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

Katanec, Antonija

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Antonija Katanec

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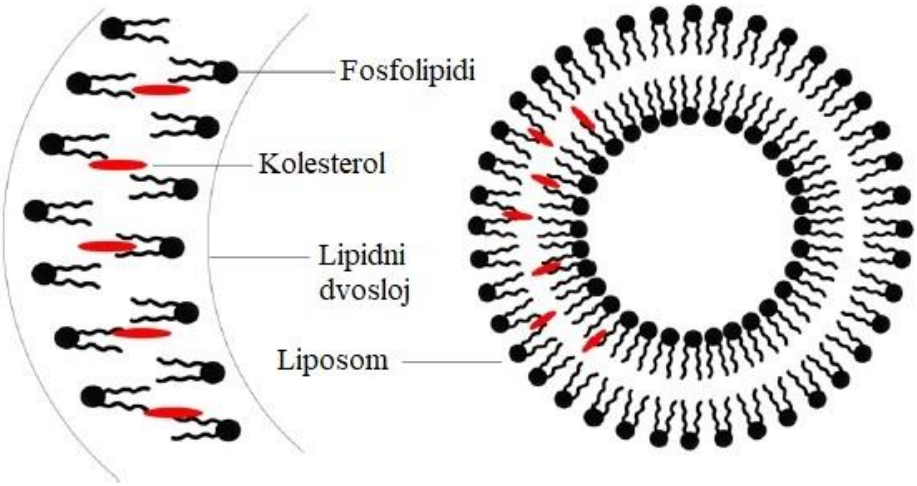
Sadržaj

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).



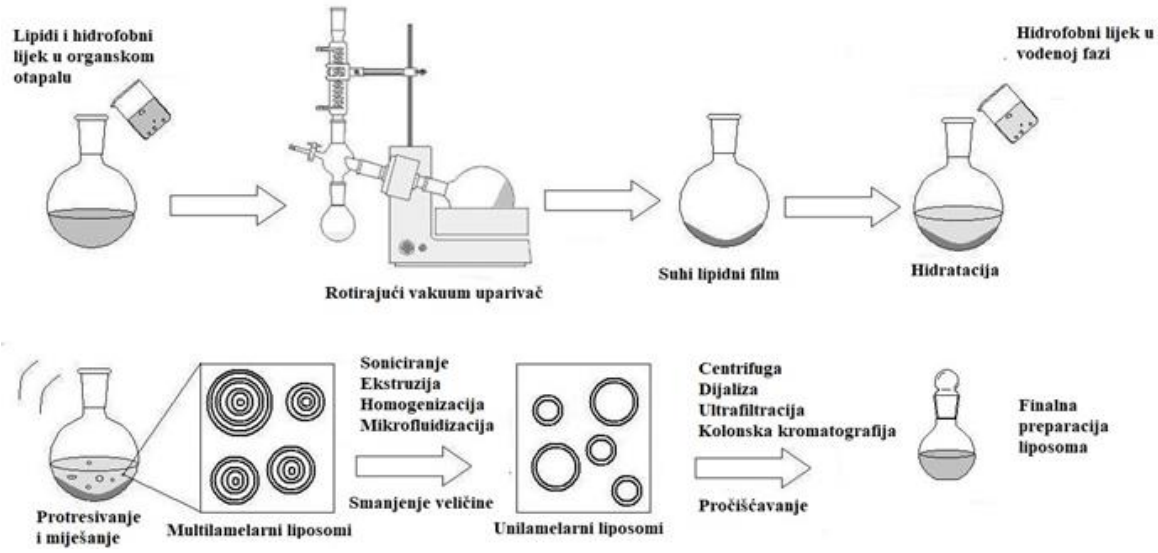
Slika 1. Struktura liposoma. Preuzeto i prilagođeno iz Mu i sur. (2016), uz dozvolu *Elsevier*-a.

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 ovojnicu (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 fosfolipidinih 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 nm), srednje-velike unilamelarne liposome (r < 100 nm), velike unilamelarne liposome (r = 100 – 1000 nm) te veoma velike unilamelarne liposome (r > 1000 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 μm), sadrže mnogo vodenih odjeljaka, separiranih nekoncentrično položenim fosfolipidnim dvoslojem (Vanić, 2012a).

Uobičajena metoda priprave 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 priprave liposoma mora biti iznad temperature faznog prijelaza (Tc) korištenih fosfolipida. Temperatura faznog prijelaza (Tc) definira se kao temperatura na kojoj se odvija prijelaz fosfolipida iz gel faze u fazu tekućih kristala. Poznavanje Tc i s tim u vezi fluidnosti membrane važno je pri proizvodnji i istraživanju formulacija lijekova zasnovanih na liposomima. Liposomi pripravljeni 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 priprave liposoma film metodom. Preuzeto i prilagođeno iz Lopes i sur. (2013), uz dozvolu *IntechOpen*-a.

1.2. Karakterizacija liposoma

Nakon priprave 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 određvanje 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. *dinamic 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).

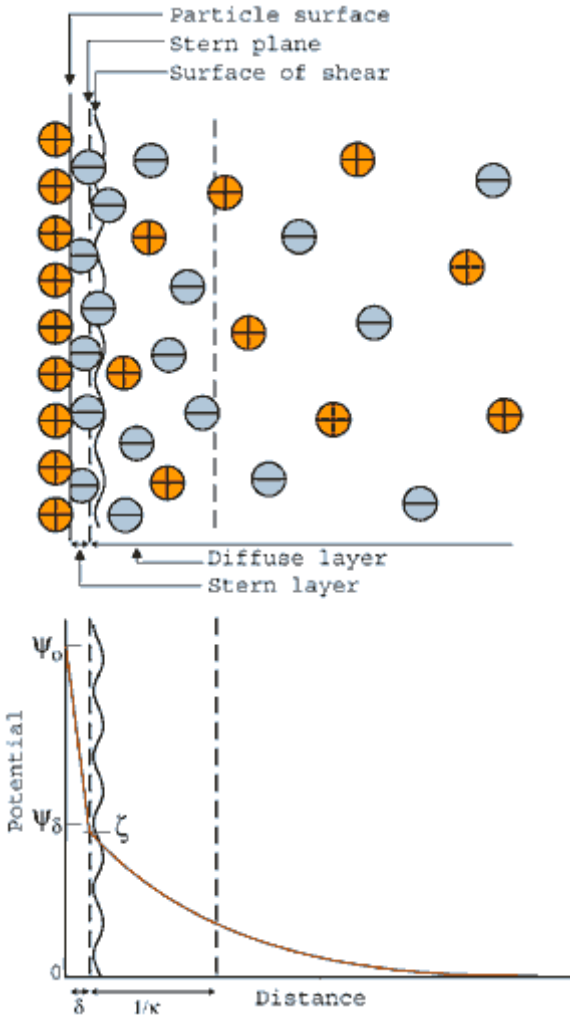
Nabijene dispergirane čestice putuju u električnom polju prema elektrodi suprotnog predznaka, i ta se pojava naziva elektroforetskom gibljivošću. Zajedno s česticama giba se i Sternov sloj, te dio „vezanih“ molekula otapala. Može se zamisliti da se zajedno s česticom giba mali volumen medija odijeljen 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 dipergirane 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 uklanjanju i dolazi do izmjene stanica iz dubljih slojeva kože. *Stratum corneum* predstavlja barijeru perkutane apsorpcije te ujedno štiti tijelo od vanjskih utjecaja. Sastoji se od nekoliko slojeva mrtvih, izduženih stanica (korneocita) koje sadrže keratin, nastao u dubljim slojevima epiderimisa. 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 *strataума 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 mehanizamu 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 adsoprpciju, 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 meticilina 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 smanjenja 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 biofilmove, 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, odosno 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).

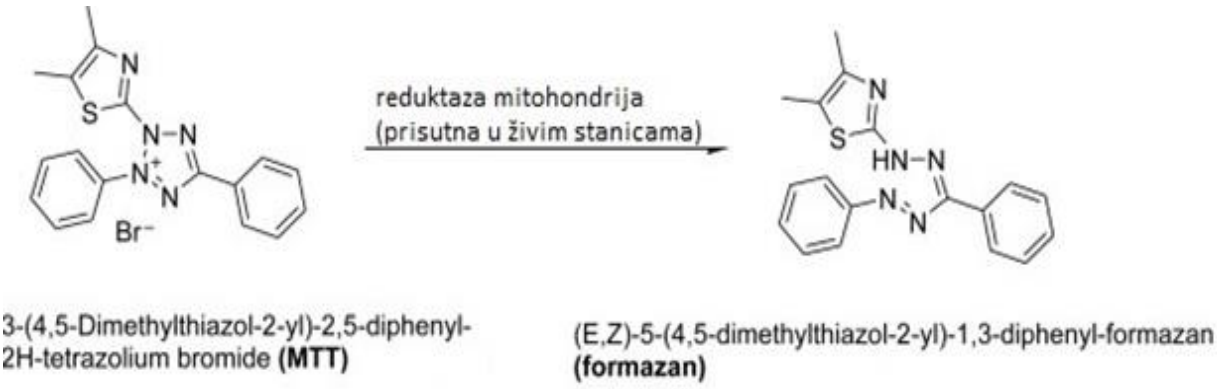
1.4.1. MTT test (test redukcije tetrazolijeve soli)

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 ºC u razdoblju od 1 do 4 sata. Količina nastalog formazana (s pretpostavkom da je proporcionalna količini živućih stanica) određuje se mjerenjem aposorbancije na 570 nm. Naime, stanice koje su žive aktivnim metabolizmom pretvaraju MTT u modro obojani formazan koji ima maksimum apsorbancije 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 apsorbancija 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 apsorbancije.

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 različte koncentracije ispitivanog spoja (Riss i sur., 2013).

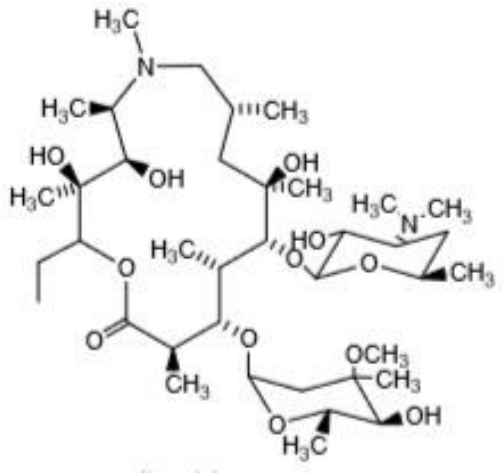
MTT test je, dakle, kolorimetrijska metoda koja se temelji na određivanju metaboličke aktivnosti mitohondrija mjerenjem redukcije topljive žute MTT tetrazolijeve soli u modri netopljivi formazan (Slika 4). Očitana apsorbancija proporcionalna je broju živih stanica u uzorku, pri čemu se preživljenje stanica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



Slika 4. Enzimska 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 ºC, a logP iznosi 4,02. Azitromicin je prvi predstavnik skupine makrolidnih antibiotika nazvane azalidi te ima širok spekatr 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 azitromcina. 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 (nekomplicirane genitalne infekcije izazvane *Chlamydiom 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. Vezanje 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), pripljemljen 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, streptomicina i amfotericina B (Lonza, Basel, Švicarska).

3.2. Metode

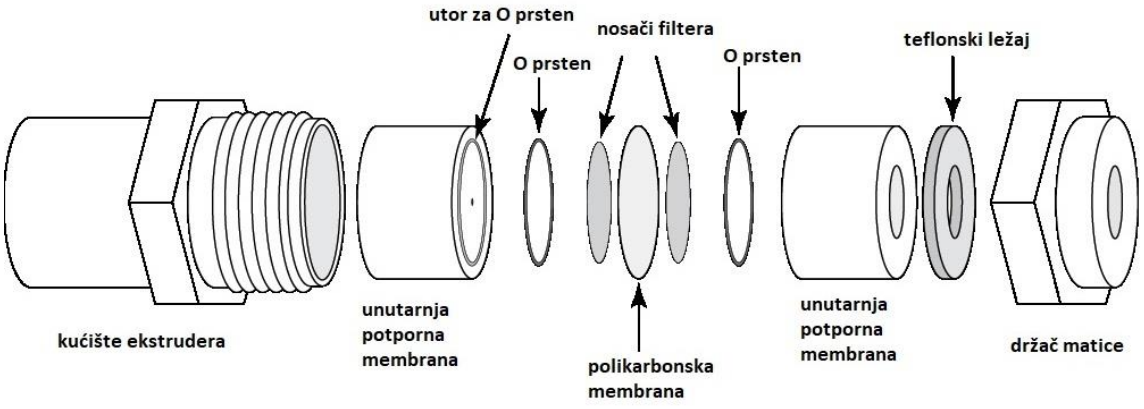
3.2.1. Priprema liposoma

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 dipalmitiolfosfatidilkolina, 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 koncetriranog etanola. Pomoću rotirajućeg vakuum uparivača potpuno je uklonjen etanol na temperaturi od 40 ºC za konvencionalne, deformabilne i propilenglikol liposome, odnosno 50 ºC 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 ºC 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 polidispreznosti

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 º i temperaturi od 25 ºC. 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 ºC.

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 ºC. 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:

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

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

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 nasađene 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 poveden 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 µl MTT otopine te su stanice inkubirane daljnjih 30 minuta na 37 ºC. Potom je uklonjen medij, a istaloženi modri formazan je ekstrahiran dodatkom 100 µl 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.

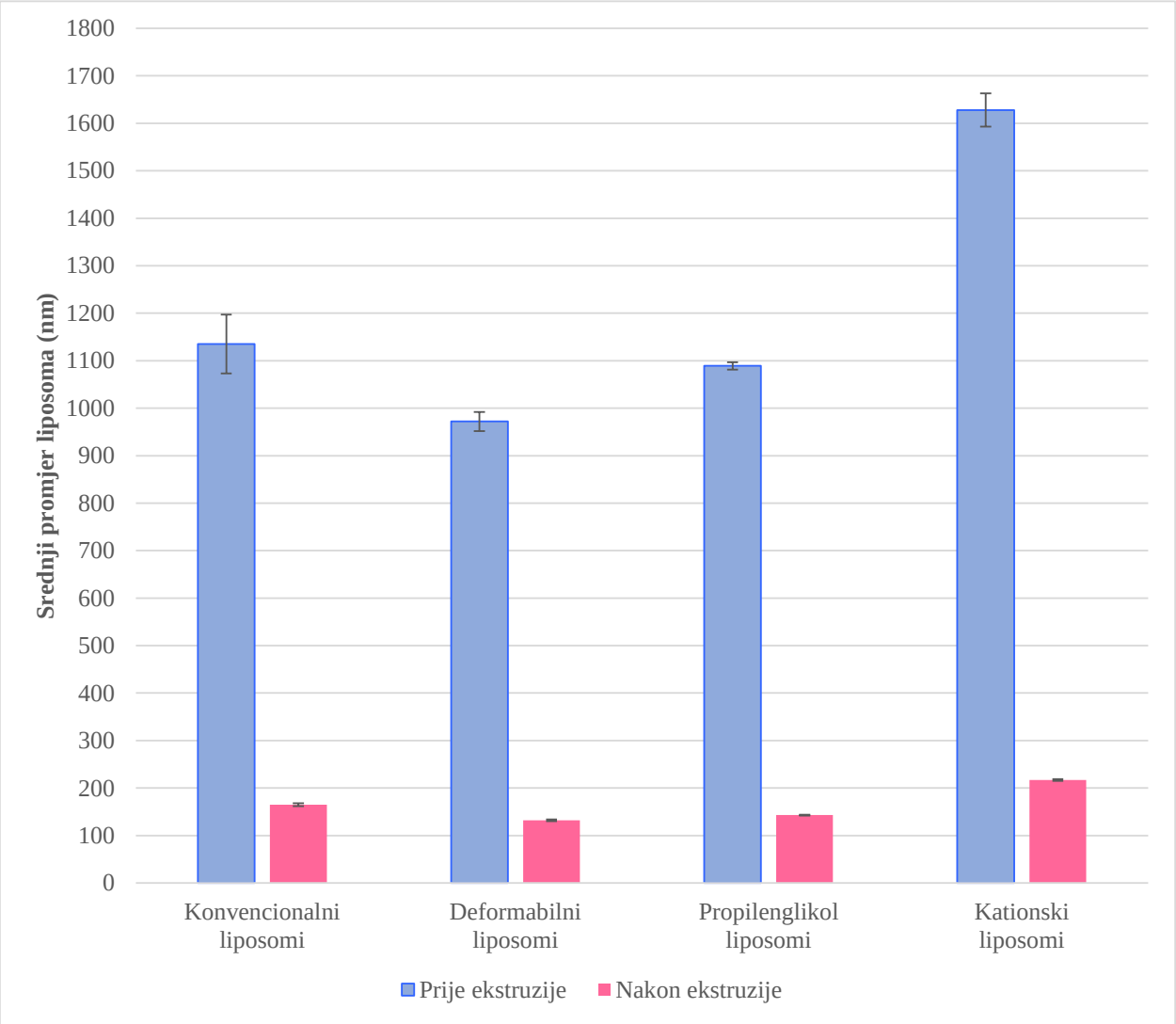
3.2.7. Statistička obrada podataka

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.

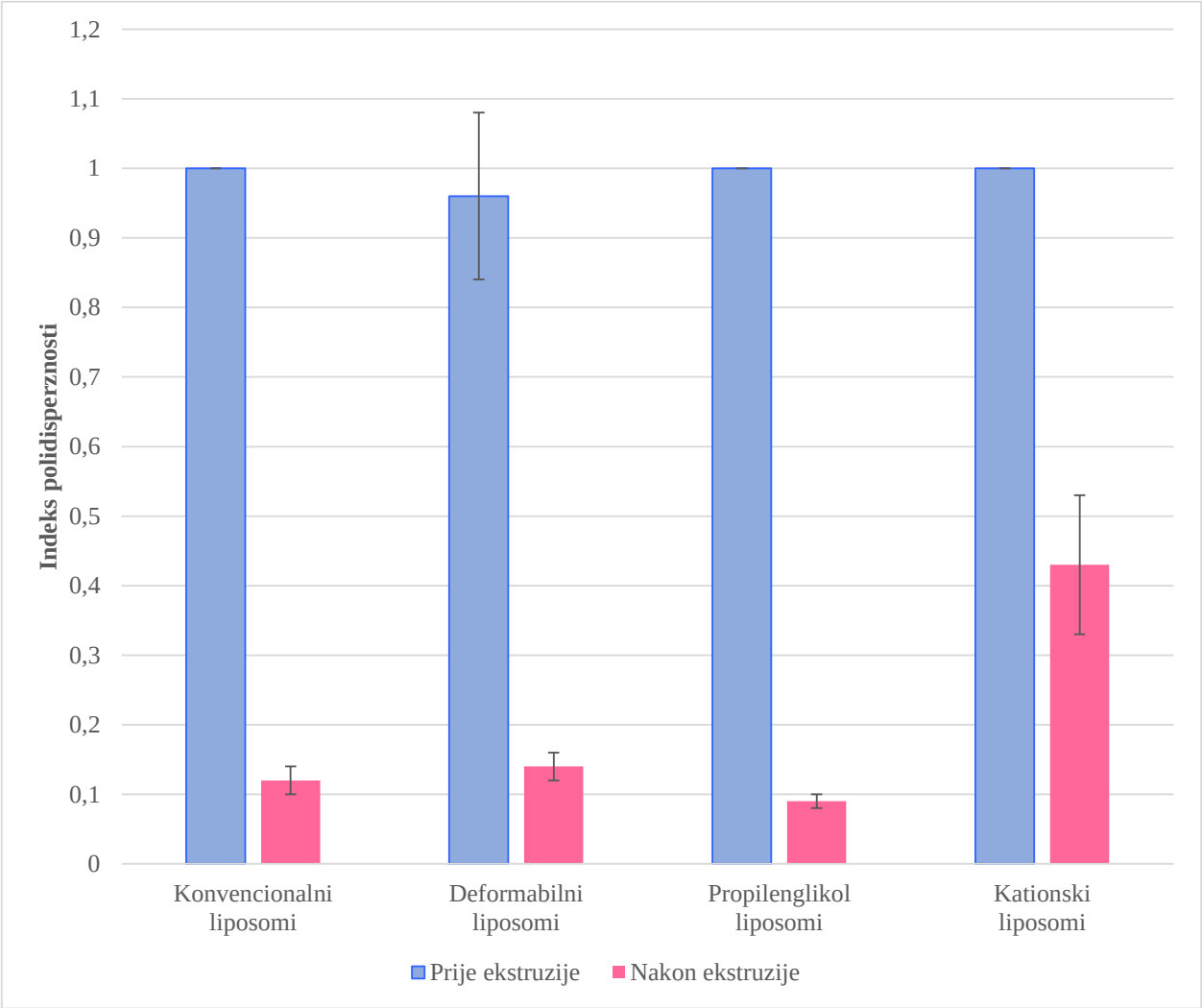
4. REZULTATI I RASPRAVA

4.1. Fizikalno-kemijske karakteristike liposoma

Konvencionalni, deformabilni, propilenglikol i kationski lipsomi 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



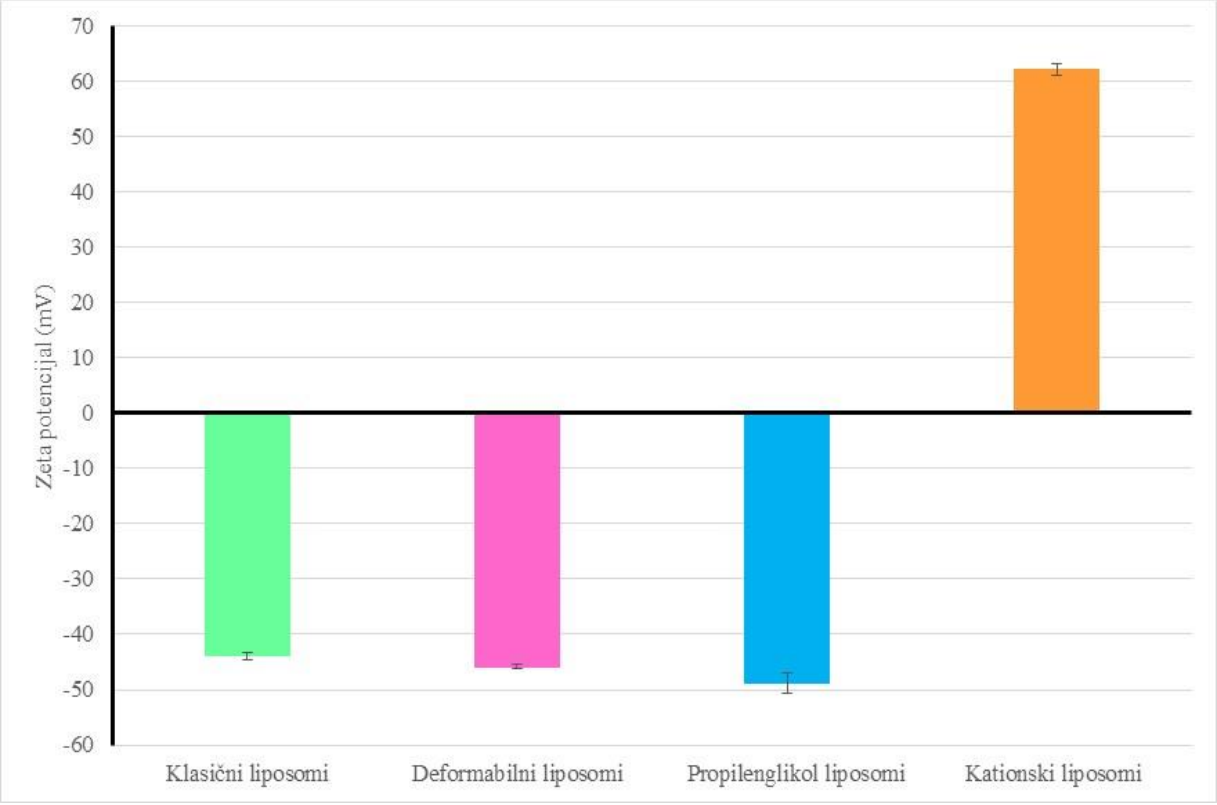
Slika 9. Grafički prikaz indeksa polidisperznosti različitih vrsta liposoma prije i nakon ekstruzije

Iz prikazanih rezultata (Slike 8 i 9) vidljivo je da su izvorno pripravljeni liposomi bili izrazito veliki i visokog indeks polidisperznosti (PDI > 0,95), što je bilo i očekivano budući su liposomi pripravljeni 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 izmjerenim 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 pripave 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 učink. Vijabilnost keratinocita iznosila je 38% za deformabilne liposome i 45% za kationske liposome. Nasuporot tome, konvencionalni i propilenglikol liposomi bili su biokompatilni (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 koncentracijima, koje odgovaraju koncentracijama lipida od 213 i 853 μg/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)	**Vijabilnost keratinocita 24 sata nakon inkubacije (%)**				
	Konvencionalni liposomi	Deformabilni liposomi	Propilenglikol liposomi	Kationski liposomi	Otopina azitromicina
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)	**Vijabilnost keratinocita 24 sata nakon inkubacije (%)**				
	Konvencionalni liposomi („prazni“)	Deformabilni liposomi („prazni“)	Propilenglikol liposomi („prazni“)	Kationski liposomi („prazni“)	Otapalo
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.

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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 nm and a significantly reduced mean diameters (< 250 nm). The (phospo)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 (phospo)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)

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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.

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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.*

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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 (phospo)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 (phospo)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

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