs were converted to CPX-liposomes, and the nonliposomal (free) drug was removed from the liposomally-entrapped CPX by ultracentrifugation at 120,000 x g during 1 h at 20 °C using Beckman Optima LE-80 K Ultracentrifuge (Beckman Coulter Inc., Fullerton, CA). To assess the effect of BSA from SWF on the liposomes' stability, 500 μL of the liposomes containing only entrapped CPX were mixed with 1.5 mL of SWF or buffer, pH 7.4. The mixtures were incubated at 32 °C and the content of

Table 1
Composition of the different CPX-VPGs.

VPG	SPC (g)	Chit (g)	P90H (g)	Chol (g)	SLPC80 (g)	PG (g)	CPX (g)	Water (g)
SPC/CPX	34						2	64
SPC/Chit/CPX	30	1					2	67
SPC/P90H/CPX*	16.5		8.5				2	73
SPC/Chol/CPX*	28.3			3.3			2	66.4
SPC/SLPC80/CPX	29.8				5.3		2	62.9
SPC/PG/CPX	35					18.9	2	44.1
SPC/SLPC80/PG/CPX	22.4				4	5.2	2	66.4

Chit, low molecular weight chitosan (added as 1.5 % chitosan solution in 0.5 % acetic acid); Chol, cholesterol; CPX, ciprofloxacin hydrochloride monohydrate; PG, propylene glycol; P90H, Phospholipon 90H; SLPC80, soybean monoacyl phosphatidylcholine; SPC, soybean lecithin with ≥ 94 % phosphatidylcholine; VPG, vesicular phospholipid gel. *Hydration and homogenization of SPC-Chol and SPC-P90H was performed at 60 °C.

entrapped CPX was determined after 24 h. The amount of the released CPX from the liposomes was detected spectrophotometrically (2.2.2) after separation of the released drug from liposomes by Vivaspin 6 centrifugal concentrator (Sartorius, Ulm, Germany).

Additionally, stability of CPX-VPGs in SWF was also assessed by determining mean diameters, polydispersity indexes and zeta potentials of CPX-liposomes following the procedure described in 2.2.2; however, instead of distilled water as dispersant, SWF was used, and the measurements were done at 32 °C.

These studies were conducted in triplicate.

2.2.5. Rheological evaluation of CPX-VPGs

The rheological properties of the different CPX-VPGs were assessed at 25 and 32 °C on a Modular Compact Rheometer MCR 102 (Anton Paar GmbH, Graz, Austria) fitted with a parallel-plate measuring system (PP25, diameter 25 mm) and the gap set to 1 mm. Two types of measurements were performed, rotational and oscillatory tests. Prior to the measurement, the sample of CPX-VPG was equilibrated for 10 min to the corresponding temperature. Viscosity curves were determined in the shear rate range from 0.1 to 1000 s^{-1}, while oscillatory amplitude sweep tests were carried out by applying an angular frequency of 10 s^{-1} in the shear stress range of 0.1–1000 Pa (Čačić et al., 2023; Rukavina et al., 2023). Both types of measurements were done in triplicate.

2.2.6. Bioadhesion assessment

The abilities of the different CPX-VPGs to adhere onto the skin surface were tested using a TA.XT Plus Texture Analyzer equipped with a gel mucoadhesion probe (Stable Micro Systems Ltd., Surrey, UK). The studies were performed using full-thickness pig ear skin.

2.2.6.1. Skin samples preparation. Pig ears were obtained from the local slaughterhouse. They were washed under running tap water and dried with a paper towel. The hair was cut by scissors, carefully shaved and the full-thickness skin was taken from the back of ears. The skin samples were carefully separated from the underlaying tissue by a scalpel, rinsed with 0.9 % NaCl, dried by a paper towel and frozen at − 20 °C. Prior to the testing, the skin was defrosted, pre-equilibrated in 0.9 % NaCl for approximately 10 min, and the face side of the skin was gently dried by a paper towel. The thickness of the skin was 1.3 mm.

2.2.6.2. In vitro bioadhesion assay. The skin was cut into discs (diameter of 10 mm) and attached to the upper probe of the instrument with cyanoacrylate glue. A sample of the CPX-VPG (0.2 mL corresponding to 0.10–0.15 g), was placed directly on the lower platform of the mucoadhesion test attachment. The measurement settings were as follows: pre-test speed 0.5 mm/s, test speed 0.1 mm/s, contact time 60 s, constant force 0.5 N, and post-test speed 0.1 mm/s. The Texture Analyzer software (Exponent Connect Version 7.0.6.0) was used to calculate the work of adhesion (the area under the force/distance curve) and maximum detachment force, which served as an indicator of the bioadhesive abilities of VPGs. The measurements were performed in pentaplicate.

2.2.7. In vitro antibacterial testing

Antibacterial activity of the CPX-VPGs was assessed toward *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 6538 and MRSA clinical isolate MFBF 10679 obtained from the microbial collection of the Department of Microbiology, Faculty of Pharmacy and Biochemistry, University of Zagreb (Croatia). Planktonic bacteria and bacterial biofilms were used to determine minimum inhibitory concentrations (MICs) and minimum biofilm inhibitory concentrations (MBICs) of the CPX-liposomes converted from CPX-VPGs. The studies were carried out in three independent experiments, and four technical replicates.

2.2.7.1. Preparation of inoculum and CPX-VPGs samples. Bacterial cultures were grown at 37 °C aerobically on appropriate agar plates. To prepare the inoculum, 24-hour bacterial cultures grown in a medium suitable for the tested bacterial strain (listed in 2.1.) were used. Bacteria were suspended in the cultivation medium to a density of 0.5 McFarland (DEN-1 densitometer, Grant Instruments, Royston, UK), which corresponded to a bacterial concentration of 1.5×10^8 CFU/mL. Before plating, the bacteria were further diluted in the fresh medium to obtain an amount of 10^6 bacterial cells.

The samples of CPX-VPGs were converted to CPX-liposomes by dilution with distilled water (1:10 ratio), followed by ultracentrifugation to remove the free CPX. The required initial concentrations for the bacterial testing were obtained by dilution of the tested CPX-liposomes with the growth media, depending on the tested bacterial strain and the type of test (MIC or MBIC). The antibacterial activity of CPX-liposomes was examined with respect to both CPX-solution and empty (CPX-free) liposomes, converted from CPX-free VPG (empty VPG).

2.2.7.2. Antibacterial activity against planktonic bacteria. A two-fold microdilution assay on a 96-well plate was applied to determine MIC values of the CPX-liposomes for the tested bacterial strains. It was performed according to the National Committee for Clinical Laboratory Standards (NCCLS) standard protocol (NCCLS, 2007). In brief, cultures of bacteria in an appropriate cultivation medium were treated with CPX-liposomes or free CPX (CPX solution) at concentrations ranging from 0.25 to 8 μg/mL. Planktonic bacteria in the cultivation medium were used as positive control (growth control), while pure cultivation medium (without addition of bacteria) represented negative control. Empty (CPX-free) liposomes (converted from empty VPGs) were tested under the same conditions to elucidate possible antibacterial effects exerted by the VPGs constituents themselves. After 24 h of incubation at 37 °C, the MICs were assessed by absorbance measurement at 570 nm (Wallac 1420 Victor 2, PerkinElmer, USA). The MIC was defined as the lowest concentration of CPX that restricted bacterial growth (Rukavina et al., 2018; Babić et al., 2010).

2.2.7.3. Antibiofilm activity. To determine MBIC values of the CPX-liposomes, a modified method according to O'Toole was applied (O'Toole, 2011). Bacterial strains, suspended in the growth medium to a density of 0.5 McFarland, were diluted (1:100) with M63 medium (for *P. aeruginosa* ATCC 27853) or with TSB supplemented with 0.25 % D-(+)-glucose (for *S. aureus* ATCC 6538 and MRSA MFBF 10679) to achieve a final concentration of $1 - 2 \times 10^6$ CFU/mL. The tested CPX samples were diluted in the cultivation medium to attain an initial CPX concentration of 16 μg/mL. The two-fold dilution assay was applied to further dilute the samples in the corresponding mediums at concentrations ranging from 0.25 – 8 μg/mL using a 96-well U-bottom plates (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). The overnight bacterial cultures were diluted (1:10) in the suitable medium and 50 μL of bacterial suspension was added to each well. Each bacterial strain was treated with CPX-loaded liposomes or CPX solution in quadruplicate. As a positive control, planktonic bacteria in the corresponding cultivation medium were used, while negative control represented pure cultivation medium (without addition of bacteria). Following incubation at 37 °C during 24 h, planktonic bacterial strains were carefully removed from the wells and 125 μL of 0.1 % crystal violet was added to the biofilm for staining (15 min). The well plate was washed with distilled water (3 times), dried, and 150 μL of 30 % acetic acid was added to dissolve

crystals. The solubilized crystal violet (125 μL) was transferred to a new 96-well plate with the flat bottom and the absorbance was determined at 540 nm using a Wallac 1420 Victor 2 microplate reader (Perkin Elmer, Waltham, MA, USA) (Perković et al., 2023). The CPX-free VPGs (i.e., CPX-free liposomes, empty liposomes) were tested under the same conditions to detect the possible effect of VPG constituents themselves on the antibacterial activity.

2.2.8. *In vitro* biocompatibility studies

In vitro biocompatibility of CPX-VPGs, converted into CPX-liposomes and containing only entrapped drug, was tested on human keratinocyte cell line HaCaT (Cell Line Services, Germany).

2.2.8.1. Cell culturing. HaCaT cells were incubated in DMEM supplemented with 10 % FBS and a mixture of penicillin, streptomycin and amphotericin B at 37 °C and 5 % CO_2. To determine the potential cytotoxicity of the liposomally-entrapped CPX, HaCaT cells were seeded onto 96-well plates at a density of 10^4 cells/well, and then allowed to reach confluence in 48 h (Rukavina et al., 2018).

2.2.8.2. Cell toxicity study. CPX-liposomes were dispersed in DMEM at concentrations of 1, 8, 16 and 32 μg/mL. For comparison, CPX-solution at the same CPX concentrations as in the liposomes was tested. Prior to the treatment with the CPX samples, the cell culture medium was removed, and the cells were washed with the PBS (pH 7.4). The cells were then treated with CPX samples at different concentrations and incubated for 24 h. The cells incubated in DMEM were used as a negative control. After 24 h, *in vitro* cytotoxicity was determined by the MTT assay, as described in detail by Rukavina et al. (2018). The experiments were also performed with CPX-free liposomes (empty VPG) to examine the possible cytotoxicity effects of the VPG-building ingredients. The cell viability of the treated cells was expressed as a percentage compared to the untreated control cells. The experiments were conducted in quadruplicate.

2.2.9. *In vitro* scratch test

The scratch assay was applied to evaluate *in vitro* healing potential of the selected CPX-VPGs. The test is based on creating a scratch on the cell monolayer and tracking the cells initially (immediately after scratch) and at certain intervals during cell division. Images of the scratch were compared to determine the rate of cell migration, i.e., healing effect (Liang et al., 2007).

In vitro healing potential of CPX-VPGs was assessed on the human keratinocyte's monolayer. HaCaT cells were seeded onto 24-well plates at a density of 10^4 cells/well and were allowed to reach confluence over one day of incubation in DMEM supplemented with 10 % FBS and the mixture of penicillin, streptomycin and amphotericin B (37 °C, 5 % CO_2). Each well was marked on the bottom by drawing a horizontal line, to ensure that an equal portion of the well with the scratch would be monitored. The culture medium was removed after 24 h and the fresh, non–supplemented DMEM was added. After 24 h, a straight scratch was made with a 200 μL pipette tip, to simulate a wound. The cell monolayer was washed with PBS (7.4) to remove detached cells. The scratches ("wounds") were exposed to selected CPX-VPG liposomes: SPC/CPX-, SPC/Chit/CPX-, SPC/P90H/CPX-, SPC/Chol/CPX- and SPC/SLPC80/PG/CPX-liposomes as well as CPX solution at the concentration of CPX being 16 μg/mL. Non-treated cells incubated in the non–supplemented DMEM served as a control. Scratch closure was monitored over 24 h using an inverted microscope (5 × magnification; Olympus CKX41) equipped with a camera (Samsung, 16 MP, f/1.9). To assess the wound healing rate, the difference between the wound surface at time 0 (initial scratch) and after 24 h was determined. The surface area of the scratch was calculated using ImageJ software (National Institutes of Health, USA). The wound closure rate (WCR) was expressed as the percentage of wound closure after 24 h compared to the initial area, according to the equation:

$$WCR\% = \frac{A(0) - A(24)}{A(0)} \cdot 100,$$

where A (0) was the initial scratch area (0 h), while A (24) represented scratch area after 24 h (Blažević et al., 2019).

2.2.10. *Ex vivo* permeability studies

A pig ear skin model was employed to evaluate skin localization of CPX from selected CPX-VPGs. Skin samples were prepared according to the procedure described in detail in subchapter 2.2.6.1. The permeation testing was performed on the automated Franz diffusion cell testing system Phoenix™ RDS (Teledyne Hanson, Chatsworth, CA, USA). The full thickness skin was sandwiched between the donor and receptor compartment of the Franz cell (surface area of 1.77 cm^2), with the *stratum corneum* facing the donor compartment. The receptor compartment was filled with 15 mL of the buffer, pH 7.4, tempered at 37 °C, and continuously stirred (400 rpm). Sample of CPX-VPG or control (CPX-solution), each corresponding to 1 mg of CPX, was placed in the donor compartment and properly sealed. The receptor medium (500 μL) was removed at certain time intervals (1, 2, 3, 4, 5, 6, 7, 8, 16 and 24 h) and immediately replaced with an equal volume of the fresh buffer. After 24 h, the skin surface was carefully rinsed with methanol (represented the drug accumulated on skin surface). The skin was then cut into small pieces, dispersed in methanol and agitated for 10 h to extract the drug localized within the skin (Rukavina et al., 2018). The content of the drug deposited on the skin surface, within the skin and penetrated through the skin into receptor medium, was determined by HPLC. The experiments were done in triplicate.

2.2.10.1. HPLC assay for CPX. Quantification of CPX, penetrated or localized on/within the skin, was determined by HPLC using 1260 Infinity II LC System (Agilent, Santa Clara, CA, USA). Prior to analysis, all samples were filtered through 0.22 μm polyethersulfone filters (Filter-Bio, Labtes Ltd.). Separation was carried out on a Kinetex® C18 column (4.6 mm x 250 mm, particle size 5 μm, Phenomenex, Torrance, USA) with SecurityGuard column protection (Phenomenex, Torrance, USA), by a mixture of 2.45 g/L phosphoric acid and acetonitrile (87/13, v/v) as the mobile phase (pH 3). The flow rate was 1.5 mL/min, and the oven temperature was maintained at 40 °C. UV detection of CPX was conducted at 278 nm at a retention time of 6.5 min, while the total run time was 10 min. Solutions of CPX in methanol at drug concentrations ranging from 1-15 μg/mL were used to construct a standard curve (R^2 = 0.9980). Quantitative analysis was performed in triplicate.

2.2.11. *Storage stability studies*

Physical stability of CPX-VPGs was estimated by determining their mean diameters, polydispersity indexes and zeta potentials during 6 months of storage at 4 °C. The measurements were conducted in distilled water, following the procedure described in Section 2.2.2. Moreover, to determine possible changes in mechanical properties of CPX-VPGs, oscillatory frequency test was carried out immediately after preparation of CPX-VPGs and after 2 months of their storage at 4 °C. The test was performed using a Modular Compact Rheometer MCR 102 (Anton Paar GmbH, Austria) equipped with a parallel-plate measuring system (PP25, diameter 25 mm) and the gap set to 1 mm. The angular frequency was in the range of 1–100 rad/s and the shear strain was 0.01 %. The measurements were conducted at 32 °C.

2.2.12. *Statistical analysis*

Statistical data analysis was assessed by one-way ANOVA and post hoc Tukey's multiple comparison test. In case of direct comparison

between two groups, *t*-test was used. All calculations were made using GraphPad Prism program, version 8.4.3 (GraphPad Software, Inc., San Diego, CA, USA). Means were considered significantly different when $p < 0.05$.

3. Results and discussion

3.1. Physicochemical characteristics of CPX-VPG liposomes

Physicochemical evaluation has a crucial role in obtaining reliable data during the development and optimization of CPX-VPGs. Namely, physicochemical properties determine pharmacokinetic characteristics of entrapped drug, stability of nanoformulation during storage and its behavior within biological milieu, indirectly influencing the effectiveness of therapy (Bhattacharjee, 2016; Vanić et al, 2019). Physicochemical characteristics are strongly related to the composition of drug nanoformulation. Therefore, CPX-VPGs composed of different (phospho)lipids, with or without addition of polymer and cosolvent, were prepared and examined.

Since VPG consists of tightly packed liposomes, the first phase in the assessment of CPX-VPGs was to determine physicochemical properties of the corresponding CPX-liposomes: size, surface charge, bilayer fluidity and entrapment efficiency.

High pressure homogenization method used in the preparation of CPX-VPGs enabled formation of liposomes with average diameters between 126 and 193 nm with polydispersity indexes mostly from 0.3 to 0.5 (Table 2). These results are consistent with those of Brandl et al. (1997), where VPG liposomes ranged from 100 to 200 nm. The ingredients of CPX-VPGs influenced the size of CPX-liposomes. Amongst the different examined CPX-liposomes, those containing hydrogenated phospholipid (P90H) were the largest ($p < 0.0001$) with the highest polydispersity index (0.69), followed by SPC/PG/CPX- and SPC/Chit/CPX-liposomes (Table 2). In contrast, CPX-liposomes embedding SLPC80 were the smallest due to the impact of the edge activator (SLPC80) on reducing the size of deformable liposomes (Vanić et al. 2019; Palac et al, 2014, Rukavina et al., 2018).

Surface charge of liposomes plays an important role in stability of liquid liposomal dispersions and interactions of liposomes with the biological environment, including cells and bacteria. The zeta potential values of CPX-liposomes were influenced by bilayer-building ingredients (phospholipids, cholesterol), presence of propylene glycol or chitosan. Slightly negative zeta potentials (−4 to −9 mV) were characteristic for all liposomes composed of SPC, P90H or SLPC80. While embedding of cholesterol slightly decreased the zeta potential (−1.5 mV) of the corresponding liposomes, presence of propylene glycol increased the zeta potentials of SPC/PG/CPX- and SPC/SLPC80/PG/CPX-liposomes up to 8 mV. These slightly negative/positive zeta potentials of liposomes could contribute to reduced penetration of the entrapped drug into the skin (Ibaraki et al., 2019), facilitating localization of CPX at the site of action. The most pronounced effect on the zeta potential change was achieved with SPC/Chit/CPX-liposomes since chitosan displays polycationic structure in acidic media (pKa approximately 6.5). This feature could be favorable for possible improvement of antimicrobial activity (Rukavina et al., 2023). Interestingly, in the presence of nonentrapped CPX (remained in the outer water phase of the liposomes after reconstitution of CPX-VPG with distilled water), all the CPX-liposomes exerted positive zeta potential (18–32 mV). Namely, CPX is positively surface charged in water solution, resulting with an overlap of the zeta potential of the CPX-liposomes (Table 2).

The thermodynamic state of the liposome bilayers also contributes to the stability of liposomes, drug release pattern and penetration into the skin (Rukavina et al., 2018). The results presented in Table 2 confirmed the impact of SLPC80 and especially propylene glycol on increasing bilayers' elasticity. Chitosan reduced fluidity of SPC bilayers, while cholesterol and particularly P90H significantly enhanced rigidity of VPGs' bilayers ($p < 0.0001$). As in previous studies (Rukavina et al., 2018; Vanić et al., 2019), the degree of bilayer elasticity (E) was dependent on the mass of extruded liposomes (J), rather than their size after passage through 100 nm pore-sized membranes.

An effective nanoformulation for dermal drug delivery should provide a high drug load to assure the appropriate amount of the drug at the site of action. A significant advantage of VPG over liposomes (liquid dispersion) is that all the drug (100 %) is loaded within semisolid VPG (Brandl et al., 1997; Brandl, 2007). Since *in vitro* antimicrobial, cytotoxicity and stability studies require that the tested sample is in a liquid form, CPX-VPGs were converted to the CPX-liposomes. The free (non-liposomal) CPX was separated to simulate conditions of CPX-VPG application, where all the drug is encapsulated within VPG.

Composition of CPX-VPGs influenced the entrapment of the drug in liposomes. It varied from 18 % (SPC/CPX- and SPC/SLPC80/CPX-liposomes) to 33 % (SPC/PG/CPX-liposomes), with the drug recovery being 94–102 %. Deformable liposomes (SPC/SLPC80/CPX-liposomes) are known to enable less entrapment of the drug, while propylene glycol in SPC/PG/CPX- and SPC/SLPC80/PG/CPX-liposomes showed an opposite effect, which is in agreement with the earlier research (Palac et al., 2014; Rukavina et al., 2018; Vanić et al., 2019). Moreover, the

Table 2
Physicochemical characteristics of the different CPX-VPG liposomes.

VPG	Size MD (nm)	PDI	Zeta potential (mV)	Bilayer elasticity $(r_v/r_p)^2$	J (g)	E	Liposomally-entrapped CPX (%)
SPC/CPX	130.0 ± 2.6	0.40 ± 0.0	−6.01 ± 0.49 +23.75 ± 0.51[a] **	28.93 ± 0.02	1.12 ± 0.08	1.59 ± 0.12	18.0 ± 2.0
SPC/Chit/CPX	179.8 ± 4.4*	0.32 ± 0.04	+26.88 ± 1.43* +26.82 ± 1.55 [a]	40.58 ± 0.23	0.34 ± 0.17	0.96 ± 0.49	28.5 ± 0.7*
SPC/P90H/CPX	193.2 ± 2.5*	0.69 ± 0.07*	−4.22 ± 2.46* +18.53 ± 0.93 [a] **	38.28 ± 0.46	0.07 ± 0.02	0.18 ± 0.09*	26.3 ± 1.0*
SPC/Chol/CPX	144.8 ± 2.2	0.49 ± 0.09	−1.58 ± 0.24* +27.06 ± 0.99 [a] **	37.65 ± 0.66	0.21 ± 0.03	0.47 ± 0.07*	22.7 ± 2.1
SPC/SPLC80/CPX	129.2 ± 1.9	0.45 ± 0.02	−8.50 ± 0.30* +32.12 ± 0.70[a] **	31.61 ± 0.09	1.28 ± 0.34	2.13 ± 0.47*	17.6 ± 2.2
SPC/PG/CPX	166.2 ± 1.8*	0.27 ± 0.01*	+8.22 ± 0.84* +29.85 ± 1.58[a] **	43.42 ± 0.37	1.21 ± 0.27	3.71 ± 0.50*	33.0 ± 1.0*
SPC/SLPC80/PG/CPX	126.8 ± 1.4	0.43 ± 0.02	+5.26 ± 1.13* +25.16 ± 0.91 [a] **	30.41 ± 0.11	2.50 ± 0.62	3.81 ± 1.13*	25.0 ± 1.4*

E, degree of bilayer elasticity; J, mass of extruded liposomes in elasticity measurements; MD, mean diameter; PDI, polydispersity index; r_v, mean diameters of CPX-liposomes after extrusion through the membrane of 100 nm, r_p, membrane pore size (100 nm); ZP, zeta potential. The results denote the average ± S.D. (n = 3). Measurements of size, zeta potential and bilayer elasticity were done with CPX-liposomes containing only entrapped CPX. [a], Zeta potentials of liposomal dispersions containing both, liposomally-entrapped and nonentrapped, CPX. *Significantly different from the SPC/CPX VPG liposomes ($p < 0.05$). **Significantly different compared to CPX-VPG liposomes containing nonentrapped CPX ($p < 0.05$).

larger size of the liposomes containing hydrogenated phospholipid or chitosan also contributed to better entrapment of CPX (Table 2).

3.2. Characterization of CPX-VPGs

When designing a dermal formulation, the anatomical and physiological properties of the skin should be taken into consideration. Since pH of the skin surface is between 4.1 and 5.8 and increases in infection to neutral or even alkaline pH (Proksch, 2018; Sim et al., 2022), the application of the dermal formulation with a slightly acidic pH may be useful in topical dermatotherapy.

The measured pH values of all CPX-VPGs (4.4 – 4.7) were aligned with the pH of the healthy skin. They were affected by CPX and are generally favorable in terms of the drug stability (Rodríguez-López et al., 2021) and assuring intact skin barrier. On the other hand, the drug free-VPGs had a pH between 5.4 and 6.4, except SPC/Chit VPG (pH 4.9) (Fig. 1). The significantly lower pH of chitosan containing VPG ($p < 0.0001$), compared to the other drug-free VPGs, was affected by acetic acid used to dissolve chitosan.

Once the drug loaded VPG is administered dermally, it should distribute along the skin and reside at the site of application long enough to allow release of the incorporated drug. These features are strongly determined by the composition of the VPG. Therefore, rheological evaluation, bioadhesion testing and *in vitro* release studies are essential to assess the characteristics of the prepared CPX-VPGs.

Rheological studies comprised rotational and oscillatory measurements. Rotational tests were performed to gather information on the effect of high-pressure homogenization on flow behavior of VPGs and to determine the effect of different CPX-VPGs-building ingredients on its viscosity. The viscosity studies were conducted at 25 °C (CPX-VPG mixtures prior high-pressure homogenization) and at 32 °C (prepared CPX-VPGs).

By applying pressure of 500 bar, the viscosity of each CPX-phospholipid mixture increased by one order of magnitude (Fig. 2A-C) due to reduction in the size of the phospholipid vesicles and the dense arrangement of numerous vesicles within the VPG. Under such circumstances intervesicular water spaces are diminished, leading to steric interactions between neighboring vesicles and formation of a gel-like consistency (Elnaggar et al. 2014). VPG ingredients responsible for bilayers rigidity (Phospholipon 90H, cholesterol) and elasticity (propylene glycol, SLPC80), did not affect the viscosity of the CPX-VPGs because the concentration of SPC was not constant for all formulations. Namely, the flow properties of CPX-VPGs were optimized to ensure that all hydrated (phosho)lipid mixtures could be processed through the high-pressure homogenizer during production of VPGs. The viscosity was primarily determined by SPC concentration (preliminary investigations), which agrees with the previous findings (Tardi et al., 1998; Elnaggar et al., 2014; Tian et al., 2010). Accordingly, the final viscosities of CPX-VPGs were optimized by adjusting the amount of SPC in each formulation.

A deeper understanding of a formulation's rheological behavior is essential to clarify the complex relationship between its chemical structure and resulting properties (Stojkov et al. 2021). Oscillatory amplitude tests are preferred in rheological characterization of semi-solid formulations as they enable assessment of the impact of formulation composition on the viscoelastic structure of gels (Yu et al. 2011). They were conducted at 32 °C to evaluate the flow properties of VPGs under application conditions (spreading of the formulation on the skin).

As displayed in Fig. 3, all CPX-VPGs exhibited the linear viscoelastic region (LVR), i.e., a constant plateau where the values of the storage modulus (G') and loss modulus (G'') are independent of shear stress and correlate only with the molecular structure (Simões et al. 2020). For all tested CPX-VPGs, G' was higher than G'', indicating that the CPX-VPGs possess a viscoelastic solid structure. This observation is consistent with the research by Qi et al. (2020) where storage modulus was also higher than loss modulus for all tested VPGs containing cytarabine.

Prolonged contact of the drug formulation on the skin surface is a prerequisite for its therapeutic effectiveness. Therefore, in the next phase of CPX-VPGs characterization, bioadhesive features of the different CPX-VPGs were assessed. The measurements were performed by a tensile test using texture analysis, where detachment force (Fig. 4A) and the work needed for detachment of the formulation from the skin (Fig. 4B) were determined.

Composition of the CPX-VPGs significantly influenced their bioadhesiveness. Thus, VPG containing hydrogenated phospholipid (P90H) displayed the highest detachment force and work of adhesion ($p < 0.001$), followed by those containing chitosan and a large amount of propylene glycol (Fig. 4). Interestingly, the bioadhesion of CPX-VPGs was not affected by their viscosity, i.e., SPC concentration. Thus, the work of adhesion for highly viscous SPC/CPX VPG (Fig. 2A) was 6-fold lower compared to similarly viscous SPC/Chit/CPX VPG and even more

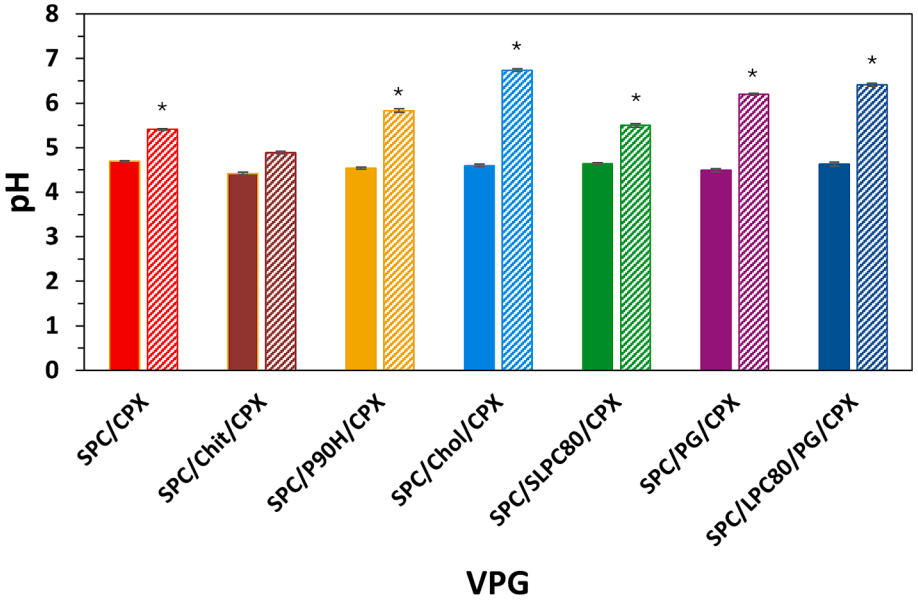

Fig. 1. pH values of CPX-VPGs (solid bars) and CPX-free VPGs (patterned bars). The results are mean ± S.D. (n = 3). *Significantly different compared to the corresponding CPX-VPG.

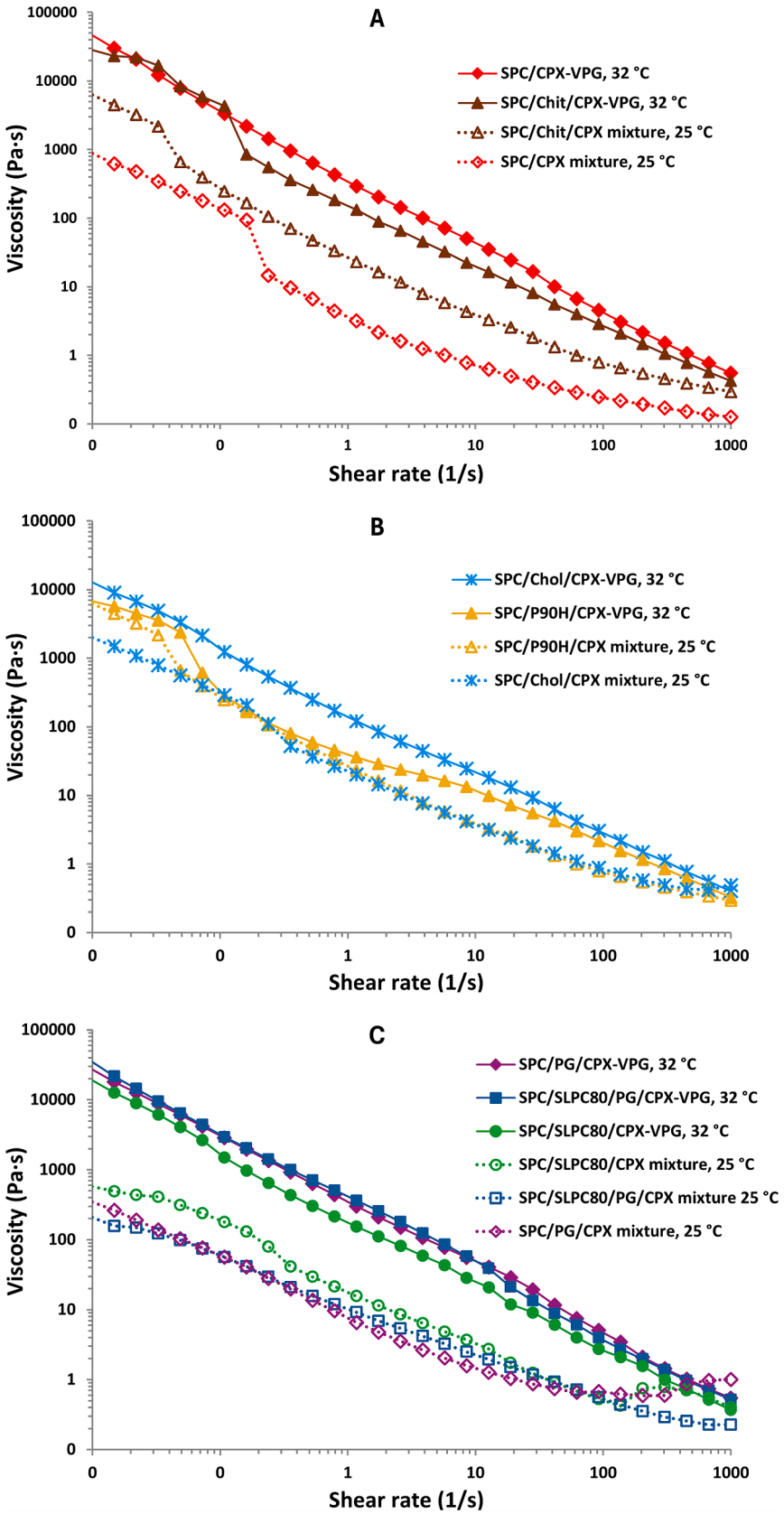

Fig. 2. Viscosity profiles of the different CPX-VPGs before (25 °C) and after (32 °C) high-pressure homogenization. A – SPC/CPX and SPC/Chit/CPX mixtures and VPGs; B – SPC/P90H/CPX and SPC/Chol/CPX mixtures and VPGs; C – SPC/SLPC80/CPX, SPC/PG/CPX and SPC/SLPC80/PG/CPX mixtures and VPGs.

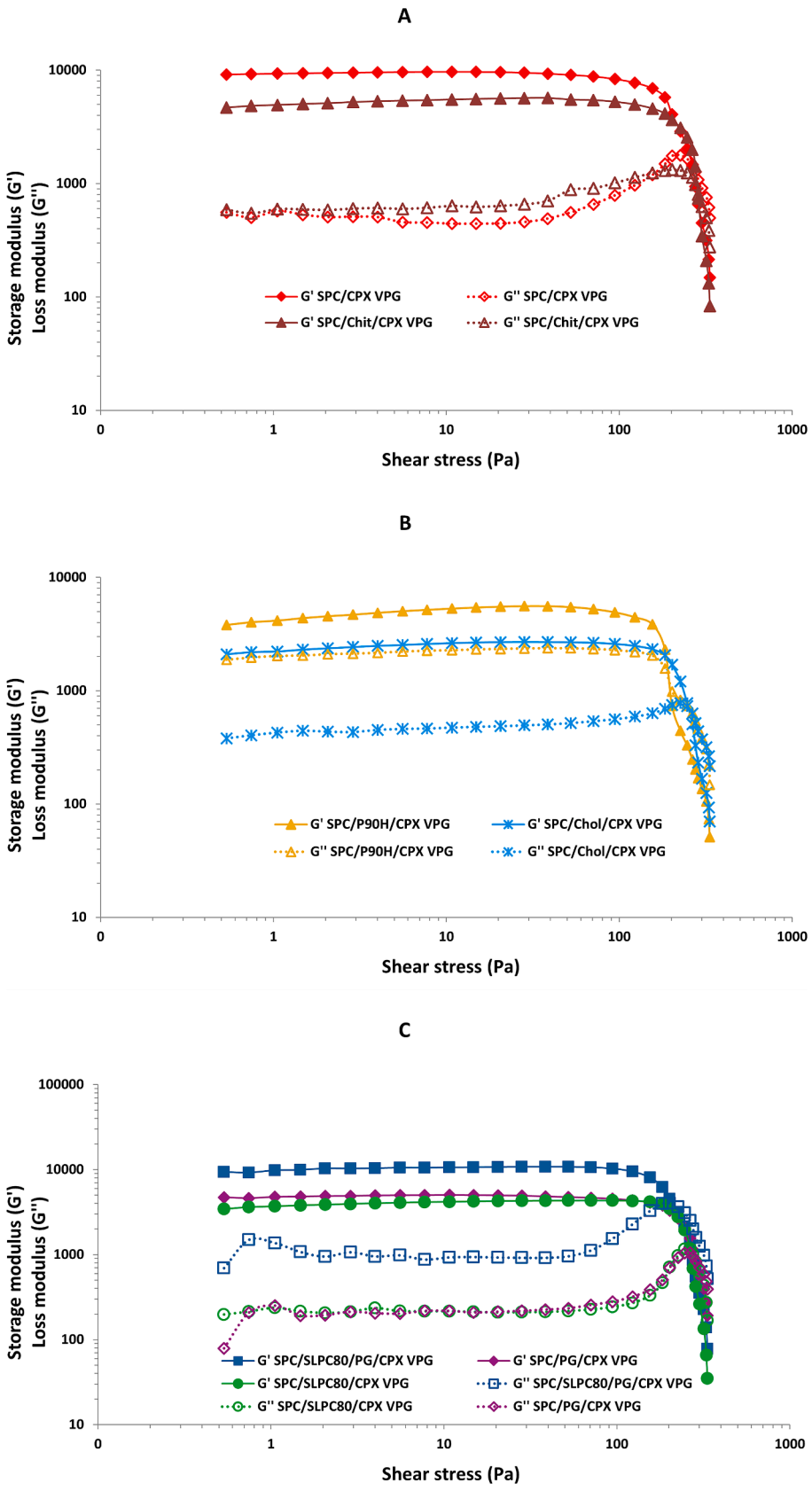

Fig. 3. Amplitude sweep curves of the different CPX-VPGs. A – SPC/CPX VPG and SPC/Chit/CPX VPG; B – SPC/P90H/CPX VPG and SPC/Chol/CPX VPG; C – SPC/SLPC80/CPX VPG, SPC/PG/CPX VPG and SPC/SLPC80/PG/CPX VPG.

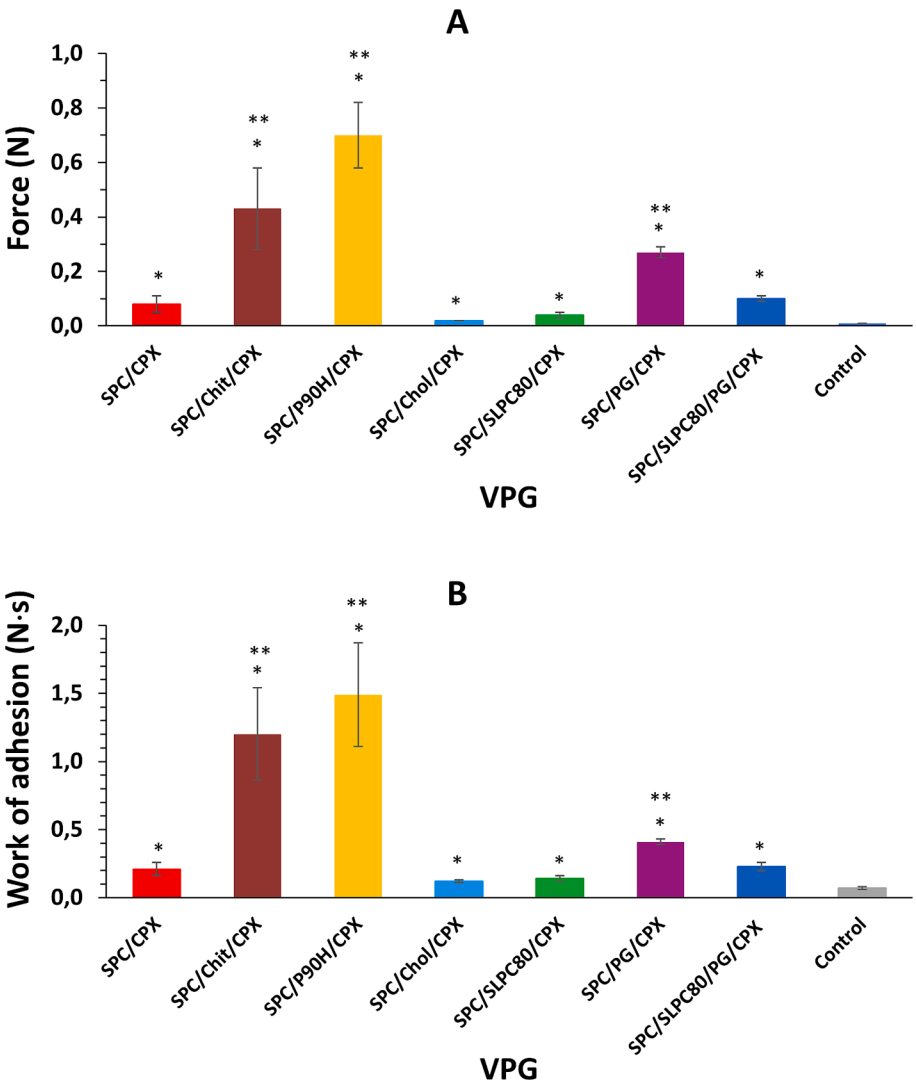

Fig. 4. Bioadhesive properties of the different CPX-VPGs: force detachment (A) and work of adhesion (B). The presented values are mean ± S.D. (n = 5). *Significantly different ($p < 0.0001$) compared to control (CPX solution). **Significantly different ($p < 0.0001$) compared to SPC/CPX VPG.

than 7-fold lower than the least viscous SPC/P90H/CPX VPG (Fig. 4). While chitosan is a well-known mucoadhesive polymer (Wang et al., 2024; Čačić et al., 2023), hydrogenated phospholipids have been reported to increase retention of the dermal formulation on the skin surface and keep the barrier function intact (Lipoid, 2022). Namely, due to high transition temperature, hydrogenated phospholipids are in the gel state when applied to the skin. Moreover, they mimic the lamellar structure of the skin's extracellular matrix, forming a protective layer over the skin (van Hoogevest and Fahr, 2019). We assume that this interaction of hydrogenated phospholipids with the skin contributes to the superior bioadhesion of SPC/P90H/CPX VPG; however, this assumption should be further evaluated.

The predictable release of the drug from the VPGs in media simulating *in vivo* environment is relevant for the possible dermatological use of VPGs as drug vehicles. It would determine the onset of the drug action, its duration and frequency of administration. *In vitro* release of CPX from the CPX-VPGs was carried out by dialysis tubing method in water (Fig. 5A) and buffer, pH 7.4 (Fig. 5B). Distilled water was chosen as the release medium to better simulate the unbuffered conditions that might be found in skin. Also, due to the acidic pH of CPF, the resulting pH of the medium was between 4.70–5.55, which corresponds to the pH of the *stratum corneum* which is 4.1–5.8 (Proksch, 2018).

The results presented by Fig. 5A demonstrate prolonged release of CPX from all the CPX-VPGs in comparison to control (CPX solution), where the total drug has been released within the first hour. Among the different CPX-VPGs, those comprising SLPC80 and characterized by elastic bilayers (Table 2) enabled the fastest CPX release (83 %, 30 h). In contrast, the slowest drug release was obtained from the rigid SPC/P90H/CPX-VPG (61 %, 30 h). A similar trend of the drug release was obtained when the experiments were conducted in buffer, pH 7.4, simulating pH of infected compromised skin, but the amount of the drug released was lower (Fig. 5B) than those obtained using distilled water as a dialysis medium (Fig. 5A). Regardless of the tested media, the differences in the CPX release from the various CPX-VPGs were affected by the bilayer fluidity and the lipid concentration. Thus, even though SPC/PG/CPX-VPG exerted very high degree of bilayer elasticity (Table 2), the release in the first 6 h was significantly lower ($p < 0.001$) than from VPG's containing SLPC80 (similar elasticity) since the lipid concentration was higher in SPC/PG/CPX-VPG (Fig. 5A). These results agree with those reported by Tardi et al. (1998) who demonstrated the effect of the VPG's lipid concentration on the drug release pattern; by increasing lipid concentration, the slower drug release was obtained and *vice versa*. Interestingly, CPX release from SPC/PG/CPX-VPG increased in period 6–24 h, and the level of the drug released after 24 h was like that obtained from VPGs containing SLPC80. Such behavior is probably a result of the slower erosion of the semisolid matrix of SPC/PG/CPX-VPG in

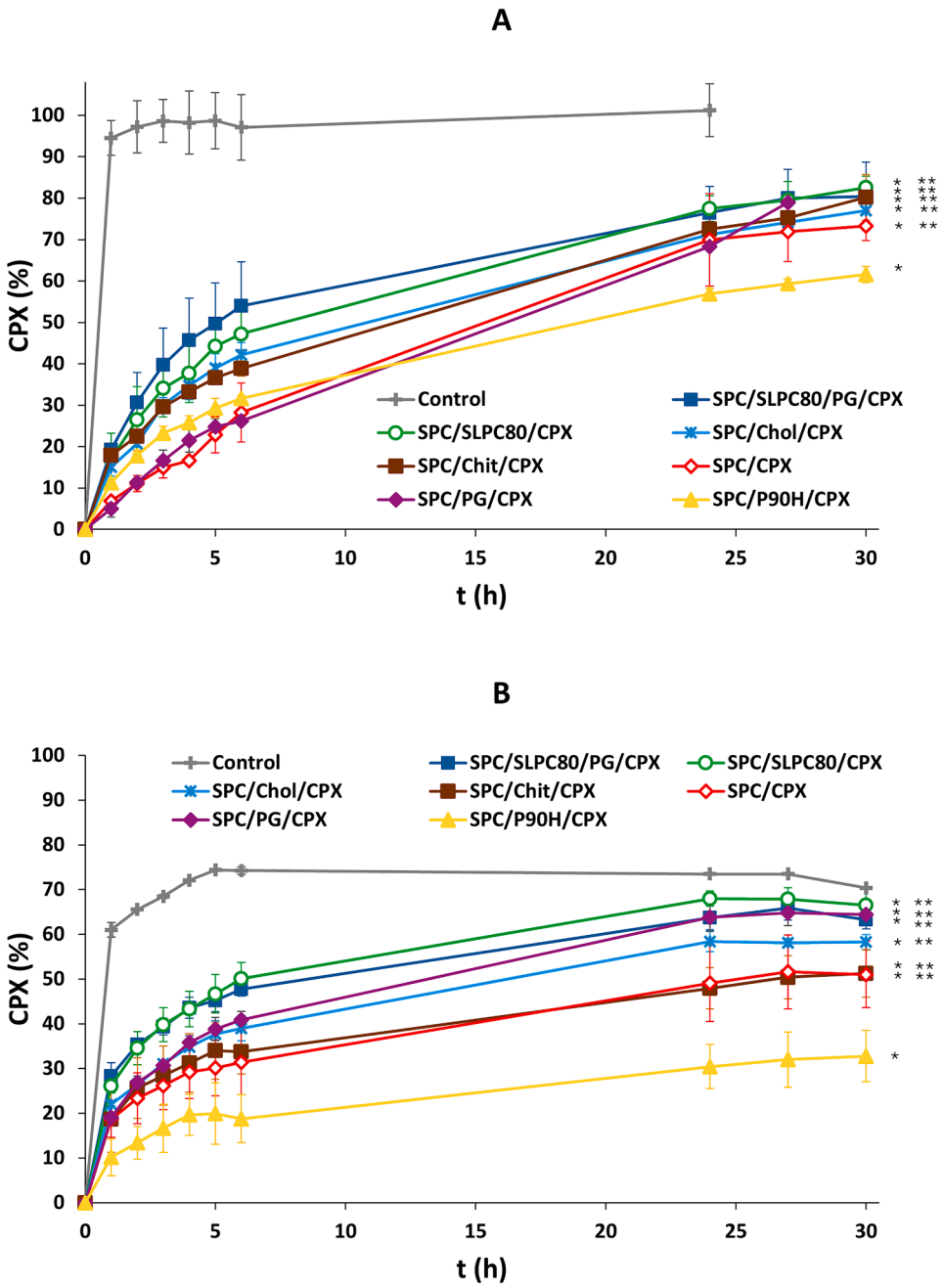

Fig. 5. The cumulative release of CPX from the various CPX-VPGs in distilled water (A) and buffer, pH 7.4 (B). Values denote the mean ± S.D. (n = 3). The values without error bars have standard deviations equal or smaller than the size of the symbols. *Significantly different ($p < 0.0001$) from the control (1–24 h, for A and 1–6 h for B). **Significantly different ($p < 0.05$) from the SPC/P90H/CPX-VPG (after 24 h).

comparison to VPGs comprising lower lipid concentration, including both VPGs with elastic and rigid bilayers. Erosion of the semisolid matrix resulted with release of CPX trapped between the liposomes within VPG, followed by release of the drug entrapped in liposomes. Bilayer fluidity influenced the release of the drug from the liposomes. Thus, the slowest release was obtained from SPC/P90H/CPX-VPG (rigid bilayers) although the total lipid concentration was the lowest in this formulation (Fig. 5A and 5B, Table 2).

Slower drug release observed with all CPX-VPGs in a period of 30 h is considered beneficial for assuring local drug effects, preventing bacterial regrowth and reducing the frequency of administration, what is particularly beneficial when the painful skin area is treated.

3.3. Stability of CPX-VPG liposomes at simulated in vivo environment

The conventional dermal formulations, particularly those for wound treatment, are commonly associated with limitations, like inappropriate drug release and/or its instability at the site of application, leading to insufficient drug concentration at the targeted site of action (Rukavina et al., 2018). To enable a high local content of the drug at the infection site, in addition to a suitable drug release profile, dermal formulation should also protect the drug from an unfavorable biological milieu. Following topical administration, erosion of the VPG's matrix will enable release of the drug trapped between the vesicles, where CPX-liposomes (responsible for slower drug release) will be exposed to injured and infected skin environment. Since the biological milieu could also affect the physical properties of the CPX-liposomes (size, surface

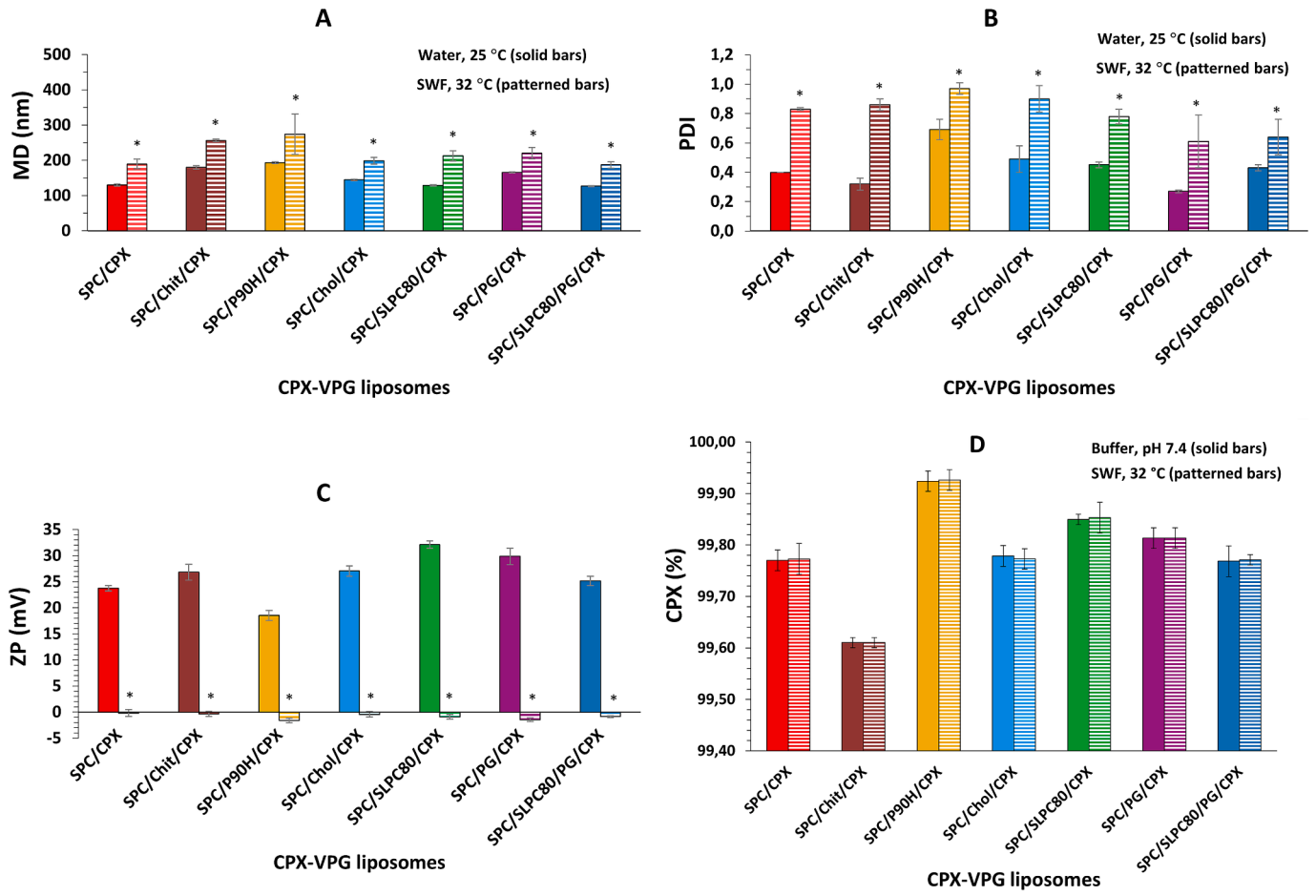

Fig. 6. Characteristics of CPX-VPG liposomes at simulated wound conditions (SWF, 32 °C). A, mean diameters (MD); B, polydispersity indexes (PDI); C, zeta potentials (ZP); D, ratio (%) of CPX retained within liposomes after 24 h of incubation at 32 °C in SWF (pH 7.4) and control (buffer, pH 7.4). CPX-liposomes (converted from the corresponding CPX-VPGs by addition of water) were dispersed in water (solid bars, 25 °C) or SWF (patterned bars, 32 °C) for determination of mean diameters, polydispersity indexes and zeta potentials. Each value represents the mean ± S.D. (n = 3). *Significantly different compared to corresponding CPX-VPG liposomes (water, 25 °C) ($p < 0.0001$).

charge) or destabilize the liposome membrane causing the drug leakage, these issues should be also considered.

To resemble more closely the environment to which CPX-VPGs will be exposed when applied *in vivo*, the size and zeta potentials of the corresponding CPX-liposomes were measured in SWF (at 32 °C). Besides, the effect of neutral pH and albumin on the liposomes' stability was determined by studying the drug leakage.

As illustrated by Fig. 6A and 6B, mean diameters and polydispersity indexes of all CPX-VPG liposomes significantly increased ($p < 0.001$), compared with those obtained after dispersing CPX-liposomes in distilled water. Likewise, the zeta potentials of the CPX-liposomes also significantly changed ($p < 0.001$) and revealed a neutral surface charge (Fig. 6C). These findings are probably a consequence of the electrostatic interaction of liposomes with albumin from the SWF, and formation of an albumin coat over liposomes surface (Taguchi et al., 2021). However, this interaction did not result in drug leakage. Namely, when CPX-VPG liposomes were incubated in SWF for 24 h (Fig. 6D), the content of the liposomally-entrapped CPX remained unchanged and was the same as in control (buffer, pH 7.4).

Interestingly, even though all the liposomes increased in their size (Fig. 6A), most of them remained below 300 nm, what is still considered acceptable for local drug effects (Hemmingsen et al., 2021). The larger size of the liposomes allows better retention of the drug in uppermost skin layers and on the skin surface (El Maghraby et al., 2006).

3.4. Storage stability studies

Physical stability of VPGs is an important feature regarding their potential use as topical formulations. Storage stability of the different CPX-VPGs was verified by rheological assessment (Fig. 7) and by monitoring the changes in size and surface charge of the CPX-liposomes during storage of CPX-VPGs at 4 °C (Fig. 8).

Knowing of rheological characteristics is relevant not only because of its impact on the formulation appearance, skin spreadability and retention at the site of application, but also provides valuable insights into possible physical instability of formulation during its shelf life. Additionally, biopharmaceutical characteristics, such as drug release, permeability, bioadhesion, and spreadability are also influenced by the rheological profile of the formulation, whereby any change in rheological property might influence these features (Simoes et al., 2020; Sivaraman et al., 2017). For these reasons, the physical stability of CPX-VPGs was estimated by evaluating their flow behavior over time using the oscillatory frequency test.

The oscillatory frequency test is a dynamic rheological test where the sample is subjected to a gradual increase in frequency during application of a constant shear strain (Korhonen et al. 2000). The optimal shear strain value for CPX-VPGs was determined using the oscillatory amplitude test (2.2.5) and was found to be 0.01 %.

As displayed in Fig. 7, there was no meaningful change in the flow behavior of the each CPX-VPG after 2 months of storage. All CPX-VPGs demonstrated a dominance of the storage modulus (G') over the loss

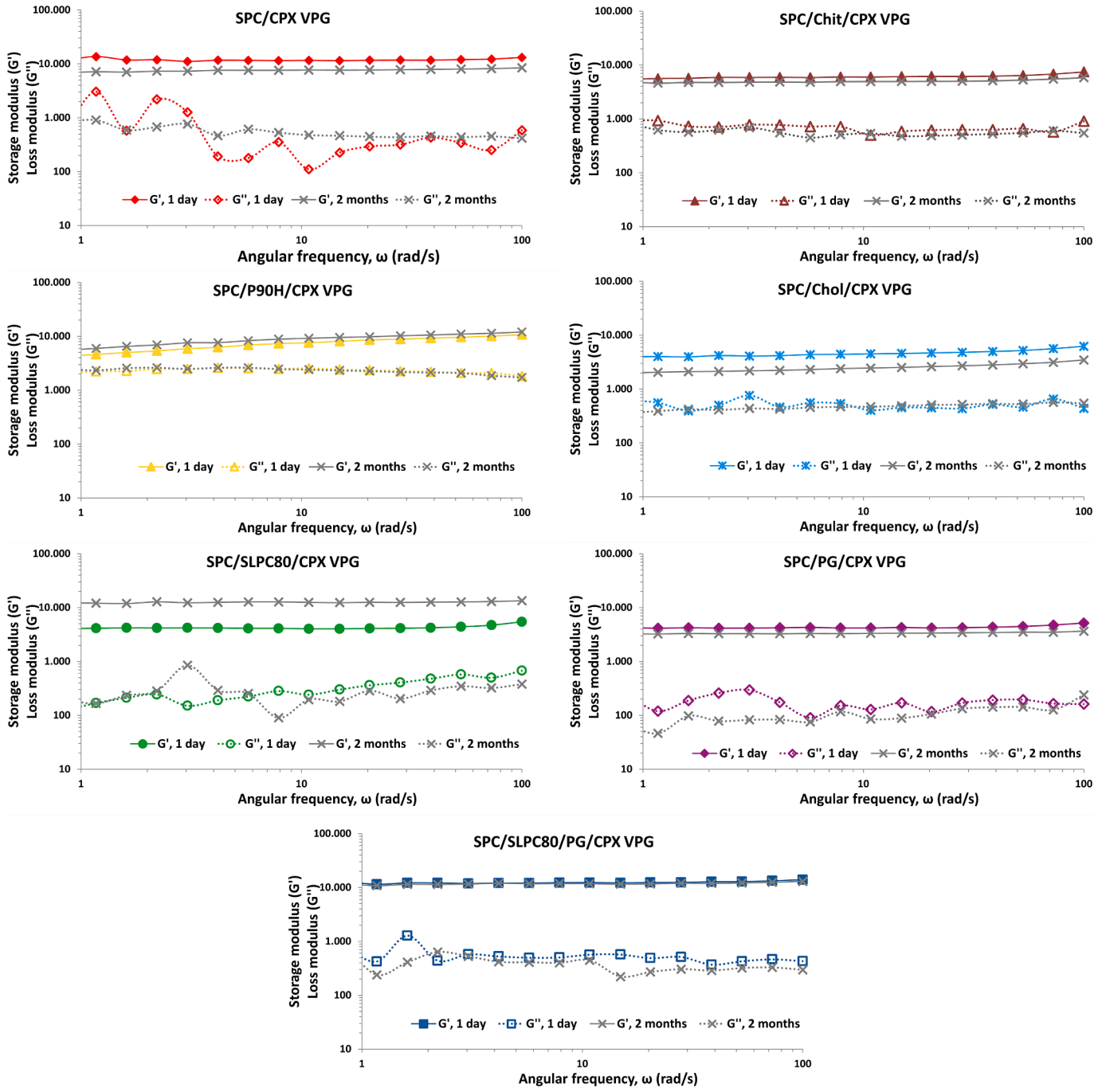

Fig. 7. Amplitude frequency curves of the different CPX-VPGs during 2 months of storage at 4 °C. The values are means of 3 consecutive measurements.

modulus (G''), both immediately after preparation of VPGs and after 2 months of storage at 4 °C. Specifically, when the ratio G' > G'' is observed for a sample, it indicates that elastic properties dominate, meaning that the material will return to its original state after the deforming force is removed. In other words, during deformation, the prevalence of elastic properties also indicates a more stable microstructure, as reversible deformations (G') exceed irreversible ones (G'') (Simões et al. 2020; Adeyeye et al. 2002).

The assessment of the physical stabilities of various CPX-VPG liposomes by monitoring the changes in their mean diameters and polydispersity indexes, showed the impact of the VPG's composition on stability of CPX-liposomes (Fig. 8). Presence of chitosan or propylene glycol within CPX-VPGs preserved the original size of the corresponding liposomes for 3 and 6 months, respectively. All the other CPX-liposomes slightly increased in their size after 3 months. However, even after 6 months of storage their average diameters remained below 200 nm. Among the different CPX-VPG liposomes, these containing hydrogenated phospholipid (P90H) significantly enhanced in their size after 3 months (264 nm). Interestingly, this formulation was the least viscous CPX-VPG (Fig. 2) with initially the largest liposomes (Table 2, Fig. 8). Nevertheless, during the following 3 months they did not further increase in size (232 nm).

Surface charges of all CPX-liposomes (Fig. 8C) remained unchanged during the examined period. Positive zeta potentials of CPX-VPG liposomes can be ascribed to chitosan, in the case of SPC/Chit/CPX VPG, and CPX entrapped in all VPGs. Namely, presence of CPX in the outer water phase of all VPGs affected the zeta potentials of CPX liposomes formed after dispersing CPX-VPGs in water. We assume that such a positive

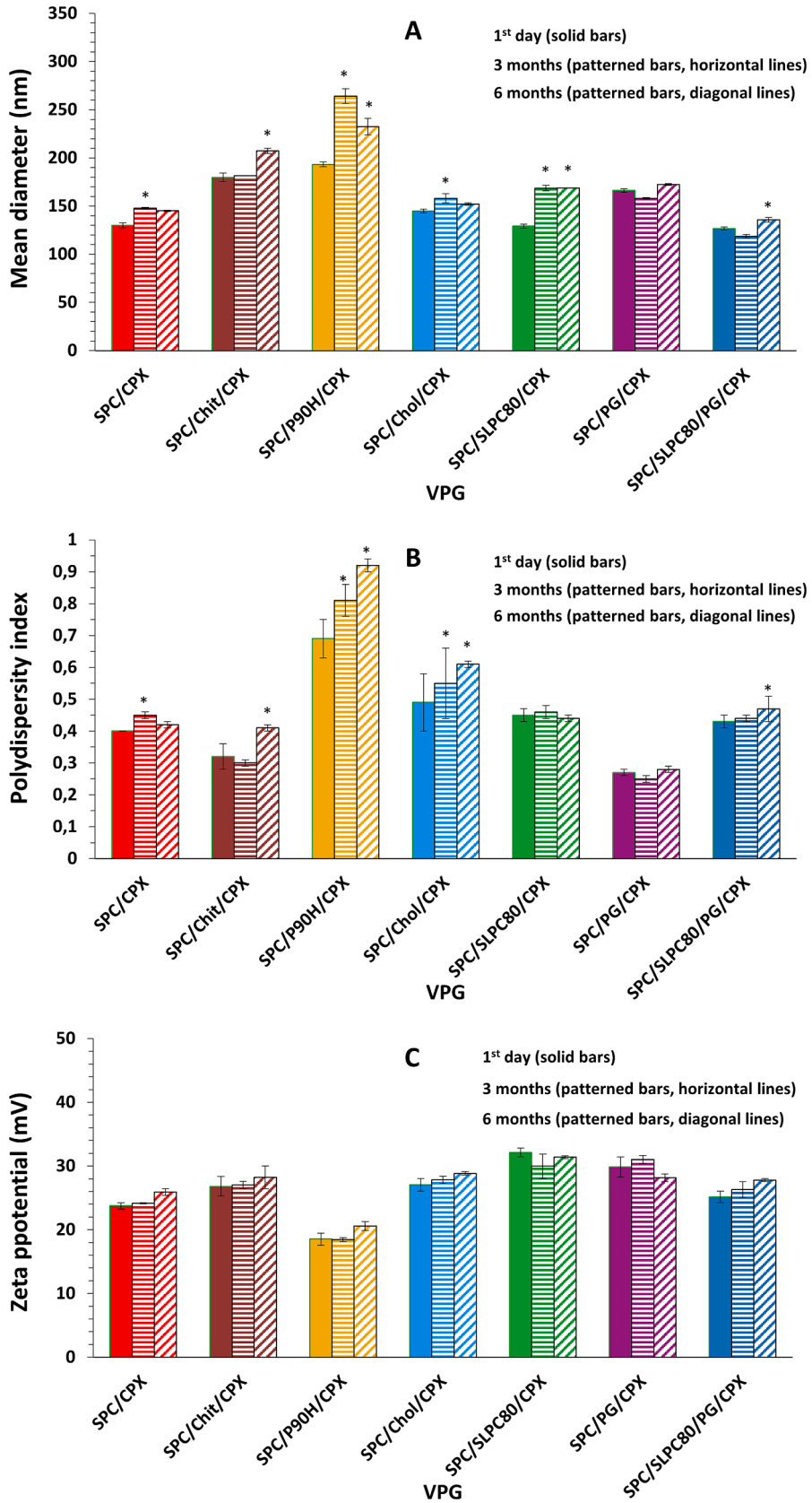

Fig. 8. Physical stability of CPX-VPG liposomes for 6 months storage at 4 °C: mean diameters (A), polydispersity indexes (B) and zeta potentials (C). The results indicate the mean ± S.D. (n = 3). *Significantly different compared to initial values ($p < 0.0001$).

surface charge in the outer water phase of VPG probably cause electrostatic interaction between the neighboring vesicles within VPG, contributing formulation stability (Fig. 7).

3.5. *In vitro* antibacterial activity

Efficient topical treatment of skin infections should allow a complete elimination of pathogenic bacteria and prevent their regrowth, thus reducing the necessity for systemic use of high doses of antibiotics. To examine whether entrapment of CPX in VPG preserve the drug activity, the antibacterial effects of CPX-VPG liposomes were tested against the most common bacteria found in infected wounds: *S. aureus*, *P. aeruginosa* and MRSA. Their efficacy was explored against the planktonic bacterial strains (Fig. 9A) and the corresponding biofilms (Fig. 9B).

As shown by Fig. 9A the entrapment of CPX in VPGs did not hamper the CPX activity. Moreover, the drug activity was even increased against *P. aeruginosa* ATCC 27853, while against *S. aureus* ATCC 6538 and MRSA clinical isolate (MFBF 10679) CPX-VPG liposomes exerted the same antibacterial effects as the free CPX. The results are even more significant if we consider that *P. aeruginosa* ATCC 27853 was the most resistant to CPX than the other two tested bacterial species. Amongst the tested bacterial strains, *S. aureus* ATCC 6538 was the most sensitive to CPX-VPGs (MIC 0.2 μg/mL). CPX-free VPG liposomes were also tested as control to explore the possible antibacterial effect of VPG liposomes alone. However, they did not affect the bacterial growth (Figure S1A).

Insufficiently effective topical antimicrobials can lead to delay in the healing process, resulting in formation of biofilms and development of chronic wounds (Daeschlein, 2013). To explore the antibiofim activities of CPX-VPG liposomes, the tested bacteria were exposed to the liposomally-entrapped or free CPX at concentrations 0.25 – 8 μg/mL during biofilm formation. The results illustrated by Fig. 9B demonstrate the increased efficacy of all CPX-VPG liposomes in preventing *P. aeruginosa* ATCC 27853 and MRSA MFBF 10679 biofilms. The MBIC values were 2-fold lower than those of free CPX. Equivalent to results against planktonic bacteria (Fig. 9A), the efficacy of CPX-VPG liposomes in inhibiting *S. aureus* ATCC 6538 biofilm was the same as with the free

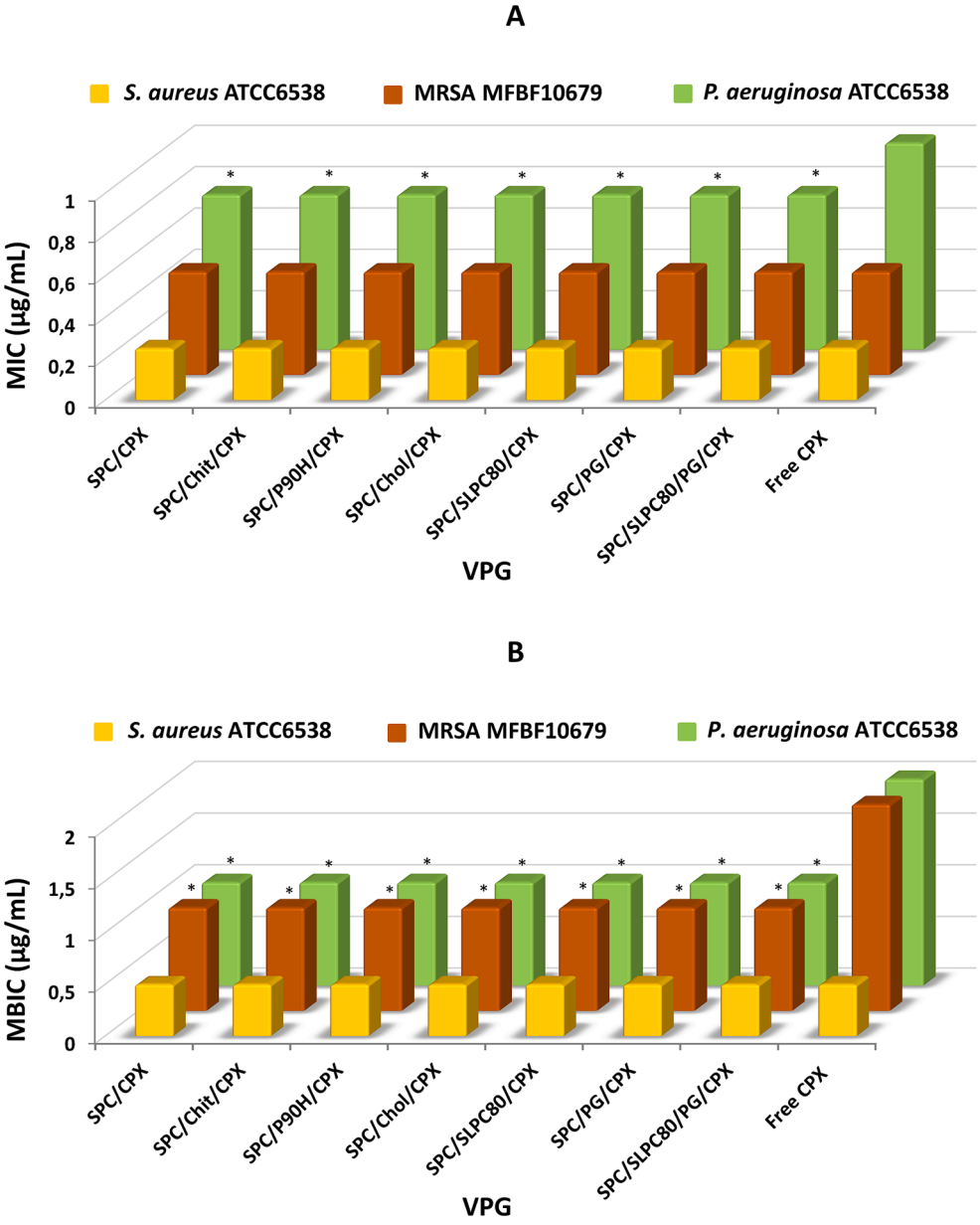

Fig. 9. *In vitro* antibacterial activities of CPX-VPGs against planktonic and biofilm forming *S. aureus* ATCC 6538 (yellow bars), MRSA MFBF 10679 (brown bars) and *P. aeruginosa* ATCC 27853 (green bars): minimum inhibitory concentrations, MIC (A), minimum biofilm inhibitory concentrations, MBIC (B).

drug (Fig. 9B). Interestingly, CPX-free (empty) VPGs liposomes displayed certain effects on preventing biofilm formation (Figure S1B), especially SPC/Chit-VPG and SPC/SLPC80/PG-VPG liposomes. However, they were not able to completely inhibit bacterial growth as did corresponding VPG liposomes containing CPX.

The antibacterial efficiencies of CPX-VPGs liposomes are consistent with the earlier investigations of antibiotic-loaded liposomes, demonstrating increased or same activity relative to that of the free drug (Rukavina et al., 2018; Vanić et al. 2019; Hemmingsen et al., 2023; Ghosh et al., 2019; Alzahrani et al., 2022). Interestingly, the composition of CPX-VPGs appeared to have no effect on antibacterial activities of the corresponding liposomes. On the contrary, previous studies on azithromycin-loaded liposomes (Rukavina et al., 2018; Vanić et al., 2019; Bogdanov et al., 2021) showed a strong impact of the physicochemical properties of liposomes on antibacterial effect of azithromycin, with cationic liposomes and elastic propylene glycol liposomes as the most perspective. We hypothesize that the cationic nature of CPX in this study has a predominant effect on the activity of the CPX-VPG liposomes and requires to be further investigated.

According to the literature review, CPX-liposomes have not been studied for the treatment of skin infections, but other topical formulations have. For instance, Rancan et al. (2019) developed polyvinylpyrrolidone foils and nanofiber mats as delivery vehicles for poorly soluble ciprofloxacin base. Using a wound-infection model on *ex vivo* human skin, both formulations successfully prevented growth of *P. aeruginosa* biofilms, while foils loaded with high amount of the drug (500 μg) enabled complete eradication of the formed biofilm. In another study, chitosan bandages embedding CPX- and fluconazole-loaded nanoparticles were evaluated for the topical treatment of polymicrobial infections. The developed formulations permitted sustained release of ciprofloxacin for up to two weeks and demonstrated antimicrobial activity both *in vitro* and *in vivo* (Thattaruparambil Raveendran et al., 2019).

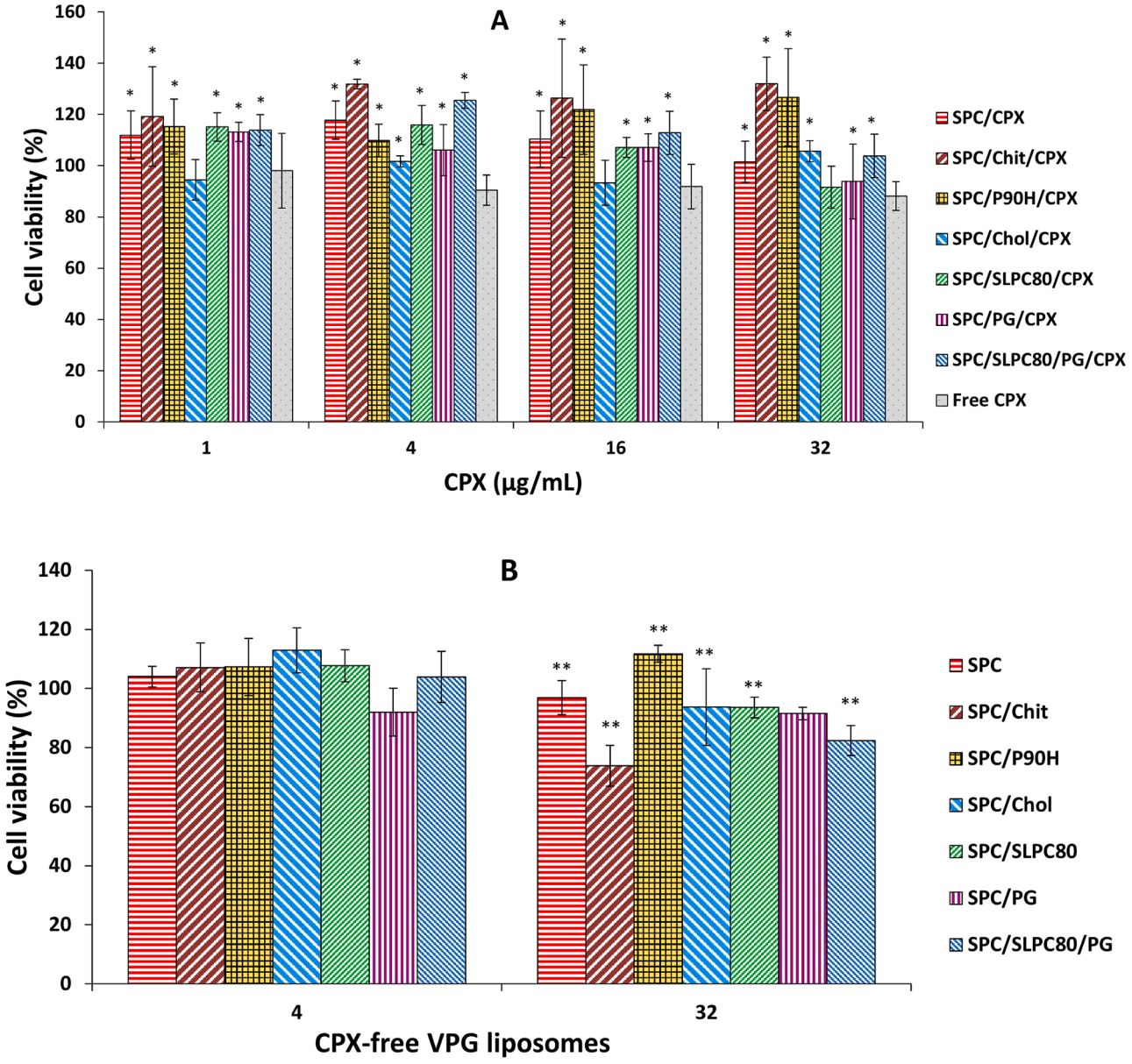

Fig. 10. HaCaT cells viability (%) after 24 h of incubation at 37 °C with the different CPX-VPG liposomes or free CPX (A) or the corresponding CPX-free VPG liposomes (B). The results are expressed as mean ± S.D. (n = 4). *Significantly different ($p < 0.0001$) compared to free CPX (control) at the tested concentration. **Significantly different ($p < 0.0001$) compared to the respective CPX-free VPG liposomes at the lowest concentration.

3.6. Biocompatibility of CPX-VPG liposomes with human keratinocytes

Biocompatibility is an important requirement that should be satisfied when developing drug formulation. Apart from the drug molecules, each formulation ingredient can exert toxic effects when applied to living cells, especially to injured and inflamed skin. To examine the possible cytotoxic effects of the CPX-VPGs on the skin cells, human keratinocytes cell line (HaCaT) was exposed to the CPX-VPG liposomes at the concentrations 1–32 μg/mL CPX. As controls, free CPX and the drug-free VPG liposomes were tested at concentrations corresponding to the CPX-VPG liposomes.

As shown in Fig. 10A, all CPX-liposomes were completely biocompatible with the keratinocytes at the tested concentrations. Moreover, a proliferative effect of all CPX-VPG liposomes was observed, which was even increased at the higher tested concentrations (16 and 32 μg/mL CPX). Comparison of the different CPX-liposomes demonstrates the strongest proliferative effects of the liposomes comprising chitosan or hydrogenated phospholipid (P90H), the ingredients known for their beneficial effects on the skin and in wound healing process (van Hoogevest and Fahr, 2019; Feng et al., 2021). Although cholesterol is known as biocompatible ingredient, the formulation with cholesterol was less tolerable with the keratinocytes at the lowest tested concentration, while *vice versa* effect was observed at the highest CPX concentration. These findings are likely due to the lower lipid content of SPC/Chol/CPX VPG, with the effect of the free drug dominating at the lowest concentration tested. CPX-liposomes containing larger amount of propylene glycol (SPC/PG/CPX), at the highest concentration tested, exhibited moderate tolerability with the HaCaT cells, like SPC/SLPC80/PG/CPX liposomes. Among the examined CPX-liposomes with fluid membranes (elastic, deformable liposomes), those containing monoacylphosphatidaylcholine (SLPC80) and smaller proportion of propylene glycol (SPC/SLPC80/PG/CPX) enhanced the cell growth.

Neither one of the drug-free (empty) liposomes exhibited toxic effects towards HaCaT cells, although the viability marginally decreased for all liposomes at the highest tested concentration of liposomes, except SPC/P90H liposomes and SPC/Chit liposomes (Fig. 10B). SPC/P90H-liposomes significantly improved cell proliferation ($p < 0.001$), while the viability of the cells treated with SPC/Chit liposomes decreased to 73 %. Although it is a significant decrease, the cell viability above 70 % is still considered non-toxic (ISO, 2009). This result is a consequence of the neutral pH of the empty liposomal dispersion, where chitosan is not in active (ionizable) form. Namely, all CPX-VPG liposomes exhibited slightly acidic pH, due to the acidic nature of the drug.

Proliferative effect of liposomes is highly appreciated for the treatment of skin disorders with damaged skin barrier and especially in wound healing. Moreover, hydrophilic nature of liposomes also contributes to an increase in the skin hydration effect, which is also valuable in most dermatological treatments.

Based on the results achieved in this study the following VPGs were selected for further *in vitro* scratch assay: SPC/CPX, SPC/Chit/CPX, SPC/P90H/CPX, SPC/Chol/CPX and SPC/SLPC80/PG/CPX VPG.

3.7. In vitro wound healing effect

To examine the effect of CPX-VPG liposomes on reepithelization of the injured skin, *in vitro* cell migration test (scratch test) was performed. Scratch test is an easy and fast *in vitro* method for verifying migration of cells during healing process. It is based on the creation of an artificial gap (scratch) on a confluent cell monolayer and monitoring the gap closure due to cells migration from the edge of the gap to the opening of the scratch until new contacts between cells are reached (Liang et al., 2007).

The healing effect of the selected CPX-VPG liposomes was assessed on the human keratinocytes monolayers (HaCaT). Among the tested CPX-VPG liposomes, those containing chitosan or hydrogenated phospholipid (P90H) showed complete gap closure after 24 h (Fig. 11), consistent with a cell proliferation effect observed (Fig. 10A). Chitosan is well known for its beneficial effects in wound healing process (Feng et al., 2021), while hydrogenated phosphatidylcholine is reported to have advantageous effects in skin reepithelization (van Hoogevest and Fahr, 2019, Lautenschläger, 2006).

Wound healing rate was negligibly reduced for SPC/CPX liposomes, while it was 90 % after scratch treatment with CPX/Chol/CPX-VPG liposomes. SPC/SLPC80/PG/CPX-VPG liposomes failed to show healing effect on the injured keratinocytes, compared to free CPX and non-treated cells (control). Such a result is a consequence of monoacyl phospholipid exerting toxic effects on the cell bilayers (Tan et al., 2020). Interestingly, in the *in vitro* biocompatibility study (Fig. 10A) this nanoformulation did not display any toxic effect, probably due to the dominant effect of biocompatible SPC as the main ingredient, and the

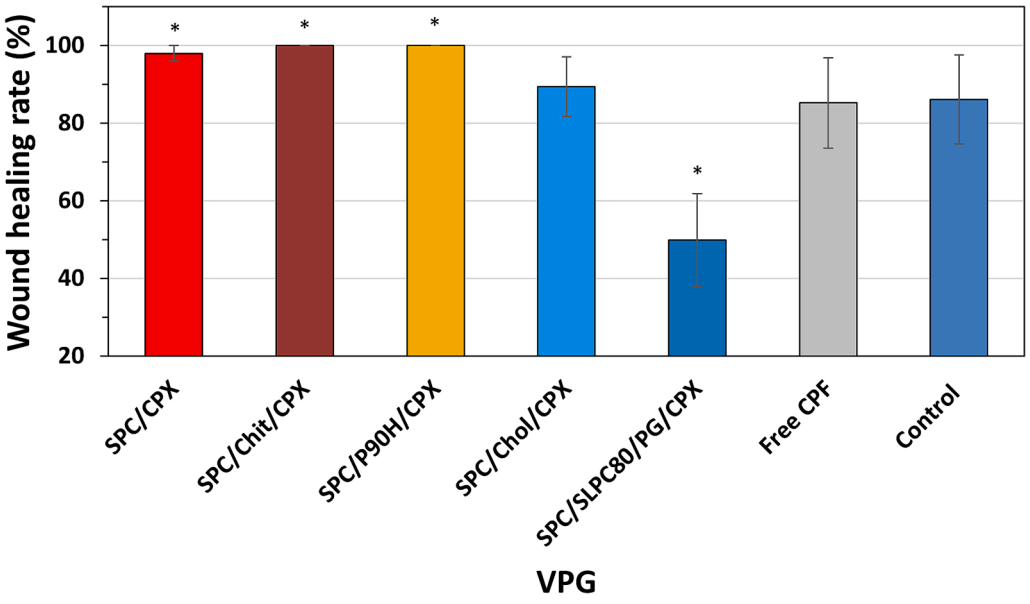

Fig. 11. Influence of CPX-VPG liposomes and free CPX on the *in vitro* wound healing rate (24 h after treatment). The wound healing rate presents the percentage of scratch closure relative to the initial scratch area. The concentration of CPX in all the samples was 16 μg/mL. Non-treated cells served as control. The results denote the mean ± S.D. (n = 3).

fact that the nanoformulation was applied on the confluent cell monolayers. Given that the scratch test is particularly suitable for testing cell-matrix interactions, it provided a more precise evaluation of individual components of the formulations, which were not clearly visible in the *in vitro* biocompatibility assessment (3.6.).

3.8. Ex vivo skin permeation studies

As a step forward towards the localized dermatotherapy, CPX-VPGs exerting superior *in vitro* healing effects were subjected to *ex vivo* skin permeation testing. They differed in bilayer rigidity, viscosity and bioadhesiveness: SPC/CPX VPG (viscous, basic VPG with moderate bilayer fluidity and bioadhesiveness), SPC/Chit/CPX VPG (viscous VPG, exhibiting moderate bilayer rigidity and increased bioadhesiveness), and SPC/P90H/CPX VPG (the least viscous VPG with extremely rigid bilayers and superior bioadhesiveness). The impact of these formulation features was examined on the drug penetration into/through the skin.

The results presented in Fig. 12A unequivocally demonstrate the ability of all the examined VPGs to reduce the penetration of CPX through the skin, compared with free CPX, thus allowing skin localization of the drug. Among the tested VPGs, the lowest penetration of CPX was achieved by CPX-VPGs incorporating hydrogenated phospholipid or chitosan, both characterized by rigid bilayers, while the highest amount of the penetrated CPX (after 24 h) was obtained with SPC/CPX/VPG exerting moderately fluid bilayers ($p < 0.0001$) (Fig. 12A). These results are expected and corroborate with the previous findings obtained with the rigid and moderately fluid liposomes containing azithromycin (Rukavina et al., 2018; Vanić et al., 2019).

Overall, the examined CPX-VPGs allowed accumulation of CPX on the skin surface and its deposition within the skin (Fig. 12B). The fluidity of the bilayers as well as the viscosity and bioadhesiveness of the respective VPGs, influenced the site of skin localization of CPX. Thus, the application of the least viscous SPC/P90H/CPX VPG characterized by superior bioadhesiveness, resulted in the highest level of the drug accumulated inside the skin, while the least amount of the drug deposited on the skin, compared to the other two formulations. The reverse effect was observed with the viscous and slightly less bioadhesive SPC/Chit/CPX VPG, characterized by moderately rigid bilayers, where a larger amount of the drug remained on the skin surface, than accumulated inside the skin. On the other hand, viscous SPC/CPX VPG, with moderately fluid bilayers, displayed less CPX accumulation inside the skin than SPC/Chit VPG, but higher deposition of CPX on the skin surface than SPC/P90H/CPX VPG (Fig. 12B).

Although viscosity affected skin localization of CPX, it did not affect penetration of the drug through the skin. Instead, fluidity of the bilayers determined the penetration potential of CPX-VPGs. Accordingly, almost the same amount of the penetrated drug was detected in the receptor compartment with both, the highly viscous SPC/Chit/CPX VPG and the least viscous SPC/P90H/CPX VPG, each characterized by rigid bilayers (Fig. 12A).

The surface charge of the liposomes within VPG may also contribute to the drug penetration. While anionic liposomes have shown to increase

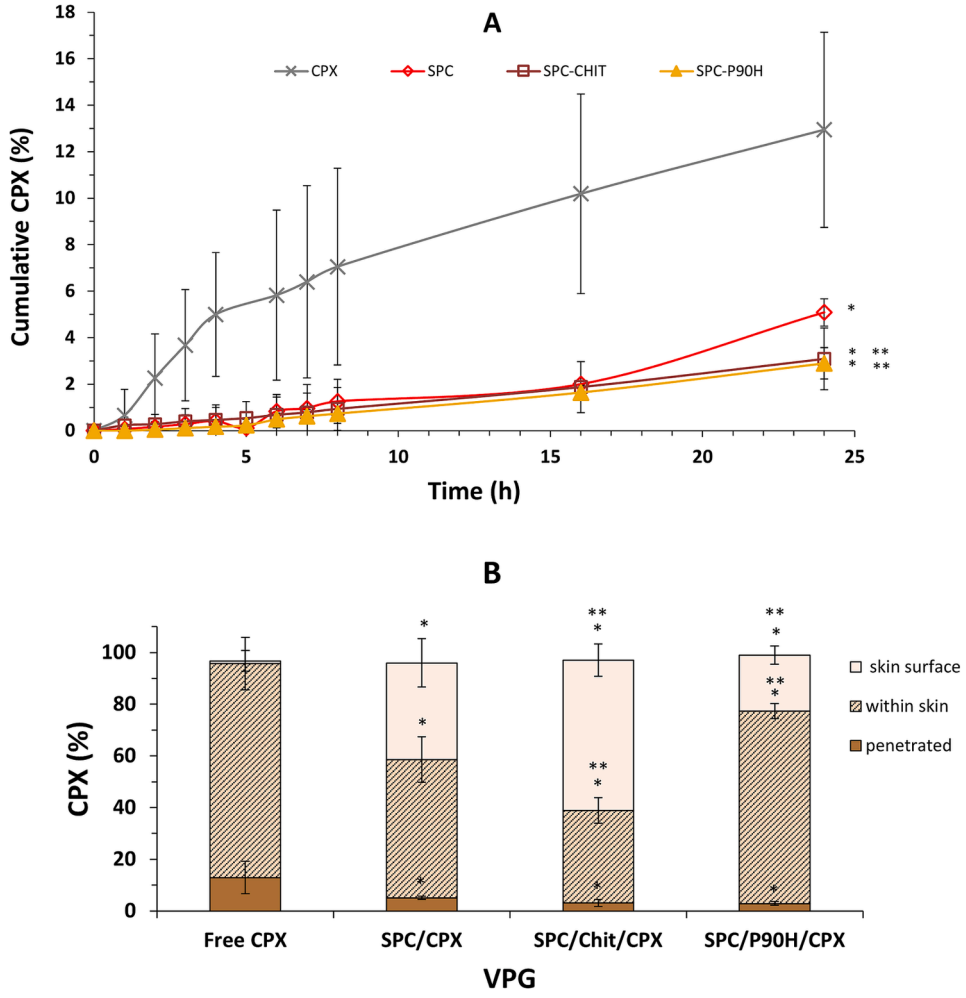

Fig. 12. Cumulative amount of penetrated CPX through the full-thickness skin (A). *Ex vivo* accumulation/deposition/penetration of CPX on/into/through the skin. The results are expressed as mean ± S.D. (n = 3). *Significantly different compared to free CPX (control). **Significantly different compared to SPC/CPX VPG.

permeation of the drug into/through the skin, opposite effects were achieved with cationic and neutral surface-charged liposomes (Ibaraki et al., 2019; Ternullo et al., 2019). All CPX-VPG liposomes in this study exerted positive surface charge because of the cationic nature of the drug overlapping the original neutral surface charge of the liposomes (Table 2). We hypothesize that this effect could also contribute to the localization effect of the drug within the skin.

Based on the results achieved in this research, SPC/Chit/CPX VPG and SPC/P90H/CPX VPG appear as the most perspective drug nanoformulations for localization of the drug in the skin and reducing its undesired penetration to the systemic circulation. An increased residence time of formulation on the skin together with a slow release of CPX for a longer period in concentrations that significantly exceed MIC and MBIC values against pathogenic bacteria at infection site, would result in superior antimicrobial treatment and prevention of chronic infections. This would allow reducing the dosing frequency, what is particularly important when treating painful areas of injured skin. Both formulations were entirely biocompatible, revealed strong proliferative effects on keratinocytes and provided superior *in vitro* wound healing rate. All these outcomes will subsequently lead to improved topical dermatotherapy. Since skin infections comprise various lesions, affecting different skin layers (from uppermost epidermal layers to subcutaneous tissue) (Augustin et al., 2017), we assume that SPC/Chit/CPX VPG would be appropriate for the treatment of superficial infections with compromised, injured skin, while SPC/P90H/CPX VPG for the treatment of the deeper skin infections.

4. Conclusions

This study is the first to examine the potentials of VPGs for topical dermatotherapy. VPGs of appropriate physicochemical properties, allowing total drug loading of hydrophilic drug were prepared without the use of organic solvents by a robust and reproducible method, also suitable for industrial production. By adjusting VPG's composition, using hydrogenated phospholipid and chitosan, completely biocompatible VPGs of adequate rheological characteristics, bioadhesiveness and bilayer features can be prepared, able to extend the drug release and control its penetration and localization within the skin. Entrapment of antimicrobial drug in VPGs did not hamper its activity, it even increased *in vitro* activity against biofilm forming *P. aeruginosa* and clinical MRSA isolate. Such superior effects combined with enhanced *in vitro* wound healing effect, would result in improved topical antimicrobial dermatotherapy, which would be worthy to explore on an *in vivo* animal model.

CRediT authorship contribution statement

Sabina Keser: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Data curation, Conceptualization. **Gordana Maravić-Vlahoviček:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Jasmina Lovrić:** Writing – review & editing, Resources, Methodology, Data curation. **Željka Vanić:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpharm.2024.124931.

Data availability

Data will be made available on request.

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Article

A Novel Approach for the Treatment of Aerobic Vaginitis: Azithromycin Liposomes-in-Chitosan Hydrogel

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Abstract: Biocompatible mucoadhesive formulations that enable a sustained drug delivery at the site of action, while exhibiting inherent antimicrobial activity, are of great importance for improved local therapy of vaginal infections. The aim of this research was to prepare and evaluate the potential of the several types of azithromycin (AZM)-liposomes (180–250 nm) incorporated into chitosan hydrogel (AZM-liposomal hydrogels) for the treatment of aerobic vaginitis. AZM-liposomal hydrogels were characterized for in vitro release, and rheological, texture, and mucoadhesive properties under conditions simulating the vaginal site of application. The role of chitosan as a hydrogel-forming polymer with intrinsic antimicrobial properties was explored against several bacterial strains typical for aerobic vaginitis as well as its potential effect on the anti-staphylococcal activity of AZM-liposomes. Chitosan hydrogel prolonged the release of the liposomal drug and exhibited inherent antimicrobial activity. Additionally, it boosted the antibacterial effect of all tested AZM-liposomes. All AZM-liposomal hydrogels were biocompatible with the HeLa cells and demonstrated mechanical properties suitable for vaginal application, thus confirming their potential for enhanced local therapy of aerobic vaginitis.

Keywords: azithromycin; liposomes; chitosan; hydrogel; aerobic vaginitis; vaginal drug delivery; antimicrobial activity; vaginal infections

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1. Introduction

Aerobic vaginitis is a form of vaginitis accompanied by the growth of abnormal vaginal microflora comprising aerobic, enteric commensals, or pathogens (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis*), in comparison to bacterial vaginosis, where mainly anaerobic microbes (*Gardnerella vaginalis*, *Prevotella species*, *Mycoplasma hominis*, and *Mobiluncus species*) are present [1,2]. Both types of vaginitis comprise a decrease in the dominance of lactobacilli and an increase in vaginal pH. However, aerobic vaginitis is accompanied by more extreme inflammatory changes and deficient epithelial maturation than bacterial vaginosis [2,3]. If untreated, aerobic vaginitis may lead to gynecological and obstetrical complications, including premature rupture of membranes, preterm delivery, fetal infections, infertility, abnormal Pap test results, and increased risk of sexually transmitted bacterial and viral infections [4,5].

Aerobic vaginitis is commonly treated with local and oral antibiotics, estrogens, non-steroidal anti-inflammatory drugs, and probiotics [2,6]. Local therapy is preferred as it enables higher drug concentration at the action site, while escaping systemic adverse effects and reducing the risk for bacterial resistance. Nevertheless, conventional formulations for vaginal administration are not always effective because of their leakage, messiness, inappropriate drug release, or short residence time inside the vagina. These limitations can be overcome by the use of nanoparticle-based mucoadhesive formulations [7–9]. Thus, liposomes encapsulating antimicrobials and incorporated into poly(acrylate) hydrogels have been shown to prolong retention of the formulation on vaginal mucosa, enabling sustained release of the liposomally-encapsulated drug [10]. Moreover, liposomes have been shown to improve delivery of the antibiotics to bacteria and biofilms as reported by decreased minimal inhibitory concentrations (MICs) and minimal biofilm inhibitory concentrations [11–13].

Hydrogels represent one of the most common vaginal dosage forms exerting a moisturizing effect, appropriate viscosity, increased residence time, and good distribution over the mucosal surface. They are well accepted by the patients and easy to produce [14,15]. Among the numerous hydrogel-forming polymers [16], chitosan is particularly interesting because of its mucoadhesiveness, biodegradability, and antimicrobial activity against various bacteria and fungi [17], which is of great importance considering the increased global trend of microbial resistance. The potentials of chitosan in enhanced vaginal drug delivery have been investigated in the form of various drug delivery nanosystems [17,18].

We previously demonstrated the suitability of liposomes with encapsulated azithromycin (AZM) for the treatment of bacterial cervicovaginal *Chlamydia trachomatis* and *E. coli* infections. All the tested liposomes were more effective against both planktonic and biofilm-forming *E. coli* than the free AZM [19]. Continuing this research line, we report here, for the first time, the potential of chitosan hydrogel (chitosan-HG) as a vehicle for AZM-liposomes intended for the local treatment of aerobic vaginitis. Specifically, incorporating AZM-liposomes into the chitosan-HG should increase their retention within the vaginal cavity, prolong drug release, and potentially improve the antimicrobial effect.

Chitosan-HG was assessed in vitro for its intrinsic antimicrobial potential against three bacterial strains (*S. aureus*, *E. coli*, *P. aeruginosa*), characteristic for aerobic vaginitis, and against yeast *Candida albicans*, whose overgrowth is associated with vulvovaginal vaginitis. Moreover, to determine whether the chitosan-HG could potentiate the antimicrobial activity of the several types of AZM-liposomes, in vitro antibacterial studies against *S. aureus* ATCC 6538 were performed, and the results were compared with the antibacterial activities of the different types of AZM-liposomes, chitosan-HG incorporating free AZM (control-HG), and the originally prepared chitosan-HG. The AZM-liposomes-in-chitosan-HG formulations (AZM-liposomal hydrogels) were further evaluated for in vitro drug release in conditions simulating the vaginal environment. In addition, the rheological, texture, and mucoadhesive properties of the AZM-liposomal hydrogels were determined to consider the applicability of the tested formulations for vaginal administration. Finally, the biocompatibility of the developed formulations was examined in vitro on the HeLa cells.

2. Materials and Methods

2.1. Materials

AZM in the form of dihydrate was a donation from PLIVA Croatia Ltd. (Zagreb, Croatia). Egg phosphatidylcholine (EPC), egg phosphatidylglycerol sodium salt (EPG), hydrogenated soybean phosphatidylcholine (SPC-3), and monoacyl phosphatidylcholine from soybean (SLPC-80) were kindly gifted by Lipoid GmbH (Ludwigshafen, Germany). Medium molecular weight (MMW) chitosan with 82.0% deacetylation degree and viscosity of 420 CPS (c = 1%, 1% acetic acid) as well as Sephadex G-50, urea, glucose, lactic acid, propylene glycol, Dulbecco's modified Eagle medium, fetal bovine serum, L-glutamine, and bovine serum albumin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile, ethanol, and methanol were of HPLC grade and procured from BDH Prolabo

(Lutterworth, UK). Tryptic Soy Agar, Sabouraud Dextrose Agar and Tryptic Soy Broth were purchased from BioMérieux (Marcy-l'Étoile, France). Dey/Engley (D/E) Neutralizing Broth and Difco Antibiotic Medium 11 were obtained from Becton Dickinson (East Rutherford, NJ, USA).

Phosphate buffer, pH 8.0 (used in microbiological studies), was prepared by dissolving 16.73 g of potassium hydrogen phosphate and 0.523 g of potassium dihydrogen phosphate in demineralized water up to 1000 mL.

Vaginal fluid simulant (VFS, pH 4.5) and phosphate buffer (pH 7.5) were prepared as previously reported [19].

2.2. Preparation of Chitosan-HG

Chitosan (4 g) was weighted in a beaker and dispersed in 44 g of 3.5% (*w*/*w*) lactic acid. Subsequently, propylene glycol (10 g) was added, and the mixture was manually stirred, followed by the addition of the appropriate amount of demineralized water to attain the final concentration of the chitosan 4% (*w*/*w*). The blend was continued to stir, and bath sonicated until a homogenous mixture was formed (approx. 45 min). The hydrogel was covered with parafilm and allowed to swell for 24 h at room temperature.

2.3. Preparation and Characterization of AZM-Liposomes

Three preparations of AZM-liposomes differing in composition (Table 1) and bilayer properties, namely, conventional liposomes (CLs) characterized by rigid bilayers and two preparations of elastic liposomes, i.e., propylene glycol liposomes (PGLs) and deformable propylene glycol liposomes (DPGLs), were prepared according to procedures described in detail by Vanić et al. [19]. All the liposomes were manually extruded (3 cycles) through 400 nm pore-sized polycarbonate membranes using LiposoFast Basic extruder (Avestin, Ottawa, ON, Canada), and stored at 4 °C prior to their use.

Table 1. Composition of AZM-liposomes.

AZM-Liposomes	EPC (mg)	EPG (mg)	SLPC-80 (mg)	SPC-3 (mg)	PG (g)	AZM (mg)	Buffer, pH 7.5 (g)
CLs	140	10	-	50	-	30	10
PGLs	190	10	-	-	1	30	9
DPGLs	160	10	30	-	1	30	9

AZM, azithromycin; CLs, conventional liposomes with azithromycin; DPGLs, deformable propylene glycol liposomes with azithromycin; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol sodium salt; PG, propylene glycol; PGLs, propylene glycol liposomes with azithromycin; SLPC-80, soybean monoacyl phosphatidylcholine; SPC-3, hydrogenated soybean phosphatidylcholine.

Mean diameters, polydispersity indexes, and zeta potentials of the AZM-liposomes were analyzed by dynamic light scattering at 25 °C on a previously calibrated Zetasizer 3000HS (Malvern Instruments, Malvern, UK). The measurements were performed using a capillary cell with an optical modulator at 1000 Hz (zeta potential) and scattering angle of 90° (size analysis). Samples of the liposomes were adequately diluted with buffer (pH 7.5) to ensure a suitable count rate.

To determine the elasticity of the liposomal bilayers, continuous extrusion of the liposomal dispersions through 100 nm pore-sized membranes was performed at constant pressure (2.5 bar) for 5 min, and the degree of the bilayer elasticity was calculated according to the previously reported formula [19].

Quantification of the liposomally-encapsulated AZM was performed by HPLC at 210 nm (C18 column; acetonitrile/phosphate buffer, pH 7.5 in ratio 70:30 *v*/*v* as mobile phase) [13] after separation of the nonencapsulated drug by the minicolumn centrifugation method [19]. Encapsulation of the drug in liposomes was expressed as μg of encapsulated AZM per mg of the phospholipids used.

2.4. Preparation of AZM-Liposomal Hydrogels

Each type of AZM-liposomes (CLs, PGLs, or DPGLs) was incorporated into the chitosan-HG by manual stirring for approximately 5 min (room temperature) until formation of a homogenous AZM-liposomal hydrogel (CLs-HG, PGLs-HG, or DPGLs-HG). The concentration of the AZM-liposomes inside the hydrogel was 30% *w*/*w* (AZM-liposomes/AZM-liposomal hydrogel).

Control-HG was prepared under the same conditions and contained a solution of the free AZM in the same concentration as in the liposomes. AZM-solution was prepared by dissolving the drug in a mixture of ethanol and water (6/4, *v*/*v*) and subsequent dilution with buffer, pH 7.5 to achieve appropriate concentration of the AZM-solution inside the hydrogel (30%, *w*/*w*). Control-HG was examined in all the studies where AZM-liposomal hydrogels were explored.

2.5. Measurements of the Hydrogels' pH Values

The pH values of all the prepared hydrogels were determined at 25 °C by a pH meter equipped with an electrode for semisolid formulations (Mettler-Toledo, Greifensee, Switzerland). The pH values were determined by direct immersion of the pH electrode for semisolid formulations inside the hydrogels. Prior to the measurement, the pH meter was calibrated at 25 °C using standard buffer solutions (pH 4.0, pH 7.0, and pH 9.0). Three measurements were performed for each sample of the hydrogel.

2.6. In Vitro Release Studies

A sample of the AZM-liposomal hydrogel (CLs-HG, PGLs-HG or DPGLs-HG) or control-HG, each corresponding to 0.5 mg AZM, was placed into a dialysis bag (Medicell Membranes, Mw cut-off 3500 Da) and dialyzed against 20 mL of VFS during constant stirring (150 rpm) at 37 °C. At certain time intervals (1, 2, 3, 4, 5, 6, and 24 h), 2 mL of the dialysis medium containing the released drug was withdrawn, filtered through 0.22 μm Minisart RC4 filters (Sartorius AG, Göttingen, Germany), and replaced with the fresh medium (VFS). The amount of the released AZM was determined by HPLC as described earlier [19].

2.7. Rheological Assessment of the AZM-Liposomal Hydrogels

The rheological characteristics of the AZM-liposomal hydrogels were determined at 25 and 37 °C using a Modular Compact Rheometer MCR 102 (Anton Paar GmbH, Graz, Austria) fitted with a parallel-plate measuring system (diameter 25 mm, PP25), and the gap set to 1 mm. Prior to the measurements, the AZM-liposomal hydrogels were equilibrated for 10 min at the corresponding temperature. Viscosity curves were determined by rotational tests performed in the shear rate range from 0.1 to 1000 s^{-1}. To simulate in vivo vaginal application, the AZM-liposomal hydrogels were mixed with VFS in 5:1 ratio (*w*/*w*), as previously reported [10], and the measurements were performed at 37 °C. Oscillatory amplitude sweep tests were also carried out with the hydrogels mixed with VFS (5:1, *w*/*w*) at 37 °C applying an angular frequency of 10 s^{-1} in the shear strain range of 0.1–100%. Control-HG was examined under the same conditions.

2.8. Texture Analysis of the AZM-Liposomal Hydrogels

Texture properties of the AZM-liposomal hydrogels were assessed by a TA.XT Plus Texture Analyser (Stable Micro Systems Ltd., Surrey, UK) using a previously reported procedure [10]. In brief, 50 g of the hydrogel was placed in a standard beaker, ensuring that no air bubbles were included and that the surface was smooth. A disk (40 mm in diameter) was pushed into the formulation (10 mm at a speed of 1 mm/s) and removed. The hardness and cohesiveness of each AZM-liposomal hydrogel were determined. The results were compared with the texture characteristics of the control-HG and chitosan-HG. Each formulation was measured five times, ensuring the same conditions for each measurement.

2.9. Mucoadhesion Test

The mucoadhesive properties of the different AZM-hydrogels (CLs-HG, PGLs-HG, DPGLs-HG, and control-HG) were evaluated using the TA.XT Plus Texture Analyser equipped with the gel mucoadhesion probe (Stable Micro Systems Ltd., Surrey, UK). The experiments were performed on porcine vaginal mucosa, which was obtained as waste in a local slaughterhouse. The vaginal mucosa was carefully separated from the underlying tissue, washed with isotonic solution (0.9% NaCl), cut into smaller pieces, and frozen at −20 °C. Immediately prior to the measurements the mucosa was defrosted at room temperature, cut into squares of approximately 6.25 cm^2, and soaked in VFS for 5 min. Each mucosal sample was attached to the lower platform and incubated at 37 °C. The tested hydrogel (1.5 mL) was attached to the gel mucoadhesion probe, facing the vaginal mucosa samples. The probe contained a conically cut area (15 mm diameter) with concentric grooves, which promoted gel attachment.

AZM-hydrogel was placed in contact with the vaginal mucosa for 30 s using a force of 0.05 N. The probe was lowered at a speed of 0.5 mm/s until it reached the sample surface. Test speed and probe withdrawing speed were both set at 0.1 mm/s. A trigger force of 0.029 N and return distance of 15 mm were applied.

The Texture Analyser software (Exponent Connect Version 7.0.6.0) was used to calculate the maximum detachment force and work of adhesion (the area under the force/distance curve), which served as an indicator of the mucoadhesive properties.

All measurements were performed in triplicate.

2.10. Antimicrobial Evaluation of the Chitosan-HG

Intrinsic antimicrobial activity of the chitosan-HG was evaluated toward *S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 8739, and *C. albicans* ATCC 10231. The assessment was performed based on the logarithmic reduction of the number of test microbes relative to their initial number using the pharmacopoeial method for testing the effectiveness of preservatives in preparations for topical administration [20].

Briefly, samples of chitosan-HGs (20 g each) were inoculated with 0.1 mL of bacterial or 0.2 mL yeast suspension. The final concentration of test microbes in each hydrogel sample was 10^5–10^6 colony-forming units/g (CFU/g) microbes.

The samples were well mixed to achieve a homogeneous distribution of the microbes within the hydrogels and were stored at 20–25 °C, protected from light, for 28 days. The number of test microbes in the inoculated chitosan-HG sample was determined immediately after inoculation and at the particular time intervals (7th, 14th, and 28th days), according to the pharmacopoeial pour-plate method [20]. For that purpose, 1 g of the inoculated hydrogel sample was weighed for each test microorganism and serial dilutions of 10^{-1} to 10^{-5} were prepared in tubes containing 9 mL of D/E neutralizing broth. The number of microbes was determined by the pour-plate method whereby 1 mL of each dilution of the test sample was placed in a Petri dish in duplicate and homogenized with 15–20 mL of the previously dissolved growth medium. Tryptic soy agar and Sabouraud's dextrose agar were used for bacterial and yeast growth, respectively. Incubation was performed for 3–5 days at 30–35 °C (bacteria) or 5 days at 20–25 °C for fungi.

During the antimicrobial activity assessment of the chitosan-HG, in parallel, negative and positive control tests were performed, too. Negative control represented the microbiological examination of the chitosan-HG not inoculated with the microbes, while the inoculum control of tested microbes served as positive control.

After incubation of the chitosan-HGs with the test microbes, grown colonies were counted and the number of the test microbes in 1 g of the tested hydrogel sample was calculated according to the following equation:

$$X \quad \frac{1}{10^{-n}},$$

where X represents the number of colonies grown in each dilution, and n denotes the dilution of the test microorganism.

Based on the number of microbes determined, the corresponding logarithmic ($\log_{10}$) value was calculated in 1 g of the tested hydrogel sample.

2.11. Antibacterial Activity of the AZM-Liposomal Hydrogels

In vitro antibacterial activities of the different AZM-liposomal hydrogels (CLs-HG, PGLs-HG, and DPGLs-HG) toward *S. aureus* ATCC 6538 were determined by the agar-diffusion method. To determine whether chitosan-HG could potentiate the antibacterial activity of the incorporated AZM-liposomes, the studies were also performed with AZM-liposomes (CLs, PGLs, and DPGLs), a solution of AZM in an ethanol/water mixture (6/4, *v*/*v*), control-HG (hydrogel containing AZM-solution instead of the AZM-liposomes in the same concentration as in the liposomes), chitosan-HG, and an ethanol/water solution (6/4, *v*/*v*) that represented the solvent control. To enable diffusion of the hydrogel into agar, all the hydrogel samples were diluted with buffer, pH 8.0.

The following procedure was applied: The inoculum of the tested bacteria in Tryptic soy broth at a concentration of 1×10^8 CFU/mL was diluted with a 0.9% saline solution (1:10) and then evenly dispersed in liquid Antibiotic medium 11 (1:100) thermostated at 37 °C. Inoculated agar (20 mL) was poured into a sterile Petri plate (100 × 15 mm) and left on a flat surface to solidify. Subsequently, six wells (each 6 mm in diameter) were drilled on each plate using sterilized stainless steel tubes and approximately 0.1 mL of the diluted tested sample (corresponding to 1.2 μg AZM) was added into the wells. The plates were incubated at 37 °C under aerobic conditions for 24 h. Following incubation, confluent growth of the bacteria on the plates was observed, and the diameters of the bacterial growth inhibition zones were measured in millimeters. If the zone of inhibition was not present around the well, the tested material was considered to have no antibacterial effect on the tested bacterial strain.

These experiments were conducted in duplicate.

2.12. In Vitro Biocompatibility Assessment

The HeLa cell line was obtained from the American Type Culture Collection (CCL-2™). HeLa cells were cultured as monolayers and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

The cells were inoculated onto a series of standard 96-well microtiter plates at concentrations of 1.6×10^4 cells/mL. The following day, prediluted AZM-liposomal hydrogels or control-HG were added at five concentrations (1, 5, 10, 20, and 40 μg/mL) and incubated with the cells for an additional 24 h (37 °C, 5% CO_2). The corresponding empty liposomal hydrogels (without AZM) and empty control-HG (without AZM) were tested under the same conditions. The cell growth rate was evaluated by detecting the dehydrogenase activity in viable cells using the MTT assay, as previously reported [19]. The cell viability of the treated cells was displayed as a percentage compared to the untreated control cells. Each result represents a mean value from at least three separate experiments performed in quadruplicate.

2.13. Statistical Analysis

Statistical data analysis was evaluated by one-way ANOVA and Tukey's multiple comparison test using the GraphPad 5.0 Prism program (GraphPad Software, Inc., San Diego, CA, USA). Means were considered significantly different when $p < 0.05$.

3. Results and Discussion

When designing a vaginal drug formulation, several factors affecting the success of therapy should be considered, ranging from precise diagnosis, selection of an effective drug in the appropriate dose, and finally, a formulation that remains long enough on the

vaginal tissue, ensuring adequate release of the drug [7,19]. Topical formulations made from biodegradable and biocompatible excipients that additionally exhibit intrinsic antimicrobial activity are particularly valuable for the treatment of aerobic vaginitis. Among them, chitosan has shown great potential because of its natural origin, ability to form hydrogel, acidity suitable for the vaginal mucosa, and diversity of biological activities including anti-inflammatory and antimicrobial properties [21].

AZM-liposomes have already shown promise for treating cervicovaginal infections [19]. However, their liquid nature and non-mucoadhesive features limit vaginal application despite their therapeutic potential. Since chitosan-HGs have shown compatibility with liposomes intended for skin therapy [22], it seemed reasonable to use chitosan-HG as a vehicle (base) for incorporating AZM-liposomes intended for the local treatment of aerobic vaginitis.

The optimized AZM-liposomes [19], i.e., CLs, PGLs, and DPGLs, were incorporated into chitosan-HG, resulting in 30% AZM liposomes-in-hydrogel (*w*/*w*). As summarized in Table 2, all liposomes were anionic (zeta potential of approximately −60 mV), allowing good physical stability with mean diameters in the range of 180–250 nm and encapsulation efficacy between 6 and 8 μg AZM/mg lipid. CLs were the biggest and characterized by rigid bilayers in comparison to PGLs and DPGLs, which had elastic bilayers. Moreover, the presence of propylene glycol in PGLs and DPGLs led to the slightly increased encapsulation of AZM in comparison to CLs, probably because of its solubilizing effect.

Table 2. Physicochemical characteristics of AZM-liposomes.

AZM-Liposomes	Mean Diameter (nm)	PDI	ZP (mV)	Degree of Elasticity	EE (μg AZM/mg Lipid)
CLs	253 ± 3	0.17 ± 0.03	−62.5 ± 0.8	0.5 ± 0.2 *	6.4 ± 0.8
PGLs	216 ± 2	0.19 ± 0.02	−56.2 ± 0.7	13.4 ± 0.5	8.0 ± 0.4
DPGLs	179 ± 1 *	0.20 ± 0.02	−54.3 ± 0.6	16.1 ± 0.7	7.4 ± 0.7

AZM, azithromycin; CLs, conventional liposomes with azithromycin; DPGLs, deformable propylene glycol liposomes with azithromycin; EE, encapsulation efficacy; PDI, polydispersity index; PGLs, propylene glycol liposomes with azithromycin; ZP, zeta potential. Each result represents the mean ± S.D. (n = 3). * Significantly different ($p < 0.05$).

3.1. Characterization of AZM-Liposomal Hydrogels

The physiological and anatomical features of the vagina should be respected when designing a vaginal formulation [7]. Since the healthy vaginal pH is slightly acidic [23] and increases during infections [2], the application of a formulation exerting a suitable pH value can be beneficial for local therapy of aerobic vaginitis.

The chitosan-HG evaluated in this research was composed of 4% (*w*/*w*) MMW chitosan and contained lactic acid, a natural compound found in vaginal fluid [24]. Lactic acid was used for dissolving the chitosan during hydrogel preparation, instead of the commonly used acetic acid [25]. The hydrogel pH value was slightly acidic (5.48) and favorable for intended administration as well as incorporation of liposomes. As shown in Figure 1, the incorporation of AZM-liposomes resulted in slightly increased pH values because of the effect of the buffer pH 7.5 in which the liposomes were prepared; however, this change was not statistically significant ($p > 0.05$).

Volume, pH, and the constituents of vaginal fluid may affect drug release characteristics as well as the distribution and retention of the hydrogel within the vagina. Once the AZM-liposomal hydrogel is administered, it should distribute along the mucosa and provide prolonged residence time to enable delivery of the incorporated drug [7,10]. Therefore, performing in vitro release studies in conditions that simulate vaginal application, rheological and texture evaluation, including mucoadhesion testing, seem necessary in the characterization of the prepared AZM-hydrogels.

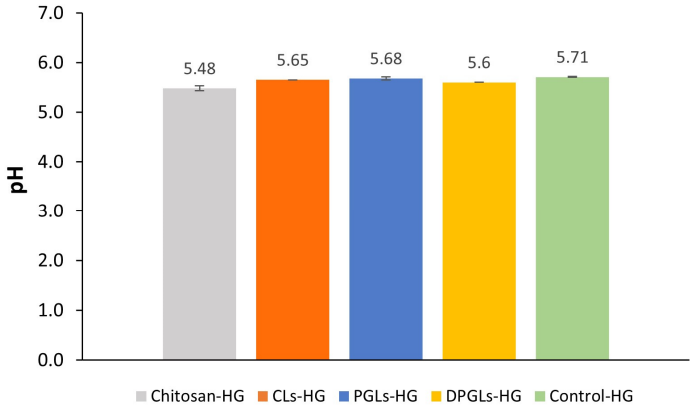

Figure 1. pH values of the originally prepared chitosan-HG and different AZM-hydrogels. The results represent the mean ± S.D. (n = 3). CLs-HG, conventional AZM-liposomes-in-chitosan-HG; control-HG, AZM-solution-in-chitosan-HG; DPGLs-HG, deformable propylene glycol AZM-liposomes-in-chitosan-HG; PGLs-HG, propylene glycol AZM-liposomes-in-chitosan-HG.

The dialysis tubing method was applied to study the release of AZM from the different AZM-liposomal hydrogels (CLs-HG, PGLs-HG, DPGLs-HG). The results displayed in Figure 2 demonstrated the prolonged release of AZM from all the liposomal hydrogels in comparison to control-HG ($p < 0.05$). Among the different types of liposomes incorporated into hydrogel, the slowest release of AZM was achieved by CLs-HG ($p < 0.05$), while somewhat faster release was obtained from PGLs-HG and DPGLs-HG. These results are associated with the bilayer properties of AZM-liposomes. Hence, with the increasing bilayer elasticity of AZM-liposomes (Table 2), a higher amount of AZM was released (Figure 2). The similar trend of AZM release was obtained from the AZM-liposomal suspensions (CLs, PGLs, and DPGLs); however, the ratio of the released drug was significantly greater [19], compared to the AZM-liposomal hydrogels because of the presence of a hydrogel barrier further extending release of the liposomal drug.

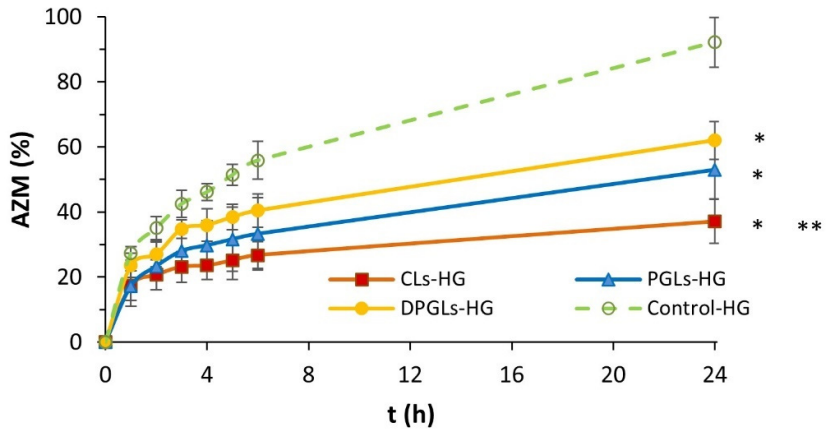

Figure 2. Cumulative in vitro release of AZM from the different AZM-hydrogels. The values represent the mean ± S.D. (n = 3). * Significantly different compared to control-HG at the 24 h time point ($p < 0.05$). ** Significantly different from DPGLs-HG at the 24 h time point ($p < 0.05$).

Assessment of rheological and texture features plays an important role in development of semisolid vaginal formulation. Specifically, rheological behavior affects the formulation appearance, its performance, stability, spreadability, and retention at the application site [26]. Moreover, understanding the rheological features of a formulation helps in explaining the relationship between its chemical structure and the resulting formulation properties [27]. For instance, viscosity measurements allow determination of the gel's resistance to structural breakdown [26].

Vaginal hydrogels are expected to maintain appropriate viscosity upon application of higher shear rates or when diluted with vaginal fluid. Interaction of the formulation with vaginal fluid as well as body temperature can change the hydrogel's initial viscosity [10]. Hence, viscosity measurements of the different AZM-hydrogels were performed at 25 °C and 37 °C, referring to storage and application temperatures, respectively.

As can be seen in Figure 3, all tested AZM-hydrogels were of similar viscosities and exhibited shear-thinning profiles like those obtained with Carbopol gels [28]. The type of AZM-liposomes incorporated in chitosan-HG did not cause significant differences in the viscosity profiles of the different AZM-liposomal hydrogels. Similar behavior was also demonstrated with Carbopol hydrogels containing metronidazole- or clotrimazole-liposomes [10]. The slightly higher initial viscosities of the AZM-liposomal hydrogels compared to control-HG (Figure 3) were probably caused by the liposome membrane ingredients known to increase the strength of the hydrogel into which they are mixed [29].

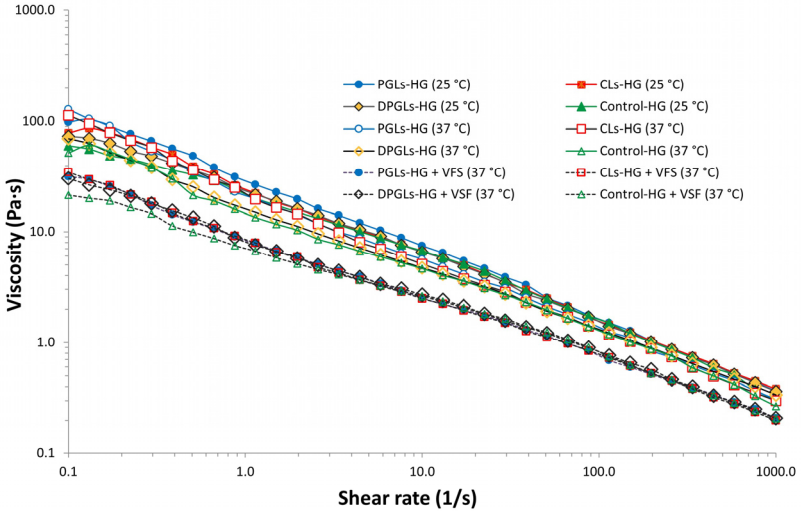

Figure 3. Viscosity profiles of different AZM-liposomal hydrogels at 25 °C and mimicked vaginal conditions (+VFS, 37 °C).

To better simulate the in vivo performance of the AZM-hydrogels, VFS was added to all tested samples and viscosity profiles were determined at 37 °C. As a result, a significant decrease in the initial viscosities of the hydrogels (at 25 °C) was obtained (Figure 3). However, this reduced viscosity of the AZM-liposomal hydrogels was still considered suitable for retaining the formulation on vaginal mucosa. Our findings were in agreement with the previous research where the viscosity of liposomes incorporated in Carbopol gel was also assessed in simulated vaginal conditions [10] and are speculated to be the result of only dilution of the AZM-hydrogels with VFS.

Oscillatory tests are preferred in rheological evaluation as they provide information on the viscoelastic character of the semisolid formulation [26,27]. Like viscosity assessment, amplitude sweep measurements of the different AZM-liposomal hydrogels were also conducted in a mimicked vaginal environment [10].

AZM-hydrogels exhibited a linear viscoelastic region, i.e., a constant plateau where storage modulus (G′) and loss modulus (G″) values do not depend on the strain and only correlate with a molecular structure [26]. As demonstrated in Figure 4, all examined hydrogels demonstrated the higher storage modulus (G′), thus proving their viscoelastic solid structure. Among the different formulations, PGLs-HG, DPGLs-HG, and control-HG exhibited similar viscoelastic behavior, while CLs-HG displayed lower values (Figure 4), indicating its stronger structure. Specifically, CLs are composed of hydrogenated phospholipids (Table 1) contributing to the rigidity of their bilayers in comparison to PGLs and DPGLs, characterized by elastic membranes (Table 2). The results were supported by the

findings of Mourtas et al. [29], where the elastic character of the gel strengthened with an increase in the concentration of hydrogenated phospholipids.

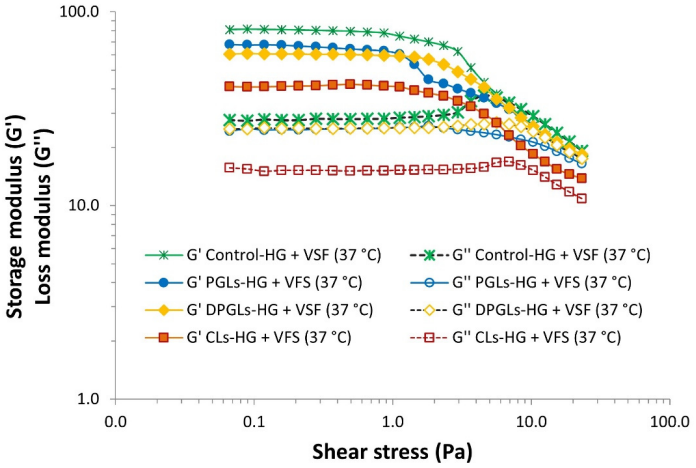

Figure 4. Amplitude sweep curves of the different AZM-liposomal hydrogels under mimicked vaginal conditions (+VFS, 37 °C).

The mechanical properties of the prepared AZM-liposomal hydrogels were also assessed by texture analysis. For this purpose, the hardness, corresponding to the easiness of administration at the application site [10], was determined as well as the cohesiveness, which refers to the recovery of the structural network within the hydrogel after application [22]. These properties can be affected by the ingredients of the formulation. In this study, the influence of incorporating different types of AZM-liposomes on the textural properties of chitosan-HG was examined, and the results were compared with the textural properties of the hydrogel containing the AZM-solution (control-HG). As expected, incorporation of 30% (*w*/*w*) AZM-liposomes into the chitosan-HG resulted in a significant decrease in the initial hardness and cohesiveness of the chitosan-HG (Figure 5). This effect was mostly caused by simple dilution of the chitosan-HG because a similar effect was observed with control-HG (which contained 30% AZM-solution, *w*/*w*). Comparison of the different AZM-liposomal hydrogels showed very similar profiles of hardness and cohesiveness regardless of the type of liposomes embedded in the hydrogel, while the cohesiveness of DPGLs-HG was negligibly higher than that obtained for CLs-HG and PGLs-HG ($p > 0.05$).

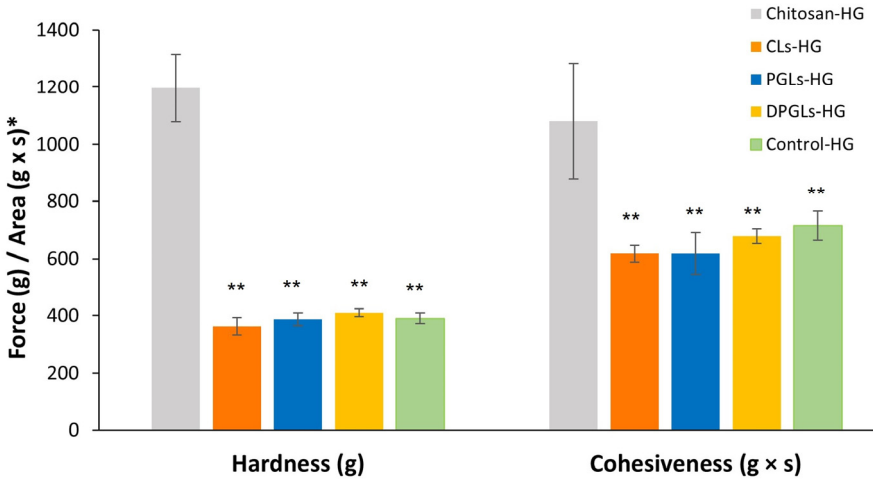

Figure 5. Texture properties of the different hydrogels, expressed as hardness and cohesiveness. * Force (g) refers to the hardness, while Area (g × s) refers to the cohesiveness. The presented values are the mean ± S.D. ($n = 5$). ** Significantly different compared to the chitosan-HG ($p < 0.05$).

All AZM-liposomal hydrogels showed prolonged release of AZM at simulated vaginal conditions (Figure 2). However, to permit prolonged contact of the liposomal drug at the vaginal mucosa, as a prerequisite for therapeutic effect, AZM-liposomal hydrogels should be sufficiently mucoadhesive. Mucoadhesion was estimated by a tensile test using a texture analyzer [30], where the measurement of maximum force, i.e., detachment force (Figure 6A), or the work required for detachment of the hydrogel sample from porcine vaginal mucosa, i.e., work of adhesion (Figure 6B), was determined.

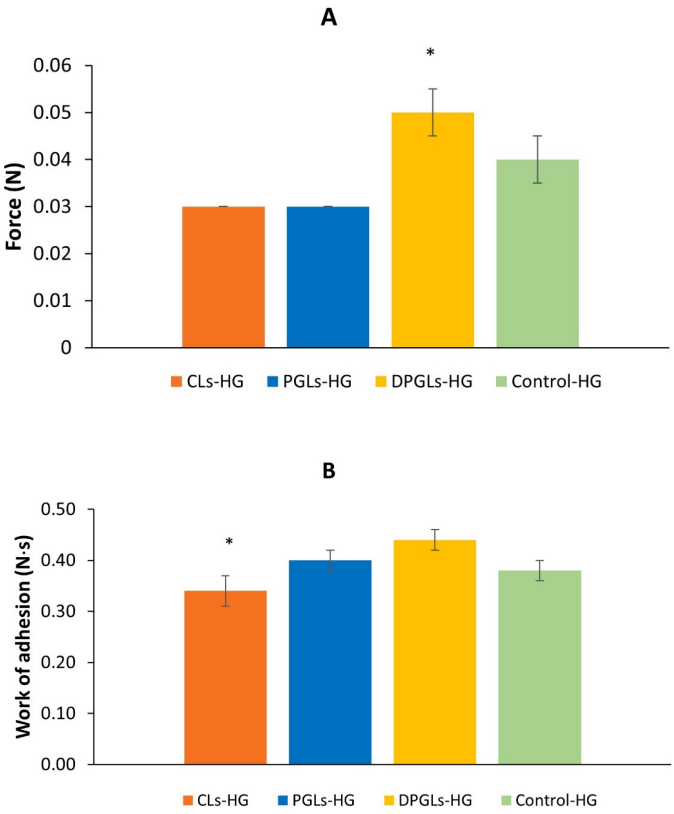

Figure 6. Mucoadhesion of different AZM-hydrogels on porcine vaginal mucosa: force detachment values (**A**) and work of adhesion (**B**). The values are the mean ± S.D. (n = 3). * Significantly different ($p < 0.05$).

The detachment force was found to be the highest for DPGLs-HG ($p < 0.05$), followed by control-HG, while the lowest values were determined for CLs-HG and PGLs-HG (Figure 6A). Therefore, it seems that the presence of only 15% lysophospholipids (SLPC-80) in bilayers of DPGLs had an impact on the mucoadhesivity of DPGLs-HG. We suppose that its contribution to mucoadhesivity is probably coupled to the role of lysophospholipids in the adhesion processes in biological tissues [31], but it should be further evaluated. The dominance of DPGLs-HG was additionally confirmed when the work of adhesion was measured (Figure 6B). However, it appeared that propylene glycol also contributed to mucoadhesivity because both PGLs and DPGLs contained the same portion of propylene glycol in comparison to CLs, which were composed of hydrogenated phospholipids and prepared without propylene glycol.

3.2. Antimicrobial Potential of the Chitosan-HG

The assessment of the intrinsic antimicrobial activity of the chitosan-HG was performed using the pharmacopeial method for determination of the effectiveness of preservatives in non-sterile formulations for topical and mucosal administration [20]. The study included all the requested bacterial strains (*S. aureus* ATCC 6538, *P. aeruginosa* ATCC 9027, *E. coli* ATCC 8739) and yeast (*C. albicans* ATCC 10231), while the efficacy against the mold

(*Aspergillus brasiliensis* ATCC 1604) was not tested, as it was not of interest as a vaginal pathogenic microbe. The tested microbes are characteristic for aerobic vaginitis (*S. aureus*, *E. coli* and *P. aeruginosa*) [2] and vulvovaginal candidiasis (*C. albicans*) [7].

As shown in Table 3, chitosan-HG exhibited a similar intrinsic antimicrobial potential regardless of whether it was Gram-positive or Gram-negative bacteria or yeasts. Logarithm reduction of the bacterial and yeast number upon incubation in chitosan-HG met the criteria of the pharmacopoeia for preservatives in topical and mucosal formulations; the number of microbes dropped from the initial inoculum for about 4.5 logs on the 2nd, 7th, and 14th days, with no change between the 14th and 28th days of incubation.

Table 3. Survival of the test microbes in the chitosan-HG incubated at 37 °C for 28 days, expressed as the logarithmic reduction of the number of microbes after a certain time of incubation.

Test Microbes		***S. aureus* ATCC 6538**	***P. aeruginosa* ATCC 9027**	***E. coli* ATCC 8739**	***C. albicans* ATCC 10231**
Inoculum CFU/mL (log_{10})		3.0×10^5 (5.5)	4.3×10^5 (5.6)	3.5×10^5 (5.5)	4.2×10^5 (5.6)
Log_{10} reduction	2nd day	>4.5	>4.6	>4.5	-
	7th day	>4.5	>4.6	>4.5	-
	14th day	>4.5	>4.6	>4.5	>4.6
	28th day	NI	NI	NI	NI

-: no growth. NI: no increase in the number of viable microbes compared to the previous day. Experiments were performed in duplicate.

The antimicrobial activity of chitosan has been linked to several mechanisms. The polycationic structure of chitosan in an acidic environment is thought to act through binding to the negatively charged bacterial cell wall components such as teichoic acid, lipopolysaccharide, and protein structures, leading to disruption of the cell wall. Furthermore, chitosan can attach to DNA and inhibit DNA replication, subsequently causing cell death [32,33]. Chitosan may also act as a chelating agent of trace metal elements causing toxin production and inhibition of microbial growth [34]. Chitosan inhibits the growth of *Candida* spp. by interfering in the action of synthase complexes responsible for the synthesis of key cell wall components (glucans and chitin). Antifungal activity in yeasts is more pronounced in limited carbon and nitrogen environments (e.g., in blood). Additionally, induction of the intracellular production of reactive oxygen species (ROS) and growth inhibition were seen under glucose starvation in *Candida glabrata*, linking limited nutrient content and ROS with the antifungal action of chitosan (reviewed in [35]).

Considering the microbial count reduction upon incubation in chitosan-HG and its slightly acidic pH (Figure 1) promoting the polycationic state of chitosan, such hydrogels can be applied as a vehicle (base) in the design of vaginal formulations without additional preservatives in the formulation and can favorably affect the vaginal mucosa because of its hydrophilic nature.

Interestingly, besides the antibacterial effect against bacteria typical for aerobic vaginitis (*E. coli*, *S. aureus*, *P. aeruginosa*), chitosan-HG exerted anticandidal activity, too. (Table 3). These effects of the chitosan-HG can be useful in the regulation of vaginal microbiota disorders and during antibacterial therapy when *C. albicans* overgrowth may occur, thus preventing its incidence.

3.3. Antimicrobial Activity of the AZM-Liposomal Hydrogels against S. aureus

The antimicrobial activity of AZM-liposomes and AZM-liposomal hydrogels was tested on *S. aureus* because this species is frequently involved in aerobic vaginitis [1,2]. Results of the agar-diffusion test (Table 4) indicated that all AZM-liposomes exhibited stronger antibacterial activity than the free drug. Comparison of the different types of AZM-liposomes demonstrated that CLs produced slightly larger inhibition zones of bacterial growth in comparison to DPGLs and/or PGLs and particularly when compared to control

(AZM-solution). These findings are probably a result of the (phospho)lipid composition and the presence of propylene glycol affecting the bilayer properties of AZM-liposomes. The empty liposomes and empty liposomal hydrogels (both without AZM), which were used as controls in this study, failed to show any anti-staphylococcal effect. This observation was in accordance with our earlier report on the activity of AZM incorporated in anionic and cationic liposomes against *S. aureus* and methicillin-resistant *S. aureus* (MRSA) clinical isolates aimed at the treatment of skin infections [13], where the activity of the drug-free liposomes did not show a direct antimicrobial effect. The same research group also demonstrated that the proportion of propylene glycol in PGLs with AZM contributed to their antibacterial potential [13].

Table 4. Antibacterial activity of the different AZM-liposomal hydrogels in comparison to corresponding AZM-liposomes.

AZM-Sample	Zone of Inhibition (mm ± S.D.)	
	AZM-Liposomes	AZM-Liposomal Hydrogel
CLs	18.47 ± 0.28	19.07 ± 0.60
PGLs	17.85 ± 0.24	18.50 ± 0.39
DPGLs	17.59 ± 0.22	18.46 ± 0.02
Control *	15.55 ± 0.20	17.05 ± 0.04

* Control denotes free AZM-solution and AZM-hydrogel. The amount of AZM corresponded to the AZM amount in liposomes and liposomal hydrogels. Experiments were performed in duplicate.

The AZM-liposomes used in this study, i.e., CLs, PGLs, and DPGLs, were previously examined against several strains of planktonic and biofilm-forming *E. coli* [19]. All types of AZM-liposomes were similarly effective against planktonic bacteria, but their activity differed against biofilm-forming *E. coli*. Thus, CLs were more effective at preventing formation of *E. coli* biofilms, while DPGLs were superior in their eradication. The greater activity of DPGLs against already formed biofilms is assumed to be a result of the presence of monoacyl phospholipids (lysophospholipids), which have been shown to play a role in adhesion processes in biological environment [31] and therefore could affect adhesion of DPGLs to biofilms. This effect was supported by this study where DPGLs-HG were shown to be the most mucoadhesive among the tested AZM-hydrogels (Figure 6).

Incorporation of AZM-liposomes into chitosan-HG improved the anti-staphylococcal activity of the corresponding AZM-liposomes. Among the different AZM-liposomal hydrogels, CLs-HG exerted the most potent activity against *S. aureus*, followed by PGLs-HG and DPGLs-HG. The observed increase in the antibacterial activity of AZM-liposomes by their incorporation into chitosan-HG can be linked to the chitosan polycationic structure discussed above. These findings were also supported by Hemmingsen et al. [22], who demonstrated that chitosan hydrogels can boost the antibiofilm effect of incorporated chlorhexidine liposomes for the treatment of chronic wounds.

3.4. In Vitro Biocompatibility Assessment

One of the requirements for pharmaceutical formulations is their safety for patients. Therefore, testing the biocompatibility of the formulation is of great importance. The biocompatibility of AZM-liposomal hydrogels was tested in vitro on the HeLa cell line to determine their potential cytotoxicity, which may be contributed by AZM, but also by the ingredients of the formulation. Therefore, in addition to testing the AZM-liposomal hydrogels, empty liposomal hydrogels and empty control-HG (all without AZM) were also tested at concentrations corresponding to the AZM-liposomal hydrogels.

Although the HeLa cell line belongs to cervical cells, it can be used in testing in vitro cytotoxicity/biocompatibility of vaginal formulations [19,36,37].

As shown in Figure 7A, all AZM-liposomal hydrogels were compatible with the HeLa cells. Even at the highest examined concentration (40 μg/mL), which was almost 40-fold higher than the MIC for *S. aureus*, the viability of the cells was greater than 90%

(regardless of the type of AZM-liposomes incorporated in chitosan-HG). Testing of the corresponding empty liposomal hydrogels (Figure 7B) confirmed the biocompatibility of all tested liposomal hydrogels. Moreover, the performed studies confirmed the safety of chitosan-HG as a vehicle for the incorporation of AZM-liposomes, where at the highest tested concentration the viability of the cells was higher than 98% (Figure 7). Comparison of the different types of liposomes embedded in chitosan-HG demonstrated that CLs and PGLs were somewhat more tolerable than DPGLs (Figure 7B), although the viability of the cells was greater than 82% at the highest tested concentration. These findings were in agreement with a previous study in which the in vitro cytotoxicity of AZM-liposomes alone was examined and is supposed to be affected also by the negative surface charge of the liposomes [19].

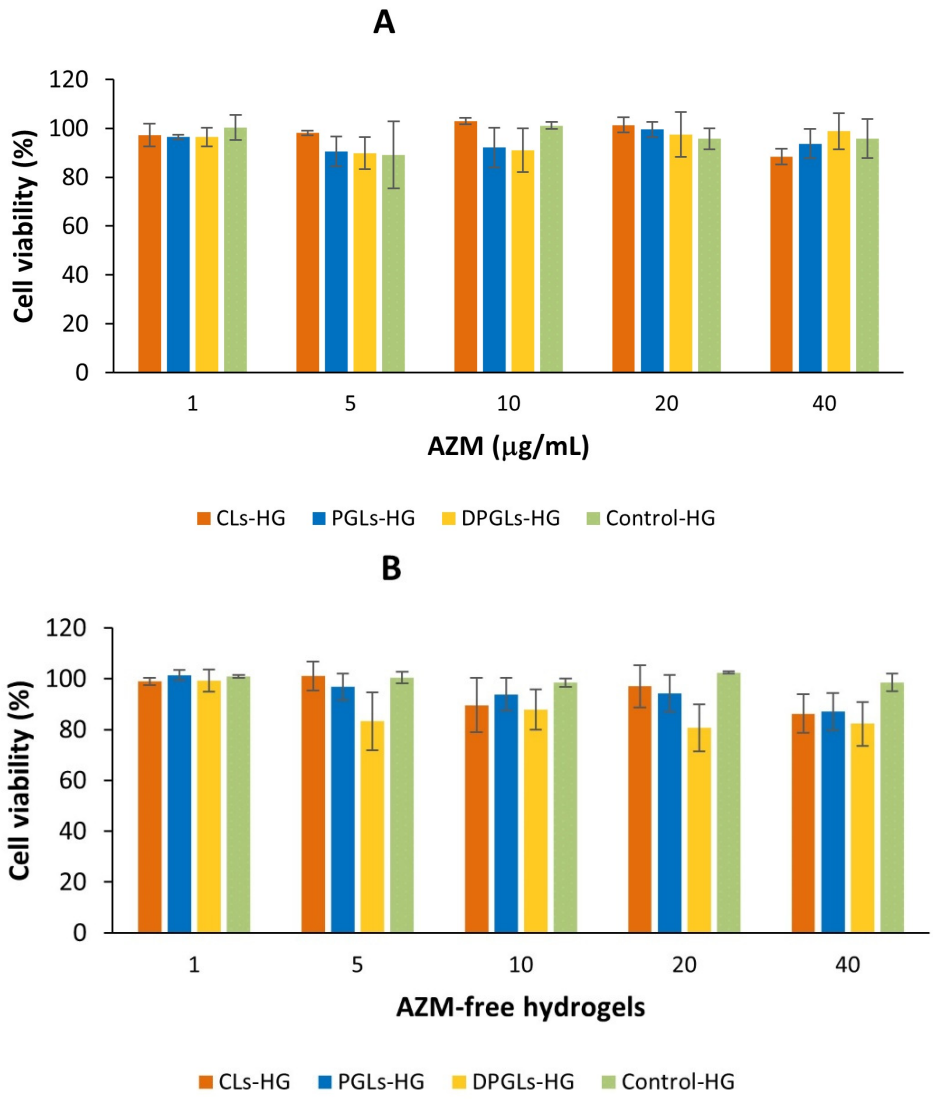

Figure 7. HeLa cell viability after incubation with the different AZM-hydrogels, expressed by AZM concentration (**A**), and with the corresponding AZM-free hydrogels (**B**). The presented values are the mean ± S.D. (n = 3).

The additional value of the AZM-liposomal hydrogels for the treatment of aerobic vaginitis lies in the immunomodulatory and anti-inflammatory effects of AZM [38,39]. Likewise, chitosan hydrogel made of MMW chitosan has also demonstrated inherent anti-inflammatory effects in vitro [22]. Because of the large amount of water in liposomal hydrogels and its moisturizing effect, in addition to their mucoadhesiveness, which enables enhanced retention of the incorporated drug at mucosal surface, they are suitable for

vaginal application and treating various vaginal conditions [7]. Since aerobic vaginitis is characterized by inflammatory changes in the vaginal epithelium, a burning sensation, and epithelial dryness in addition to the excessive growth of aerobic bacteria [2], we assume that AZM-liposomal hydrogels will exhibit multiple therapeutic activities and therefore significantly improve local therapy of aerobic vaginitis. However, these effects should be examined in future studies. Moreover, we also hypothesize that the confirmed inherent anticandidal properties of the chitosan-HG (Table 3) can be beneficial for the prevention of vulvovaginal candidiasis, which might occur as a side-effect of AZM administration.

4. Conclusions

This study is the first to report on the potential of AZM-liposomes incorporated in chitosan-HG for the local treatment of aerobic vaginitis. The chitosan-HG improved the antibacterial efficacy of the AZM-liposomes while being physiologically acceptable. By adjusting the physicochemical properties of the AZM-liposomes, prolonged and controlled release of AZM from biocompatible and mucoadhesive AZM-liposomal hydrogels can be achieved, leading to an increased antibacterial effect as well as the desirable mechanical properties relevant for vaginal application. In addition to these features, considering the pathology of aerobic vaginitis (infection and inflammation), the moisturizing effect of AZM-liposomal hydrogels and the accompanying anti-inflammatory abilities of both AZM [38] and chitosan [22], improved local therapy can be achieved.

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Abbreviations

AZM, azithromycin; CFUs, colony-forming units; CLs, conventional liposomes with azithromycin; CLs-HG, conventional liposomes with azithromycin incorporated in chitosan hydrogel; control-HG, control hydrogel (azithromycin solution incorporated in chitosan hydrogel); DPGLs, deformable propylene glycol liposomes with azithromycin; DPGLs-HG, deformable propylene glycol liposomes with azithromycin incorporated in chitosan hydrogel; EE, encapsulation efficacy; EPC, egg phosphatidylcholine; EPG, egg phosphatidylglycerol sodium salt; HG, hydrogel; MIC, minimum inhibitory concentration; MMW, medium molecular weight; PDI, polydispersity index; PGLs, propylene glycol liposomes with azithromycin; PGLs-HG, propylene glycol liposomes with azithromycin incorporated in chitosan hydrogel; SLPC-80, monoacyl phosphatidylcholine from soybean; SPC-3, hydrogenated soybean phosphatidylcholine; VFS, vaginal fluid simulant; ZP, zeta potential.

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