

In vitro ispitivanja biokompatibilnosti liposoma različitog (fosfo)lipidnog sastava sa stanicama keratinocita

Katanec, Antonija

Master's thesis / Diplomski rad

2019

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Pharmacy and Biochemistry / Sveučilište u Zagrebu, Farmaceutsko-biokemijski fakultet**

Permanent link / Trajna poveznica: <https://um.nsk.hr/um:nbn:hr:163:440457>

Rights / Prava: [In copyright](#) / [Zaštićeno autorskim pravom](#).

Download date / Datum preuzimanja: **2025-01-22**



Repository / Repozitorij:

[Repository of Faculty of Pharmacy and Biochemistry University of Zagreb](#)



Antonija Katanec

***In vitro* ispitivanja biokompatibilnosti liposoma
različitog (fosfo)lipidnog sastava sa stanicama
keratinocita**

DIPLOMSKI RAD

Predan Sveučilištu u Zagrebu Farmaceutsko-biokemijskom fakultetu

Zagreb, 2019.

Ovaj diplomski rad prijavljen je na kolegiju Oblikovanje lijekova, Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta i izrađen na Zavodu za farmaceutsku tehnologiju, pod stručnim vodstvom izv. prof. dr. sc. Željke Vanić.

Zahvala:

Srdačno se zahvaljujem svojoj mentorici izv. prof. dr. sc. Željki Vanić i asistentici mag. pharm. Zori Rukavina na uloženom vremenu, strpljenu, nesebičnom dijeljenju znanja te stručnom vodstvom pri izradi ovog rada.

Zahvaljujem i svim djelatnicima Zavoda za farmaceutsku tehnologiju na pomoći i ugodnoj radnoj atmosferi.

Hvala svim mojim prijateljima koji su uvijek bili tu, vjerovali u mene i slali poruke ohrabrenja prije ispita.

Veliko hvala mojoj obitelji, a posebice roditeljima koji su mi svojom ljubavlju i razumijevanjem uvijek bili najveća podrška.

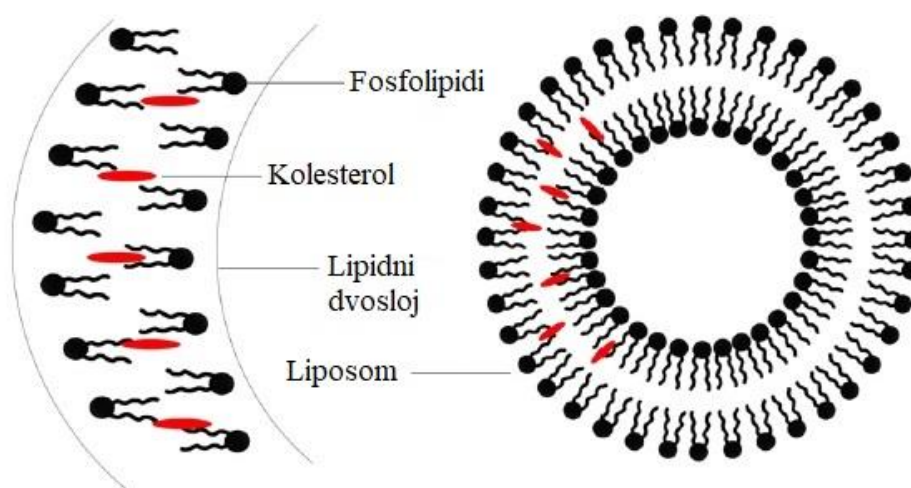
Sadržaj

1. UVOD	1
1.1. Liposomi: struktura, svojstva i priprava	1
1.2. Karakterizacija liposoma	3
1.2.1. Srednji promjer liposoma	3
1.2.2. Zeta potencijal liposoma	4
1.3. Liposomi u topikalnoj primjeni na kožu	6
1.3.1. Liječenje kožnih infekcija: potencijali primjene liposoma	7
1.4. Ispitivanja citotoksičnosti na staničnim kulturama HaCaT keratinocita	8
1.4.1. MTT test (test redukcije tetrazolijeve soli)	9
1.5. Azitromicin	11
2. OBRAZLOŽENJE TEME	13
3. MATERIJALI I METODE	14
3.1. Materijali	14
3.2. Metode	14
3.2.1. Priprema liposoma	14
3.2.2. Određivanje veličine liposoma i indeksa polidisperznosti.....	16
3.2.3. Određivanje zeta potencijala liposoma	16
3.2.4. Određivanje uspješnosti uklapanja azitromicina u različitim vrstama liposoma	17
3.2.5. Ispitivanja citotoksičnosti/biokompatibilnosti <i>in vitro</i>	17
3.2.6. Test redukcije tetrazolijeve soli (MTT test).....	18
3.2.7. Statistička obrada podataka.....	18
4. REZULTATI I RASPRAVA	19
4.1. Fizikalno-kemijske karakteristike liposoma	19
4.2. Uklapanje azitromicina u liposome	22
4.3. Ispitivanje biokompatibilnosti liposoma s HaCaT stanicama <i>in vitro</i>	22
5. ZAKLJUČCI	27
6. LITERATURA	28
7. SAŽETAK	32
SUMMARY	33
8. PRILOZI	
9. TEMELJNA DOKUMENTACIJSKA KARTICA/BASIC DOCUMENTATION CARD	

1. UVOD

1.1. Liposomi: struktura, svojstva i priprava

Liposomi su sferične, zatvorene tvorevine u kojima je unutarnja vodena faza obavijena jednom ili više koncentrično položenih dvoslojeva fosfolipidnih membrana (Slika 1). Veličina im se kreće od pedesetak nanometara do nekoliko mikrometara. Osnovu dvosloja (ovojnice) liposoma čine molekule fosfolipida koje su složene tako da su polarne, hidrofilne „glave“ orijentirane prema vanjskoj i unutarnjoj vodenoj fazi, zaklanjajući nepolarne, lipofilne „repove“ (lanci masnih kiselina) jednog prema drugome (Banović i sur., 2011).



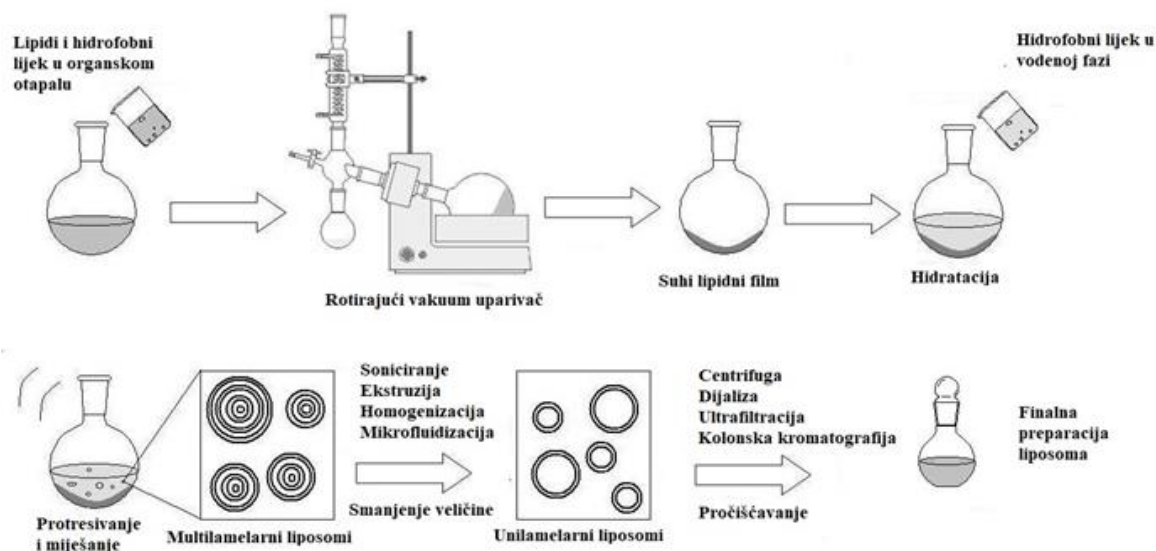
Slika 1. Struktura liposoma. Preuzeto i prilagođeno iz Mu i sur. (2016), uz dozvolu Elsevier-a.

U neutralnom pH području fosfolipidi mogu biti u obliku „zwitter“ iona ili su nabijeni negativno. Zbog toga se i klasificiraju u neutralne (fosfatidilkolin i fosfatidiletanolamin) te negativno nabijene fosfolipide (fosfatidilglicerol, fosfatidilserin, fosfatidilinozitol i fosfatidna kiselina). Strukturna obilježja liposoma razlog su da se u njih mogu uklapati različiti lijekovi (djelatne tvari): lipofilni u ovojnici (fosfolipidni dvosloj), hidrofilni u unutarnju vodenu jezgru, a amfipatski između vodene i lipidne regije.

Liposomi su strukturom (ovojnica liposoma) poprilično slični s biološkim membranama. Biorazgradljivi su, neimunogeni i netoksični što im omogućuje primjenu u različitim terapijskim područjima: infektivna oboljenja (virusna, bakterijska, gljivična, parazitska), dijagnostika, hormonska terapija, onkologija, stimulacija imunološkog odgovora, vakcinacija itd. (Vanić, 2012a).

Mogu se klasificirati prema veličini i broju fosfolipidnih dvoslojeva te prema strukturnim svojstvima i načinu oslobađanja uklopljenog sadržaja. Prema veličini i broju fosfolipidnih dvoslojeva razlikuju se skupine unilamelarnih, multilamelarnih, oligolamelarnih i multivezikularnih liposoma. Unilamelarni liposomi sadrže jednu fosfolipidnu ovojnicu, a prema veličini se dijele na: male unilamelarne liposome ($r = 20 - 100 \text{ nm}$), srednje-velike unilamelarne liposome ($r < 100 \text{ nm}$), velike unilamelarne liposome ($r = 100 - 1000 \text{ nm}$) te veoma velike unilamelarne liposome ($r > 1000 \text{ nm}$). Oligolamelarni liposomi sadrže nekoliko, dok multilamelarni liposomi imaju mnogo koncentrično postavljenih fosfolipidnih dvoslojeva između kojih su vodeni odjeljci. Multivezikularni liposomi su izrazito veliki ($r > 20 \text{ }\mu\text{m}$), sadrže mnogo vodenih odjeljaka, separiranih nekoncentrično položenim fosfolipidnim dvoslojem (Vanić, 2012a).

Uobičajena metoda pripreme liposoma je hidratacija suhog fosfolipidnog filma. Temelji se na pripremi tankog fosfolipidnog sloja te dodatku vodenog medija uz snažno protresivanje. Postupak se provodi u okruglim tikvicama većeg volumena, da bi nakon otparavanja organskog otapala, na stijenkama tikvice nastao suhi fosfolipidni film velike površine. Dodatkom vodenog medija dolazi do hidratacije fosfolipida i spontanog formiranja liposoma (Slika 2). Pozornost valja obratiti na temperaturu koja tijekom pripreme liposoma mora biti iznad temperature faznog prijelaza (T_c) korištenih fosfolipida. Temperatura faznog prijelaza (T_c) definira se kao temperatura na kojoj se odvija prijelaz fosfolipida iz gel faze u fazu tekućih kristala. Poznavanje T_c i s tim u vezi fluidnosti membrane važno je pri proizvodnji i istraživanju formulacija lijekova zasnovanih na liposomima. Liposomi pripremljeni film metodom su multilamelarne strukture i zbog toga su prikladni za uklapanje lipofilnih lijekova. Poprilično su veliki i imaju visok indeks polidisperznosti pa je potrebna njihova daljnja obrada u svrhu homogenizacije. Homogenizacija se može provesti ekstruzijom kroz polikarbonske membrane određene veličine pora ili soniciranjem (ultrazvučna kupelj, sonda). Nedostatak ekstruzije je što redukcija veličine može rezultirati značajnim gubitkom sadržaja uklopljenog hidrofilnog lijeka (Vanić, 2012b).



Slika 2. Prikaz pripreme liposoma film metodom. Preuzeto i prilagođeno iz Lopes i sur. (2013), uz dozvolu *IntechOpen*-a.

1.2. Karakterizacija liposoma

Nakon pripreme liposoma, provodi se njihova karakterizacija. Osnovni fizikalni parametri koji se procjenjuju su veličina (srednji promjer) liposoma, indeks polidisperznosti i zeta potencijal.

1.2.1. Srednji promjer liposoma

Postoje mnoge tehnike za određivanje veličine liposoma. Prikladnije su one koje se temelje na mjerenju raspršenja svjetlosti liposomske disperzije jer ne dovode do narušavanja sustava i pokrivaju veliki raspon veličina (0,1 do nekoliko mikrometara). Fotonska korelacijska spektroskopija (eng. *photon correlation spectroscopy*) se temelji na mjerenju promjena intenziteta raspršenja svjetlosti uzrokovane uzorkom, tj. disperzijom liposoma (Ostrowsky, 1993). Poznata je i pod nazivom dinamičko raspršenje svjetlosti (eng. *dynamic light scattering*) jer mjeri promjenu intenziteta raspršenja svjetlosti u ovisnosti o vremenu, a pojavljuje se kao posljedica Brownovog gibanja čestica (<https://www.ugent.be/en>). Brownovo gibanje je kaotično gibanje čestica male veličine, zbog kojeg dolazi do čestih promjena smjera i međusobnog sudaranja čestica. Brzina Brownovog gibanja povezana je s veličinom čestica. Analizom razlika intenziteta rasipanja svjetlosti određuje se koeficijent difuzije čestica koji se onda transformira u parametar koji opisuje raspodjelu veličina čestica (www.malvern.com).

1.2.2. Zeta potencijal liposoma

Površina čestica dispergirane faze može biti električki nabijena zbog suviška iona, što je prouzročeno adsorpcijom nekog iona iz otopine ili disocijacijom površinskih skupina. Na Slici 3 prikazane su negativno nabijene površine čestica čvrste faze. Uz negativno nabijenu površinu nalaze se pozitivno nabijeni ioni u otopini, tako da ih je uz samu površinu čestice najviše, a zatim se, s udaljenošću čestice prema dubini otopine, broj pozitivnih i negativnih iona izjednačava. Opisana pojava naziva se dvostruki električnim slojem. Pojednostavljeno zamišljeno dvostruki se sloj sastoji od triju dijelova. Prvi sloj čine, u ovom slučaju, adsorbirani negativni ioni na površini, neposredno uz njih je drugi sloj pozitivno nabijenih iona, koji čine tzv. Sternov sloj kojeg je debljina reda veličine iona. Treći dio čini Gouy-Chapmanov sloj (difuzijski dio dvostrukog sloja). Debljina difuzijskog sloja (δ) prikazana je recipročnom vrijednošću Debye-Hückelovog parametra, χ :

$$\delta = 1/\chi.$$

Dvostruki električni sloj može se predočiti kao električni kondenzator od dviju suprotno nabijenih površina. Potencijal na površini čestice (ψ) je maksimalan, potom naglo opada (Sternov sloj), a zatim eksponencijalno (Gouy-Chapmanov sloj).

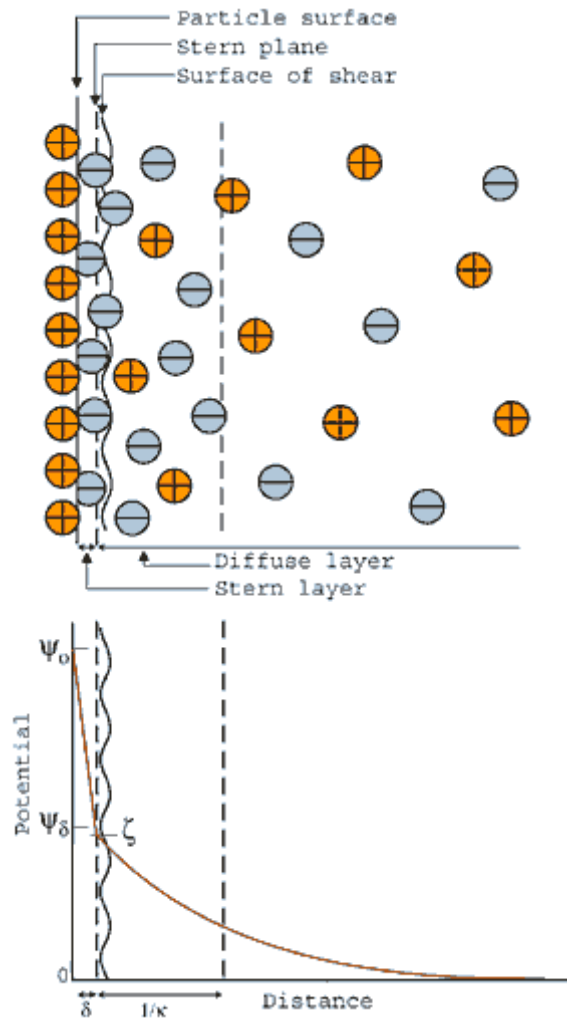
Nabijene dispergirane čestice putuju u električnom polju prema elektrodi suprotnog predznaka, i ta se pojava naziva elektroforetskom gibljivošću. Zajedno s česticama giba se i Sternov sloj, te dio „vezanih“ molekula otapala. Može se zamisliti da se zajedno s česticom giba mali volumen medija odijeljen od ostalih molekula vode tzv. plohom smicanja. Potencijal na udaljenosti plohe smicanja zove se elektrokinetički (ξ) zeta- potencijal. Zeta potencijal je važan zbog toga jer se može relativno lako odrediti iz elektroforetske gibljivosti čestica, a o njemu i naboju na površini dispergirane faze ovisi stabilnost pripremljenih disperzija (Jalšenjak i sur., 1998).

Ukoliko sve čestice u suspenziji posjeduju veliki negativni, odnosno vrlo pozitivan zeta potencijal težit će obijanju pa neće doći do aglomeracije čestica. Međutim, ako čestice imaju niske vrijednosti zeta potencijala tada ne postoji dovoljno velika odbojna sile koja bi sprječila međusobno spajanje i dolazi do aglomeracije i fluktuacije. Kao granične vrijednosti stabilnih i nestabilnih suspenzija uzeto je +30 mV i -30 mV, dakle čestice sa zeta potencijalom većim od +30 mV ili manjim od -30 mV smatraju se stabilnima (Larsson i sur., 2012).

Nanočestice s vrijednostima zeta potencijala od -10 mV do +10 mV smatraju se neutralnima, no nanočestice čije su vrijednosti zeta potencijala veće od +30 mV, odnosno

manje od -30 mV pokazuju snažan kationski, odnosno anionski karakter. S obzirom da je većina staničnih membrana negativno nabijeno, zeta potencijal može utjecati na samu permeabilnost nanočestica. Čestice kationskog karaktera češće pokazuju toksičnost izazvanu oštećenjem staničnog zida (McNeil, 2011).

Istraživanjima je utvrđeno da nanočestice koje posjeduju zeta potencijal od -43 mV imaju veći afinitet ulaska u stanicu od onih koje posjeduju pozitivniji to jest negativniji zeta potencijal (Honary i sur., 2013).



Slika 3. Shematski prikaz zeta-potencijala.

Preuzeto s <https://www.brookhaveninstruments.com/what-is-zeta-potential>.

1.3. Liposomi u topikalnoj primjeni na kožu

Koža kao najveći ljudski organ zauzima ukupno 16% tjelesne mase čovjeka, a građena je od različitih slojeva, koji čine barijeru (granicu) između tijela i okoliša. Međutim, ova barijera je permeabilna za pojedine tvari iz okoliša i omogućuje izmjenu topline, zraka i fluida koji sadrže čestice niske molekulske mase (Verma, 2003).

Koža se sastoji od 3 sloja: epidermisa, dermisa i supkutanog sloja. Vanjski sloj epidermisa, *stratum corneum* prekriva cijelo tijelo. Stanice rožnatog sloja konstantno se uklanjaju i dolazi do izmjene stanica iz dubljih slojeva kože. *Stratum corneum* predstavlja barijeru percutane apsorpcije te ujedno štiti tijelo od vanjskih utjecaja. Sastoji se od nekoliko slojeva mrtvih, izduženih stanica (korneocita) koje sadrže keratin, nastao u dubljim slojevima epidermisa. U slučaju nedostatka keratina zbog određenih stanja (bolesti) kože, apsorpcija lijeka kroz kožu može biti povećana (Cevc i sur., 1992).

Mnogi čimbenici utječu na dostavu lijeka i kozmeceutika u dublje slojeve kože pri topikalnoj primjeni, poput molekulske mase, lipofilnosti molekule, tipu formulacije, prisutnosti pojačivača penetracije i fizikalnog stanja *stratuma corneuma* (Verma, 2003).

Liposomi se dugi niz godina istražuju za poboljšanu dostavu aktivnih tvari u/kroz kožu. Pritom je utvrđeno da lamelarnost, lipidni sastav, naboj na površini liposoma, ukupna primijenjena koncentracija lipida te način aplikacije (okluzivno ili neokluzivno) utječu na odlaganje uklopljenog lijeka (djelatne tvari) iz liposoma u dublje slojeve kože (Cevc i sur., 1992; Weiner i sur., 1989). Osim konvencionalnih liposoma, veliki broj opisanih istraživanja dermalne i transdermalne primjene liposoma provedena su s elastičnim liposomima (deformabilni liposomi, propilenglikol liposomi, invasomi, etosomi) (Vanić i sur., 2015).

Opisano je pet mogućih mehanizama kojim liposomi povećavaju unos lijeka u/kroz kožu. Prema prvom mehanizmu lijek samostalno prodire kroz kožu nakon oslobađanja iz liposoma na površini kože. Smatra se da je uloga tog procesa u dostavi lijeka u kožu zanemariva. Drugi mehanizam je poticanje prolaska lijeka kroz kožu djelovanjem fosfolipida iz liposoma na razrahljivanje intercelularnog lipidnog matriksa. Treći mehanizam predstavlja adsorpciju, odnosno fuziju liposoma na površini kože. Prolazak intaktnih liposoma (četvrti mehanizam) kroz rožnati sloj u epidermis svojstven je deformabilnim (elastičnim) liposomima i moguć je jedino uz primjenu u neokluzivnim uvjetima, dok je za ostale elastične tipove liposoma moguća i okluzivna i neokluzivna primjena. Peti mehanizam podrazumijeva prolazak intaktnih liposoma putem dlačnih folukula i preko žlijezda lojnica, ali je njegova

uloga zbog gustoće dlačnih folikula u koži manje dominantan put u odnosu na ostale moguće mehanizme (interakcije liposoma s kožom) (Banović i sur., 2011).

1.3.1. Liječenje kožnih infekcija: potencijali primjene liposoma

Mnogobrojni pacijenti svakodnevno oboljevaju od bakterijskih infekcija kože i mekih tkiva, a kao jedan od glavnih uzročnik navedenih stanja otkriven je *Staphylococcus aureus* s incidencijom od 32 infekcije na 100.000 ljudi u SAD-u (Creech i sur., 2015). Prilikom liječenja kožnih infekcija izazvanih sa *S. aureusom* najveći izazov predstavlja antibiotska rezistencija. Naime, ubrzo nakon uvođenja penicilina i metecilina u kliničku praksu otkrivene su vrste *S. aureusa* koje su razvile toleranciju na navedene antibiotike. Tako je meticilin rezistentni *S. aureus* (MRSA) postao jedan od vodećih uzročnika komplikacija brojnih post-operativnih oporavaka, dijaliza i kroničnih bolesti, a kompleksne kožne infekcije poput celulitisa, impetiga, folikulitisa, dermatitisa dovele su do porasta hospitalizacija povećavajući time ekonomske troškove za zdravstveni sustav, a u najtežim, kroničnim oblicima bolesti mogu dovesti i do smrti pacijenta (Creech i sur., 2015; Lee i sur., 2013).

Topikalna primjena antibiotika temeljni je način liječenja kožnih infekcija. Međutim, učinkovitost antibiotika često je smanjena zbog nedovoljne koncentracije lijeka na željenom mjestu djelovanja, čime dolazi do povećanja antibiotske rezistencije i formiranja bakterijskog biofilma ili nemogućnosti dopremanja lijeka do mjesta djelovanja. Nadalje, takve kožne infekcije zahtjevaju liječenje s visokim dozama antibiotika koji se primjenjuju oralno ili parenteralno i dovode do brojnih nuspojava poput alergijskih reakcija, a nerijetko povećavaju i rizik od razvoja rezistencije na antibiotike. Stoga je opravdana potreba razvoja formulacija lijekova za učinkovitu lokalnu antimikrobnu terapiju. Pregledom znanstvene literature utvrđeni su brojni potencijali korištenja nanočestica kao nosača antimikrobnih djelatnih tvari za topikalnu primjenu (Goyal i sur., 2015; Wang i sur., 2017; Yah i sur., 2015; Zhang i sur., 2010). Među brojnim istraživanim nanosustavima (različite polimerne, anorganske i lipidne nanočestice), liposomi su od velikog značaja. Optimizacijom lipidnog sastava, veličine, površinskog naboja i stupnja elastičnosti dobivaju se liposomi sa željenim farmakokinetičkim svojstvima uklopljenog antimikrobnog lijeka. Velika prednost liposoma kao nosača antimikrobnih tvari je njihova sposobnost fuzije s bakterijama ili adsorpcija na razvijene biofilme, čime se olakšava dostava uklopljenog sadržaja u unutrašnjost biofilma ili citoplazmu bakterija (Forier i sur., 2014).

Kako bi se poboljšao antimikrobni učinak liposomskih antibiotika, u posljednje vrijeme velika pažnja je usmjerena na istraživanje kationskih antimikrobnih peptida, čiji derivati ugrađeni u lipidni dvosloj liposoma značajno doprinose antimikrobnom učinku liposoma, a time predstavljaju i moćno oružje u prevladavanju antibiotske rezistencije. Nasuprot tradicionalnih antibiotika koji direktno ubijaju uzročnike infekcije, kationski antimikrobni peptidi djeluju poput imunomodulatora, odnosno dovode do uništenja prokariotske membrane, modulirajući time nespecifičnu imunost domaćina. U svrhu liječenja infekcija izazvanih MRSA-om, Liu i suradnici (2016) sintetizirali su kationski antimikrobni peptid DP7-C kojeg su ugradili u lipidni dvosloj liposoma s uklopljenim azitromicinom. Pokazali su kako DP7-C posjeduje sinergističko djelovanje s uklopljenim lijekom. *In vitro* ispitivanjem je utvrđeno kako se liposomima, koji su u lipidnom sloju sadržavali DP7-C, postiže kontrolirano oslobađanje lijeka te djeluju imunomodulatorno, ali bez direktne antibakterijske aktivnosti *in vitro* kao i citotoksičnog učinka na stanice sisavaca. *In vivo* istraživanjem je pokazano da liposomi s kationskim antimikrobnim peptidom značajno smanjuju koncentraciju bakterija te ne uzrokuju nuspojave niti toksičnost. Dakle, liposomi s modificiranim DP7-C u lipidnom dvosloju, posjeduju povoljniji terapijski profil u odnosu na „prazne“ liposome koji sadrže DP7-C, odnosno nemodificirane liposome s uklopljenim azitromicinom. Liposomi s uklopljenim azitromicinom koji u lipidnom dvosloju sadrže modificirani DP7-C pokazali su veliki potencijal za liječenje MRSA infekcija jer DP7-C potiče nespecifični imuni odgovor, ali ne posjeduje direktnu antimikrobnu aktivnost *in vitro* te zbog toga ne dovodi do razvoja rezistencije (Liu i sur., 2016).

1.4. Ispitivanja citotoksičnosti na staničnim kulturama HaCaT keratinocita

HaCaT je spontano nastala stanična linija ljudskih keratinocita koja se razvila iz kulture ljudskih keratinocita u uvjetima smanjene koncentracije kalcija i povišene temperature (Boukamp i sur., 1988). Stanična linija nosi naziv HaCaT gdje Ha označava odrasle ljudske keratinocite, Ca označava kalcij, a T temperaturu, kako bi se nazivom ukazivalo na podrijetlo kulture stanica i uvjete nastanka (Lehmann i sur., 1993).

Prednosti uporabe *in vitro* testova toksičnosti su niža cijena u odnosu na *in vivo* testove, visok stupanj standardizacije, reproducibilnost i brzina izvođenja pri čemu nastaje manja količina toksičnog otpada te se izbjegava korištenje pokusnih životinja. Također, primjenom staničnih kultura se u kratkom vremenu može analizirati veliki broj tvari u širokom rasponu koncentracija što je svakako dobra preliminarna smjernica za planiranje *in*

vivo studija. Nedostaci primjene *in vitro* testova su nepotpuna ili u potpunosti odsutna metabolička aktivacija ispitivane tvari u staničnim sustavima, budući da te stanice ipak imaju izmijenjena svojstva u odnosu na ishodne *in vivo* stanice, te mogućnost reagiranja ispitivane tvari sa sastojcima medija za uzgoj. Najčešće korišten *in vitro* test za određivanje citotoksičnosti kemikalija primjenom kultura stanica je test redukcije tetrazolijeve soli (MTT) (Radojčić Redovniković i sur., 2016).

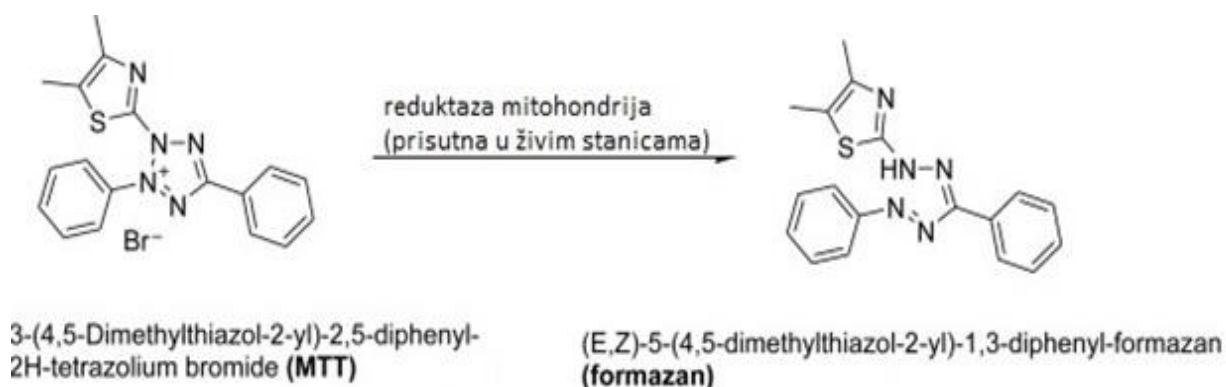
1.4.1. MTT test (test redukcije tetrazolijeve soli)

MTT, odnosno (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid) test redukcije tetrazolijeve soli, jedan je od najčešće korištenih testova prilikom ispitivanja citotoksičnosti. MTT supstrat obično se priređuje u fiziološki izbalansiranoj otopini i dodaje staničnoj kulturi, uglavnom u koncentraciji od 0,2-0,5 mg/ml, te inkubira pri temperaturi od 37 °C u razdoblju od 1 do 4 sata. Količina nastalog formazana (s pretpostavkom da je proporcionalna količini živućih stanica) određuje se mjerenjem apsorbanacije na 570 nm. Naime, stanice koje su žive aktivnim metabolizmom pretvaraju MTT u modro obojani formazan koji ima maksimum apsorbanacije pri 570 nm. S druge strane stanice koje su mrtve, izgubile su sposobnost prevođenja MTT-a u formazan. Formazan, koji je produkt redukcije tetrazolijeve soli, stvara netopljivi talog u stanici. Kako bi se mogla mjeriti apsorbanacija potrebno je ponovno otopiti formazan. Pritom se koriste različite metode kako bi se dobila stabilna boja, izbjeglo isparavanje i smanjila interferencija s crveno obojenim fenolom ili drugim komponentama stanične kulture. Metode solubilizacije najčešće uključuju: zakiseljeni izopropanol, dimetilformamide i kombinacije detergensa i organskih otapala. Zakiseljevanjem otopine za solubilizaciju postiže se promjena boje fenola iz crvene u žutu čime se smanjuje mogućnost interferencije pri mjerenju apsorbanacije.

Količina detektiranog signala ovisi o nekoliko parametara: koncentraciji MTT-a, duljini inkubacijskog perioda, broju živućih stanica i njihovoj metaboličkoj aktivnosti. Svi ti parametri se trebaju uzeti u obzir prilikom optimizacije ispitivanja, kako bi nastala odgovarajuća količina produkta koji se može odrediti spektrofotometrijski. Redukcija MTT-a u formazan u staničnoj kulturi vremenski je ovisna i zbog toga inkubacijom kroz dulji vremenski period dolazi do nakupljanja boje i povećanja osjetljivosti. No, vrijeme inkubacije je ipak ograničeno zbog citotoksične prirode reagensa koji troši energiju stanica za nastanak signala. Uvjeti kulture stanica koji mijenjaju metabolizam utječu i na redukciju MTT-a u formazan. Na primjer, kada se adherentne stanice u kulturi počinju preklapati i njihov daljnji

rast postane kontaktno inhibiran, dolazi do usporavanja metabolizma i usporavanja redukcije MTT-a. U takvoj situaciji dolazi do gubitka linearnosti između apsorbancije i broja stanica. Drugi nepovoljni uvjeti kulture stanica, poput promijenjenog pH ili nedostatka esencijalnih nutrijenata, na primjer glukoze, također dovode do smanjenje redukcijske sposobnosti stanica. Toksičnost MTT-a raste proporcionalno s koncentracijom koja je dodana stanicama te se optimizacijom koncentracije postiže manja toksičnost. Redukcijske tvari, također mogu dovesti do interferencija s testom redukcije tetrazolijeve soli. Kemikalije poput askorbinske kiseline, ili supstancija koje sadrže sulfhidril, poput glutationa, koenzima A, ditiotreitola, mogu neenzimatski reducirati tetrazolijeve soli i dovesti do povišenja vrijednosti apsorbancije. Nadalje, medij u kulturi, povišena vrijednost pH ili direktno izlaganje reagensa svjetlosti može ubrzati redukciju tetrazolijevih soli i dovesti do lažno pozitivnih rezultata apsorbancije. Takav tip kemijskih interferencija može se izbjeći mjerenjem apsorbancije kontrolnih jažica, koje ne sadrže stanice, već samo stanični medij, MTT i različite koncentracije ispitivanog spoja (Riss i sur., 2013).

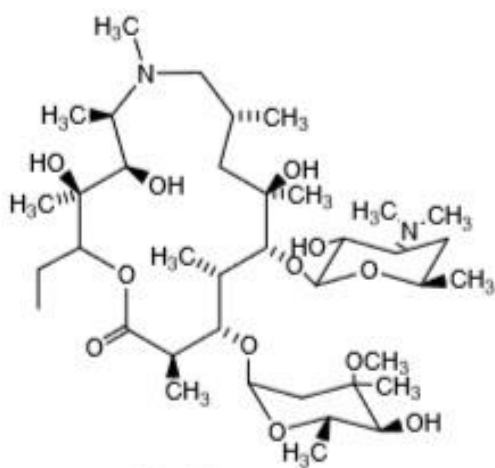
MTT test je, dakle, kolorimetrijska metoda koja se temelji na određivanju metaboličke aktivnosti mitohondrija mjerenjem redukcije topljive žute MTT tetrazolijeve soli u modri netopljivi formazan (Slika 4). Očitana apsorbancija proporcionalna je broju živih stanica u uzorku, pri čemu se preživljenje stanica izražava kao postotak omjera apsorbancije tretiranih i netretiranih (kontrolnih) stanica (Radojčić Redovniković i sur., 2016).



Slika 4. Enzimaska redukcija žuto obojanog MTT-a do modrog formazana. Preuzeto i prilagođeno prema Kuete i sur., 2017, uz dozvolu *Elsevier*-a.

1.5. Azitromicin

Azitromicin (Slika 5), kemijskog imena 9-deokso-9a-aza-9a-metil-9a-homoeritromicin A, je prvi antibiotik sintetiziran u Republici Hrvatskoj (Sumamed[®]), a sintetizirala ga je skupina istraživača iz PLIVA-e 1981. godine. Riječ je o bijelom amorfnom prahu, gorka okusa i bez mirisa. Slabe je topljivosti u vodi, a vrlo dobre u kloroformu i metanolu. Molekularna masa (M_r) iznosi 748,97, temperatura tališta nalazi se između 118 i 122 °C, a logP iznosi 4,02. Azitromicin je prvi predstavnik skupine makrolidnih antibiotika nazvane azalidi te ima širok spektar djelovanja. Molekula je nastala dodavanjem metiliranog dušika u položaj 9a na laktonski prsten eritromicina. Mehanizam djelovanja azitromicina temelji se na vezanju na 50S podjedinicu ribosoma bakterija, na isti receptor kao i eritromicin, ali s većim afinitetom vezanja, čime se remeti sinteza bjelančevina i translokacija peptida (Zorc i Butula, 1995).



Slika 5. Struktura azitromicina. Preuzeto iz Zorc i Butula (1995).

Azitromicin je indiciran za liječenje infekcija: gornjih dišnih putova (faringitis/tonzilitis, sinusitis, otitis media), donjih dišnih putova (akutna egzacerbacija kroničnog bronhitisa i izvanbolnička stečena pneumonija), kože i potkožnog tkiva (*erythema migrans*, erizipel, impetigo i sekundarna piodermija), spolno prenosivih bolesti (nekomplikirane genitalne infekcije izazvane *Chlamydom trachomatis*), urogenitalne infekcije (endometritis i salpingitis uzrokovane s *Chlamydia trachomatis* ili s gonokokom) te infekcije želuca i dvanaesnika uzrokovane s *Helicobacter pylori*.

Bioraspoloživost azitromicina nakon oralne primjene je oko 37%. Vršne koncentracije u plazmi postižu se za 2-3 sata nakon uzimanja te se lijek brzo raspodjeljuje iz plazme u tkiva i organe. Farmakokinetičkim ispitivanjima se pokazalo da azitromicin u tkivima postiže koncentracije i do 50 puta više nego u plazmi, što ukazuje da se lijek snažno deponira u tkiva. Vežanje za proteine u serumu varira ovisno o koncentraciji u plazmi i u rasponu je od 12% pri 0,5 µg/ml do 52% pri 0,05 µg/ml seruma. Završno poluvrijeme eliminacije iz plazme odražava poluvrijeme eliminacije iz tkiva i iznosi 2-4 dana. Oko 12% i.v. primijenjenog azitromicina izlučuje se nepromijenjeno u urinu tijekom sljedeća 3 dana. Osobito visoke koncentracije nepromijenjenog azitromicina prisutne su u žuči. Također je u žuči nađeno deset metabolita, koji su nastali N- i O-demetilacijom, hidroksilacijom dezozamina i aglikonskog prstena te cijepanjem kladinoznih konjugata. Metaboliti nisu mikrobiološki aktivni. Otkriveno je da se veće koncentracije azitromicina oslobađaju za vrijeme aktivne fagocitoze nego za vrijeme inaktivne fagocitoze (www.halmed.hr). Sposobnost prodiranja u stanice omogućuje djelovanje na intracelularne patogene, nakupljanje u neutrofilnim granulocitima, monocitima i makrofagima, koji ga onda procesom kemotaksije usmjeravaju na mjesto upale (Zorc i Butula, 1995).

2. OBRAZLOŽENJE TEME

Azitromicin je danas na tržištu dostupan u ljekovitim oblicima namijenjenim za peroralnu (filmom obložene tablete, suspenzija), oftalmičku (kapi, otopina) i intravensku primjenu. Zbog širokog spektra terapijskih indikacija javlja se potreba za daljnjim razvojem formulacija za topikalnu primjenu na kožu, za liječenje kompliciranih infekcija uzrokovanih *S. aureus*-om i MRSA-om poput *erythema migrans*, erizipela, impetiga te umjerenih do težih oblika *acne vulgaris* i piodermija. Ograničavanjem djelovanja antibiotika na lokalizirano područje infekcije, smanjila bi se sistemska apsorpcija i mogućnost razvoja rezistencije, čime bi se značajno unaprijedila terapija. Uklapanjem azitromicina u liposome, mogla bi se poboljšati topljivost lijeka, omogućiti produljeno i lokalizirano oslobađanje lijeka te poboljšati antimikrobni učinak. Takav sustav trebao bi biti fiziološki prihvatljiv, tj. biokompatibilan sa tkivom na koje se primjenjuje.

Svrha ovog istraživanja bila je ispitati biokompatibilnost nekoliko različitih tipova liposoma s uklopljenim azitromicinom sa stanicama HaCaT keratinocita *in vitro*. Liposomske formulacije međusobno su se razlikovale po fizičkim svojstvima; naboju na površini, elastičnosti/rigidnosti fosfolipidnih dvoslojeva i sadržaju uklopljenog azitromicina.

3.MATERIJALI I METODE

3.1. Materijali

Supstancije, otapala i puferi koji su korišteni u ovom ispitivanju bili su:

- Acetonitril (BDH Prolabo, Lutterworth, Velika Britanija)
- Aposolutni etanol i metanol (BDH Prolabo, Lutterworth, Velika Britanija)
- Azitromicin dihidrat (PLIVA d.o.o., Zagreb, Republika Hrvatska)
- Dimetildioktadecilamonijev bromid (DODAB) (Sigma-Aldrich Company, St. Louis, Sjedinjene Američke Države)
- Dipalmitoilfosfatidilkolin (DPPC) (Lipoid GmbH, Ludwigshafen, Njemačka)
- Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermo, Paisley, Velika Britanija)
- Keratinociti stanične linije HaCaT (Cell Line Services, Njemačka)
- Natrijev deoksikolat (SDCh, Sepadex G-50) (Sigma-Aldrich Company, St. Louis, Sjedinjene Američke Države)
- 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazol bromid (MTT) (Sigma-Aldrich Company, St. Louis, Sjedinjene Američke Države)
- Propilenglikol (Kemika, Zagreb, Republika Hrvatska)
- Sojin lecitin (Lipoid S75) (Lipoid GmbH, Ludwigshafen, Njemačka)
- Fosfatni pufer (PBS 0,01 M), pripljmljen otapanjem 1,3609 g KH_2PO_4 u destiliranoj vodi, u tikvici od 1000 ml i podešavanjem pH s 10 M KOH
- 10 %-tni fetalni goveđi serum (Gibco, Thermo Fisher Scientific,)
- mješavina penicilina, streptomocina i amfotericina B (Lonza, Basel, Švicarska).

3.2. Metode

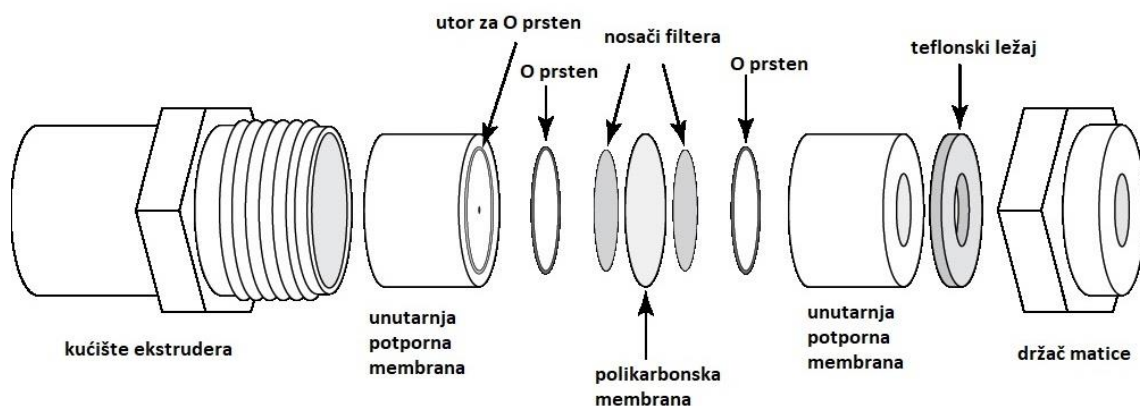
3.2.1. Priprema liposoma

Metodom hidratacije suhog fosfolipidnog sloja (filma) pripremljene su različite preparacije liposoma s azitromicinom korištene u ovom istraživanju: konvencionalni liposomi, deformabilni liposomi, propilenglikol liposomi i kationski liposomi.

Konvencionalni liposomi su pripremljeni koristeći 100 mg sojinog lecitina i 15 mg azitromicina. Deformabilni liposomi su u lipidnoj fazi sadržavali 85 mg sojinog

fosfatidilkolina, 15 mg natrijevog deoksikolata i 15 mg azitromicina. Propilenglikol liposomi su pripremljeni koristeći 100 mg sojinog lecitina, 1500 mg propilenglikola i 15 mg azitromicina, dok su kationski liposomi u lipidnoj fazi sadržavali 73 mg dipalmitoilfosfatidilkolina, 27 mg dioktadecildimetilamonijev bromid (DODAB) i 15 mg azitromicina. Izvagane količine (fosfo)lipida i azitromicina za svaku pojedinu preparaciju liposoma prenešene su u tikvice okruglog dna i otopljene u 3 ml koncentriranog etanola. Pomoću rotirajućeg vakuum uparivača potpuno je uklonjen etanol na temperaturi od 40 °C za konvencionalne, deformabilne i propilenglikol liposome, odnosno 50 °C za kationske liposome. Suhi, lipidni film koji je nastao na stijenkama tikvica, hidratiziran je dodatkom 5 ml fosfatnog pufera, na sobnoj temperaturi za pripremu konvencionalnih i deformabilnih liposoma, te na 50 °C za pripremu kationskih liposoma. Za pripremu propilenglikol liposoma lipidnom filmu je dodana 30%-tna (v/v) otopina propilenglikola u fosfatnom puferu. Tako pripravljene disperzije homogenizirane su ručnim ekstruderom (Slika 6) protiskivanjem liposoma kroz polikarbonske membrane od 400 nm (3 puta) i jednaput kroz membrane od 200 nm. Postupak je proveden na sobnoj temperaturi za konvencionalne, deformabilne, propilenglikol i kationske liposome.

„Prazni“ liposomi, odnosno liposomi bez uklopljene djelatne tvari (azitromicina), pripremljeni su istim postupkom i pod istim uvjetima kao liposomi s azitromicinom, a služili su kao kontrola u ispitivanjima citotoksičnosti.



Slika 6. Shematski prikaz dijelova ekstrudera. Preuzeto i prilagođeno s

<https://avantilipids.com/divisions/equipment-products>.

3.2.2. Određivanje veličine liposoma i indeksa polidisperznosti

Srednji promjer i indeks polidisperznosti različitih liposoma s azitromicinom određeni su metodom fotonske korelacijske spektroskopije pri čemu je korišten uređaj Zetasizer 3000 HS (Malvern Instruments, Malvern, Velika Britanija, Slika 7). Mjerenje se izvodilo 24 sata nakon pripreme liposoma, pod kutem raspršenja od 90 ° i temperaturi od 25 °C. Uzorci liposoma su razrijeđeni fosfatnim puferom, koji je prethodni filtriran kroz Minisart filtere veličine pora 200 nm. Mjerenje veličine liposoma izvodilo se prije i nakon ekstruzije samih preparacija.



Slika 7. Zetasizer 3000HS. Preuzeto s

<http://www.etseq.urv.cat/dinamic/english/presentacio/equips/zsizer.htm>.

3.2.3. Određivanje zeta potencijala liposoma

Mjerenje zeta potencijala liposoma izvršeno je pomoću Zetasizer 3000 HS (Malvern Instruments, Malvern, Velika Britanija) koristeći protočnu kivetu s optičkim modulatorom u radnom području od 1000 Hz. Kako bi mjerenje bilo valjano, uređaj je prethodno kalibriran standardnom disperzijom koja je imala deklariranu vrijednost zeta potencijala od -50 ± 5 mV (Malvern Zeta Potential Transfer Standard, Malvern Instruments, Malvern, Velika Britanija). Mala količina uzorka liposoma (nekoliko kapi) razrijeđena je s 1 mM otopine NaCl. Mjerenja su provedena na temperaturi od 25 °C.

3.2.4. Određivanje uspješnosti uklapanja azitromicina u različitim vrstama liposoma

Kako bi se odredila uspješnost uklapanja bilo je potrebno odijeliti uklopljenu od neuklopljene frakcije azitromicina. Pritom je korišten postupak ultracentrifugiranja koji je proveden tako da je od svake preparacije liposoma uzet 1 ml i razrijeđen s 4 ml fosfatnog pufera. Uzorci su ultracentrifugirani (Beckman Optima LE-80 K Ultracentrifuge, Beckman Coulter Inc., Fullerton, SAD) u vremenskom periodu od 1 sata na 120.000 g pri temperaturi od 20 °C. Supernatant (neuklopljeni azitromicin) je oprezno uklonjen, a pelet je ispran fosfatnim puferom i zatim ponovno ultracentrifugiran pod gore navedenim uvjetima. Nakon drugog ultracentrifugiranja, dobiveni talog (pelet) je resuspendiran s 1 ml fosfatnog pufera na početni volumen disperzije.

Količina slobodnog i uklopljenog lijeka određena je pomoću HPLC-a. Količina azitromicina uklopljenog u liposomima određena je nakon otapanja lipidnih komponenti liposoma u metanolu. Uspješnost uklapanja lijeka i analitički prinos (eng. *recovery*) izraženi su pomoću izraza:

$$\text{Uspješnost uklapanja (\%)} = \frac{\text{lijek u liposomima (LL)}}{\text{lijek u liposomima (LL)} + \text{slobodan lijek (SL)}} \cdot 100$$

$$\text{Analitički prinos (\%)} = \frac{\text{lijek u liposomima (LL)} + \text{slobodan lijek (SL)}}{\text{ukupna količina lijeka u disperziji liposoma (UK)}} \cdot 100.$$

Ukupna količina lijeka u liposomskoj disperziji sadržavala je uklopljeni i neuklopljeni azitromicin, a određena je tako da je liposomskoj disperziji dodan metanol za otapanje lipidnih komponenti i oslobađanje uklopljenog azitromicina. Analitički prinos iznosio je od 96,1-109,9 % za sve uzorke.

3.2.5. Ispitivanja citotoksičnosti/biokompatibilnosti *in vitro*

Keratinociti stanične linije HaCaT (Cell Line Services, Njemačka) su kultivirani u DMEM-u uz dodatak 10 %-tnog fetalnog goveđeg seruma i mješavinu penicilina, streptomicina i amfotericina B. Stanice su nasadene na ploče s 96 jažica uz gustoću od 10^4 odnosno $1,5 \cdot 10^4$ stanica po jažici, te su inkubirane 48 sati kako bi se postigla potpuna prekrivenost dna jažica sa stanicama (100%-tna konfluentnost). Liposomi, kojima je prethodno uklonjen neuklopljeni azitromicin, ili etanolno-vodena otopina azitromicina (6/4, v/v) su suspendirani u DMEM-u u koncentracijama azitromicina od 0,25, 1, 16, 64 i 256 µg/ml. Potom je keratinocitima iz jažica uklonjen hranidbeni medij, pažljivo su isprane s

fosfatnim puferom te su dodani uzorci liposoma s azitromicinom ili otopine azitromicina. Nakon 24 sata inkubacije mjerena je *in vitro* citotoksičnost korištenjem testa redukcije tetrazolijeve soli. „Prazni“ liposomi i otapalo za izradu otopine azitromicina služili su kao kontrole te su podvrgnuti identičnom, gore opisanom, ispitivanju.

3.2.6. Test redukcije tetrazolijeve soli (MTT test)

MTT test korišten je kako bi se utvrdila metabolička aktivnost HaCaT stanica nakon što su bile izložene različitim tipovima liposoma s uklopljenim azitromicinom ili otopini azitromicina. Postupak je poveljen tako da je 24 sata nakon tretiranja stanica s liposomima, odnosno otopinom azitromicina, stanicama uklonjen medij sa suspendiranim ispitivanim uzorcima, te su dva puta isprane fosfatnim puferom i inkubirane sa svježim hranidbenim medijem (24 sata). Nakon toga, u svaku pojedinu jažicu dodano je 10 μ l MTT otopine te su stanice inkubirane daljnjih 30 minuta na 37 °C. Potom je uklonjen medij, a istaloženi modri formazan je ekstrahiran dodatkom 100 μ l kiselog izopropanola u svaku jažicu te su izmjerene apsorbancije na 570 nm (Victor, PerkinElmer, SAD). Mitohondrijska aktivnost HaCaT stanica izražena je u odnosu na aktivnost netretiranih stanica, odnosno onih koje su sadržavale samo hranjivi medij.

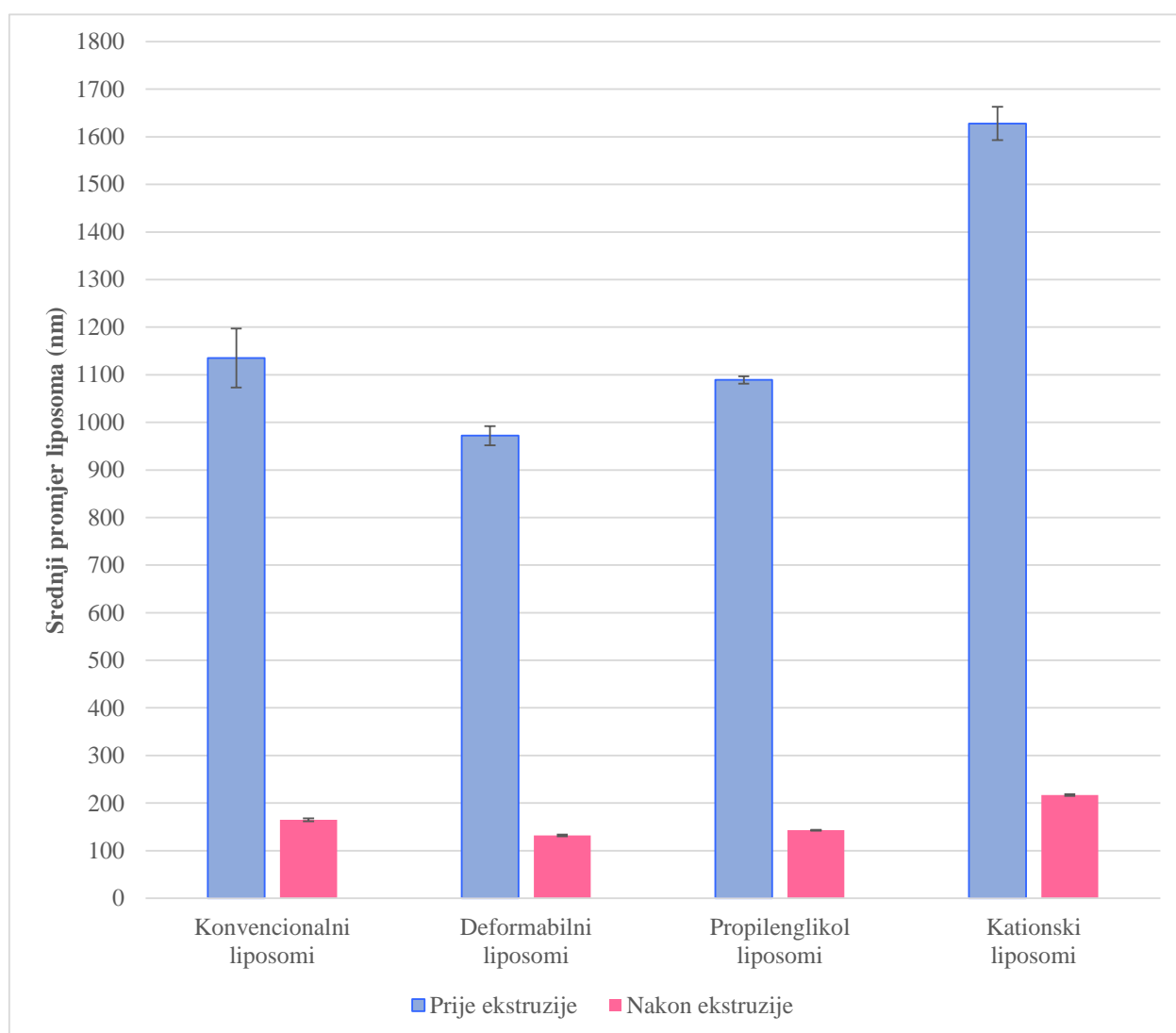
3.2.7. Statistička obrada podataka

Statistička analiza provedena je koristeći *one-way ANOVA* i *Tukey's multiple comparison test* za usporedbu tri ili više skupina podataka. Razina značajnosti iznosila je 0,05 tj. 5%. Ako je $p < 0,05$ postoji statistički značajna razlika među skupinama, dok za $p > 0,05$ ne postoji statistički značajna razlika među skupinama. Razine značajnosti testova izračunate su koristeći *GraphPad 8 Prism* program.

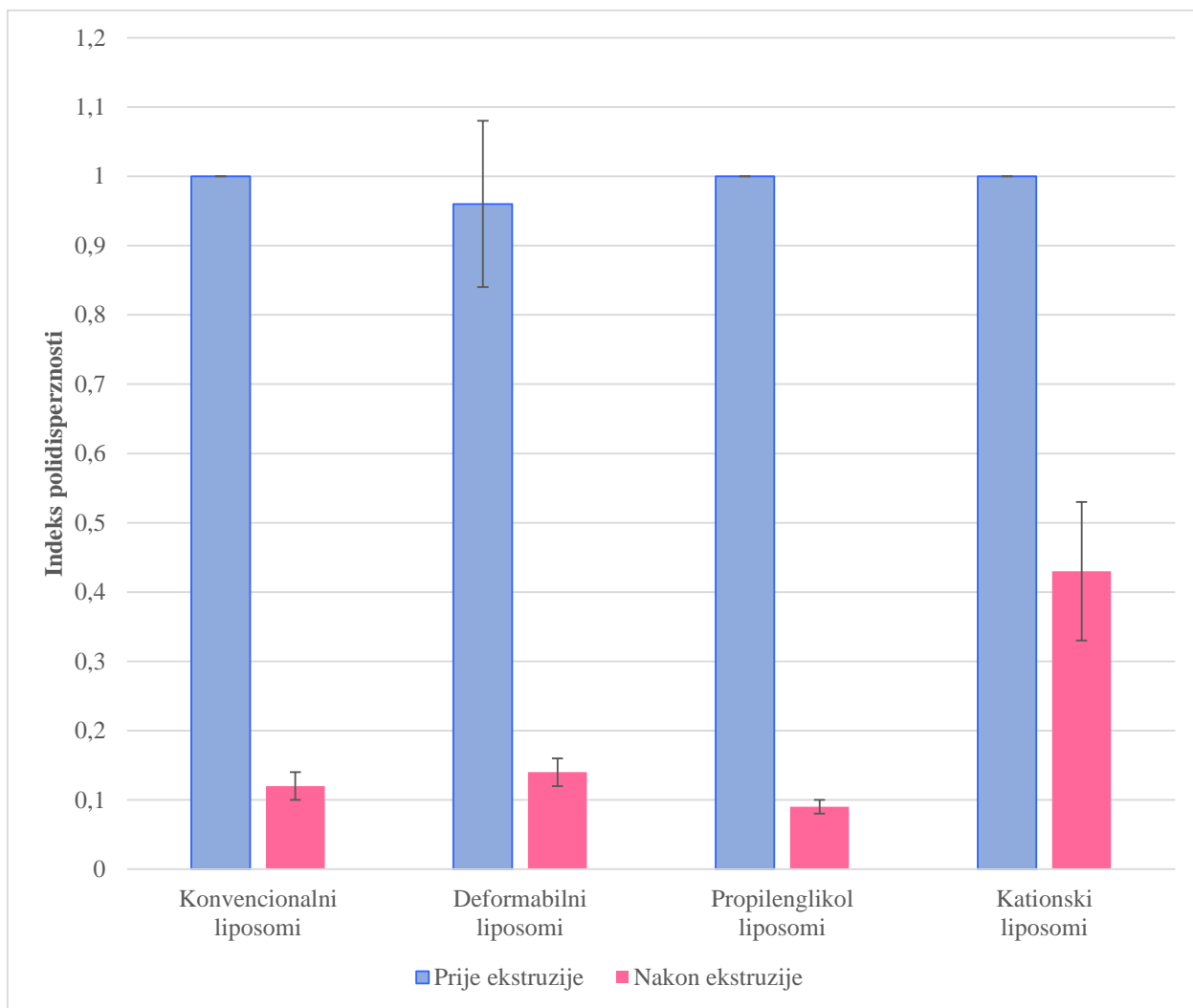
4. REZULTATI I RASPRAVA

4.1. Fizikalno-kemijske karakteristike liposoma

Konvencionalni, deformabilni, propilenglikol i kationski liposomi pripremljeni su film metodom uz homogenizaciju ekstruzijom. Rezultati određivanja srednjeg promjera i indeksa polidisperznosti različitih tipova liposoma s azitromicinom prikazani su Slikama 8 i 9, zeta potencijala Slikom 10, a uspješnosti uklapanja Tablicom 1.



Slika 8. Grafički prikaz srednjeg promjera različitih vrsta liposoma prije i nakon ekstruzije



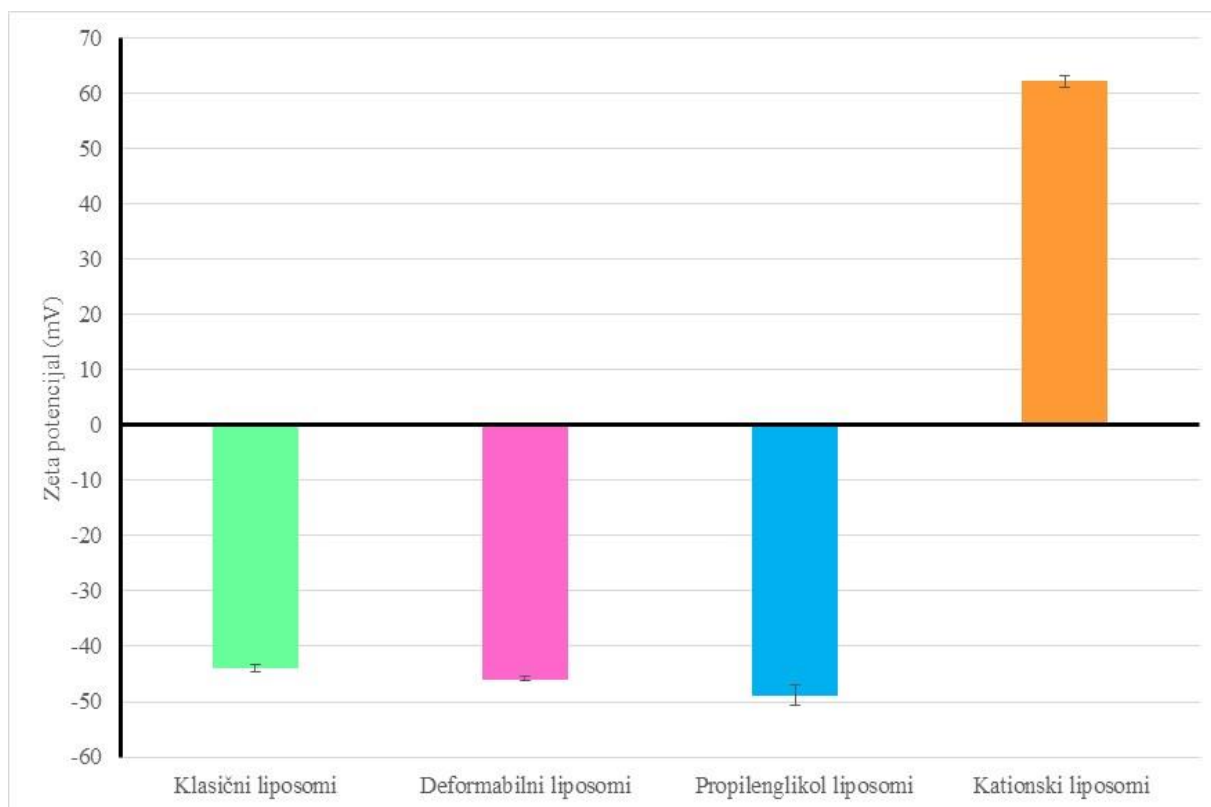
Slika 9. Grafički prikaz indeksa polidisperznosti različitih vrsta liposoma prije i nakon ekstruzije

Iz prikazanih rezultata (Slike 8 i 9) vidljivo je da su izvorno pripremljeni liposomi bili izrazito veliki i visokog indeks polidisperznosti ($PDI > 0,95$), što je bilo i očekivano budući su liposomi pripremljeni film metodom. Visok indeks polidisperznosti (0,96-1,0) upućuje na prisutnost liposoma široke distribucije veličina, tj. promjera, no s druge strane upućuje i da izmjereni srednji promjeri nisu apsolutno točni tim više jer je instrument kojim je provedeno mjerenje prikladan za nanometarsko mjerno područje. Raspon izmjerenih srednjih promjera kretao se od 972 nm (deformabilni liposomi) preko 1089 nm (propilenglikol liposomi) do 1135 nm (konvencionalni liposomi), dok su s druge strane kationski liposomi bili znatno veći od svih ostalih (1628 nm).

Kako bi se smanjio indeks polidisperznosti, disperzije liposoma su homogenizirane ekstruzijom. Time su dobiveni liposomi značajno manjeg srednjeg promjera (132-165 nm za

deformabilne, propilenglikol i kationske liposome, a 217 nm za kationske liposome) i nižih vrijednosti indeksa polidisperznosti (0,43 za kationske, 0,12 za konvencionalne, 0,14 za deformabilne i 0,09 za propilenglikol liposome) (Slika 9). Kationski liposomi bili su veći od ostalih jer je ekstruzija izvršena na sobnoj temperaturi. U njihovom sastavu prisutni su sintetski lipidi s višom Tc (New, 1990), te je membrana kationskih liposoma u datim uvjetima mjerenja bila poprilično rigidna (čvrsta) u usporedbi s ostalim ispitivanim liposomima što se odrazilo na izmjenjenim vrijednostima srednjeg promjera.

Raznolikost u fizičko-kemijskim svojstvima (fosfo)lipida prisutnih u lipidnim dvoslojevima liposoma s azitromicinom reflektirala se na vrijednosti zeta potencijala. Konvencionalni, deformabilni i propilenglikol liposomi bili su anionskog karaktera s vrijednostima zeta potencijala između -40 i -50 mV. Kationski liposomi su zbog prisutnog DODAB-a imali visoke vrijednosti zeta potencijala (> 60 mV) (Slika 10). Takve vrijednosti zeta potencijala ukazuju na dobru fizičku stabilnost liposoma.



Slika 10. Grafički prikaz vrijednosti zeta potencijala ispitivanih liposoma

4.2. Uklapanje azitromicina u liposome

Kako bi se primjenom liposoma postigao željeni terapijski učinak nužno je da je u njima uklopljena adekvatna količina lijeka (Vanić i sur., 2012a). Zato je tijekom optimizacije postupka pripreme liposoma nužno dobiti liposome sa zadovoljavajućim sadržajem uklopljene djelatne tvari.

Uspješnost uklapanja azitromicina u liposome različitog sastava određena je nakon odjeljivanja neuklopljenog lijeka ultracentrifugiranjem i njegove kvantifikacije HPLC-metodom. Rezultati prikazani Tablicom 1 pokazuju da je uspješnost uklapanja azitromicina u različite liposomske preparacije iznosila 45-64%. Elastični liposomi, pogotovo deformabilni liposomi su pokazali bolje uklapanje azitromicina od konvencionalnih i kationskih liposoma, najvjerojatnije zbog učinka natrijevog deoksikolata (deformabilni liposomi) i propilenglikola (propilenglikol liposomi) na solubilizaciju azitromicina unutar fosfolipidnih dvoslojeva. Bolje uklapanje u propilenglikol liposome i deformabilne propilenglikol liposome pokazano je u ranijim istraživanjima s hidrofilnim i lipofilnim djelatnim tvarima (Palac i sur., 2014; Vanić i sur. 2014). Nešto niže, ali i dalje dovoljno visoko uklapanje azitromicina postignuto je s konvencionalnim liposomima (52%), dok je najniže bilo u kationskim liposomima (45%).

Tablica 1. Uspješnost uklapanja azitromicina

Vrsta liposoma	Azitromicin (%)
Deformabilni liposomi	64,20 ± 1,34
Kationski liposomi	45,00 ± 1,24
Konvencionalni liposomi	52,10 ± 1,34
Propilenglikol liposomi	56,10 ± 3,38

Rezultati su prikazani kao srednja vrijednost ± S.D. (n=4).

4.3. Ispitivanje biokompatibilnosti liposoma s HaCaT stanicama *in vitro*

Mjerenjem stanične metaboličke aktivnosti HaCaT stanica, nakon tretiranja s uzorcima liposoma, utvrđena je citotoksičnost/biokompatibilnost različitih liposomskih preparacija azitromicina s keratinocitima. HaCaT stanice su tijekom 24 sata bile izložene liposomima s uklopljenim azitromicinom ili otopini azitromicina u koncentracijama 0,25 do 256 µg/ml. Pritom su testirane koncentracije bile značajno veće od minimalnih biofilm inhibitornih

koncentracija azitromicina za *S. aureus* ATCC 29213 i MRSA kliničke izolate: MRSA 10674, MRSA 10676, MRSA 10677, MRSA 10679 i MRSA 10680 (Rukavina i sur., 2018).

Rezultati prikazani Tablicom 2 potvrđuju biokompatibilnost svih ispitivanih tipova liposoma s azitromicinom s keratinocitima. Čak i pri koncentraciji azitromicina od 64 µg/ml, koja je 16-256 puta bila veća od minimalnih biofilm inhibitornih koncentracija za ispitivane liposome (Rukavina i sur., 2018), vijabilnost stanica je bila veća od 70%. Tek pri dvostruko-većoj testiranoj koncentraciji azitromicina (256 µg/ml) deformabilni i kationski liposomi pokazali su citotoksičan učinak. Vijabilnost keratinocita iznosila je 38% za deformabilne liposome i 45% za kationske liposome. Nasuprot tome, konvencionalni i propilenglikol liposomi bili su biokompatibilni (vijabilnost > 70%), pri čemu je bolja kompatibilnost s HaCaT stanicama postignuta s propilenglikol liposomima. Nadalje, otopina azitromicina pokazala je citotoksičan učinak na HaCaT stanice pri koncentraciji 64 µg/ml. To znači da se uklapanjem azitromicina u liposome značajno smanjuje njegova citotoksičnost.

Utvrđeno je da ne postoji statistički značajna razlika (ANOVA, $p > 0,05$) u vijabilnosti keratinocita nakon tretiranja s različitim vrstama liposoma.

Kako bi se utvrdilo koliko sastavnice liposoma, prisustvo suotapala i otapalo korišteno za izradu otopine azitromicina doprinose biokompatibilnosti/citotoksičnosti, provedena su ispitivanja s „praznim“ liposomima i otapalom, pod istim uvjetima i pri koncentraciji lipida/otapala koje odgovaraju koncentracijama lipida/otapala u preparacijama liposoma s azitromicinom, odnosno otopinom azitromicina.

Rezultati prikazani Tablicom 3 pokazuju da je vijabilnost HaCaT stanica pri tretiranju konvencionalnim i propilenglikol liposomima u cijelom rasponu koncentracija, uključujući i najveću koncentraciju (853 µg/ml), bila veća od 70%, što potvrđuje biokompatibilnost konvencionalnih i propilenglikol liposoma s HaCaT stanicama. Prema ISO10993-5 odredbi, vijabilnost stanica manja od 70% smatra se citotoksičnom (ISO10993-5). Tako je vijabilnost keratinocita nakon 24-satnog tretiranja s ispitivanim konvencionalnim i propilenglikol liposomima u koncentraciji lipida od 853 µg/ml iznosila čak 88%. Nasuprot tome, vijabilnost keratinocita nakon tretiranja s deformabilnim i kationskim liposomima je bila veća od 70% samo u rasponu koncentracija lipida 0,83 µg/ml do 213 µg/ml. Vijabilnost HaCaT stanica je nakon tretiranja s deformabilnim i kationskim liposomima pri najvišoj koncentraciji lipida (853 µg/ml) iznosila 56% za deformabilne i 65% za kationske liposome. Takav učinak posljedica je prisutnog natrijevog deoksikolata (iz deformabilnih liposoma) i DODAB-a iz

kationskih liposoma. Naime, za DODAB je poznato da može pri visokim koncentracijama izazivati toksične učinke (Mamizuka i Carmona-Ribeiro, 2007).

Nasuprot liposomima, ispitivanja provedena s otapalom korištenim za otapanje azitromicina (otopina azitromicina), pokazala su citotoksičan učinak otapala pri većim testiranim koncentracijama, koje odgovaraju koncentracijama lipida od 213 i 853 $\mu\text{g/ml}$ (Tablica 3). Takvi rezultati su na neki način i bili očekivani uzimajući u obzir veći udio etanola u otapalu pri većim ispitivanim koncentracijama otapala.

Statističkom analizom rezultata prikazanih u Tablici 3, uvrđeno je da postoji statistički značajna razlika u vijabilnosti keratinocita nakon tretiranja različitim liposomima, odnosno uočena je statistički značajna razlika u vijabilnosti HaCaT stanica nakon tretiranja s konvencionalnim i propilenglikol liposomima u odnosu na otapalo (ANOVA, $p < 0,05$).

Imajući u vidu rezultate ispitivanja biokompatibilnosti prikazane Tablicama 2 i 3, očito je da sastav liposoma i koncentracija azitromicina značajno utječu na njihovu kompatibilnost s keratinocitima. Međutim, prilikom donošenja zaključaka potrebno je uzeti u obzir koncentracije tvari s kojima su izvedena ispitivanja biokompatibilnosti/citotoksičnosti i koncentraciju azitromicina na kojoj su liposomi pokazali zadovoljavajući antimikrobni učinak. Koncentracije lipida i azitromicina korištene u ovom radu su bile poprilično visoke. One su primjerice za kationske liposome, koji su pokazali citotoksičan učinak pri najvećoj testiranoj koncentraciji, bile čak 256 puta veće od minimalne biofilm inhibitorne koncentracija za MRSA kliničke izolate (Rukavina i sur., 2018). Ukoliko bi se formulacija kationskih liposoma primijenivala *in vivo*, koncentracija azitromicina u liposomima bila bi daleko niža od te koncentracije. Slično vrijedi i za deformabilne liposome.

Tablica 2. Biokompatibilnost liposoma s HaCat stanicama *in vitro*

Azitromicin u liposomima (µg/ml)	<u>Vijabilnost keratinocita 24 sata nakon inkubacije (%)</u>				
	<u>Konvencionalni liposomi</u>	<u>Deformabilni liposomi</u>	<u>Propilenglikol liposomi</u>	<u>Kationski liposomi</u>	<u>Otopina azitromicina</u>
0,25	97,76 ± 3,58	87,27 ± 8,40	99,42 ± 3,22	90,83 ± 13,49	83,25 ± 5,10
1	99,18 ± 5,81	82,90 ± 1,71	92,16 ± 1,64	85,28 ± 6,94	81,13 ± 0,76
4	90,79 ± 1,03	80,73 ± 1,36	97,40 ± 9,75	80,52 ± 3,39	68,48 ± 9,23
16	83,27 ± 1,31	70,50 ± 1,23	87,68 ± 8,20	73,88 ± 4,97	65,62 ± 7,53
64	85,40 ± 8,35	69,59 ± 2,74	81,46 ± 2,58	70,10 ± 2,77	40,79 ± 8,67
256	75,97 ± 5,93	38,28 ± 2,18	81,58 ± 3,10	45,35 ± 7,94	9,56 ± 2,51

Vijabilnost stanica manja od 70%

Tablica 3. Biokompatibilnost „praznih“ liposoma s HaCaT stanicama *in vitro*

Lipidi u liposomima (µg/ml)	<u>Vijabilnost keratinocita 24 sata nakon inkubacije (%)</u>				
	<u>Konvencionalni liposomi („prazni“)</u>	<u>Deformabilni liposomi („prazni“)</u>	<u>Propilenglikol liposomi („prazni“)</u>	<u>Kationski liposomi („prazni“)</u>	<u>Otapalo</u>
0,83	96,70 ± 4,64*	90,56 ± 5,02	107,50 ± 5,75*	101,17 ± 9,51	87,03 ± 5,10
3,3	103,50 ± 3,63*	78,83 ± 2,40	97,76 ± 4,96*	98,66 ± 8,15	81,15 ± 0,85
13,3	96,66 ± 5,38*	77,22 ± 3,74	85,82 ± 9,47*	87,73 ± 4,33	73,99 ± 9,23
53,3	89,91 ± 2,53*	73,01 ± 6,61	96,72 ± 7,07*	76,89 ± 7,27	72,83 ± 7,53
213,3	86,21 ± 8,47*	73,01 ± 4,87	85,05 ± 5,66*	75,40 ± 7,44	55,06 ± 8,67
853,2	87,93 ± 6,97*	55,91 ± 8,76	88,38 ± 7,04*	65,34 ± 8,08	41,95 ± 3,55

Vijabilnost stanica manja od 70%; * Statistički značajna razlika u usporedbi s otapalom (ANOVA, p < 0,05)

5. ZAKLJUČCI

Na temelju rezultata provedenih istraživanja mogu se izvesti sljedeći zaključci:

- Film metodom priređeni konvencionalni, deformabilni, propilenglikol i kationski liposomi s azitromicinom su bili velikog srednjeg promjera i visokog indeksa polidisperznosti.
- Ekstruzijom izvornih liposomskih disperzija dobivene su preparacije liposoma homogenije distribucije veličina i srednjeg promjera do 220 nm.
- Fosfolipidni sastav utjecao je na naboj na površini liposoma. Konvencionalni, deformabilni i propilenglikol liposomi su bili anionskog karaktera, dok su oni s DODAB-om bili kationskog karaktera.
- Izrazito negativne i pozitivne vrijednosti zeta potencijala svih liposoma s azitromicinom ukazuju na potencijalnu dobru fizičku stabilnost formulacija tijekom uskladištenja.
- Ispitivanja citotoksičnosti liposoma *in vitro* provedena na HaCaT stanicama potvrđuju da se uklapanjem u liposome značajno smanjuje citotoksičnost lijeka.
- (Fosfo)lipidni sastav i suotapalo iz liposoma utjecalo je na biokompatibilnost liposoma.
- Svi ispitivani liposomi su bili biokompatibilni s keratinocitima pri koncentracijama značajno većim od onih u kojima su formulacije pokazale antimikrobni učinak.

6. LITERATURA

Azitromicin, Halmed, <http://www.halmed.hr/Lijekovi/Baza-lijekova/>, pristupljeno 8.4.2019.

Banović J, Bego M, Cuković N, Vanić Ž. Lipidne vezikule za (trans)dermalnu primjenu lijekova. *Farm Glas*, 2011, 76, 229-244.

Brownovo gibanje, www.malvern.com, pristupljeno 12.4.2019.

Boukamp P, Popp S, Altmeyer S, Hulsen A, Fasching C, Cremer T, Fusenig NE. Sustained nontumorigenic phenotype correlates with a largely stable chromosome content during long-term culture of the human keratinocyte line HaCaT. *Genes Chromosomes Cancer*, 1997, 19, 201-214.

Cevc G, Schatzein A, Blume G. Transdermal drug carriers, basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. *J Control Release*, 1995, 36, 3-16.

Creech CB, Al-Zubeidi DN, Fritz SA. Prevention of Recurrent Staphylococcal Skin Infections. *Infect Dis Clin North Am*, 2015, 29, 429-464.

Dinamičko raspršenje svjetlosti, <https://www.ugent.be/en>, pristupljeno 20.3.2019.

Forier K, Raemdonck K, De Smedt SC, Demeester J, Coenye T, Braeckmans K. Lipid and polymer nanoparticles for drug delivery to bacterial biofilms. *J Control Release*, 2014, 190, 607-623.

Grada ekstrudera, <https://avantilipids.com/divisions/equipment-products>, pristupljeno 20.2.2019.

Goyal R, Macri LK, Kaplan HM, Kohn J. Nanoparticles and nanofibers for topical drug delivery. *J Control Release*, 2016, 240, 77-92.

Honary S, Zahir F. Effect of Zeta potential on the Properties of Nano – Drug Delivery Systems – A Review (Part 1). *Trop J Pharm Res*, 2013, 12, 255-264.

ISO 10993-5, 1992. Biological Evaluation of Medical Devices-Part 5: Tests for cytotoxicity: *In vitro* methods.

Jalšenjak I, Jalšenjak V, Filipović-Grčić J. Farmaceutika. Zagreb, Školska knjiga, 1998, 2930, 129-130.

Kuete V, Karaosmanoğlu O, Sivas H. Anticancer Activities of African Medicinal Spices and Vegetables. U: Medicinal Spices and Vegetables from Africa. Kuete V, urednik, London, Academic Press, 2017, 271–297.

Larsson M, Hill A, Duffy J. Suspension Stability, Why particle size, Zeta Potential and Rheology are Important. Annual transactions of the nordic rheology society, 2012.

Lee BY, Singh A, David MZ, Bartsch SM, Slayton RB, Huang SS, Zimmer SM, Potter MA, Macal CM, Lauderdale DS, Miller LG, Daum RS. The economic burden of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA). *Clin Microbiol Infect*, 2013, 19, 528–536.

Lehmann TA, Modali R, Boukamp P, Stanek J, Bennett WP, Welsh JA, Metcalf RA, Stampfer MR, Fusenig NE, Rogan EM, Harriss CC. p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis*, 1993, 14, 833-839.

Liu X, Li Z, Wang X, Chen Y, Wu F, Men K, Luo Y, Xu T, Yang, L. Novel antimicrobial peptide–modified azithromycin-loaded liposomes against methicillin-resistant *Staphylococcus aureus*. *Int J Nanomedicine*, 2016, 11, 6781–6794.

Lopes SCA, Giuberti CS, Rocha TGR, Ferreira DS, Leite EA, Oliveira MC. Liposomes as carriers of anticancer drugs. U: Cancer Treatment Conventional and Innovative Approaches. Rangel R, urednik, InTech, 2013, 85-124.

Mamizuka EM, Carmona-Ribeiro AM. Cationic liposomes as antimicrobial agents. U: Communicating Current Research and Educational Topics and Trends in Applied Microbiology. Mendez-Vilas A, urednik, Bajadoz, Formatex, 2007, 636-647.

McNeil SE. Characterization of Nanoparticles Intended for Drug Delivery. McNeil SE, urednik, New York, Humana Press, 2011, 63-78.

Mu L-M, Ju R-J, Liu R, Bu Y-Z, Zhang J-Y, Li X-Q, Lu W-L. Dual-functional drug liposomes in treatment of resistant cancers. *Advanced Drug Delivery Reviews*, 2016, 115, 46–56.

New R.R.C. Liposomes: A practical approach. Oxford, IRL Press, 1990.

Ostrowsky N. Liposome size measurements by photon correlation spectroscopy. *Chem Phys Lipids*, 2013, 64, 45–56.

Radojčić Redovniković I, Bubalo M C, Gaurina Srček M, Radošević K. Primjena kultura stanica za određivanje biološke aktivnosti spojeva iz biljaka. *Croatian Journal of Food Technology, Biotechnology and Nutrition*, 2016, 11, 169-175.

Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, Minor L. Cell Viability Assays. Sittampalam GS, Coussens NP, Brimacombe K, urednici, Assay Guidance Manual. Bethesda, Eli Lilly & Company and the National Center for Advancing Translational Sciences, 2013.

Palac Z, Engesland A, Flaten GE, Škalko-Basnet N, Filipović-Grčić J, Vanić Ž. Liposomes for (trans)dermal drug delivery: the skin-PVPA as a novel in vitro stratum corneum model in formulation development. *J Liposome Res*, 2014, 24, 313–322.

Rukavina Z, Šegvić-Klarić M, Filipović-Grčić J, Lovrić J, Vanić Ž. Azithromycin-loaded liposomes for enhanced topical treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections. *Int J Pharm*, 2018, 553, 109-119.

Vanić Ž. Liposomi kao nosači lijekova: strukturalna svojstva i klasifikacija. *Farm Glas*, 68, 2012a, 391-400.

Vanić Ž. Liposomi kao nosači lijekova: metode pripreme. *Farm Glas*, 68, 2012b, 457-466.

Vanić Ž. Phospholipid Vesicles for Enhanced Drug Delivery in Dermatology. *J Drug Discov Develop and Deliv*, 2015, 1-9.

Vanić Ž, Hurler J, Ferderber K, Golja Gašparović P, Škalko-Basnet N, Filipović-Grčić J. Novel vaginal drug delivery system: deformable propylene glycol liposomes-in-hydrogel. *J Liposome Res*, 2014, 24, 27–36.

Verma D. Particle size of liposomes influences dermal delivery of substances into skin. *Int J Pharm*, 2003, 258, 141–151.

Wang L, Hu C, Shao L. The antimicrobial activity of nanoparticles: present situation and prospects for the future. *Int J Nanomedicine*, 2017, 12, 1227–1249.

Weiner N, Williams N, Birch G, Ramachandran C, Shipman C, Flynn G. Topical delivery of liposomally encapsulated interferon evaluated in a cutaneous herpes guinea pig model. *Antimicrob Agents Chemother*, 1989, 33, 1217– 1221.

Yah CS, Simate GS. Nanoparticles as potential new generation broad spectrum antimicrobial agents. *DARU*, 2015, 23.

Zeta potencijal, <https://www.brookhaveninstruments.com/what-is-zeta-potential>, pristupljeno 2.4.2019.

Zeta-sizer Malvern 3000 HS,
<http://www.etseq.urv.cat/dinamic/english/presentacio/equips/zsizer.htm>, pristupljeno 3.2.2019.

Zhang L, Pornpattananankul D, Hu C-M, Huang C-M. Development of Nanoparticles for Antimicrobial Drug Delivery. *Curr Med Chem*, 2010, 17, 585–594.

Zorc B, Butula I. Vježbe iz farmaceutske kemije. Sveučilište u Zagrebu, Farmaceutsko-biokemijski fakultet, Zagreb, 1995, 12-16.

7. SAŽETAK

Različite vrste liposoma, konvencionalni, deformabilni, propilenglikol i kationski liposomi s uklopljenim azitromicinom, pripremljeni su film metodom. Ekstruzijom kroz polikarbonske membrane, formulacije liposoma su homogenizirane (indeks polidisperznosti $< 0,43$), a srednji promjeri značajno smanjeni (< 250 nm). Fosfolipidni sastav liposoma imao je utjecaja na površinski naboj liposoma. Konvencionalni, deformabilni i propilenglikol liposomi imali su zeta potencijale od -48 do -44 mV, dok je zeta potencijal kationskih liposoma iznosio $+ 62$ mV. *In vitro* ispitivanja biokompatibilnosti liposoma s uklopljenim azitromicinom provedena na HaCaT stanicama procijenjena su pomoću MTT testa, mjerenjem metaboličke aktivnosti keratinocita 24 sata nakon inkubacije liposoma s HaCaT stanicama. Uklapanjem u liposome citotoksičnost azitromicina je smanjena. Fosfolipidni sastav i prisustvo suotapala u liposomima su imali utjecaja na biokompatibilnost. Propilenglikol je pokazao povoljan učinak na stanice keratinocita, dok su natrijev deoksikolat i DODAB pokazali potencijalnu citotoksičnost, ali tek pri izuzetno visokim koncentracijama lipida. Imajući u vidu koncentracije azitromicina u liposomima s kojima je postignut odgovarajući antibakterijski učinak, svi ispitivani tipovi liposoma su bili biokompatibilni s HaCaT stanicama.

SUMMARY

Conventional, deformable, propylene glycol and cationic liposomes encapsulating azithromycin were prepared by the film hydration method. Extrusion of the liposomal dispersions through the polycarbonate membranes resulted with liposomes of more homogenous size distributions with a polydispersity indexes $< 0,43$ and a significantly reduced mean diameters (< 250 nm). The (phospho)lipid composition affected the surface charge of the liposomes. Zeta potential values for the conventional, deformable and propylene glycol liposomes ranged from -48 mV to -44 mV, while the cationic liposomes were shown to have a positive zeta potential of $+62$ mV. *In vitro* biocompatibilities of the different types of liposomes containing azithromycin with the human HaCaT cells were evaluated 24 h after the incubation of the cells with the liposomes using the cellular metabolic activity (MTT assay). The encapsulation of azithromycin in liposomes reduced the cytotoxicity of the drug, whereas the (phospho)lipid composition and the presence of the surfactant and cosolvent affected the biocompatibility. Propylene glycol was well tolerated by the keratinocytes in comparison to sodium deoxycholate and DODAB, where potential cytotoxicities were observed. All types of the liposomes were biocompatible with the keratinocytes even at the very high concentrations of azithromycin tested.

8. PRILOZI

Prilog 1. Dozvola *Elsevier-a* za preuzimanje i prilagodbu slike iz Mu i suradnici (2016)

ELSEVIER LICENSE TERMS AND CONDITIONS

Jul 04, 2019

This Agreement between Miss. Antonija Katanec ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4621400077083
License date	Jul 03, 2019
Licensed Content Publisher	Elsevier
Licensed Content Publication	Advanced Drug Delivery Reviews
Licensed Content Title	Dual-functional drug liposomes in treatment of resistant cancers
Licensed Content Author	Li-Min Mu,Rui-Jun Ju,Rui Liu,Ying-Zi Bu,Jing-Ying Zhang,Xue-Qi Li,Fan Zeng,Wan-Liang Lu
Licensed Content Date	Jun 1, 2017
Licensed Content Volume	115
Licensed Content Issue	n/a
Licensed Content Pages	11
Start Page	46
End Page	56
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	Yes, without English rights

Number of languages	1
Languages	croatian
Original figure numbers	Fig.1.
Title of your thesis/dissertation	In vitro biocompatibility study of liposomes differing in (phospho)lipid composition with the keratinocyte cells
Expected completion date	Jul 2019
Estimated size (number of pages)	34
Requestor Location	Miss. Antonija Katanec Milana Pavelića 31 Zagreb, Grad Zagreb 10000 Croatia Attn: Miss. Antonija Katanec
Publisher Tax ID	GB 494 6272 12
Total	0.00 USD
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.

3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."

4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and

conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. **Revocation:** Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation:** This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website:** The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> or the Elsevier homepage for books at <http://www.elsevier.com>; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at <http://www.elsevier.com>. All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal

publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
 - via their non-commercial person homepage or blog
 - by updating a preprint in arXiv or RePEc with the accepted manuscript
 - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
 - directly by providing copies to their students or to research collaborators for their personal use
 - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
 - via non-commercial hosting platforms such as their institutional repository
 - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a [CrossMark logo](#), the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's [posting policy](#) for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation:** If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our [open access license policy](#) for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant

DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at <http://creativecommons.org/licenses/by/4.0>.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at <http://creativecommons.org/licenses/by-nc-sa/4.0>.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at <http://creativecommons.org/licenses/by-nc-nd/4.0>. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.9

Questions? customer@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Prilog 2. Dozvola Elsevier-a za preuzimanje i prilagodbu slike iz Kuete i suradnici (2017)

**ELSEVIER LICENSE
TERMS AND CONDITIONS**

Jul 04, 2019

This Agreement between Miss. Antonija Katanec ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number 4621400578940

License date Jul 03, 2019

Licensed Content Publisher Elsevier

Licensed Content Publication Elsevier Books

Licensed Content Title Medicinal Spices and Vegetables from Africa

Licensed Content Author V. Kuete, O. Karaosmanoğlu, H. Sivas

Licensed Content Date Jan 1, 2017

Licensed Content Pages 27

Start Page 271

End Page 297

Type of Use reuse in a thesis/dissertation

Intended publisher of new work other

Portion figures/tables/illustrations

Number of figures/tables/illustrations 1

Format both print and electronic

Are you the author of this Elsevier chapter? No

Will you be translating? Yes, without English rights

Number of languages 1

Languages croatian

Original figure numbers Figure 10.1 Enzymatic reduction of MTT to formazan

Title of your thesis/dissertation In vitro biocompatibility study of liposomes differing in (phospho)lipid composition with the keratinocyte cells

Expected completion date Jul 2019

Estimated size (number of pages) 34

Requestor Location Miss. Antonija Katanec, Milana Pavelića 31, Zagreb, Grad Zagreb 10000, Croatia

Attn: Miss. Antonija Katanec

Publisher Tax ID GB 494 6272 12

Total 0.00 USD

Terms and Conditions

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions

apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at <http://myaccount.copyright.com>).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows: "Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.

10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.

11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.

12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).

13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.

14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:

15. **Translation:** This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.

16. **Posting licensed content on any Website:** The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at <http://www.sciencedirect.com/science/journal/xxxxx> or the Elsevier homepage for books at <http://www.elsevier.com>; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at <http://www.elsevier.com> . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following

clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
 - via their non-commercial person homepage or blog
 - by updating a preprint in arXiv or RePEc with the accepted manuscript
 - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
 - directly by providing copies to their students or to research collaborators for their personal use
 - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
 - via non-commercial hosting platforms such as their institutional repository
 - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a [CrossMark logo](#), the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's [posting policy](#) for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation:** If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our [open access license policy](#) for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at <http://creativecommons.org/licenses/by/4.0>.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at <http://creativecommons.org/licenses/by-nc-sa/4.0>.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at <http://creativecommons.org/licenses/by-nc-nd/4.0>. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.9

Questions? customer care@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Prilog 3. Dozvola *IntechOpen*-a za preuzimanje i prilagodbu slike iz Lopes i suradnici (2013)

Creative Commons Legal Code

Attribution 3.0 Unported

CREATIVE COMMONS CORPORATION IS NOT A LAW FIRM AND DOES NOT PROVIDE LEGAL SERVICES. DISTRIBUTION OF THIS LICENSE DOES NOT CREATE AN ATTORNEY-CLIENT RELATIONSHIP. CREATIVE COMMONS PROVIDES THIS INFORMATION ON AN "AS-IS" BASIS. CREATIVE COMMONS MAKES NO WARRANTIES REGARDING THE INFORMATION PROVIDED, AND DISCLAIMS LIABILITY FOR DAMAGES RESULTING FROM ITS USE.

License

THE WORK (AS DEFINED BELOW) IS PROVIDED UNDER THE TERMS OF THIS CREATIVE COMMONS PUBLIC LICENSE ("CCPL" OR "LICENSE"). THE WORK IS PROTECTED BY COPYRIGHT AND/OR OTHER APPLICABLE LAW. ANY USE OF THE WORK OTHER THAN AS AUTHORIZED UNDER THIS LICENSE OR COPYRIGHT LAW IS PROHIBITED.

BY EXERCISING ANY RIGHTS TO THE WORK PROVIDED HERE, YOU ACCEPT AND AGREE TO BE BOUND BY THE TERMS OF THIS LICENSE. TO THE EXTENT THIS LICENSE MAY BE CONSIDERED TO BE A CONTRACT, THE LICENSOR GRANTS YOU THE RIGHTS CONTAINED HERE IN CONSIDERATION OF YOUR ACCEPTANCE OF SUCH TERMS AND CONDITIONS.

1. Definitions

- a. **"Adaptation"** means a work based upon the Work, or upon the Work and other pre-existing works, such as a translation, adaptation, derivative work, arrangement of music or other alterations of a literary or artistic work, or phonogram or performance and includes cinematographic adaptations or any other form in which the Work may be recast, transformed, or adapted including in any form recognizably derived from the original, except that a work that constitutes a Collection will not be considered an Adaptation for the purpose of this License. For the avoidance of doubt, where the Work is a musical work, performance or phonogram, the synchronization of the Work in timed-relation with a moving image ("synching") will be considered an Adaptation for the purpose of this License.
- b. **"Collection"** means a collection of literary or artistic works, such as encyclopedias and anthologies, or performances, phonograms or broadcasts, or other works or subject matter other than works listed in Section 1(f) below, which, by reason of the selection and arrangement of their contents, constitute intellectual creations, in which the Work is included in its entirety in unmodified form along with one or more other contributions, each constituting separate and independent works in themselves, which together are assembled into a collective whole. A work that constitutes a Collection will not be considered an Adaptation (as defined above) for the purposes of this License.

- c. **"Distribute"** means to make available to the public the original and copies of the Work or Adaptation, as appropriate, through sale or other transfer of ownership.
- d. **"Licensor"** means the individual, individuals, entity or entities that offer(s) the Work under the terms of this License.
- e. **"Original Author"** means, in the case of a literary or artistic work, the individual, individuals, entity or entities who created the Work or if no individual or entity can be identified, the publisher; and in addition (i) in the case of a performance the actors, singers, musicians, dancers, and other persons who act, sing, deliver, declaim, play in, interpret or otherwise perform literary or artistic works or expressions of folklore; (ii) in the case of a phonogram the producer being the person or legal entity who first fixes the sounds of a performance or other sounds; and, (iii) in the case of broadcasts, the organization that transmits the broadcast.
- f. **"Work"** means the literary and/or artistic work offered under the terms of this License including without limitation any production in the literary, scientific and artistic domain, whatever may be the mode or form of its expression including digital form, such as a book, pamphlet and other writing; a lecture, address, sermon or other work of the same nature; a dramatic or dramatico-musical work; a choreographic work or entertainment in dumb show; a musical composition with or without words; a cinematographic work to which are assimilated works expressed by a process analogous to cinematography; a work of drawing, painting, architecture, sculpture, engraving or lithography; a photographic work to which are assimilated works expressed by a process analogous to photography; a work of applied art; an illustration, map, plan, sketch or three-dimensional work relative to geography, topography, architecture or science; a performance; a broadcast; a phonogram; a compilation of data to the extent it is protected as a copyrightable work; or a work performed by a variety or circus performer to the extent it is not otherwise considered a literary or artistic work.
- g. **"You"** means an individual or entity exercising rights under this License who has not previously violated the terms of this License with respect to the Work, or who has received express permission from the Licensor to exercise rights under this License despite a previous violation.
- h. **"Publicly Perform"** means to perform public recitations of the Work and to communicate to the public those public recitations, by any means or process, including by wire or wireless means or public digital performances; to make available to the public Works in such a way that members of the public may access these Works from a place and at a place individually chosen by them; to perform the Work to the public by any means or process and the communication to the public of the performances of the Work, including by public digital performance; to broadcast and rebroadcast the Work by any means including signs, sounds or images.
- i. **"Reproduce"** means to make copies of the Work by any means including without limitation by sound or visual recordings and the right of fixation and reproducing fixations of the Work, including storage of a protected performance or phonogram in digital form or other electronic medium.

2. Fair Dealing Rights. Nothing in this License is intended to reduce, limit, or restrict any uses free from copyright or rights arising from limitations or exceptions that are provided for in connection with the copyright protection under copyright law or other applicable laws.

3. License Grant. Subject to the terms and conditions of this License, Licensor hereby grants You a worldwide, royalty-free, non-exclusive, perpetual (for the duration of the applicable copyright) license to exercise the rights in the Work as stated below:

- a. to Reproduce the Work, to incorporate the Work into one or more Collections, and to Reproduce the Work as incorporated in the Collections;
- b. to create and Reproduce Adaptations provided that any such Adaptation, including any translation in any medium, takes reasonable steps to clearly label, demarcate or otherwise identify that changes were made to the original Work. For example, a translation could be marked "The original work was translated from English to Spanish," or a modification could indicate "The original work has been modified.";
- c. to Distribute and Publicly Perform the Work including as incorporated in Collections; and,
- d. to Distribute and Publicly Perform Adaptations.
- e. For the avoidance of doubt:
 - i. **Non-waivable Compulsory License Schemes.** In those jurisdictions in which the right to collect royalties through any statutory or compulsory licensing scheme cannot be waived, the Licensor reserves the exclusive right to collect such royalties for any exercise by You of the rights granted under this License;
 - ii. **Waivable Compulsory License Schemes.** In those jurisdictions in which the right to collect royalties through any statutory or compulsory licensing scheme can be waived, the Licensor waives the exclusive right to collect such royalties for any exercise by You of the rights granted under this License; and,
 - iii. **Voluntary License Schemes.** The Licensor waives the right to collect royalties, whether individually or, in the event that the Licensor is a member of a collecting society that administers voluntary licensing schemes, via that society, from any exercise by You of the rights granted under this License.

The above rights may be exercised in all media and formats whether now known or hereafter devised. The above rights include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. Subject to Section 8(f), all rights not expressly granted by Licensor are hereby reserved.

4. Restrictions. The license granted in Section 3 above is expressly made subject to and limited by the following restrictions:

- a. You may Distribute or Publicly Perform the Work only under the terms of this License. You must include a copy of, or the Uniform Resource Identifier (URI) for, this License with every copy of the Work You Distribute or Publicly Perform. You may not offer or impose any terms on the Work that restrict the terms of this License or the ability of the recipient of the Work to exercise the rights granted to that recipient under the terms of the License. You may not sublicense the Work. You must keep intact all notices that refer to this License and to the disclaimer of warranties with every copy of the Work You Distribute or Publicly Perform. When You Distribute or Publicly Perform the Work, You may not impose any effective technological measures on the Work that restrict the ability of a recipient of the Work from You to exercise the rights granted to that recipient under the terms of the License. This Section 4(a) applies to the Work as incorporated in a Collection, but this does not require the Collection apart from the Work itself to be made subject to the terms of this License. If You create a Collection, upon notice from any Licensor You must, to the extent

practicable, remove from the Collection any credit as required by Section 4(b), as requested. If You create an Adaptation, upon notice from any Licensor You must, to the extent practicable, remove from the Adaptation any credit as required by Section 4(b), as requested.

- b. If You Distribute, or Publicly Perform the Work or any Adaptations or Collections, You must, unless a request has been made pursuant to Section 4(a), keep intact all copyright notices for the Work and provide, reasonable to the medium or means You are utilizing: (i) the name of the Original Author (or pseudonym, if applicable) if supplied, and/or if the Original Author and/or Licensor designate another party or parties (e.g., a sponsor institute, publishing entity, journal) for attribution ("Attribution Parties") in Licensor's copyright notice, terms of service or by other reasonable means, the name of such party or parties; (ii) the title of the Work if supplied; (iii) to the extent reasonably practicable, the URI, if any, that Licensor specifies to be associated with the Work, unless such URI does not refer to the copyright notice or licensing information for the Work; and (iv) , consistent with Section 3(b), in the case of an Adaptation, a credit identifying the use of the Work in the Adaptation (e.g., "French translation of the Work by Original Author," or "Screenplay based on original Work by Original Author"). The credit required by this Section 4 (b) may be implemented in any reasonable manner; provided, however, that in the case of a Adaptation or Collection, at a minimum such credit will appear, if a credit for all contributing authors of the Adaptation or Collection appears, then as part of these credits and in a manner at least as prominent as the credits for the other contributing authors. For the avoidance of doubt, You may only use the credit required by this Section for the purpose of attribution in the manner set out above and, by exercising Your rights under this License, You may not implicitly or explicitly assert or imply any connection with, sponsorship or endorsement by the Original Author, Licensor and/or Attribution Parties, as appropriate, of You or Your use of the Work, without the separate, express prior written permission of the Original Author, Licensor and/or Attribution Parties.
- c. Except as otherwise agreed in writing by the Licensor or as may be otherwise permitted by applicable law, if You Reproduce, Distribute or Publicly Perform the Work either by itself or as part of any Adaptations or Collections, You must not distort, mutilate, modify or take other derogatory action in relation to the Work which would be prejudicial to the Original Author's honor or reputation. Licensor agrees that in those jurisdictions (e.g. Japan), in which any exercise of the right granted in Section 3(b) of this License (the right to make Adaptations) would be deemed to be a distortion, mutilation, modification or other derogatory action prejudicial to the Original Author's honor and reputation, the Licensor will waive or not assert, as appropriate, this Section, to the fullest extent permitted by the applicable national law, to enable You to reasonably exercise Your right under Section 3(b) of this License (right to make Adaptations) but not otherwise.

5. Representations, Warranties and Disclaimer

UNLESS OTHERWISE MUTUALLY AGREED TO BY THE PARTIES IN WRITING, LICENSOR OFFERS THE WORK AS-IS AND MAKES NO REPRESENTATIONS OR WARRANTIES OF ANY KIND CONCERNING THE WORK, EXPRESS, IMPLIED, STATUTORY OR OTHERWISE, INCLUDING, WITHOUT LIMITATION, WARRANTIES OF TITLE, MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, NON-INFRINGEMENT, OR THE ABSENCE OF LATENT OR OTHER

DEFECTS, ACCURACY, OR THE PRESENCE OF ABSENCE OF ERRORS, WHETHER OR NOT DISCOVERABLE. SOME JURISDICTIONS DO NOT ALLOW THE EXCLUSION OF IMPLIED WARRANTIES, SO SUCH EXCLUSION MAY NOT APPLY TO YOU.

6. Limitation on Liability. EXCEPT TO THE EXTENT REQUIRED BY APPLICABLE LAW, IN NO EVENT WILL LICENSOR BE LIABLE TO YOU ON ANY LEGAL THEORY FOR ANY SPECIAL, INCIDENTAL, CONSEQUENTIAL, PUNITIVE OR EXEMPLARY DAMAGES ARISING OUT OF THIS LICENSE OR THE USE OF THE WORK, EVEN IF LICENSOR HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES.

7. Termination

- a. This License and the rights granted hereunder will terminate automatically upon any breach by You of the terms of this License. Individuals or entities who have received Adaptations or Collections from You under this License, however, will not have their licenses terminated provided such individuals or entities remain in full compliance with those licenses. Sections 1, 2, 5, 6, 7, and 8 will survive any termination of this License.
- b. Subject to the above terms and conditions, the license granted here is perpetual (for the duration of the applicable copyright in the Work). Notwithstanding the above, Licensor reserves the right to release the Work under different license terms or to stop distributing the Work at any time; provided, however that any such election will not serve to withdraw this License (or any other license that has been, or is required to be, granted under the terms of this License), and this License will continue in full force and effect unless terminated as stated above.

8. Miscellaneous

- a. Each time You Distribute or Publicly Perform the Work or a Collection, the Licensor offers to the recipient a license to the Work on the same terms and conditions as the license granted to You under this License.
- b. Each time You Distribute or Publicly Perform an Adaptation, Licensor offers to the recipient a license to the original Work on the same terms and conditions as the license granted to You under this License.
- c. If any provision of this License is invalid or unenforceable under applicable law, it shall not affect the validity or enforceability of the remainder of the terms of this License, and without further action by the parties to this agreement, such provision shall be reformed to the minimum extent necessary to make such provision valid and enforceable.
- d. No term or provision of this License shall be deemed waived and no breach consented to unless such waiver or consent shall be in writing and signed by the party to be charged with such waiver or consent.
- e. This License constitutes the entire agreement between the parties with respect to the Work licensed here. There are no understandings, agreements or representations with respect to the Work not specified here. Licensor shall not be bound by any additional provisions that may appear in any communication from You. This License may not be modified without the mutual written agreement of the Licensor and You.

- f. The rights granted under, and the subject matter referenced, in this License were drafted utilizing the terminology of the Berne Convention for the Protection of Literary and Artistic Works (as amended on September 28, 1979), the Rome Convention of 1961, the WIPO Copyright Treaty of 1996, the WIPO Performances and Phonograms Treaty of 1996 and the Universal Copyright Convention (as revised on July 24, 1971). These rights and subject matter take effect in the relevant jurisdiction in which the License terms are sought to be enforced according to the corresponding provisions of the implementation of those treaty provisions in the applicable national law. If the standard suite of rights granted under applicable copyright law includes additional rights not granted under this License, such additional rights are deemed to be included in the License; this License is not intended to restrict the license of any rights under applicable law.

Creative Commons Notice

Creative Commons is not a party to this License, and makes no warranty whatsoever in connection with the Work. Creative Commons will not be liable to You or any party on any legal theory for any damages whatsoever, including without limitation any general, special, incidental or consequential damages arising in connection to this license. Notwithstanding the foregoing two (2) sentences, if Creative Commons has expressly identified itself as the Licensor hereunder, it shall have all rights and obligations of Licensor.

Except for the limited purpose of indicating to the public that the Work is licensed under the CCPL, Creative Commons does not authorize the use by either party of the trademark "Creative Commons" or any related trademark or logo of Creative Commons without the prior written consent of Creative Commons. Any permitted use will be in compliance with Creative Commons' then-current trademark usage guidelines, as may be published on its website or otherwise made available upon request from time to time. For the avoidance of doubt, this trademark restriction does not form part of this License.

Creative Commons may be contacted at <https://creativecommons.org/>.

Temeljna dokumentacijska kartica

Sveučilište u Zagrebu
Farmaceutsko-biokemijski fakultet
Studij: Farmacija
Zavod za farmaceutsku tehnologiju
Domagojeva 2, 10000 Zagreb, Hrvatska
ili druga adresa

Diplomski rad

***In vitro* ispitivanja biokompatibilnosti liposoma različitog (fosfo)lipidnog sastava sa stanicama keratinocita**

Antonija Katanec

SAŽETAK

Različite vrste liposoma, konvencionalni, deformabilni, propilenglikol i kationski liposomi s uklopljenim azitromicinom, pripremljeni su film metodom. Ekstruzijom kroz polikarbonske membrane, formulacije liposoma su homogenizirane (indeks polidisperznosti $< 0,43$), a srednji promjeri značajno smanjeni (< 250 nm). Fosfolipidni sastav liposoma imao je utjecaja na površinski naboj liposoma. Konvencionalni, deformabilni i propilenglikol liposomi imali su zeta potencijale od -48 do -44 mV, dok je zeta potencijal kationskih liposoma iznosio $+ 62$ mV. In vitro ispitivanja biokompatibilnosti liposoma s uklopljenim azitromicinom provedena na HaCaT stanicama procijenjena su pomoću MTT testa, mjerenjem metaboličke aktivnosti keratinocita 24 sata nakon inkubacije liposoma s HaCaT stanicama. Uklapanjem u liposome citotoksičnost azitromicina je smanjena. Fosfolipidni sastav i prisustvo suotapala u liposomima su imali utjecaja na biokompatibilnost. Propilenglikol je pokazao povoljan učinak na stanice keratinocita, dok su natrijev deoksikolat i DODAB pokazali potencijalnu citotoksičnost, ali tek pri izuzetno visokim koncentracijama lipida. Imajući u vidu koncentracije azitromicina u liposomima s kojima je postignut odgovarajući antibakterijski učinak, svi ispitivani tipovi liposoma su bili biokompatibilni s HaCaT stanicama.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 33 stranica, 10 grafičkih prikaza, 3 tablice i 40 literaturnih navoda. Izvornik je na hrvatskom jeziku.

Ključne riječi: Liposomi, azitromicin, elastičnost, površinski naboj, propilenglikol, biokompatibilnost, keratinociti

Mentor: **Dr. sc. Željka Vanić**, *izvanredna profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*

Ocjenjivači: **Dr. sc. Željka Vanić**, *izvanredna profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*
Dr. sc. Anita Hafner, *izvanredna profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*
Dr. sc. Dubravka Vitali Čepo, *izvanredna profesorica Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.*

Rad prihvaćen: srpanj 2019.

Basic documentation card

University of Zagreb
Faculty of Pharmacy and Biochemistry
Study: Pharmacy
Department of pharmaceutical technology
Domagojeva 2, 10000 Zagreb, Croatia

Diploma thesis

In vitro biocompatibility study of liposomes differing in (phospho)lipid composition with the keratinocyte cells

Antonija Katanec

SUMMARY

Conventional, deformable, propylene glycol and cationic liposomes encapsulating azithromycin were prepared by the film hydration method. Extrusion of the liposomal dispersions through the polycarbonate membranes resulted with liposomes of more homogenous size distributions with a polydispersity indexes $< 0,43$ nm and a significantly reduced mean diameters (< 250 nm). The (phospho)lipid composition affected the surface charge of the liposomes. Zeta potential values for the conventional, deformable and propylene glycol liposomes ranged from -48 mV to -44 mV, while the cationic liposomes were shown to have a positive zeta potential of $+62$ mV. In vitro biocompatibilities of the different types of liposomes containing azithromycin with the human HaCaT cells were evaluated 24 h after the incubation of the cells with the liposomes using the cellular metabolic activity (MTT assay). The encapsulation of azithromycin in liposomes reduced the cytotoxicity of the drug, whereas the (phospho)lipid composition and the presence of the surfactant and cosolvent affected the biocompatibility. Propylene glycol was well tolerated by the keratinocytes in comparison to sodium deoxycholate and DODAB, where potential cytotoxicities were observed. All types of the liposomes were biocompatible with the keratinocytes even at the very high concentrations of azithromycin tested.

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

Thesis includes: 33 pages, 10 figures, 3 tables and 40 references. Original is in Croatian language.

Keywords: Liposomes, azithromycin, elasticity, propylene glycol, surface charge, biocompatibility, keratinocytes

Mentor: **Željka Vanić, Ph.D.** Associate Professor, University of Zagreb Faculty of Pharmacy and Biochemistry

Reviewers: **Željka Vanić, Ph.D.** Associate Professor, University of Zagreb Faculty of Pharmacy and Biochemistry
Anita Hafner, Ph.D. Associate Professor, University of Zagreb Faculty of Pharmacy and Biochemistry
Dubravka Vitali Čepo, Ph.D. Associate Professor, University of Zagreb Faculty of Pharmacy and Biochemistry

The thesis was accepted: July 2019