

UNIVERZITET U BEOGRADU
TEHNOLOŠKO-METALURŠKI FAKULTET

Marija N. Jovanović

**ADHEZIVNI FILMOVI I IMPLANTATI NA BAZI
ŽELATINA ZA BUKALNU PRIMENU I
REGENERACIJU TKIVA**

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UNIVERSITY OF BELGRADE
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Marija N. Jovanović

**GELATIN BASED ADHESIVE FILMS AND
SCAFFOLDS FOR BUCCAL APPLICATION
AND TISSUE REGENERATION**

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Mentor:

Dr Petar Uskoković, red. prof. Univerziteta u
Beogradu, Tehnološko-metalurški fakultet

Članovi komisije:

Dr Svetlana Ibrić, red. prof. Univerziteta u
Beogradu, Farmaceutski fakultet

Dr Bojana Obradović, red. prof. Univerziteta u
Beogradu, Tehnološko-metalurški fakultet

Dr Dušica Stojanović, naučni savetnik
Univerziteta u Beogradu, Tehnološko-
metalurški fakultet

Dr Miloš Petrović, van. prof. Univerziteta u
Beogradu, Tehnološko-metalurški fakultet

Datum odbrane: _____

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Marija Jovanović

Adhezivni filmovi i implantati na bazi želatina za bukalnu primenu i regeneraciju tkiva

SAŽETAK

U okviru ove disertacije izvedena su istraživanja u pravcu razvoja multifunkcionalnog materijala na bazi želatina, koji se koristi za bukalne filmove i implantate za dostavu aktivnih supstanci, kao i regeneraciju koštanog tkiva. Karakterisane su različite formulacije blendi želatina, sintetičkih polimera i aktivnih supstanci.

U prvoj fazi, razvijeni su bukalni filmovi sa poboljšanom bioraspoloživošću propranolol hidrohlorida (PRH), pri čemu su filmovi na bazi želatina A, koji se dobija kiselom hidrolizom kolagena, pokazali bolja mehanička svojstva i brže otpuštanje PRH u poređenju sa filmovima na bazi želatina B, koji se dobija baznom hidrolizom kolagena.

U drugoj fazi, izrađeni su bukalni filmovi 3D štampom, gde su blende želatina A sa sintetičkim polimerima polivinilpirolidonom (PVP) i polivinil alkoholom omogućile poboljšanje mehaničkih svojstava i produženo oslobađanje PRH. Na osnovu rezultata, bukalni film blende želatina A sa PVP pokazao je bolju biodostupnost, mehanička i mukoadhezivna svojstva.

Treća faza obuhvatila je razvoj implantata na bazi blende želatina A, glicerola i PVP, gde je genipin korišćen za umrežavanje materijala. Ispitivan je uticaj sadržaja genipina na mehanička svojstva implantata. Implantati sa 1 mas% genipina su pokazali poboljšana mehanička svojstva i odsustvo citotoksičnosti. U poslednjem delu, razvijen je implantat za dostavu natrijum alendronat trihidrata, koji je takođe pokazao dobru biokompatibilnost i produženo oslobađanje aktivne supstance.

Rezultati disertacije ukazuju na to da kombinacija želatina sa sintetičkim polimerima i umrežavanje genipinom predstavlja perspektivan pristup razvoju sistema za dostavu aktivnih supstanci i regeneraciju koštanog tkiva, sa značajnim potencijalom za primenu u farmaciji i regenerativnoj medicini.

Ključne reči: želatin, polivinil pirolidon, genipin, bukalni filmovi, implantati za dostavu aktivne supstance, 3D štampa ekstruzijom polučvrstih materijala, propranolol hidrahlorid, natrijum alendronat trihidrat, mukoadhezija, mehanička svojstva.

Naučna oblast: Tehnološko inženjerstvo

Uža naučna oblast: Inženjerstvo materijala

Gelatin based adhesive films and scaffolds for buccal application and tissue regeneration

ABSTRACT

This doctoral dissertation focuses on the development of a multifunctional gelatin based material for use as buccal films and implants for the delivery of active substances and bone tissue regeneration. Various formulations of gelatin blends, synthetic polymers, and active substances were characterized.

In the first phase, buccal films with improved bioavailability of the propranolol hydrochloride (PRH) were developed, with films based on gelatin A, product of acid hydrolysis of collagen, demonstrating better mechanical properties and faster release of PRH compared to films based on gelatin B, product of base hydrolysis of collagen.

In the second phase, a 3D printing method was applied to create buccal films, where blends of gelatin type A with the synthetic polymers polyvinylpyrrolidone (PVP) and polyvinyl alcohol enabled the enhancement of mechanical properties and prolonged release of PRH. Based on the results, the buccal film blend of gelatin A, glycerol with PVP (GAG-PVP) exhibited better bioavailability, mechanical, and mucoadhesive properties.

The third phase involved the development of implants based on the GAG-PVP blend, where genipin was used for crosslinking the material. The impact of genipin content on the mechanical properties of the implants was examined. Implants with 1 wt% genipin showed improved mechanical properties and no cytotoxicity. In the final part, an implant for the delivery of bisphosphonate sodium alendronate trihydrate was developed, which also demonstrated good biocompatibility and prolonged release of the active substance.

The results of the dissertation indicate that the combination of gelatin with synthetic polymers and crosslinking with genipin represents a promising approach to the development of systems for the delivery of active substances and bone tissue regeneration, with significant potential for application in pharmacy and regenerative medicine.

Key words: gelatin, polyvinyl pyrrolidone, genipin, buccal films, implants for active substance delivery, semisolid extrusion 3D printing, propranolol hydrochloride, sodium alendronate trihydrate, mucoadhesion, mechanical properties.

Scientific field: Technological Engineering

Scientific subfield: Materials Engineering

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1. Uvod

Predmet istraživanja ove doktorske disertacije obuhvata razvoj i karakterizaciju novih adhezivnih bukalnih filmova i implantata na bazi želatina.

U farmaceutskoj praksi definisano je nekoliko sistema za dostavu aktivne supstance grupisanih po načinu primene (engl. *route of administration*): oralni, intramuskularni, transdermalni, potkožni, intraokularni, intranazalni, intrauterini i transmukozni (plućni, sublingvalni, bukalni, intravaginalni i rektalni) [1]. Oralna bukalna sluzokoža (mukoza) se smatra jednim od najpogodnijih i najšire prihvaćenih načina primene lekova. Zbog svoje razuđene površine, oralna sluzokoža omogućava lekovima male molekulske mase da brže prodru u epitel sluzokože nego okularnim i nazalnim putevima [2,3]. Mukoadhezivni bukalni filmovi pogodni su kako za lokalnu, tako i za sistemsku dostavu lekova. Njihova prednost ogleda se u tome što zaobilaze gastrointestinalni trakt, čime se smanjuje mogućnost oštećenja želudačne sluzokože i degradacije aktivne supstance pod dejstvom želudačne kiseline. Takođe, bukalna primena omogućava izbegavanje efekta prvog prolaza kroz jetru i povećava biodostupnost leka direktnim ulaskom u sistemsku cirkulaciju. U poređenju sa tradicionalnim formulacijama leka, bukalni filmovi nude brojne prednosti, kao što su poboljšana adhezenca (saradnja i razumevanje između lekara i pacijenta), brže dejstvo leka, kao i pogodnost za pedijatrijsku i gerijatrijsku populaciju.

Implantati za dostavu aktivne supstance (leka) predstavljaju medicinska sredstva, uređaje ili polimerne strukture koje precizno i uz duži vremenski period dostavljaju aktivnu supstancu do ciljanog tkiva. U okviru implantata, biomaterijali imaju ulogu nosača ćelija ili aktivnih supstanci čime doprinose rekonstrukciji, regeneraciji kao i očuvanju ili poboljšanju funkcije tkiva. Morfologija implantata ima značajnu ulogu jer omogućava precizno prilagođavanje mestu implantacije. Zahvaljujući mehaničkom integritetu kao i mogućnosti resorpcije, implantati mogu da se koriste kao privremeni nosači i fiksatori bez potrebe za dodatnim hirurškim uklanjanjem pri tome oni mogu da sadrže inkorporirane aktivne supstance koje stimulišu regeneraciju, smanjuju bol ili modulišu imunski odgovor [4-6].

U skladu sa svojom primenom bukalni filmovi i implantati treba da poseduju određena fizičko-hemijska svojstva, strukturnu stabilnost i morfologiju koja odgovara ulozi dostave aktivne supstance kao i odgovarajuća mehanička svojstva. Adhezija takođe predstavlja jedno od ključnih svojstava, jer omogućava stabilno vezivanje za tkivo i dostavu aktivne supstance. Prekomerna adhezija može izazvati iritaciju tkiva, dok nedovoljna adhezija može smanjiti terapijsku efikasnost [7-11].

Želatin predstavlja atraktivan materijal za primenu u farmaciji i biomedicini zbog svojih povoljnih svojstava, kao što su biokompatibilnost, adhezivnost, dostupnost i niska cena. Zbog slabijih mehaničkih svojstava, želatin se često ili kombinuje sa drugim polimerima formirajući blende ili se umrežava (engl. *crosslinking*) određenim agensima. [4, 12,13].

Cilj ove disertacije je razvoj adhezivnih filmova i implantata za dostavu aktivnih supstanci primenom metoda izlivanja iz rastvora i 3D štampe ekstruzijom polučvrstih materijala. Karakterizacija polaznih komponenti kao i prelaznih formi daje uvid u svojstva određenih formulacija, kao što su njihova morfološka, termička, mehanička i adhezivna svojstva, kao i citotoksičnost. Na taj način moguće je postići željene profile oslobađanja aktivnih supstanci, poboljšati mehanička svojstva i omogućiti personalizovan pristup terapiji i tretmanu pacijenata.

U okviru ove disertacije izvedena je optimizacija sastava, strukture i morfologije kao i procesiranje bukalnih filmova i implantata na bazi želatina namenjenih otpuštanju aktivnih supstanci

za primenu u farmaciji i biomedicini. Kao metoda procesiranja izabrana je 3D štampa ekstruzijom polučvrstih materijala koja je pogodna jer nema termičkog ili UV tretmana tokom procesa, čime se sprečava degradacija inkorporiranih aktivnih supstanci. S tim ciljem, u prvoj fazi istraživanja izveden je odabir vrste želatina koji odgovara kako samom procesu 3D štampe, tako i vrstama aktivnih supstanci radi dobijanja optimalnog profila otpuštanja propranolol hidrohlorida iz bukalnih filmova. Nakon toga je optimizovan sastav blende želatina i polimera za razvoj bukalnih filmova sa željenim profilom otpuštanja. Kao kandidati za blendu sa želatinom ispitivani su polivinilpirolidon (PVP) i polivinil alkohol (PVA). U daljim fazama istraživanja razvijan je implantat za otpuštanje natrijum alendronat trihidrata za primenu u regeneraciji koštanog tkiva i tretmanu osteoporoze. Za umrežavanje želatina i poboljšanje mehaničkih svojstava, izabran je genipin, prirodno jedinjenje koje se dobija iz glikozida prisutnog u plodovima biljke *Gardenia jasminoides*. Izabran je zbog znatno manje citotoksičnosti u odnosu na druge, sintetičke agense za umrežavanje. Nakon toga je izvedena 3D štampa implantata sa direktnom inkorporacijom i genipina i natrijum alendronat trihidrata u mastilo. U okviru istraživanja ispitivan je uticaj sadržaja genipina na fizičko-mehanička svojstva, profil otpuštanja natrijum alendronat trihidrata i citotoksičnost dobijenih implantata. Rezultati istraživanja prikazani su kroz četiri istraživačke faze koje su rezultirale objavljivanjem četiri naučna rada koji predstavljaju sastavni deo ove doktorske disertacije.

2. Teorijski deo

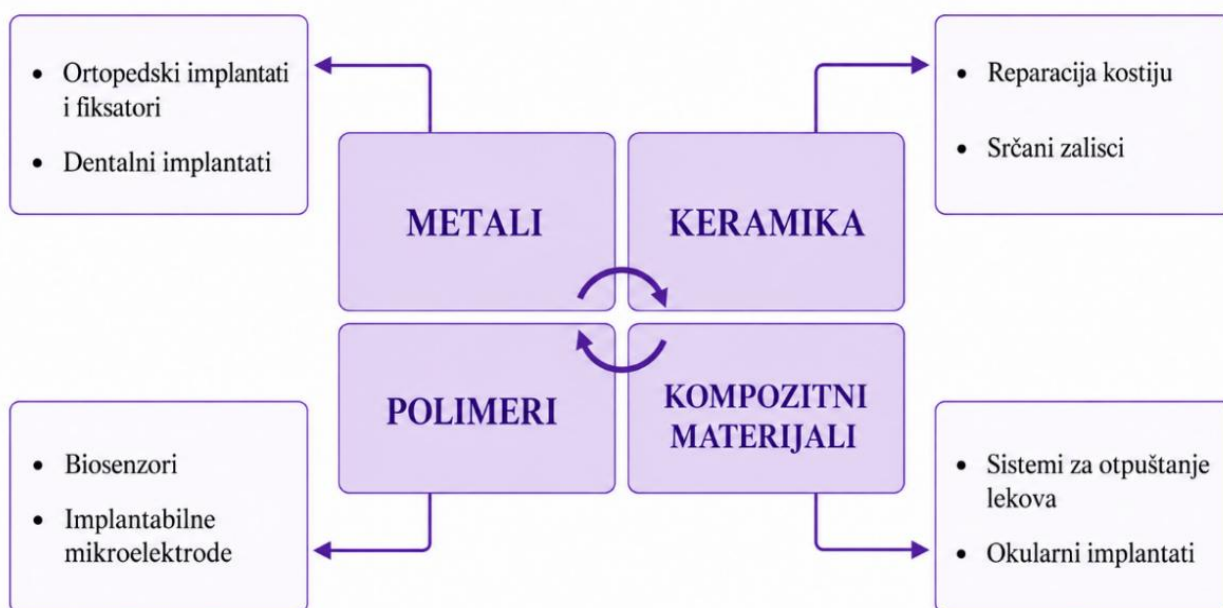
2.1. Biomaterijali

Biomaterijali su materijali prirodnog ili sintetičkog porekla koji dolaze u kontakt sa biološkim sistemima kako bi lečili, zamenili ili regenerisali tkivo ili organe. Oni moraju da ispune određene kriterijume kao što su biokompatibilnosti, biofunktionalnosti i mehaničkih karakteristika, a često su i biorazgradivi, odnosno resorbuju se u ljudskom organizmu [14,15].

Jedna od osnovnih podela biomaterijala prema sastavu (slika 2.1.) je na:

- metalne biomaterijale kao što su na primer titanijum, magnezijum, legure kobalta i nerđajući čelik koji se koriste za ortopedske i stomatološke implantate,
- keramičke biomaterijale kao na primer hidroksiapatit i aluminijum oksid koji su pogodni za koštane i stomatološke implantate,
- polimerne biomaterijale prirodnog (npr. kolagen, želatin, alginat i dr.) ili sintetičkog porekla (npr. polimlečna kiselina, poliglikolna kiselina, poli(ϵ -kaprolakton) i dr.) koji se koriste za kontrolisano otpuštanje lekova i u regeneraciji tkiva,
- kompozitne biomaterijale koji predstavljaju kombinaciju više komponenata radi postizanja optimalnih svojstava i mogu se koristiti u vidu premaza, vlakana, filmova, pena, netkanih ili tkanih struktura u biomedicinskim uređajima i proizvodima.

Biomaterijali se primenjuju u različitim granama medicine, imunologiji, neurologiji, kardiohirurgiji, ortopediji, oftalmologiji kao i u stomatologiji, farmaciji i veterinarskoj medicini. Osnovna svojstva koje biomaterijal treba da ispuni su otpornost na koroziju i habanje, žilavost, elastičnost, odgovarajuća mehanička čvrstoća, odlična biokompatibilnost, adhezija, biofunktionalnost i netoksičnost. Biokompatibilnost je ključno svojstvo biomaterijala i predstavlja sposobnost materijala da obavlja svoju funkciju bez izazivanja neželjenih lokalnih ili sistemskih reakcija organizma i da ne izazove imunski odgovor organizma [16,17].

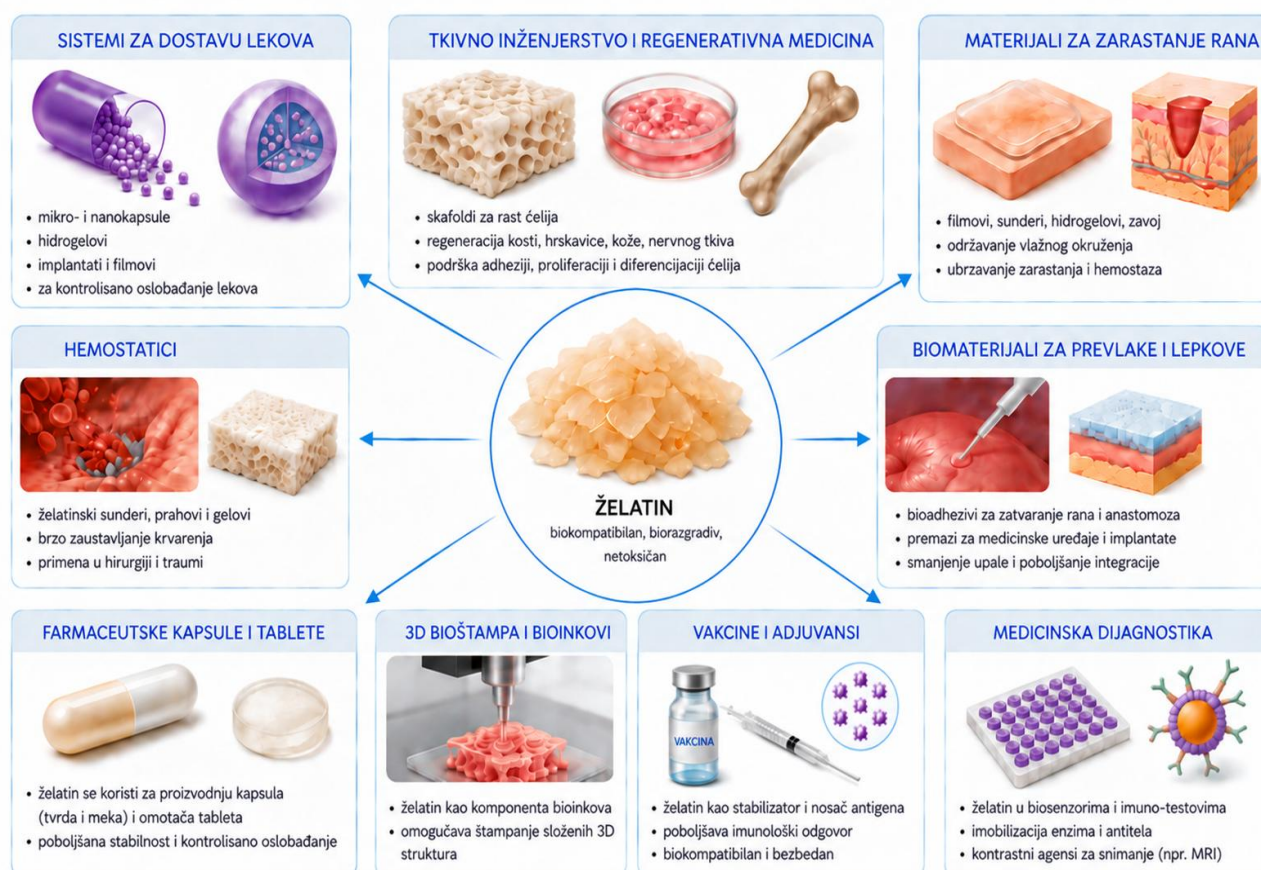


Slika 2.1. Klasifikacija i neke od primena biomaterijala (preuzeto iz [14]).

Biomedicinsko inženjerstvo kombinuje principe prirodnih (biologija, fizika, hemija) i inženjerskih nauka (nanotehnologija, inženjerstvo materijala, hemijsko i biohemijsko inženjerstvo, mašinstvo, elektrotehnika) u cilju razvijanja biomimetičnih struktura, implantata za regeneraciju i inženjerstvo tkiva, kao i sistema za dostavu lekova. Ključna faza u tom razvoju je praćenje interakcija između biomaterijala i bioloških sistema i optimizacija biokompatibilnosti. Uz to, u savremenim istraživanjima se teži razvoju naprednih biomaterijala koji odgovaraju na fiziološke signale. Ovakav multidisciplinarni pristup u kombinaciji sa inovativnim tehnologijama za procesiranje materijala omogućava razvoj savremenih terapijskih sistema.

2.2. Želatin

Želatin je jedan od prirodnih biomaterijala koji se često koristi u savremenoj farmaceutskoj i biomedicinskoj industriji zbog svoje biokompatibilnosti, biorazgradivosti, male imunogenosti, bioaktivnosti i niske cene [18]. To je jedan od značajnih pomoćnih materijala u farmaciji koji se koristi za proizvodnju tvrdih i mekih kapsula, tableta, mikrosfera, nanočestica, hidrogelova, oralnih filmova i implantata za dostavu lekova (slika 2.2). U regeneraciji tkiva želatin je posebno pogodan zbog sličnosti sa prirodnim kolagenom, dobre biokompatibilnosti i mogućnosti kombinovanja sa drugim polimerima i bioaktivnom keramikom.



Slika 2.2. Primena želatina u farmaciji i biomedicini

Želatin je prirodni adhezivni polimer dobijen delimičnom hidrolizom kolagena, najzastupljenijeg strukturnog proteina u ljudskom organizmu. U zavisnosti od načina hidrolize kolagena, razlikuju se dva osnovna tipa želatina: želatin A i želatin B. Želatin A je poreklom iz svinjske kože i dobija se kiselom hidrolizom kolagena, ima izoelektričnu tačku (pI) između 7 i 9, veći

sadržaj pozitivno naelektrisanih grupa pri fiziološkoj pH vrednosti, bolju sposobnost interakcije sa anjonskim molekulima, veću transparentnost i često bolju sposobnost geliranja. Želatin B dobija se iz govedih kostiju ili kože alkalnom hidrolizom kolagena, ima izoelektričnu tačku između 4,7 i 5,3, negativno površinsko naelektrisanje pri fiziološkoj pH vrednosti, veći sadržaj karboksilnih grupa i pogodnost vezivanja katjonskih biomolekula. Razlike između ova dva tipa želatina su prvenstveno zbog načina dobijanja i zato imaju različita fizičko-hemijska svojstva i interakcije sa aktivnim supstancama, što utiče na izbor odgovarajućeg tipa za specifičnu primenu u 3D štampi i dobijanju farmaceutskih oblika i implantata za dostavu lekova [4,18].

Nedostaci želatina su slaba mehanička svojstva i brza razgradnja u ljudskom organizmu. Da bi se to prevazišlo, želatin se kombinuje sa različitim polimerima ili česticama i primenjuju se fizičke ili hemijske modifikacije. Ovim metodama se dobijaju materijali sa poboljšanim mehaničkim, fizičko-hemijskim i biološkim svojstvima što ih čini pogodnim za razvoj sistema za dostavu aktivnih supstanci i za primenu u regeneraciji tkiva.

2.2. Bukalni filmovi

Adhezivni filmovi za bukalnu primenu (bukalni filmovi) su farmaceutski oblik za dostavu aktivne supstance koji se postavlja na unutrašnju stranu obraza odnosno na bukalnu mukozu [19,20]. Ovi filmovi predstavljaju tanke, fleksibilne polimerne formulacije i mogu da se koriste u terapiji pacijenata za postizanje lokalnog efekta u usnoj duplji ukoliko se tu oslobodi aktivna supstanca, ali i za postizanje sistemskog efekta zbog apsorpcije aktivne supstance direktno u krvotok kroz sluzokožu obraza. Bukalni filmovi mogu biti jednoslojni ili višeslojni. Kod jednoslojnih filmova, otpuštanje aktivne supstance je kroz obe strane filma, dok kod višeslojnih filmova zbog postojanja zaštitnog sloja (engl. *backing layer*) oslobađanje aktivne supstance je jednosmerno [19].

Prednost apsorpcije aktivne supstance preko bukalne mukoze je brza dostupnost, dobra permeabilnost zbog vaskularizacije, zaobilazi se gastrointestinalni trakt i potencijalna degradacija aktivne supstance, a takođe se postiže bolja biodostupnost aktivnih supstanci koje podležu efektu prvog prolaska kroz jetru (engl. *first-pass metabolism*). Dobri su da se koriste kod osoba koje imaju problema sa gutanjem, kod pedijatrijske i gerijatrijske populacije, kao i kod osoba koje imaju gastrointestinalne poremećaje [21,22]. Pored toga, bukalni filmovi imaju prednosti kao što su jednostavna primena, dobra prihvatljivost od strane pacijenata, mogućnost preciznog doziranja i brzo oslobađanje aktivne supstance.

Polimeri predstavljaju osnovnu komponentu bukalnih filmova i formiraju matricu u koju se inkorporiraju aktivne supstance. Obezbeđuju mehaničku stabilnost filma i kontrolišu profil oslobađanja aktivne supstance. Izbor odgovarajućeg polimera značajno utiče na fizičko-hemijska, biofarmaceutska i mehanička svojstva filma, uključujući fleksibilnost, čvrstoću, sposobnost adhezije na sluzokožu, kao i na profil oslobađanja aktivne supstance. Dobra mukoadhezija omogućava blizak kontakt između filma i sluzokože, čime se produžava vreme zadržavanja filma i povećava efikasnost apsorpcije aktivne supstance. Mukoadhezivni polimeri poseduju sposobnost vezivanja za površnu sluzokožu i tako ostvaruju produžen kontakt adhezivnog filma sa bukalnom mukozom [23,24]. Polimeri koji se koriste u formulaciji bukalnih filmova treba da budu netoksični, biokompatibilni, mukoadhezivni i da mogu da formiraju homogen i fleksibilan film. Prirodni polimeri su posebno značajni zbog svoje biokompatibilnosti, biodegradabilnosti i male toksičnosti, dok se sintetički polimeri koriste u formulaciji bukalnih filmova zbog odlične mogućnosti formiranja filmova, dobre stabilnosti i mehaničkih svojstava. Najčešće korišćeni polimeri su kolagen, želatin, derivati celuloze (posebno hidroksipropil metilceluloza (HPMC)), hitozan, natrijum-alginat, poli(ϵ -kaprolakton), polivinil alkohol (PVA), polivinilpirolidon (PVP). Pojedinačni polimer retko može da ispunji sve zahteve za formiranjem optimalnog bukalnog filma, tako da se često koriste blende različitih

polimera. U formulacijama bukalnih filmova bitna je kombinacija polimera kako bi se postigla ravnoteža između mehaničke čvrstoće i fleksibilnosti, dobra mukoadhezivna svojstva i odgovarajuća brzina oslobađanja aktivne supstance [25,26].

Mehanička svojstva predstavljaju jedan od najvažnijih kriterijuma kvaliteta bukalnih filmova. Film mora biti dovoljno čvrst, da izdrži manipulaciju tokom proizvodnje, pakovanja i primene, ali istovremeno i dovoljno fleksibilan da se prilagodi pokretima usne duplje bez deformacije. Optimalni balans između čvrstoće i fleksibilnosti ovih filmova se obično postiže dodavanjem plastifikatora, kao što su npr. glicerol, propilen-glikol ili polietilen-glikol. Pored mehaničkih svojstava, kod bukalnih filmova bitno je i efikasno oslobađanje aktivne supstance. U zavisnosti od terapijskog cilja, filmovi mogu da imaju: brzo oslobađanje (engl. *immediate release*), produženo oslobađanje (engl. *sustained release*) ili kontrolisano oslobađanje (engl. *controlled release*). Kinetika oslobađanja zavisi od sastava polimerne matrice, rastvorljivosti aktivne supstance, debljine filma, kao i prisustva pomoćnih supstanci.

Mukoadhezivna svojstva, odnosno mogućnost vezivanja filma za bukalnu mukozu, predstavljaju takođe jednu od važnih karakteristika bukalnih filmova koje utiču na profil i kinetiku otpuštanja aktivne supstance. Bukalna mukozna je vlažna unutrašnja sluzokoža obraza u usnoj duplji, koja obuhvata epitel i vezivno tkivo ispod epitela (*lamina propria*) koje sadrži vlakna kolagena, fibroblaste, krvne sudove, nerve i imunske ćelije. Mukus predstavlja zaštitni sloj mukoze u čiji sastav ulaze voda, enzimi, proteini, elektroliti i mucin [27]. Mucin, najvažnija komponenta mukusa, se sastoji od glikoproteina visoke molekulske mase koji formiraju trodimenzionalni kohezivni gel koji se vezuje za epitel bukalne sluzokože i odgovoran je za adhezivna svojstva mukoze [28,29]. Mukoadhezija predstavlja sposobnost materijala da se veže za površinu mukoze. Ova veza se ostvaruje u dve faze: kontakt, a zatim konsolidacijom [30,31]. U prvoj fazi kontakta dve površine dolaze u dodir, a u drugoj mucin kvasi površinu materijal. Tada se površina materijala vezuje za površinu mukoze privlačnim fizičko-hemijskim silama i dolazi do mukoadhezije. U okviru nekoliko razvijenih teorija o fenomenu mukoadhezije [31- 33], teorija kvašenja objašnjava da se veza ostvaruje adhezijom kao posledica prodiranja materijala kroz nesavršenosti površine mukoze. Ova teorija je primenljiva za materijale sa malom viskoznošću. Prema elektrostatičkoj teoriji dolazi do prelaza elektrona između materijala i mukoze i stvara se dvostruki elektrostatički sloj na međupovršini uz pojavu privlačnih sila koje održavaju mukoadheziju. Po difuzionoj teoriji dolazi do interakcije polimernih lanaca filma sa lancima glikoproteina u mucinu i kao rezultat difuzije postiže se delimično trajna veza koja ostvaruje adheziju. Teorija loma definiše jačinu adhezije kao silu koja je potrebna da se dve površine razdvoje i ova teorija omogućava *in vitro* ispitivanja uz kvantitativne rezultate.

Razvijeno je nekoliko tehnika za dobijanje bukalnih filmova, ali se izdvajaju izlivanje iz rastvora, ekstruzija iz rastopa, štampa brizganjem i 3D štampa.

Izlivanje iz rastvora je najrasprostranjenija metoda zbog svoje jednostavnosti i niske cene. Nakon pripreme rastvora polimera (najčešće vodeni) obično se dodaju aktivne supstance i pomoćna sredstva (ekspicijensi). Rastvor se izliva na ravnu površinu i kada se film osuši, seče se na određenu dimenziju koja osigurava propisanu dozu aktivne supstance [34,35]. Dodatna prednost ove metode je što se može koristiti u industrijskim uslovima. Nedostaci su poteškoće u postizanju uniformnosti raspodele aktivne supstance, mogućnost njene kristalizacije nakon završetka procesa, kao i pojava mehurića vazduha.

Ekstruzija iz rastopa je kontinualan proces pri kome se rastopljena smeša polimera, aktivne supstance i pomoćnih sredstava izvlači kroz diznu. Ovaj proces ima dobru reproduktivnost i mogućnost automatske kontrole pa se često koristi u industrijskim uslovima. Na ovaj način se postiže bolja uniformnost raspodele aktivne supstance, radi se bez rastvarača koji bi mogao zaostati nakon sušenja, a moguće je inkorporirati teže rastvorne aktivne supstance [36]. Nedostaci ove metode su

moгуćnost rekristalizacije aktivne supstance i bubrenje filma nakon procesa kao i ograničena primena kod aktivnih supstanci osetljivih na visoke temperature koje se koriste tokom procesiranja.

Štampa brizganjem (engl. *inkjet*) je proces u kome se rastvor aktivne supstance u vidu kapljica brizga preko supstrata koji je obično polimerni film dobijen drugim tehnikama (izlivanjem iz rastvora, ekstruzijom iz rastopa ili 3D štampom) [37]. Prednosti ove metode su sprečena rekristalizacija aktivne supstance, očuvana mehanička svojstva usled nanošenja aktivne supstance na film umesto inkorporiranja i mogućnost personalizacija doze. Glavni nedostaci su što zahteva pripremu filma drugom metodom, ograničenost na male zapremine i moguća neravnomernost nanošenja.

3D štampa ili aditivna tehnologija je tehnika kod koje se sloj po sloj materijala dodaje prema softverski zadatom obliku. Ova tehnika bi mogla da prevaziđe ograničenja drugih metoda za dobijanje bukalnih filmova sa malim dozama aktivne supstance. Dodavanjem slojeva moguće je povećati količinu inkorporiranog leka po jedinici zapremine i povećati aktivnu površinu kontakta sa bukalnom mukozom [38,39]. Primenom ove tehnike sloj po sloj moguće je i rešiti inkompatibilnost pojedinih sastojaka bukalnog filma koji bi se štampali u posebnim slojevima [40]. Prednosti 3D štampe su personalizacija terapije i povećana preciznost proizvodnje. Nedostaci su mali obim proizvodnje, ali sa druge strane vodi ka personalizaciji, pa i nisu potrebne velike serije, kao i otpor u kliničkom usvajanju ove tehnike [34].

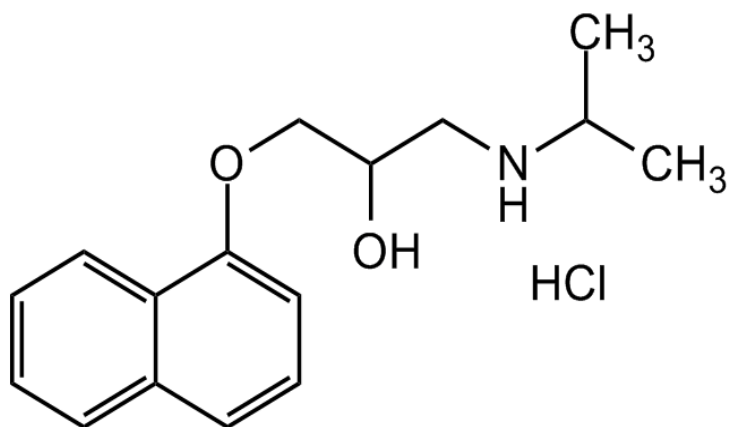
Za razvoj odgovarajućeg bukalnog filma, kojim će se postići lokalni ili sistemski terapijski efekat određene aktivne supstance, potrebno je izabrati pogodne polimere, odnosno blende polimera, kao i druge pomoćne supstance koje će podržati mukoadhezivnost, mehanička svojstva i određen profil otpuštanja.

2.3. Propranolol hidrohlorid

Propranolol hidrohlorid (PRH) (slika 2.3) je neselektivni β -adrenergički blokator koji kompetitivno inhibira β_1 i β_2 adrenergičke receptore, čime se smanjuje srčana frekvencija i kontraktilnost miokarda. Primenuje se u terapiji arterijske hipertenzije, angine pektoris, određenih srčanih aritmija, kongestivne srčane insuficijencije, esencijalnog tremora, migrene i anksioznih poremećaja. PRH ima visoku rastvorljivost pri fiziološkim pH vrednostima i visoku permeabilnost, tako da prema biofarmaceutskom sistemu klasifikacije (BSK) pripada I klasi [41].

Nakon oralne primene, PRH se dobro apsorbuje iz gastrointestinalnog trakta (GIT), pri čemu se najveće koncentracije u krvi postižu nakon 1–2 sata posle primene [10]. Ipak, značajan deo PRH se metaboliše pri prvom prolasku kroz jetru, što dovodi do niske biološke raspoloživosti odnosno u sistemsku cirkulaciju dospeva samo 30–40% primenjene doze [42]. Zbog izraženog efekta prvog prolaza, farmakokinetika PRH zavisi od načina primene, tako da bi primena PRH putem bukalne ili sublingvalne sluzokože mogla da smanji ovaj efekat i poveća bioraspoloživost PRH u organizmu.

Inkorporacija PRH u bukalne filmove posebno je značajna zbog mogućnosti postizanja kontrolisanog ili produženog oslobađanja, poboljšanja terapijske efikasnosti i povećanja komfora primene kod pacijenata [43,44]. PRH zauzima značajno mesto u pedijatrijskoj farmakoterapiji jer je potvrđena njegova efikasnost i bezbednost u lečenju infantilnih hemangioma, gde se koristi kao terapija prvog izbora. Njegova široka primena u pedijatriji podstakla je razvoj novih farmaceutskih oblika (kao npr. bukalni filmovi) prilagođenih deci jer kod njih čvrsti oralni farmaceutski oblici često nisu pogodni [45,46].



Slika 2.3. Molekulska formula propranolol hidrohlorida (PRH)

Ispitivanja bukalnih filmova sa PRH pokazala su da polimerna matrica značajno utiče na mehanička, mukoadhezivna i biofarmaceutska svojstva, kao i da se primenom savremenih tehnologija, uključujući i 3D štampu, mogu dobiti filmovi sa pogodnim karakteristikama za produženo oslobađanje PRH [43-46].

2.4. Implantati za dostavu aktivne supstance i regeneraciju tkiva

Oralni put primene i dalje predstavlja najpopularniji i najpraktičniji način dostave aktivne supstance, sa brojnim prednostima. Međutim, zajedno sa drugim uobičajenim putevima primene, kao što su transdermalni put ili intravenska (IV) injekcija/infuzija, imaju i određene nedostatke i izazove. Atraktivna metoda dostave lekova je primena polimernih implantata sa oslobađanjem aktivne supstance. Ovi implantati omogućavaju ciljanu i lokalnu primenu aktivne supstance i mogu postići terapijski efekat pri manjim koncentracijama leka [47,48], zbog toga se mogu smanjiti potencijalni neželjeni efekti terapije uz mogućnost povećanja adherence odnosno saradnje između pacijenta i lekara [36]. Kod implantata za dostavu aktivne supstance adherence se povećava zato što pacijent ne mora svakodnevno da uzima lek te se smanjuje mogućnost zaboravljanja uzimanja doze, obezbeđuje se kontinuirano oslobađanje leka tokom dužeg perioda i terapija ne zavisi od discipline pacijenta. Ovakvi implantati imaju potencijal da omoguće primenu lekova koji su inače nepogodni za oralnu primenu, jer zaobilaze metabolizam prvog prolaza i hemijsku degradaciju u gastrointestinalnom traktu, čime povećavaju bioraspoloživost [49].

Regeneracija velikih koštanih defekata predstavlja jedan od najvećih izazova savremene regenerativne medicine. Tradicionalni pristupi, kao što su autologni i alogeni koštani graftovi, povezani su sa brojnim nedostacima: ograničena dostupnost tkiva, rizik od infekcije i imunološke reakcije. Kao perspektivna alternativa ovim konvencionalnim metodama razvila se oblast regeneracije koštanog tkiva motivisana sve većim globalnim porastom bolesti i povreda kostiju [50,51]. Među njima, osteoporoza je najrasprostranjenija i predstavlja značajan zdravstveni problem zbog svoje povezanosti sa hroničnim bolom, invaliditetom i povećanom smrtnošću [52,53]. Prema definiciji Svetske zdravstvene organizacije (SZO), osteoporoza je sistemska, progresivna bolest skeleta, koju karakteriše smanjena koštana masa, sa narušenom morfologijom i mikrostrukturom što za posledicu ima povećanje krstosti i podložnost prelomima [54]. U tretmanu osteoporoze postoje dve vrste aktivnih supstanci: antiresorptivni agensi koji sprečavaju razgradnju postojeće kosti tako što inhibiraju osteoklastnu aktivnost i anabolički koji povećavaju formiranje kosti. Oni deluju na potpuno suprotne načine kako bi ojačali kost i smanjili rizik od preloma.

Implantati za regeneraciju tkiva se mogu podeliti u dve kategorije: nedegradabilne i biodegradabilne. Nedegradabilni implantati su dizajnirani da ostanu trajno u telu i pružaju strukturnu podršku. To su implantati na bazi metala, legura i keramičkih materijala, kao i kompozita [55]. Materijali kao što su čelik, titanijum ili hrom-kobaltne legure pružaju dobru mehaničku podršku koštano tkivu, ali imaju ograničenja kao što su emisija jona i pojava zapaljenskih procesa u organizmu. S druge strane biodegradabilni implantati obezbeđuju vremenski ograničenu mehaničku podršku stvarujući poroznu strukturu za migraciju ćelija i obezbeđuju terapijski tretman [56]. Za dobijanje trodimenzionalnih implantata za koštano tkivo može se koristiti širok spektar biomaterijala kao što su biokeramika [57] (trikalcijum fosfat, hidroksiapatit, bio staklo i drugi) i biodegradabilni prirodni ili sintetički polimeri.

U procesu izrade implantata značajnu ulogu imaju polimeri i njihove blende koje omogućavaju preciznu kontrolu kinetike oslobađanja aktivne supstance, poboljšanu biokompatibilnost i odgovarajuća mehanička svojstva. Neki od najviše korišćenih polimera su hitozan, alginat, kolagen, želatin, poli(ϵ -kaprolaktona), polimlečna kiselina, poliglikolna kiselina i njihov kopolimer poli(mlečna-ko-glikolna kiselina). Kombinovanje prirodnih i sintetičkih polimera omogućava dobijanje materijala sa unapređenim biološkim i mehaničkim svojstvima. Izbor blende polimera dodatno zavisi od fizičko-hemijskih svojstava aktivne supstance i željenog profila oslobađanja.

Fizičko-hemijska svojstva aktivne supstance određuju izbor odgovarajućeg sistema za dostavu u skladu sa njegovim fizičkim, hemijskim, morfološkim i mehaničkim svojstvima, što vodi do mogućeg profila otpuštanja i interakcije u organizmu. U tretmanu defekata koštanog tkiva postoji čitav niz sistema za dostavu aktivne supstance različite strukture:

- Sistem zasnovan na nanočesticama - Nanočestice, veličine 1-100 nm, intenzivno se razvijaju u ovoj oblasti zbog njihovih malih dimenzija, velikog odnosa aktivne površine prema zapremini i mogućnosti modifikacije površine. Ovaj sistem uključuje nanočestice za dostavu aktivne supstance u koštano tkivo. Istraživanja koja su sprovedena ispitivala su korišćenje nanočestica silicijum dioksida, hidroksiapatita, zlata, titanijum nanocevi, kao i polimernih nanočestica želatina i hitozana i poliuretanskih nanomicela [58].
- Sistemi na bazi lipozoma - Lipozomi poseduju sfernu šupljinu ograničenu dvostrukom membranom sličnoj ćelijskoj, i u toj šupljini mogu da nose različite aktivne supstance. Zbog njihove amfifilične prirode kompatibilni su i sa hidrofilnim i sa hidrofobnim aktivnim supstancama [59,60]. Lipozomi se smatraju najmanje toksičnim nanočesticama što ih čini pogodnim za primenu u medicini [61]. Što se tiče koštanog tkiva i tretmana osteoporoze, još uvek čeka kliničku potvrdu [62].
- Sistemi na bazi nanočestica čvrstih lipida - Ovaj sistem predstavlja alternativu prethodnom pošto su nanočestice čvrstog lipida otpornije i mogu poslužiti kao nosači velikog broja aktivnih supstanci. Predstavlja disperziju čvrstih, biodegradabilnih lipida u vodi. Odlikuje se povećanom biodostupnošću, produženim profilom otpuštanja i povećanom efikasnošću enkapsulacije [63]. Kada se poveže sa injektivnom sol-gel tehnikom, predstavlja vrlo efikasan način dostave aktivne supstance. Tako je objavljeno istraživanje o potkožno aplikovanom gelu sa lipidnim česticama natrijum alendronat trihidrata uz dobru biokompatibilnost [64]. Tu su naravno ograničenja u količini doze, s obzirom na nano dimenzije, i u vezi sa ponovljivošću i mogućnošću podizanja kapaciteta proizvodnje.
- Sistemi na bazi hidrogelova - Hidrogelovi predstavljaju trodimenzionalne umrežene strukture uglavnom hidrofilnih polimera koje u sebi zadržavaju i jedan deo vode. Ovakva struktura pogodna je za enkapsulaciju aktivnih supstanci čime se obezbeđuje produženo ili odloženo

otpuštanje. Profil otpuštanja može da se modifikuje stepenom umrežavanja polimera [65]. Primena biopolimera i inkorporacija nanočestica u matricu hidrogela mogu dodatno da poboljšaju mehanička svojstva i efikasnost enkapsulacije aktivne supstance. Izazov za ovu vrstu sistema je da se postigne integritet strukture, postojana mehanička svojstva, kontrolisana degradacija i precizno otpuštanje aktivne supstance.

- Sistemi na bazi funkcionalnih segmenata - Ovo su sistemi uglavnom na bazi vodorastvornih polimera kod kojih se ciljano dostavlja aktivna supstanca do obolelog tkiva [66]. To se postiže tako što se molekulima aktivne supstance dodaju funkcionalni segmenti koji će sprovesti supstancu do ciljnog mesta u tkivu.
- Sistemi na bazi stimulisanog odgovora - Razvijen je veliki broj strategija kako da se sprovede terapija u prostornom i vremenskom okviru do ciljanog tkiva primenom funkcionalnih grupa koje reaguju na stimulans. Ovi sistemi imaju sposobnost da reaguju na spoljašnje ili unutrašnje okidače, uključujući mehaničke sile, električna ili magnetna polja, fluktuacije temperature, promene pH vrednosti ili enzimsku aktivnost u mikrookruženju obolelog tkiva [67].
- Sistemi na bazi makromolekularne terapije - U ovim sistemima se koriste makromolekuli vodorastvornih polimera koji vezuju aktivne supstance putem fizičke adsorpcije, hemijske konjugacije ili prosto inkorporacijom i ostvaruju kontrolisano otpuštanje. Prirodni polimeri imaju značajnu ulogu u ovim sistemima zbog njihove dostupnosti i bioobnovljivosti [68].
- Sistemi sa dualnom terapijom - Ovi sistemi istovremeno dostavljaju i anaboličke i antiresorptivne agense do različitih delova kosti i na taj način podstiču terapeutsko dejstvo bez neželjenih efekata. Sprovedeno je i nekoliko kliničkih studija koje nisu pokazale kumulativni efekat ovog pristupa, ali je svakako potvrđeno da nema neželjenih efekata u poređenju sa ne ciljanom terapijom anaboličkim aktivnim supstancama [69].
- Sistemi na bazi vezikula od makrofaga - Ovi sistemi imaju izuzetnu biokompatibilnost pošto su izvedeni od prirodnih ćelija i zadržavaju unutrašnje ćelijske funkcije, tako da su odlični nosači aktivnih supstanci. Neka istraživanja su izvedena sa belim ili crvenim krvnim zrnima, trombocitima i drugim ćelijama obezbeđujući produženu cirkulaciju i efektivniju dostavu aktivne supstance [70].
- Sistemi na bazi trodimenzionalnih implantata - Ovaj sistem predstavlja inovativnu alternativu tradicionalnim formulacijama. Razvijene su različite strategije integracije aktivnih supstanci u implantat kao što su inkorporacija u pore biokeramike, direktna inkorporacija u polimernu matricu ili prevlačenje površina mezoporoznih nanočestica da bi se postigla što veća doza i odgovarajući profil otpuštanja. Ovi sistemi imaju veliku perspektivu jer pored otpuštanja aktivne supstance, mogu omogućiti i zasejavanje ćelija i njihovu adheziju, migraciju i proliferaciju povećavajući regenerativnu efikasnost. [71].

Metod proizvodnje ima značajnu ulogu u razvoju implantata za dostavu aktivne supstance i svojstvima dobijenog implantata. Izbor metode zavisi od materijala koji ulaze u sastav implantata [72]. Savremene metode za izradu implatata uključuju presovanje (kompresiju), livenje iz rastvora, ekstruziju iz rastopa, elektropredenje i 3D štampu.

Direktno presovanje omogućuje procesiranje smeše aktivne supstance sa ekspicijensima (pomoćnim supstancama) ili samo aktivne supstance ukoliko ima dobru kompresibilnost. Kod ove metode se primenjuje mehaničko opterećenje na materijal koji se istiskuje kroz diznu ili u kalup koji

mu daje oblik. Prednost ove metode je što se ne koristi termička obrada i što nema prisutnog rastvarača, što je povoljno za manje stabilne aktivne supstance. Kod ovog procesa se postiže nešto brže otpuštanje, što može biti njegovo ograničenje. Površina implantata je nepravilna, a i pore su neregularnog oblika, često i sa kanalima pa je i otpuštanje aktivne supstance teško kontrolisati [73]. Dodatno poboljšanje predstavlja oblaganje površine odgovarajućom prevlakom.

Ekstruzija iz rastopa je metoda kod koje se kontrolisanom temperaturom topljenja i mešanja aktivne supstance i polimera dobro homogenizovani rastop istiskuje kroz diznu. Prednost ove metode je što se povećava bioraspoloživost teže rastvornih aktivnih supstanci, a i profil otpuštanja se može kontrolisati. Takođe je povoljno što se ne koriste rastvarači što povećava biokompatibilnost dobijenih implantata. Ovaj proces se lako može prebaciti na industrijsku proizvodnju. Glavni nedostatak je rad sa višim temperaturama i stoga nije pogodna za termosenzitivne aktivne supstance. Takođe se mora voditi računa i o stabilnosti polimera na višim temperaturama, pa je rad ograničen samo na termostabilne polimere [72].

Izlivanje iz rastvora je process u kom se homogenizovan rastvor aktivne supstance i polimera izliva u kalup određenog oblika i ostavlja se da rastvarač ispari, a implantat zadržava oblik kalupa. Prednost ove metode je jer se dobija homogenizovan sastav i željeni oblik implantata. Ograničenje je prisustvo rastvarača koji može biti toksičan [74].

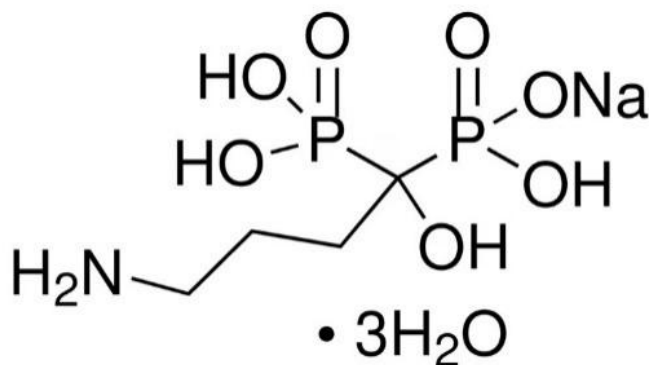
Brizganje u kalup kombinuje toplotu i pritisak da rastopljenu smešu aktivne supstance i polimera brizga u kalup određenog oblika. Nakon hlađenja smeše, implantat finalnog oblika se vadi iz kalupa. Glavna prednost ove metode je mogućnost izrade različitih oblika i odsustvo rastvarača. Pored toga moguće je da se pređe na veći obim proizvodnje. Primena toplote i pritiska dovodi do intenzivnije interakcije polimera i aktivne supstance što utiče i na profil otpuštanja. Sa druge strane, visoke temperature mogu uticati na stabilnost aktivne supstance i polimera, pa bi to bilo glavno ograničenje brizganja u kalup [75].

Elektropredenje je proces dobijanja finih vlakana prečnika od nekoliko nanometara do mikrometara. Rastop ili rastvor polimera sa aktivnom supstancom se istiskuje iz šprica i raspršuje iz igle uz primenu visokog elektrostatičkog napona. Pod uticajem električnog polja dolazi do formiranja Tajlerovog konusa i brzog formiranja finih vlakana koja se skupljaju na kolektoru. Jedna vrsta ove metode je elektropredenje iz rastopa uz korišćenje toplote pa se i ovde javlja problematika termosenzitivnih aktivnih supstanci i termostabilnost polimera. Vrsta elektropredenja iz rastvora sa druge strane koristi rastvarače i to je takođe ograničenje za ovu metodu zbog moguće citotoksičnosti. Izbor koja vrsta elektropredenja će se koristiti zavisi od svojstava aktivne supstance i polimera, kao i profila otpuštanja koji se želi postići. Elektropredenje iz rastopa omogućava duže, linijsko otpuštanje aktivne supstance, dok je kod elektropredenja iz rastvora češće početno naglo oslobađanje aktivne supstance [76].

3D štampa predstavlja tehnologiju u kojoj se dodaje sloj po sloj materijala da bi se formirao konačan oblik implantata. Postoji nekoliko metoda ovog načina procesiranja i svima je zajedničko da se oblici dobijaju prema zadatom softverskom modelu. Tradicionalne metode za izradu implantata često ne mogu da omoguće preciznu kontrolu veličine, oblika i poroznosti, niti da obezbede zadovoljavajuću reproduktivnost i konzistentnost procesa izrade. Zato je 3D štampa dobila na značaju kao metoda za procesiranje implantata. Primena 3D štampe omogućava proizvodnju složenih oblika i struktura sa velikom preciznošću i kontrolisanim karakteristikama. Izbor metode zavisi od svojstava polaznih komponenata implantata, fizičko-mehaničkih svojstava dobijenog implantata i profila otpuštanja aktivne supstance koji se žele postići. Ova tehnologija pruža mogućnost izrade specifičnih implantata prilagođenih pacijentu, čime se unapređuje uspešnost terapije i regeneracije tkiva [77].

2.5. Natrijum alendronat trihidrat

Alendronska kiselina pripada grupi amino-bisfosfonata, lekova koji se široko primenjuju u terapiji bolesti kostiju praćenih povećanom resorpcijom koštanog tkiva. Bisfosfonati deluju na strukturu kosti i njenu mineralizaciju. Natrijum alendronat trihidrat (ALN) (slika 2.4) predstavlja natrijumovu so alendronske kiseline koja se koristi kao aktivna supstanca u brojnim lekovima namenjenim lečenju osteoporoze, Pagetove bolesti kostiju i drugih poremećaja metabolizma kostiju [78-80].



Slika 2.4. Molekulska formula natrijum alendronat trihidrata

ALN je jedan od najznačajnijih aminobisfosfonata u savremenoj terapiji osteoporoze. Njegovo snažno antiresorptivno dejstvo, dokazana efikasnost u prevenciji preloma i dugogodišnja klinička primena učinili su ga standardom u lečenju bolesti povezanih sa povećanom resorpcijom kostiju. ALN predstavlja izazovnu aktivnu supstancu zbog svoje velike polarnosti, higroskopsnosti i male permeabilnosti kroz biološke membrane. Zbog toga je razvoj inovativnih farmaceutskih oblika usmeren ka poboljšanju njihove stabilnosti, bioraspoloživosti i terapijske efikasnosti. Potrebno je razviti alternativne pristupe za lokalnu i bezbedniju primenu ALN jer je veoma koristan u lečenju defekata kostiju [81]. Posebnu pažnju privlače bioaktivna stakla i 3D štampani implantati koji služe kao nosači za lokalnu dostavu ALN. Lokalna primena ALN putem implantata pruža nekoliko značajnih prednosti: postizanje visokih koncentracija leka direktno na mestu defekta, smanjenje sistemskih neželjenih efekata, produženo oslobađanje tokom procesa zarastanja, poboljšana osteointegracija implantata i smanjenje resorpcije novostvorene kosti [82].

ALN selektivno deluje na koštano tkivo, naročito na mesta aktivne resorpcije. Mehanizam dejstva ALN zasniva se na apoptozi pre-osteoklasta i inhibiciji enzima farnesil-pirofosfat sintaze, čime se smanjuje aktivnost osteoklasta i usporava resorpcija koštanog tkiva [83]. Pored antiresorptivnog efekta, istraživanja su pokazala da ALN može da stimuliše osteoblaste i na taj način se povećava ekspresija osteogenih gena što doprinosi formiranju nove kosti [84]. Kao rezultat smanjuje se razgradnja koštanog tkiva, povećava mineralna gustina kostiju i smanjuje rizik od preloma.

Nakon oralne primene na prazan želudac, ALN ima lošu apsorpciju što je povezano i sa veoma niskom bioraspoloživošću (0,9–1,8%) usled nestabilnosti ALN u gastrointestinalnom traktu [85]. Prisustvo hrane, napitaka ili preparata koji sadrže kalcijum značajno smanjuju bioraspoloživost ovog leka. Takođe, oralne tablete ALN mogu da dovedu do smetnji u traktu kao što su lokalna iritacija sluzokože gornjeg dela trakta, ezofagitis, disfagija, mučnina ili bol u abdomenu. Dugotrajna terapija kod nekih pacijenata može da izazove i ozbiljnije komplikacije kao što su osteonekroza vilice, tipični prelomi femura, oštećenje bubrega i hipokalcemija [86,87].

Iako ALN pokazuje značajan potencijal u regeneraciji koštanog tkiva, neophodno je pažljivo optimizovati njegovu koncentraciju i kinetiku oslobađanja. Previsoke lokalne koncentracije mogu izazvati inflamatorne reakcije i usporiti proces regeneracije. Poseban problem predstavlja činjenica da visoke koncentracije alendronata mogu izazvati citotoksične efekte i usporiti osteogenezu, što ukazuje na značaj precizne kontrole kinetike oslobađanja. Zbog toga se posebna pažnja posvećuje razvoju implantata koji omogućavaju kontrolisano i produženo oslobađanje leka.

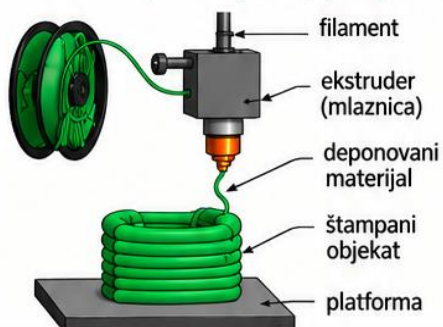
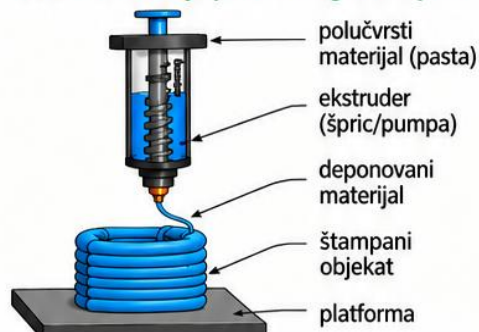
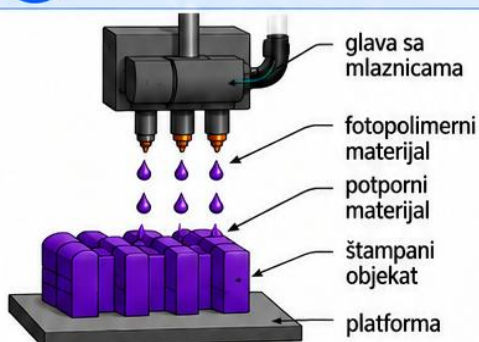
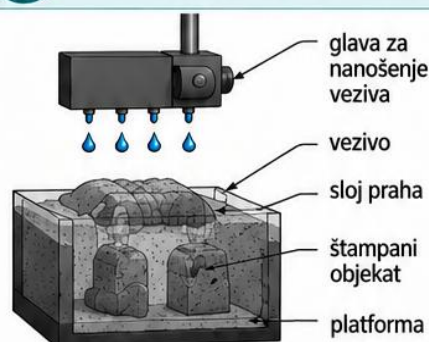
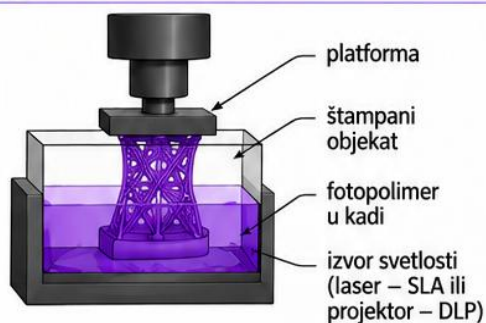
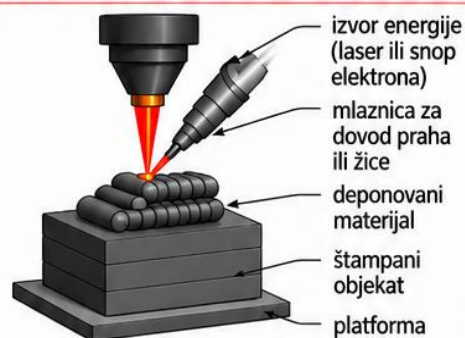
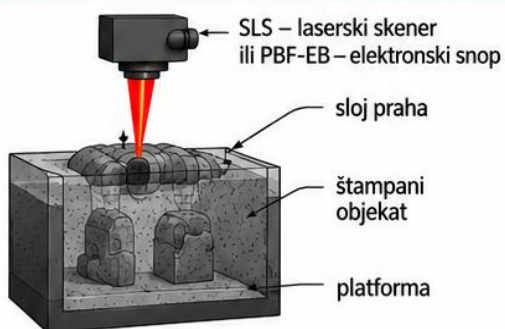
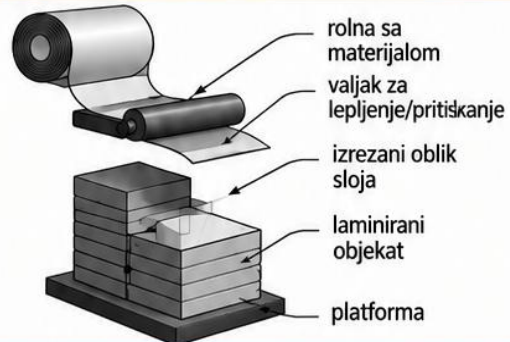
2.6. 3D štampa

Trodimenzijska štampa (3D štampa, engl. *3D printing*) predstavlja inovativnu aditivnu tehnologiju koja je našla primenu u različitim oblastima kao što su medicina, farmacija, stomatologija, elektronika, automobilska industrija, vazduhoplovstvo, prehrambena industrija i mnoge druge. Na osnovu kompjuterski potpomognutog dizajna (engl. *Computer Aided Design, CAD*) modela vrši se nanošenje materijala sloj po sloj do dobijanja finalnog proizvoda. Ovaj način proizvodnje omogućava preciznu kontrolu sastava, dimenzija, oblika i strukture. Upravo zbog toga je posebno značajna primena 3D štampe u farmaciji i biomedicini jer postoji mogućnost modifikacije i personalizacije terapije svakom pacijentu ponaosob što je jedan od ciljeva savremene medicine. Zahvaljujući mogućnosti prilagođavanja sastava formulacije i dizajna proizvoda dobijaju se trodimenzijske strukture željenog oblika i dimenzija prema specifičnim potrebama. 3D štampani farmaceutski oblici, kao i implantati sa inkorporiranim aktivnim supstancama, mogu da omoguće platformu za ciljanu dostavu i kontrolisano otpuštanje aktivne supstance tokom dužeg vremenskog perioda, čime se smanjuje učestalost doziranja [88].

Postoji više tipova i podtipova 3D štampa (slika 2.5), a neke od najviše zastupljenih su:

- ekstruzija materijala (engl. *material extrusion*) koja obuhvata metodu deponovanja istopljenog filameta (engl. *fused deposition modelling, FDM*) i metodu ekstruzije polučvrstog materijala (engl. *semi-solid extrusion, SSE*),
- brizganje materijala (engl. *material jetting*),
- vezivna 3D štampa (engl. *binder jetting*),
- fotopolimerizacija (engl. *vat photopolymerisation*) koja obuhvata stereolitografiju (engl. *stereolithography, SLA*) i digitalnu obradu svetlom (engl. *digital light processing, DLP*),
- nanošenje materijala primenom usmerene energije (engl. *directed energy deposition*) u obliku lasera ili snopa elektrona,
- fuzija praškastog sloja (engl. *powder bed fusion, PBF*) koja obuhvata selektivno lasersko sinterovanje (engl. *selective laser sintering, SLS*) i fuziju praškastog sloja mlazom elektrona (engl. *electron beam powder bed fusion, PBF-EB*),
- nanošenja slojeva laminiranjem (engl. *sheet lamination*).

Svaka od ovih tehnologija 3D štampe ima posebne karakteristike procesa, način formiranja slojeva, vrste materijala koje koristi i određene karakteristike gotovog proizvoda, a na osnovu toga i svoju posebnu primenu. U zavisnosti od potreba i svojstava finalnih proizvoda, primenom odgovarajuće tehnike 3D štampe, podešavanjem procesnih parametara, izborom vrsta materijala i inkorporiranih aktivnih supstanci, dobijaju se personalizovani farmaceutski oblici: tablete, oralni filmovi, transdermalni filmovi i drugo, kao i implantati što omogućava da se odgovori individualnim potrebama pacijenta i poveća efikasnost terapije [89].

1**EKSTRUZIJA MATERIJALA (FDM i SSE)****FDM – deponovanje istopljenog filamenta****SSE – ekstruzija polučvrstog materijala****2****BRIZGANJE MATERIJALA****3****VEZIVNA 3D ŠTAMPA****4****FOTOPOLIMERIZACIJA (SLA i DLP)****5****NANOŠENJE MATERIJALA PRIMENOM USMERENE ENERGIJE****6****FUZIJA PRAŠKASTOG SLOJA (SLS i PBF-EB)****7****NANOŠENJA SLOJEVA LAMINIRANJEM**

Slika 2.5. Vrste 3D štampe

2.6.1. 3D štampa ekstruzijom polučvrstih materijala

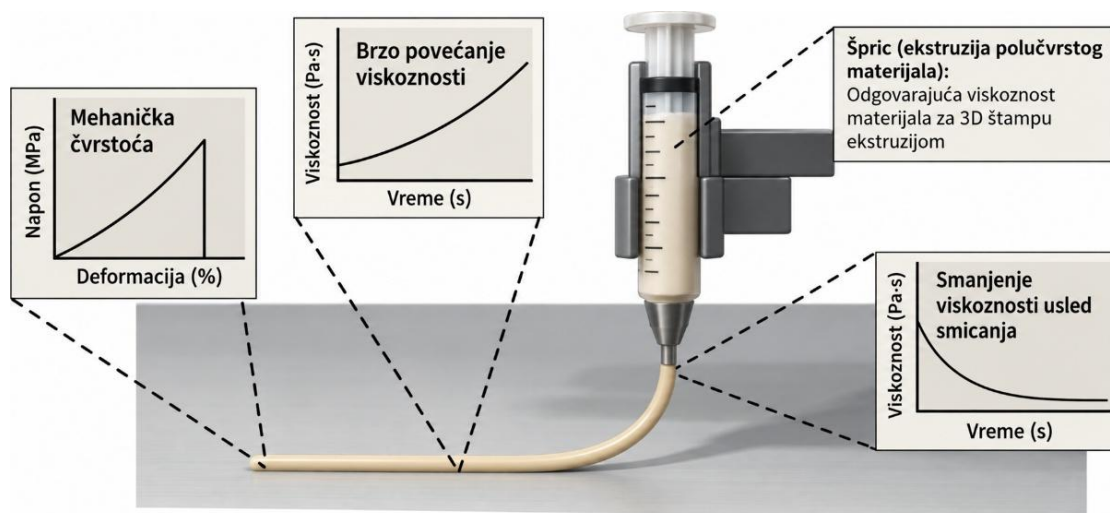
U poslednje vreme značajnu pažnju privlači 3D štampa ekstruzijom polučvrstih materijala (engl. *semi-solid extrusion*, SSE) koja predstavlja specifičnu podgrupu aditivne tehnologije u kojoj se materijal (mastilo, engl. *ink*) u obliku paste ili gela istiskuje kroz mlaznicu pri relativno blagim uslovima okoline i deponuje sloj po sloj prema prethodno napravljenom digitalnom modelu. Posebno je interesantna za primenu u farmaciji i inženjerstvu tkiva zbog toga što proces štampanja ne zahteva visoke temperature i UV zračenje, a koji bi mogli da dovedu do degradacije aktivnih supstanci ili bioloških materijala. Zbog toga je ova metoda pogodna za skoro sve vrste aktivnih supstanci, čak i za termolabilne i fotosenzitivne. Takođe je odgovarajuća i za štampanje bioloških materijala (npr ćelija ili ćelijskih agregata) odnosno njihovo inkorporiranje u materijal za štampu, čime se dobija posebna vrsta biomastila (engl. *bioink*). Zbog polučvrste konzistencije mastila (pasta) za štampanje mogu da se koriste prirodni polimeri kao što su želatin [90-92], alginat [93,94], hitozan [95,96] i drugi. Nakon procesa 3D štampe, očvršćavanje formulacije, odnosno proizvoda, može se ostvariti pri sobnoj ili nižoj temperaturi, bez dodatne obrade. U nekim slučajevima mogu da se koriste i naknadni postupci obrade kao što su umrežavanje (fizičko ili hemijsko) ili liofilizacija, što omogućava brže formiranje stabilne strukture i unapređenje funkcionalnih karakteristika proizvoda ukoliko je potrebno [97-99].

Polazno mastilo sadrži aktivnu supstancu, polimer ili blendu polimera, rastvarač i druge pomoćne supstance čija su svojstva prilagođena zahtevima primene finalnog proizvoda [100]. Kombinacijom navedenih komponenti formira se polučvrsta formulacija ili pasta pogodna za proces ekstruzije. Od svih supstanci koje ulaze u sastav formulacije, izbor polimernog nosača ima važnu ulogu, jer direktno utiče na podesnost materijala za štampu (engl. *printability*), mehanička svojstva odštampanog proizvoda, stabilnost aktivne supstance, kao i na profil njenog oslobađanja [95]. Zato je dobar izbor polimera od ključnog značaja za uspešnu primenu 3D štampe ekstruzijom polučvrstih materijala pri izradi personalizovanih doznih oblika i postizanju željenih karakteristika finalnog proizvoda.

Osnovni princip ove vrste 3D štampe zasniva se na kontrolisanom protoku materijala pod dejstvom pritiska (mehaničkog ili pneumatskog). Parametri procesa uključuju viskoznost formulacije, brzinu ekstruzije, prečnik mlaznice i njenu brzinu kretanja (slika 2.6). Dobra podesnost materijala za štampu podrazumeva da istovremeno on ima i zadovoljavajuću ekstrudabilnost, kao i sposobnost održavanja strukture nakon štampanja, pri čemu su ova dva zahteva često međusobno suprotstavljena [101]. Materijal mora imati dovoljno veliku viskoznost da bi zadržao oblik nakon deponovanja, ali ne sme biti previše viskozno jer bi to otežalo njegovo istiskivanje kroz mlaznicu štampača [102,103].

Nakon što se potvrdi mogućnost kontinuirane ekstruzije formulacije, pristupa se optimizaciji ključnih parametara štampe, kao što su visina sloja, brzina štampe i način deponovanja materijala, budući da oni značajno utiču na uspešnost procesa štampe, kao i na kvalitet i rezoluciju odštampanih struktura [104]. Posebno važan parametar predstavlja rastojanje između vrha mlaznice i platforme za štampu. Ukoliko je ovo rastojanje preveliko, može doći do neadekvatnog deponovanja materijala, dok premalo rastojanje može negativno uticati na preciznost štampe [101,105,106]. Visina sloja predstavlja još jedan značajan parametar koji utiče na rezoluciju i kvalitet površine odštampanih proizvoda, a zavisi od prečnika mlaznice, brzine kretanja glave štampača i viskoznosti mastila. Smanjenje visine sloja može doprineti formiranju glatkijih i preciznijih površina, čime se poboljšava ukupni izgled i kvalitet štampanih oblika [107]. Međutim, veoma male vrednosti visine sloja mogu dovesti do problema u poravnanju i povezivanju uzastopno nanetih slojeva, pri čemu stepen izraženosti ovog efekta zavisi od karakteristika same formulacije i njenog ponašanja tokom procesa štampe [108]. Prema drugim istraživanjima, među najvažnije parametre optimizacije ubrajaju se

brzina kretanja glave štampača, prečnik mlaznice i brzina ekstruzije materijala koji utiču na proces ekstruzije formulacije kroz mlaznicu, a samim tim i na preciznost, reproduktivnost i kvalitet finalnog proizvoda [97,109]. Formulacije manje viskoznosti obično zahtevaju veće brzine štampe i mlaznice manjeg prečnika kako bi se postigla odgovarajuća rezolucija i preciznost odštampanih struktura i oblika [107]. S druge strane, formulacije velike viskoznosti najčešće zahtevaju manje brzine štampe i upotrebu mlaznica većeg prečnika kako bi se omogućila nesmetana ekstruzija materijala.



Slika 2.6. Neki od ključnih parametara procesa i karakteristika dobijenih proizvoda 3D štampom ekstruzijom polučvrstih materijala (preuzeto i modifikovano iz [101])

Iako postoje određene razlike među istraživanjima u pogledu parametara koji se smatraju najznačajnijim za proces ovog tipa 3D štampe, prisutan je opšti konsenzus o njihovom značajnom uticaju na kvalitet i karakteristike odštampanih proizvoda. Ipak, važno je naglasiti da su saznanja o optimalnim vrednostima svih ovih parametara još uvek ograničena, budući da njihov uticaj u velikoj meri zavisi od karakteristika same formulacije. Na primer, optimalni parametri štampe značajno variraju u zavisnosti od viskoznosti materijala namenjenog ekstruziji. Stoga se kvalitet odštampanih proizvoda može pouzdano proceniti tek tokom samog procesa 3D štampe, pri čemu je neophodno kontinuirano prilagođavanje i optimizacija procesnih parametara u skladu sa svojstvima formulacije, kako bi se postigli željeni kvalitet, preciznost i reproduktivnost finalnog proizvoda [109,110].

Primena 3D štampe ekstruzijom polučvrstih materijala u farmaciji obuhvata izradu oralnih tableta sa trenutnim otpuštanjem [108,111-113], oralnih tableta sa kontrolisanim otpuštanjem [101-104], višekomponentnih farmaceutskih oblika (engl. *poly pills*) [103,107,110], farmaceutskih oblika za žvakanje [107,109,114,115] i oralnih filmova [49,116,117]. Istraživanja su pokazala da se ovaj tip 3D štampe može uspešno koristiti u razvoju personalizovanih farmaceutskih oblika koji imaju različite profile oslobađanja aktivnih supstanci štampane u posebnim odeljcima, kao i mogućnost izrade formulacija sa velikim sadržajem aktivne supstance [97,106,118,119].

Ovaj tip 3D štampe u regeneraciji tkiva može da se koristi za izradu individualno prilagođenih oblika implantata, namenjenih lečenju ili prevenciji infekcija kostiju i okolnih tkiva [120]. Zbog toga su u većem broju istraživanja za 3D štampu ovih implantata korišćeni antimikrobni lekovi, kao što su izoniazid, rifampicin, minociklin, gentamicin i vankomicin [59,121,122]. Takođe, u ove proizvode ugrađivani su i glukokortikoidi, poput deksametazona i prednizolona [124,125].

Do danas nisu razvijene standardizovane smernice za proizvodnju personalizovanih farmaceutskih oblika i implantata primenom 3D štampe [125]. Takođe još uvek nisu jasno definisani optimalni parametri procesa štampe, niti su u potpunosti usaglašene metode karakterizacije

[126,127]. Ipak, 3D štampa ekstruzijom polučvrstih materijala predstavlja inovativnu tehnologiju sa značajnim potencijalom da transformiše budući razvoj i proizvodnju u farmaceutskoj industriji i oblasti inženjerstva tkiva.

Buduća istraživanja usmerena su ka razvoju pametnih biomaterijala sa oslobađanjem aktivne supstance pomoću stimulansa u organizmu, nanokompozitnih implantata, personalizovanih 3D štampanih farmaceutskih oblika i implantata, sistema koji kombinuju matične ćelije, faktore rasta i aktivne supstance. Očekuje se da će integracija biomaterijala, naprednih tehnologija proizvodnje i kontrolisane dostave aktivnih supstanci i bioaktivnih molekula omogućiti razvoj farmaceutskih oblika i implantata nove generacije sa značajno boljim terapijskim potencijalom.

2.6.2. Pregled primene 3D štampe u razvoju bukalnih filmova i implantata

Otkako je 1996. godine prvi put primenjena 3D štampa za dobijanje sistema za dostavu i kontrolisano otpuštanje aktivne supstance [128], ova metoda se ubrzano razvija i počinje da se primenjuje u različitim oblastima farmacije i medicine. U ovom radu pokazano je da je moguće kontrolisati makrostrukturu, mikrostrukturu i prostornu raspodelu aktivne supstance i na taj način da se utiče na profil otpuštanja. Korišćeni su poli(etilen oksid) (PEO) i poli(ε-kaprolakton) za izradu slojevite strukture u koju su inkorporirali model aktivne supstance: boje metilen plavo i alizarin žuto.

Prva primena 3D štampe za izradu oralnih farmaceutskih oblika - tableta objavljena je 2000. godine [129]. Mastilo za 3D štampu bilo je na bazi celuloze avicel u kombinaciji s različitim pomoćnim supstancama, a aktivna supstanca je bio hlorfeniramin maleat. U ovim prvim radovima korišćeni su 3D štampani koji su namenski konstruisani i izrađeni u laboratorijskim uslovima. Prvo istraživanje u procesiranju mukoadhezivnih bukalnih filmova 3D štampom objavljeno je 2019. godine [130]. Izvedena je 3D štampa tehnikom deponovanja istopljenog filameta. Procesirani su bukalni filmovi na bazi PVA i hitozana, sa i bez pozadinskog zaštitnog sloja od etil celuloze, sa aktivnom supstancom natrijum diklofenakom (NaD). Zaštitni pozadinski sloj je obezbeđivao jednosmerno otpuštanje NaD u pravcu bukalne mukoze. Trenutno otpuštanje NaD je uočeno kod filmova bez zaštitnog sloja (45% za 2,5 min) dok je kod filmova sa zaštitnim slojem znatno sporije otpuštanje. Prisustvo hitozana nije značajno uticalo na profile otpuštanja, ali je popravilo mukoadhezivna svojstva dobijenih filmova.

U istraživanju [131] iz 2020. godine postavljena je platforma za 3D štampu termosenzitivnih polimera metodom ekstruzije polučvrstih materijala. Na primeru želatina proučen je uticaj temperature, koncentracije želatina u polaznom mastilu, i procesnih parametara 3D štampe na stabilnost oblika i profil otpuštanja aktivne supstance. Za primer trenutnog otpuštanja primenjen je ibuprofen u smeši želatin-glicerol-voda, a štampani oblici su bili u vidu pahuljice i tkane mreže. Postignuto je potpuno otpuštanje za 10 minuta iz oba oblika. Za produženo otpuštanje je korišćena aktivna supstanca natrijum diklofenak, a smeša želatin-glicerol-HPMC za oblaganje. U zavisnosti od sadržaja HPMC i debljine obloge postignuti su različiti profili otpuštanja.

Bukalni mukoadhezivni filmovi na bazi polietilen oksida (PEO) sa aktivnom supstancom bupropion hidrohloridom su dobijeni primenom metode 3D štampe pomoću LCD ekrana uz umrežavanje UV zračenjem [132]. Ovi filmovi su pokazali dobra mehanička i mukoadhezivna svojstva, uz postojanost - nerastvorljivost u testu bubrenja. U *in vitro* testu pokazali su trenutno otpuštanje aktivne supstance (kompletno otpuštanje za manje od 10 minuta) koje je zavisno od dimenzija filma, to jest od površine izložene medijumu, dok su u ispitivanju *ex vivo* pokazali dobru permeabilnost kroz bukalnu mukozu.

U naučnom radu [133] poređena su svojstva orodisperzivnih filmova na bazi polivinil alkohola (PVA) sa aripripazolom dobijenih izlivanjem iz rastvora i 3D štampom metodom deponovanja istopljenog filameta. U okviru istraživanja optimizovan je proces dobijanja filamenata za 3D štampu. Dobijeni profili otpuštanja u testu *in vitro* pokazali su da je kompletan sadržaj aktivne supstance otpušten kod 3D filmova za 15 minuta, a kod izlivenih za 45 minuta. Takođe je uočeno da se u 3D štampanim filmovima aktivna supstanca nalazi u amorfnom stanju, dok je kod izlivenih bila prisutna u delimično kristalnom obliku, što je i uzrok takve razlike u profilima otpuštanja. Mehanička svojstva 3D štampanih filmova su nešto slabija od izlivenih. U svom sledećem istraživanju ovaj tim je istraživanjima [134] dodao i metodu elektropredenja, kao i poređenje svojstava dobijenih filmova nakon mesec dana skladištenja. U pogledu profila otpuštanja najveću brzinu otpuštanja su pokazali izliveni filmovi, pa elektropredeni, a zatim i 3D štampani, mada je to razlika od nekih 6%. Nakon skladištenja od mesec dana najveću promenu u brzini otpuštanja pokazali su 3D štampani filmovi.

Pored direktne inkorporacije aktivne supstance u polimere, razvijene su i metode u kojima su dodavani nosači sa aktivnom supstancom. Tako je grupa istraživača procesirala bukalne filmove na bazi hidroksietilceluloze sa nanostrukturiranim lipidnim nosačima kanabidiola [135]. Filmovi su štampani metodom ekstruzije polučvrstih materijala. Tokom *in vitro* testa pokazali su sporo i produženo otpuštanje (84% za 6 h) koje zavisi i od veličine i koncentracije nanostruktuiranih lipidnih nosača aktivne supstance.

U oblasti izrade implantata za regeneraciju tkiva, kao i za dostavu aktivne supstance, 3D štampa je napravila veliki napredak zbog preciznosti, mogućnosti kontrole strukture i poroznosti, primene širokog spektra materijala i različitih tehnika, obezbeđivanja odgovarajućih mehaničkih svojstava, mogućnosti povećavanja doze aktivne supstance i, naravno personalizacije pogodne za pacijenta. U okviru istraživanja u ovoj doktorskoj disertaciji fokus je bio na implantatima za dostavu lekova u tretmanu koštanog tkiva i ova oblast je izuzetno pogodna za primenu 3D štampe. Kod implantata od prirodnih polimera, slaba mehanička svojstva se popravljaju umrežavanjem, blendom sa sintetičkim polimerom, ugradnjom nanočestica i pravljenjem kompozita. Tako je u istraživanjima [136] izvedena 3D štampa ekstruzijom implantata želatin-PVA i umrežavanje 1-etil-3-(3-dimetilaminopropil) karbodiimid hidrohloridom (EDC). Sa PVA:želatin odnosom 1:1 postignuta su bolja mehanička svojstva u odnosu na čist želatin, dobijena je dobra poroznost i poboljšana ćelijska aktivnost.

Implantati za regeneraciju hrskavice dobijeni su kombinacijom ribljeg želatina sa alginatom primenom metode 3D štampe ekstruzijom polučvrstih materijala [137]. Nakon štampe implantat je dvostruko umrežavan radi postizanja boljih mehaničkih svojstava: CaCl_2 za alginat i EDC-NHS (N-hidroksisukinimid) za želatin. Optimizovan je odnos želatina i alginata, a dobijeni implantati su pokazali dobru mikrostrukturu i poroznost sa povezanim porama, dobra mehanička svojstva, biodegradabilnost i odsustvo citotoksičnosti, sa dobrim potencijalom za ugradnju aktivnih supstanci.

3D štampom ekstruzijom polučvrstih materijala izrađeni su implantati na bazi poli(mlečno-ko-glikolne kiseline) i beta kalcijum fosfata sa inkorporiranim ikaritinom (ICT), fitomolekulom i sekretomom dobijenog iz humanih fetalnih mezenhimskih matičnih ćelija (HFS) [138]. Postignuto je odloženo otpuštanje ICT i HFS koje je promovisalo regeneraciju oštećenog koštanog tkiva. Sprovedena je 3D štampa ekstruzijom polučvrstih materijala sa mastilom od pikering fazne emulzije poli(ϵ -kaprolaktona) i nanogline [139]. Na ovaj način su dobili hijerarhijski organizovanu poroznost koja se može kontrolisati koncentracijom PCL. Takođe je ispitivano *in vitro* otpuštanje iboprufera, i postignuto je odloženo otpuštanje koje je u korelaciji sa količinom dodate nanogline.

Za dobijanje implantata na bazi hidroksiapatita (HAP) primenjena je 3D štampa koristeći mastilo na bazi kalcijum fosfatnog cementa (tetrakalcijum fosfat, dikalcijum fosfat anhidrovani i poli(vinil butiral) rastvoren u alkoholu [140]. Primenjena je modifikovana 3D štampa polučvrstih

materijala ekstruzijom u sud sa vodenim rastvorom Na_2HPO_4 koji je delovao kao katalizator. Na taj način je bilo moguće kontrolisati sadržaj HAP koji je uticao na mehanička svojstva, poroznost i ćelijsku aktivnost.

3D štampa deponovanja istopljenog filameta je korišćena za dobijanje implantata na bazi polimlečne kiseline i hidroksi apatita (HAP) [141]. Prvo su 3D štampom polimlečne kiseline formirali strukturu sa različitim orijentacijama vlakna i rešetke, koju su naknadno površinski modifikovali sa HAP potapanjem u rastvor i termičkim tretmanom. Optimizovali su orijentaciju strukture i postigli dobra mehanička svojstva, poroznost i poboljšanu ćelijsku aktivnost jer su nanočestice HAP bile dostupne na površini pora i vlakana.

U istraživanjima [143] su ekstruzijom napravljeni kompozitni filamenti polimlečne kiseline i HAP koje su se zatim koristili za 3D štampu implantata metodom deponovanja istopljenog filameta. Dobijena je dobra i precizna poroznost, a mehanička svojstva su zavisila od sadržaja HAP u filamentima. Uloga u regeneraciji kosti je ispitana *in vivo* i dobijeni su dobri rezultati. 3D štampom digitalnom obradom svetlom dobijen je implantat na bazi polimlečne kiseline i hidroksi apatita (HAP) sa kontrolisanom poroznošću i veličinom pora, dobrim mehaničkim svojstvima na osnovu modelovanja metodom konačnih elemenata i sa dobrom citokompatibilnošću [143].

3. Cilj istraživanja

Cilj ove doktorske disertacije je razvoj adhezivnog multifunkcionalnog materijala koji se može koristiti i za mukoadhezivne filmove i implantate za dostavu aktivnih supstanci primenom metoda izlivanja iz rastvora i 3D štampe ekstruzijom polučvrstih materijala. Sa tim ciljem istraživanja u okviru ove disertacije organizovana su u nekoliko faza:

- ispitivanje i izbor odgovarajuće vrste želatina koji je pogodan za formulaciju bukalnog filma sa propanolol hidrohloridom (PRH) na osnovu ispitivanja termičkih, mehaničkih i adhezivnih svojstava ovih filmova, kao i poređenja profila otpuštanja PRH,
- optimizacija parametara 3D štampe ekstruzijom polučvrstih materijala za dobijanje bukalnih filmova na bazi želatina sa inkorporiranim PRH u mastilo; izbor blende želatina i sintetičkog polimera (PVA iili PVP) da bi se postiglo modifikovano oslobađanje PRH uz optimalna mehanička i adhezivna svojstva,
- evaluacija profila otpuštanja iz dobijenih bukalnih filmova primenom *in silico* modela za predviđanje biološke raspoloživosti PRH,
- optimizacija procesa dobijanja implantata na bazi blende želatina i PVP metodom 3D štampe ekstruzijom polučvrstih materijala sa direktnom inkorporacijom agensa za umrežavanje (genipina) u mastilo,
- uspostavljanje korelacije između sadržaja genipina i fizičko-mehaničkih svojstava dobijenih implantata,
- razvoj procesa dobijanja implantata za kontrolisano otpuštanje natrijum alendronat trihidrata metodom 3D štampe ekstruzijom polučvrstih materijala i ispitivanje uticaja ALN i genipina na mehanička svojstva i citotoksičnost neumreženih i umreženih implantata.

Osnovna hipoteza ovog rada je da je kroz postepene promene procesnih parametara, sastava i strukture materijala, uz prateću odgovarajuću karakterizaciju, polazni želatin moguće modifikovati i prilagoditi za razvoj filmova namenjenih bukalnoj isporuci aktivnih supstanci, kao i implantata za regeneraciju koštanog tkiva.

4. Naučni radovi proizašli iz doktorske disertacije

4.1. Mucoadhesive Gelatin Buccal Films with Propranolol Hydrochloride: Evaluation of Mechanical, Mucoadhesive, and Biopharmaceutical Properties

Article

Mucoadhesive Gelatin Buccal Films with Propranolol Hydrochloride: Evaluation of Mechanical, Mucoadhesive, and Biopharmaceutical Properties

Marija Jovanović ^{1,*}, Nataša Tomić ², Sandra Cvijić ³, Dušica Stojanović ¹, Svetlana Ibrić ³ and Petar Uskoković ¹

¹ Department of Materials Science and Engineering, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11120 Belgrade, Serbia; duca@tmf.bg.ac.rs (D.S.); puskokovic@tmf.bg.ac.rs (P.U.)

² Innovation Center of Faculty of Technology and Metallurgy, Karnegijeva 4, 11120 Belgrade, Serbia; ntomic@tmf.bg.ac.rs

³ Department of Pharmaceutical Technology and Cosmetology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia; gsandra@pharmacy.bg.ac.rs (S.C.); svetlana.ibric@pharmacy.bg.ac.rs (S.I.)

* Correspondence: marija.jovanovic@tmf.bg.ac.rs; Tel.: +38-11-1330-3616

Abstract: This study processes and characterizes propranolol hydrochloride/gelatin mucoadhesive buccal films. Two types of gelatin are used: Gelatin from porcine skin, type A (GA), and gelatin from bovine skin (GB). The influence of gelatin type on mechanical, mucoadhesive, and biopharmaceutical characteristics of buccal films is evaluated. Fourier-Transfer infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) analysis show that GA with propranolol hydrochloride (PRH) in the film (GAP) formed a physical mixture, whereas GB with PRH (GBP) form a compound-complex. Results of mechanical testing (tensile test, hardness) revealed that GAP films exhibit higher elastic modulus, tensile strength, and hardness. A mucohesion test shows that GBP has higher adhesion strength, while GAP shows higher work of adhesion. Both in vitro release study and in silico simulation indicated that processed films can provide effective drug transport through the buccal mucosa. In silico simulation shows improved bioavailability from buccal films, in comparison to the immediate-release tablets—indicating that the therapeutic drug dose can be markedly reduced.

Keywords: mucoadhesion; buccal films; gelatin; propranolol hydrochloride; in silico simulation



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1. Introduction

Buccal mucosa is one of the promising drug administration sites that is becoming attractive for both local and systemic drug delivery [1,2]. Buccal mucosa is relatively permeable and has good vascularization [3,4]. This route offers many advantages compared to the oral route, such as avoiding gastrointestinal irritation and drug degradation and first-pass liver metabolism, which means it ensures better drug bioavailability [2,5,6]. Moreover, buccal mucosa is accessible, so that a dosage form can be easily administered, leading to better patient compliance compared to other drug dosing routes [5,6]. Buccal administration is particularly suitable for the pediatric and geriatric population, as well as patients that have problems with swallowing [6]. There are many different dosage forms for buccal administration, such as tablets, films, gels, patches, sprays, pastes [7]. Among them, mucoadhesive buccal films offer several advantages, due to the high flexibility and larger surface area for drug absorption. They ensure more accurate drug dosing compared to gels, which can be easily washed away by the saliva [2,7,8]. Also, in comparison to conventional buccal tablets, films can be more comfortable because they are thin, flexible, and also have good mechanical properties with a resistance to breakage caused by mouth

movements [9,10]. Mucoadhesive films should have good tensile strength, mucoadhesive properties, and compatibility with the active substance [11].

Mucoadhesive natural polymers have received significant attention as carriers for buccal films, due to their ability to make close and prolonged contact with mucosa and to optimize drug bioavailability [10,12]. In this study, gelatin, the natural bioadhesive polymer, was selected to develop buccal mucoadhesive films. Gelatin is already widely used in pharmaceutical and medical applications and has been recognized as a GRAS (Generally Regarded as Safe) material by the United States Food and Drug Administration [13]. Gelatin has been used in the pharmaceutical field because of its excellent biodegradability, biocompatibility, non-toxicity, non-immunogenicity, affordability, etc. Gelatin, a natural protein derived from partially denatured collagen, is readily soluble in hot water and forms physically crosslinked hydrogels that are stable below its gelation temperature ($\approx 23^\circ\text{C}$) [14,15]. Polyelectrolyte complexes readily form between polyanions and polycations. These complex compounds are formed by the ionic association of repeating units on the polymer chains [16]. The stability of complex compounds is dependent on many environmental factors, such as a solvent's nature, pH, and ionic strength [17,18]. There are two types of gelatin: Type A gelatin (GA), derived from acid-treated processes; and type B gelatin (GB), derived from alkali-treated processes [19]. Different production processes affect the isoelectric point (pI), pH, and other properties of GA and GB. GA has a pI between 8 and 9 (positive charge at neutral pH), while GB has a pI between 4.8 and 5.4 (negative charge at neutral pH) [20].

Mucoadhesive strength points to the feasibility of films in terms of their adherence to mucosal membrane. Excessive adhesion may cause harm (irritation) to mucosa, whereas inadequate adhesion will adversely affect therapeutic efficacy, and at the same time, it may cause patient non-compliance. Hence, an optimized mucoadhesion is required for a film to be effectively used as a drug delivery system [21]. There are four possible general interactions between mucoadhesive polymers and glycoproteins: (1) Covalent attachment; (2) electrostatic interaction, which requires matching of charge groups between the polymer and the mucus; (3) hydrogen bonding; and (4) hydrophobic interactions [22].

Propranolol hydrochloride (PRH) is a non-selective β -adrenergic blocking agent (β -blocker) and has been used in the treatment of hypertension, arrhythmias, angina pectoris, and many other cardiovascular diseases; moreover, it can be used for migraine prophylaxis, tremor, and anxiety treatment. PRH has low bioavailability after oral administration, due to the extensive first-pass metabolism. This can be a problem because it is reported that approximately only 25% of PRH reaches systemic circulation [2,23]. PRH shows pH-dependent solubility; solubility at pH 1.2 is 225 mg/mL, while at pH 6.8 it is 130 mg/mL [24,25]. In addition, the drug is stable when the pH level is acidic and decomposes rapidly when it is alkaline. Solutions are most stable at pH 3; in aqueous solutions, propranolol decomposes, due to the oxidation of the isopropylamine side-chain [25].

In this study, PRH has been chosen as a model drug because it has suitable physico-chemical properties (MW 259.34 g/mol, $\log P = 3.22$), it is a BCS class 1 drug (it has high solubility and permeability), and since it undergoes the first-pass metabolism, patients may benefit from buccal administration [10]. There are many papers that describe the research of PRH in different buccal formulations. Several polymer blends were used. Arbuzo et al. investigated matrices of chitosan and gelatin for buccal delivery of propranolol hydrochloride. Their group made PRH buccal films [4] with different ratios of these polymers; buccal tablets with chitosan/gelatin microparticles [26]; bilayered buccal films based on polyvinylpyrrolidone or polyvinylalcohol with different weight ratios of gelatin or chitosan [10]. Kraisit et al. developed mucoadhesive buccal film based on hydroxypropyl methylcellulose and polycarbophil loaded with PRH nanoparticles [27]. Patel et al. prepared buccal films with different ratios of chitosan and polyvinylpyrrolidone K-30 [28]. Salehi and Boddohi developed mucoadhesive buccal film for co-delivery of rizatriptan benzoate and propranolol hydrochloride using kollicoat[®] IR, polyethylene oxide, and hydroxypropyl methylcellulose [2].

Based on our best knowledge, the comparison of two types of gelatin A and gelatin B as drug carriers for buccal film has not been performed. Aramwit et al. investigated gelatin A and gelatin B nanoparticles as carriers for controlled drug release [19].

This study aims to comprehensively characterize propranolol hydrochloride/gelatin buccal mucoadhesive films prepared with two types of gelatin: Gelatin from porcine skin, type A (GA), and gelatin from bovine skin (GB). The study was focused on possible drug/carrier interactions and their impact on the mechanical, mucoadhesive, and biopharmaceutical characteristics of the film. Additionally, the bioavailability of the obtained mucoadhesive films was compared with commercially available PRH IR tablets using in silico simulation in GastroPlus™ software. The results from this study will allow researchers the choice of suitable gelatin type to obtain the desired drug release profile.

2. Materials and Methods

2.1. Materials

Propranolol hydrochloride (PRH) (Galenika a.d., Belgrade, Serbia), which was used as a model substance, was Ph. Eur. 10 grade. Type A gelatin from porcine skin (~300 g Bloom) and type B gelatin from bovine skin (~225 g Bloom) were obtained commercially from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Glacial hydrochloric acid ($M = 60.05 \text{ g/mol}$), which was used as a solvent, was obtained from Zorka Pharma (Zorka Pharma HEMIJA d.o.o., Sabac, Serbia). Deionized water (DI) (resistance of $18 \text{ M}\Omega \text{ cm}$) was used to prepare the solutions. For the mucoadhesion studies, the mucin from a porcine stomach, Type II (Sigma-Aldrich Co., St. Louis, MO, USA), was used. Potassium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, and purified water were used to prepare simulated saliva (pH 6.8 was adjusted with 0.1 M hydrochloric acid) [4].

2.2. Methods

2.2.1. Preparation of Gelatin Films

Four series of films were prepared: Pure gelatin A (GA), gelatin A with propranolol hydrochloride (GAP), pure gelatin B (GB), and gelatin B with propranolol hydrochloride (GBP). The films were produced by solvent casting method, as shown in Figure 1. Gelatin A or gelatin B was added to the water solution of the glacial acetic acid (20% *w/w*) to create a 20% polymer solution. It was then stirred on a magnetic stirrer for 3 h at room temperature. The procedure for PRH loaded films was the same: After obtaining 20% polymer solution, the specific amount of propranolol hydrochloride was added and stirred for 3 h at room temperature to obtain a homogeneous solution. After that, it was poured into a mold (Figure 2) and dried at room temperature for 72 h for the solvent to evaporate. After that, it was dried in a hot air oven at 30°C for 6 h to evaporate any residual solvent. After the films were dried, they were removed from the mold, cut in an appropriate sample size ($15 \text{ mm} \times 15 \text{ mm} \times 0.6 \text{ mm}$), packed in aluminum foil, and stored in a desiccator (NaBr saline saturated solution, relative humidity = 58%) at 25°C for at least 48 h until analysis [29]. The amount of gelatin in the film was determined as follows: The films (dimensions $15 \text{ mm} \times 15 \text{ mm} \times 0.6 \text{ mm}$) were weighed, then dried in a hot air oven at 100°C to constant weight. After drying, the films were weighed, and the amount of gelatin was calculated by subtracting the drug content from the mass of the film. For each formulation, assays were performed in triplicate. The composition of the sample film was 30 mg of PRH and 130 mg of gelatin A or B.

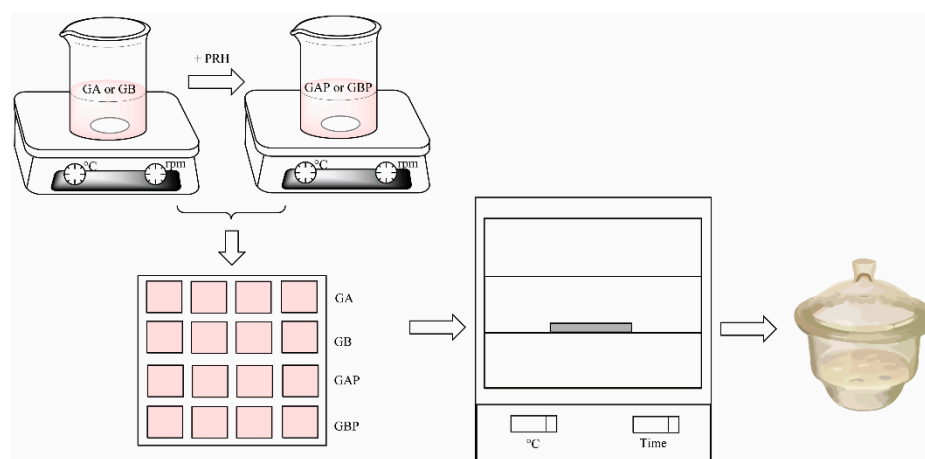


Figure 1. Scheme of the film preparation.

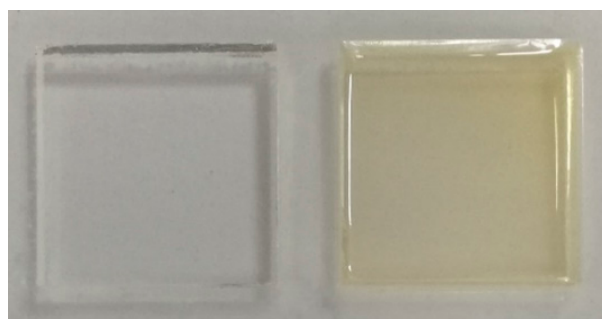


Figure 2. Mold (left) and the sample of the film (right).

2.2.2. Fourier-Transform Infrared Spectroscopy (FT-IR)

Single-beam Fourier-Transfer infrared spectroscopy (FTIR) using a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in the attenuated total reflectance (ATR) mode using a single bounce 45 °F Golden Gate ATR accessory with a diamond crystal, and an electronically cooled DTGS detector. The spectra were the co-addition of 64 scans at 4 cm^{-1} spectral resolution, and were ATR corrected. The Nicolet 6700 FT-IR spectrometer was equipped with OMNIC software (Thermo Fisher Scientific, Waltham, MA, USA) and recorded the spectra in the wavelength range from $2.5\text{ }\mu\text{m}$ to $20\text{ }\mu\text{m}$ (i.e., 4000 cm^{-1} to 500 cm^{-1}).

2.2.3. Differential Scanning Calorimetry (DSC)

The thermal analysis of films was performed on a device for differential scanning calorimetry (DSC) in a temperature range from $25\text{ }^{\circ}\text{C}$ to $185\text{ }^{\circ}\text{C}$ (Q10, TA Instruments, Crawley, UK) under a dynamic nitrogen flow of 50 mL/min . Samples of 7–9 mg were investigated. The samples were heated up at a rate of $10\text{ }^{\circ}\text{C/min}$. The glass transition temperature (T_g) was determined at the midpoint of the step-transition for each sample. The T_g values were confirmed by the use of the derivative curve [30].

2.2.4. Field Emission Scanning Electron Microscope (FESEM)

An observation of film morphology was performed using FESEM TESCAN MIRA 3 (Tescan Orsay Holding, a.s., Brno – Kohoutovice, Czech Republic), with fractured surfaces of the films sputtered with gold.

2.2.5. Mechanical Characterization

Tensile Strength

The uniaxial tensile mechanical test was performed using Shimadzu Servopulser (Shimadzu Corporation, Kyoto, Japan) according to ASTM D638 [31]. Measurements were

taken with a cross-head speed of 2 mm/min at 25 °C. The samples were in the shape of film strips 50 × 10 × 0.6 mm. The distance between the jaws was 22 mm. Young's modulus (modulus of elasticity) was calculated from the slope of the linear part of the tensile curve [32]. Three measurements were made for each sample and tested. The average values of the tensile strength, modulus of elasticity, and elongation at break were determined.

Hardness Measurement (Vickers)

The hardness test was performed to investigate the mechanical response of films to indentation. Vickers method consists of indenting the test material with a diamond indenter, in the form of a right pyramid with a square base and an angle of 136 °. The indent was in the shape of a pyramid sheath resembling a square, and the diagonal length was then measured. The micro-hardness of the samples was also measured, using Vickers micro-hardness tester DURIMET I Kleinhartepreuer (Leitz, Wetzlar, Germany) with a load of 4.9 N according to ASTM E384-16 [33]. Three indentations were performed, obtaining six diagonal lengths for the calculation of hardness.

2.2.6. Determination of Mucoadhesive Properties of Films

Mucoadhesion studies of formulated films were performed on Texture Analyzer Shimadzu EZ Test LX (Shimadzu Corporation, Kyoto, Japan). Mucin disks were used as a mucosal substrate. They were made from 250 mg of mucin powder that was compressed into a 13 mm diameter disk [34]. The simulated saliva was heated and maintained at 37 °C. A mucin disk was placed in the center hole of the acrylic holder, and attached with a double adhesive tape. The film (circle, 10 mm diameter) was fixed onto the metal rod of the texture analyzer. Mucin disk was submerged in 3 mL of the simulated saliva for 120 s, and then the excess was drawn-out with a syringe [8,35]. The holder with soaked mucin disk was being placed below metal rod of texture analyzer on which our sample was attached. Experimental conditions were: Sixty seconds of contact time of mucin disk and our sample with the force of 1 N, and then the movable metal rod was being lifted at a speed of 0.1 mm/s until the sample was totally separated from the mucin disk. The used program Trapezium X (Shimadzu Corporation, Kyoto, Japan) automatically determines the force of adhesion (maximum force required to detach sample from a mucin disk) and work of adhesion (the area under the force/time curve) [34].

The data obtained through experimental results were analyzed by using a two-way analysis of variance (ANOVA, OriginLab Corporation, Northampton, MA, USA) at a confidence level of $p < 0.05$. Polymer type and drug presence were used as factors in the analysis as independent variables, and force and work were used as dependent variables. The statistical analysis was performed in the Origin software.

2.2.7. Drug Content Uniformity

The casted films were cut from three different places to evaluate drug content uniformity [2]. Then drug content measurement was carried out by completely dissolving each sample in 250 mL of distilled water (pH = 6.8). After filtration of solutions through 0.45 µm filter paper, the absorbance of PRH was measured at 319 nm. The calibration curve was used for the determination of PRH concentrations in each film. The following equation was used for the calculation of drug content uniformity, and the average value was reported. The limit of uniformity of drug content should be in the range of 85 to 115% [2,11].

Drug's content uniformity = (Actual amount of drug in film * 100)/Theoretical amount of drug in the film)

2.2.8. In Vitro Drug Release Studies

Rotating paddle over disk apparatus Erweka DT70 (Erweka GmbH, Langen, Germany) was used, at the rotating speed of 50 rpm. 200 mL of deionized water (pH 6.8) served as a dissolution medium, at 37 ± 0.5 °C [8,28,36–41]. A single film (15 × 15 × 0.6 mm)

was placed on a metal mesh disc (Figure 3) fixed with the help of a two-sided adhesive tape to avoid floating of the film during testing. The film was put on the upper side of the disc, together with the disc, immersed in the dissolution vessel [28,42,43]. Samples of 4 mL were taken from the medium at fixed time intervals (5, 10, 15, 30, 45, 60 min) and replaced with a fresh dissolution medium [44]. All samples were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA). The concentration of PRH was determined by UV/VIS spectrophotometer Evolution 300 (Thermo Fisher Scientific, Cambridge, UK) at 319 nm. The results were shown as the medium value of three repeated measurements for each sample [45].



Figure 3. (a) Samples on metal mesh disc; (b) set up for dissolution test.

2.2.9. Physiologically-Based Simulations

Propranolol absorption and the concomitant disposition were simulated using GastroPlus™ software (v. 9.7.0009, Simulations Plus Inc., Lancaster, CA, USA). The software accounts for the three types of input data: Drug physicochemical and pharmacokinetic properties, along with physiological data stored with the software integrated Advanced Compartmental Absorption and Transit (ACAT) model and the Oral Cavity Compartmental Absorption and Transit (OCCAT) model. Both models use a system of differential equation to simulate drug transport and absorption, whereas the ACAT model describes drug performance in the gastrointestinal tract (nine consecutive segments from the stomach to the ascending colon), and the OCCAT model depicts drug transit and absorption through the oral cavity mucosa (conforming of six compartments: Buccal, gingival, palate, the top and the bottom of the tongue, the floor of the mouth). Details about the ACAT and OCCAT models are described in the literature [46,47]. We have used our previously generated and validated propranolol-specific model [48]. The selected drug-related input parameters were literature values, experimentally determined, or *in silico* predicted data. The selection of input data is shown in Table 1. The ACAT model parameters were software default values for human adult fasted physiology. The OCCAT model physiological parameters were also default values, except for the saliva production rate, which was optimized at 0.6 mL/min, as explained in our previous paper [48]. The increased saliva production rate in comparison to default 0.04 mL/min can be justified by the presence of a “foreign body” in the oral cavity [38]. Namely, published data demonstrate that saliva production rate can be increased up to 2.34 mL/min [49] or even 6.17 g/min [50], depending on the type of stimulus and the exposure time, justifying the increase in saliva production rate value considered in our model. Formulation-specific input data describing the designed formulations GAP and GBP included the drug dose (30 mg), formulation type (“buccal patch”), and experimentally determined drug release profiles. We need to note that “buccal patch” is currently the only software option that allows the input of *in vitro* dissolution data for dosage forms absorbed from the oral cavity, and therefore, it was selected to estimate the *in vivo* performance of buccal films. However, the assumption that the contact surface area of the film with buccal mucosa is constant may be somewhat erroneous.

Table 1. Model input parameters.

Parameter	Value
Molecular weight	259.34 g/mol
logP	3.22 ^a
pKa (base)	9.09 ^b
Solubility	225 mg/mL (pH 1.2); 130 mg/mL (pH 6.8) ^c
Diffusion coefficient	$0.8304 \times 10^{-5} \text{ cm}^2/\text{s}$ ^d
Human jejunal permeability	$2.7 \times 10^{-4} \text{ cm/s}$ ^e
Mean precipitation time	900 s ^f
Effective particle radius	25 μm ^f
Drug particle density	1.2 g/mL ^f
Blood/plasma concentration ratio	0.78 ^g
Unbound percent in plasma	10% ^g
First pass extraction	62.89% ^h
Clearance	0.7 l/h/kg ⁱ
Volume of distribution	0.493 l/kg ⁱ
Distribution rate constant k_{12}	11.40 1/h ⁱ
Distribution rate constant k_{21}	1.91 1/h ⁱ
Elimination half-life	3.71 h ^d
OCCAT Parameters	
Formulation type	“Buccal patch” ^j
Drug dose	30 mg ^j
Contact surface area	2.25 cm^2 ^k
Transit model	Normal swallowing ^j
Saliva production rate	0.6 mL/min ^l

^a literature value is taken from Reference [51]; ^b literature value is taken from Reference [52]; ^c literature values taken from Reference [24]; ^d GastroPlus™ calculated; ^e literature value is taken from Reference [53]; ^f GastroPlus™ default values; ^g literature values taken from Reference [54]; ^h literature value is taken from Reference [55]; ⁱ calculated from the in vivo data [56] using software PKPlus™ module; ^j applies for the investigated buccal films; ^k corresponds to the film surface area exposed to dissolution medium under the applied dissolution test conditions; ^l optimized value [48].

3. Results and Discussion

3.1. FTIR Analyses

The drug–polymer interaction was checked by comparing the FTIR spectra mixture of the drug containing the GA and GB, with the FTIR spectrum of the pure drug and the pure GA and GB (Figure 4.).

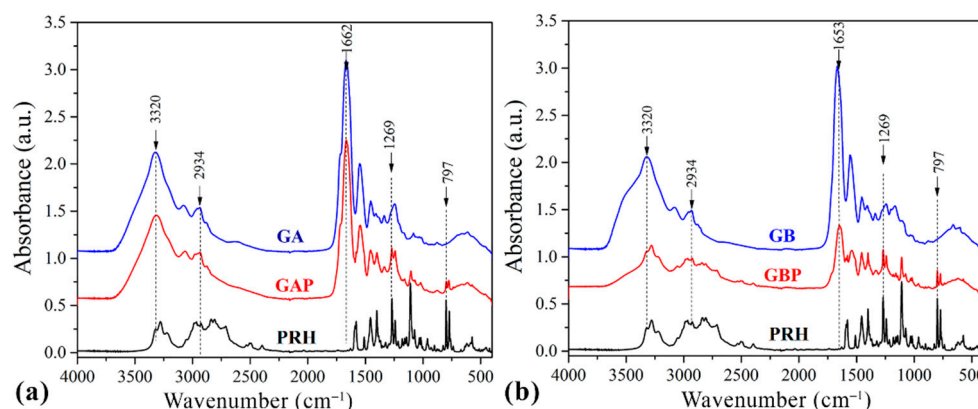


Figure 4. Fourier-Transfer infrared spectroscopy (FTIR) spectrums of (a) pure gelatin A film (GA), propranolol hydrochloride (PRH), and gelatin A film loaded with PRH (GAP); (b) pure gelatin B film (GB), propranolol hydrochloride (PRH), and gelatin B film loaded with PRH (GBP).

Pure GA showed the bands at 1662 cm^{-1} relative to the vibration of the amide carbonyl group; at 1543 cm^{-1} it was associated with stretching of the free amino groups [4,15,57]. The peak at 1243 cm^{-1} corresponds to CN stretching, NH in-plane bending, and CH_2 wagging vibrations [58,59]. The broad absorption band at 3320 cm^{-1} is assigned to the OH stretching and internal hydrogen bonds, which overlap with N–H stretching [57].

The spectrum of GB showed the bands at 1653 cm^{-1} corresponding to the vibration of the amide carbonyl; at 1557 cm^{-1} it was associated with the stretching of the free amino groups [4,15], 1234 cm^{-1} was attributed to combination peaks between C–N stretching vibrations, N–H and the wagging vibrations from CH_2 groups stem from close amino acid residues [60,61]. These peaks are known as Amides I, II, and III.

The spectrum of PRH shows peaks at 2965 cm^{-1} , due to the presence of a secondary amine group; 3283 cm^{-1} is due to the hydroxyl group (secondary), the C–O–C stretching in aryl alkyl ether display a stretching band at 1108 cm^{-1} , and the peak at 797 cm^{-1} , due to a-substituted naphthalene [2,24,62]. Frequencies of the functional groups of the pure drug remained intact in the mixture with GA (GAP); hence, there was no major interaction between the drug and the GA.

In this part, the electrolytic nature of gelatin and PRH could be considered. Their electrically charged ions could interact in solution in direction to form polyelectrolytic complex (PEC), and Coulomb forces are responsible for this behavior.

The FTIR spectrum of the complex in comparison with the physical mixture is shown in the spectrum for PRH with the GB (GBP). The characteristic carbonyl absorption in 1596 cm^{-1} is replaced by the band in the $1550\text{--}1560\text{ cm}^{-1}$ region. This band corresponds to the auto-symmetrical vibrations of the COO-structure and is used as a diagnosis for the COO-group [16,61]. This ionization leads to the possible formation of anion-cation interaction. According to the literature, the formation of PEC should be confirmed with FTIR, DSC, and drug release profile [4,8,16,56,63,64].

FTIR analysis revealed that in GAP, there is no interaction between PRH and GA, while for GBP some results indicate ion-ion reactions. This should be confirmed after DSC analyses and dissolution test.

3.2. DSC Analyses

The results of the DSC analysis of processed series and powders of Gelatin A and Gelatin B (as received) are presented in Figure 5 and Table 2. The curve of the pure PRH shows the melting point at $163\text{ }^{\circ}\text{C}$ [4,10]. The curves for powders Gelatin A and Gelatin B show the points of transition temperatures at $30.41\text{ }^{\circ}\text{C}$ and $32.35\text{ }^{\circ}\text{C}$, respectively. Also, there are endothermic peaks for Gelatin A and Gelatin B powders at $121.06\text{ }^{\circ}\text{C}$ and $115.82\text{ }^{\circ}\text{C}$, respectively. For pure GA film, the points of transition temperature at $44.52\text{ }^{\circ}\text{C}$ and endothermic peak at $85.75\text{ }^{\circ}\text{C}$ were observed [65]. The glass-to rubber transition associated with polymer molecules started to move and vibrate, and viscoelastic behavior instead of the solid body ensued [66–68]. The endothermic peak is associated with the melting of the triple-helix crystalline structure. This peak is followed by processes, such as water evaporation, melting, and recrystallization, of small and/or imperfect gelatin crystallites. Also, because gelatin consists of peptides and proteins produced by partial hydrolysis of collagen, this peak could be obtained, due to overlapping of the glass transition of α -amino acid blocks in the polypeptide chain [68–72]. The appearance of this peak is depended on the film preparation conditions (drying). For GB film, those relevant temperatures associated with T_g and endothermic peak were $48.79\text{ }^{\circ}\text{C}$ and $87.89\text{ }^{\circ}\text{C}$. With the addition of PRH, these temperatures were raised to $53.11\text{ }^{\circ}\text{C}$ and $86.09\text{ }^{\circ}\text{C}$ for GAP and for GBP $60.09\text{ }^{\circ}\text{C}$ and $94.26\text{ }^{\circ}\text{C}$. There was also the third transition with peak temperature associated with the isomerization of the peptide bonds in gelatin from the trans to the cis configuration and marked as T_i [73–75]. For GA and GB films, the values of T_i were $163.33\text{ }^{\circ}\text{C}$ and $177\text{ }^{\circ}\text{C}$, respectively. It is also found that T_i is correlated with the moisture of gelatin film, and raised when moisture decreased [75,76]. In thermograms for films with PRH, T_i was shifted

to lower temperatures, 147.32 °C for GAP and 152.55 °C for GBP. The irregularity and shifting of peaks could be attributed to the presence of acetic acid [60].

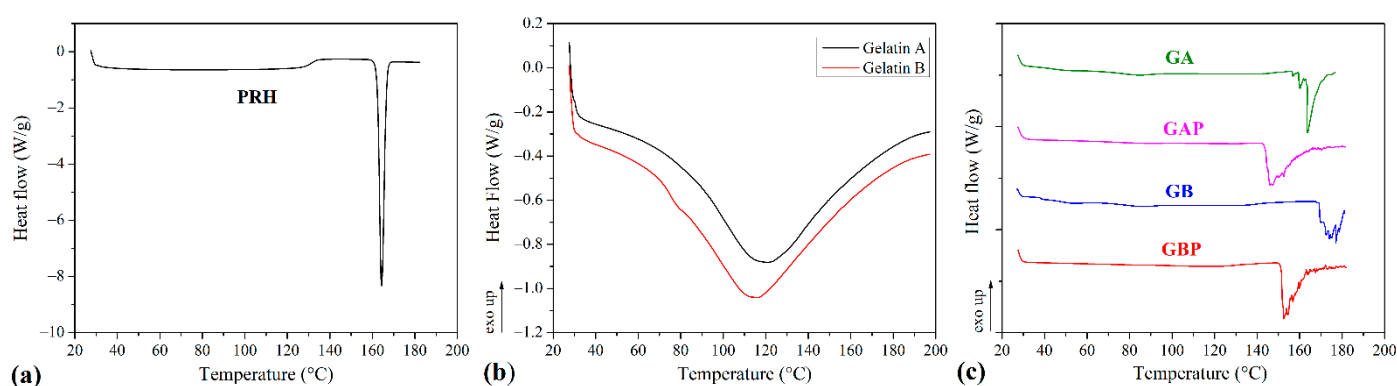


Figure 5. Differential scanning calorimetry (DSC) diagrams of (a) propranolol hydrochloride (PRH); (b) gelatin A and gelatin B powder, (c) pure gelatin A film (GA), gelatin A film loaded with PRH (GAP), pure gelatin B film (GB) and gelatin B film loaded with PRH (GBP).

Table 2. Temperatures from DSC analysis.

Sample	T_g , °C	T_m , °C	T_i , °C
GA powder	30.41	121.06	n.a.
GB powder	32.35	115.82	n.a.
GA	44.52	85.75	163.33
GB	48.79	87.89	177
GAP	53.11	86.09	147.32
GBP	60.09	94.26	152.55
P		164.33	

According to FTIR analysis, GAP is a physical mixture, while GAB is a complex compound. The DSC results have not provided clear confirmation of the interaction between gelatin and PRH. The rising of characteristic temperatures could be due to the crosslinking of polymer chains with PRH. [16,68]. The higher shift of T_g for GAP, then for GBP (accompanying with FTIR analysis), could lead to distinguish the nature of the interaction between gelatin A or B and PRH.

In both samples with the drug (GAP and GBP), a PRH crystallization peak was not found. The amorphous form was therefore obtained, indicating better solubility and bioavailability for both films [10].

3.3. SEM Analysis

In Figure 6, film cross-sections of pure gelatin A and gelatin B and films loaded with PRH are presented. The images show that films are smooth and nonporous. It can also be seen that films have good homogeneity and no presence of PRH powder.

3.4. Mechanical Properties

Tensile curves and the results of the tensile test are presented in Figure 7 and Table 3. The mechanical properties of natural polymers are influenced by the preparation process, temperature, and moisture, so it is difficult to compare them with other references. However, it could be seen that the pure GA and GB results are in agreement with some of the data from the literature [32]. Film GA with a higher bloom index showed higher tensile strength, elastic modulus, and elongation at break, as expected [32]. For mucoadhesive buccal films, there are no recommended specific values for tensile strength and modulus of elasticity. Results obtained in this study for GAP and GBP are in range with the other

published results for mucoadhesive buccal films with different compositions (i.e., hydroxypropyl methylcellulose (HPMC)/polycarbophil (PC) blend, polyvinylalcohol (PVA), and polyvinylpyrrolidone (PVP) blend and more complex composition [27,37,40,77–80].

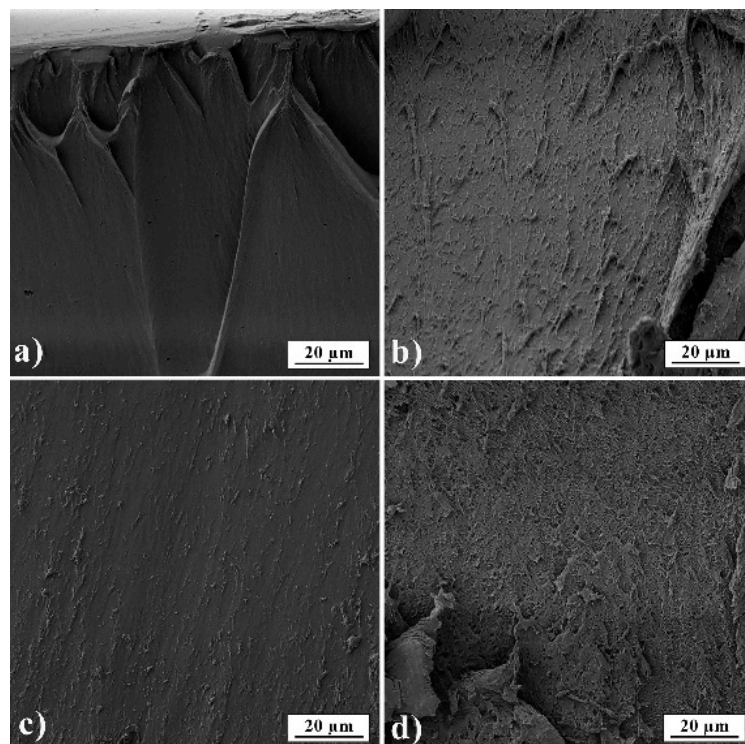


Figure 6. SEM micrographs of films' fractured surfaces (a) GA; (b) GB; (c) GAP; (d) GBP.

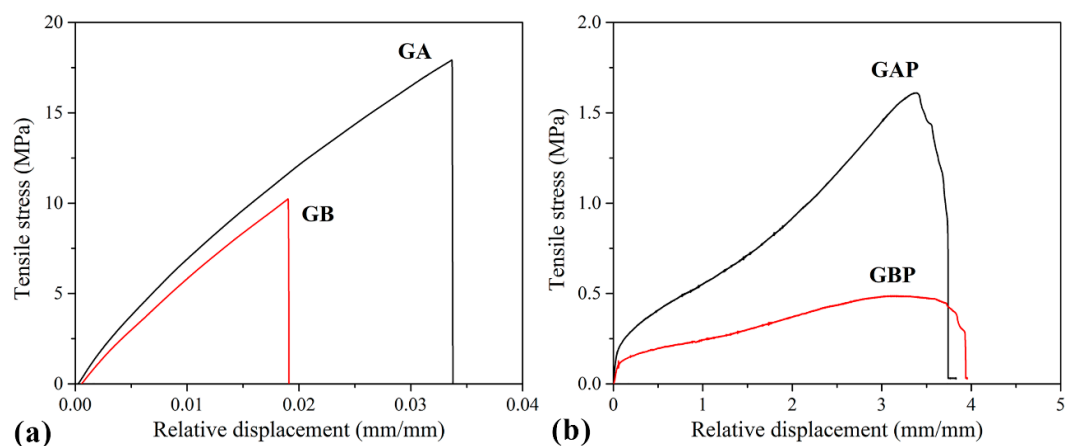


Figure 7. Tensile stress–strain curves of: (a) Pure gelatin films (GA and GB); (b) films with PRH (GAP and GBP).

Table 3. Tensile strength (σ), elongation at break (ϵ) and elastic modulus (E) of the prepared films.

Sample	E, MPa	σ_m , MPa	ϵ , mm/mm
GA	740	19	0.027
GAP	5	1.77	2.87
GB	530	16,21	0.019
GBP	2	0.4	2.85

It can be noted that the tensile strength and elastic modulus decreased with the addition of PRH both in GA and GB, while the elongation at break increased by three orders of magnitude. Also, it could be seen from the curves' shapes that the plasticity of the films with PRH was raised. The addition of drug in gelatin leads to higher free volume and make the easier moving of polymer molecules. In pure gelatin, chains are tangled. During solvent casting, drug molecules were positioned between the polymer chains and increased the possibility of film deformation.

The results of micro-hardness are presented in Figure 8. Hardness represents the ability of materials to respond to plastic deformation. GA showed a slightly lower hardness than GB. Regardless of the physical mixture in GAP in comparison to the complex in GBP, the hardness of GAP was higher than for GBP. The addition of PRH in GA acted as physical crosslinking of the polymer, while in GBP, a complex was formed [16,19].

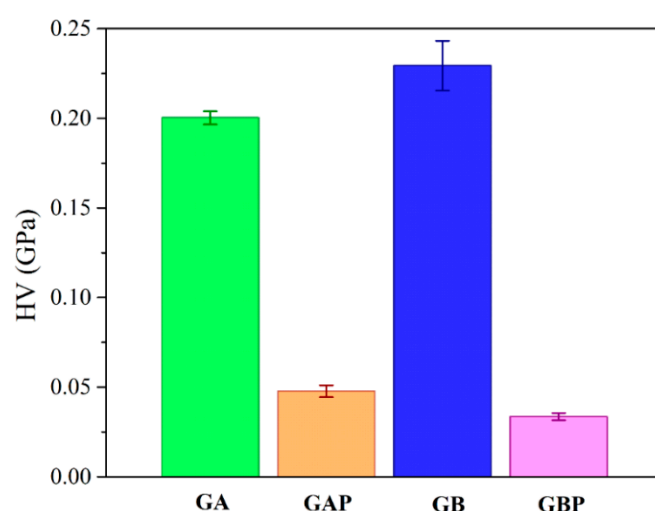


Figure 8. Results of Vickers micro-hardness test.

3.5. Mucoadhesion Studies

When two parts of material are connected together via surfaces, the force of their detachment is the measure of their adhesion [81].

A test for mucoadhesion was performed in accordance with the fracture theory, and the results are presented in Table 4. In fracture theory, the force required for the detachment of a film is related to the strength of the adhesive bond, while work of adhesion (W_{adh}) is total energy involved for this separation, and it can be calculated as the area under force–distance curve [82]. It could be seen that the strength of adhesion (detachment force, F_{adh}) of the gelatin increases with the addition of PRH. The force of adhesion for pure GB is higher than for pure GA for almost 10%. Strength of adhesion increased with the addition of PRH to the gelatin, both for GAP and GBP in relation to pure GA and GB. The energy or the work of adhesion (W_{adh}) is associated with crosslinking of polymers; it is higher when the crosslinking is reduced. Polymers with lower crosslinking easily diffuse and entangle with mucin fibers. From Table 4, it can be observed that adding PRH to GA leads to higher energy. The bonding of mucin and film may be either primary (like covalent bonds) or secondary, like van der Waals bonding, hydrogen bonding, hydrophobic inter-actions, or electrostatic forces. This result should be combined with an electrostatic theory about electro attraction between anionic polymers (GA) with negatively charged sialic acid and sulfate residues of mucin glycoprotein [10,76,81,82]. For GB, adding PRH leads to a lower value of the work of adhesion. W_{adh} is the energy associated with surface deformation in the detachment and fracture mode of adhesive material. During detachment of two surfaces occur the crack formation and its propagation. The behavior of GBP in adhesion test shows viscoelasticity, while a brittle detachment fracture occurs. Like in the case of tension, when toughness is determined as the area under stress–strain curve, it is possible to maintain

higher force with the lower area under the curve. The reversible work of adhesion could be described as the sum of the components corresponding to molecular interaction, surface deformation, and instabilities in adhesion and subsequent separation [83,84]. Moreover, it could be said that the lower W_{adh} implies a higher crosslinking of the polymer. This state causes a slower intake of fluid into the polymer and decreases the rate of polymer molecules and mucin interpenetration [22]. As the crosslinking density of the polymer is increased, the diffusion of fluid decreases. The slowdown in diffusion is more severe at the polymer-water interface [85].

Table 4. Results of the mucoadhesion test, the mucoadhesive force (F_{adh}), and the work of adhesion (W_{adh}).

Sample	F_{adh} , N	St. Dev. N	W_{adh} , μ J	St. Dev. μ J
GA	2.09	0.46	1070	150
GAP	2.14	0.44	2350	3390
GB	2.29	0.29	1300	360
GBP	2.32	0.04	910	160

The work of adhesion for GAP is the highest. The presence of a higher number of amino groups leads to a lower density of crosslinking. Due to this fact a much better intake of water into the film occurs, and all of this results in swelling. On the other hand, the highest force of adhesion occurs with GBP, but at the same time, the lowest work of adhesion can be observed. That means that, in this way, the highest density of crosslink is achieved.

Table 5 shows the results of the statistical analysis for force as the dependent variable and Table 6 for work as the dependent variable.

Table 5. Analysis of F_{adh} .

Factor	F Value	p Value
Polymer	0.03063	0.86327
Drug	4.01426	0.06235
Interaction	3.07126	0.09883

Table 6. Analysis of W_{adh} .

Factor	F Value	p Value
Polymer	1.64085	0.21846
Drug	0.88983	0.35955
Interaction	3.14981	0.09496

It can be concluded from the results of the statistical analysis that neither of the factors nor their interaction is statistically significant, as all p values are higher than 0.05.

3.6. Drug Content Uniformity

Drug content uniformity percentage is in the range of 98.61 ± 3.35 for GAP and 99.28 ± 3.74 for GBP. The results showed that there were acceptable changes in drug's content, so the films have uniform drug distribution.

3.7. In-Vitro Release Study

The release of the drug from the buccal film is determined by drug/polymer properties. It also depends on the drug solubility, the drug diffusion from the film, the swelling, and degradation of the polymer matrix [42,57,86,87]. Although propranolol hydrochloride

solubility is not expected to be the rate-limiting step for its dissolution and absorption [52], a dissolution study was performed under pH conditions that correspond to the physiological pH in the oral cavity (pH of the deionized water was 6.8). Other properties of the oral cavity fluid, such as ion concentration, osmolality, surface tension, viscosity, etc., have not been considered in this study; however, swelling and dissolution of some natural polymers may depend on these conditions. In this case, the diffusional force is much more important for the drug release than the films' degradation because of the gelatin concentration of 20% [57,87]. Therefore, it is very important to evaluate in vitro release profile of the buccal film [42].

In vitro release of PRH from GAP and GBP, films are shown in Figure 9. Drug release happened immediately out of both formulations. More than 50% of PRH was released in the first 10 min and more than 80% in the first 15 min. The release was better from GAP films because the polymer and the drug are a physical mixture in comparison to GBP, where the complex was formed, which was shown on the FTIR spectra and the DSC analysis. There was a complete release of PRH from GAP, and a bit less from GBP because of the complex formation [4,10]. Moreover, the density of crosslink has a role in the release of PRH, as was previously mentioned in the mucoadhesion discussion.

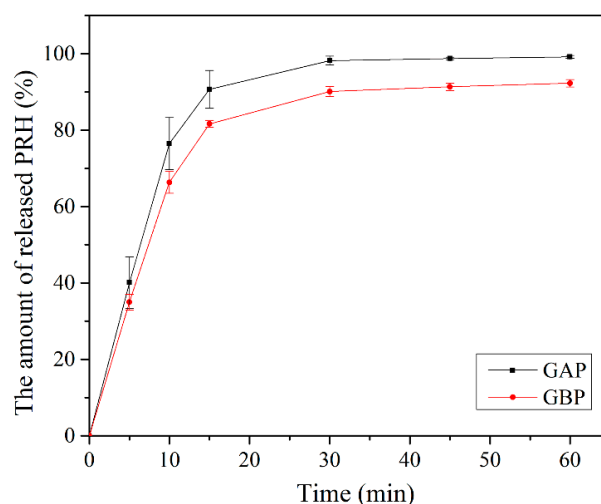


Figure 9. In vitro release study.

Here, we also need to note that under the in vivo conditions, a drug can diffuse through both sides of the film, but since the designed formulations were intended for immediate drug release, as confirmed by our dissolution test results, the applied test conditions can be considered acceptable.

3.8. Physiologically-Based Simulations

Using the input data noted in Table 1, together with the drug dissolution profiles, shown in Figure 9, the expected propranolol absorption and disposition pattern has been predicted following the buccal application of the designed films. Here we adjusted the simulation setup (i.e., contact surface area) to comply with the dissolution test conditions, which is justified by the obtained fast drug release profiles, but as implied earlier, the drug diffusion can occur through both sides of the film under in vivo conditions.

The simulated plasma concentration-time profiles are depicted in Figure 10, and the corresponding pharmacokinetic parameters are given in Table 7.

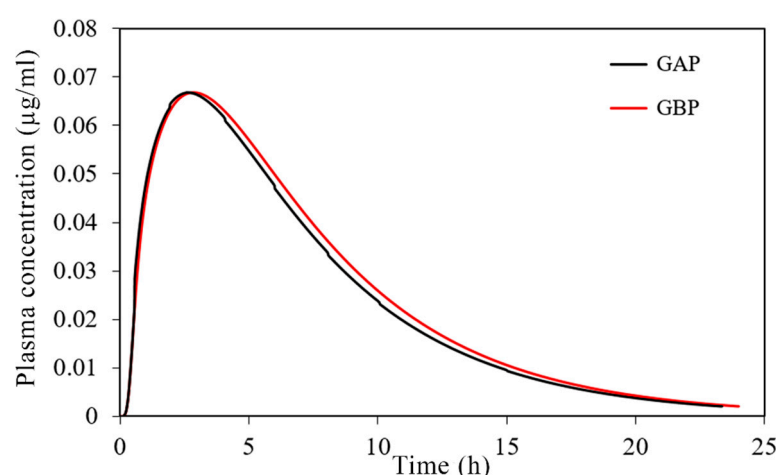


Figure 10. Predicted plasma concentration-time profiles for the tested 30 mg PRH buccal films.

Table 7. Predicted pharmacokinetic parameters following administration of the tested buccal films and the conventional immediate-release (IR) tablets.

Formulation	Dose (mg)	C_{max} (ng/mL)	t_{max} (h)	$AUC_{0-\infty}$ (ng h/mL)	F_a (%)	F (%)
GAP	30	66.80	2.80	600.99	99.99	99.99
GBP	30	66.80	2.88	612.20	99.99	99.99
IR tablet	80	98.24	1.04	644.23	99.90	35.96

C_{max} —maximum plasma concentration; t_{max} —time to reach C_{max} ; AUC —area under the plasma concentration-time curve; F_a —percent absorbed (entered into the enterocytes/epithelial cells); F —bioavailability.

The obtained results indicate negligible differences in the rate and extent of propranolol absorption from the tested formulations, with only about a 3% decrease in t_{max} and a 2% decrease in area under the plasma concentration-time curve ($AUC_{0-\infty}$) for the formulation GAP in comparison to GBP. These slight differences are caused by a somewhat slower drug dissolution rate from the formulation GBP, but in general, the profiles can be considered similar. The same congruency applies for the simulated regional drug absorption from the tested films, indicating complete absorption from the buccal region (Figure 11). Such high bioavailability can be considered a substantial benefit of propranolol buccal films [5,7,88–90]. Namely, in comparison to commercially available oral dosage forms, e.g., 80 mg propranolol IR tablets, the drug absorption is complete for both buccal and oral dosing routes (Figure 11 and Table 5), but due to the extensive first-pass metabolism in the liver, the drug bioavailability following oral dosing is notably decreased (Table 7). Due to the improved propranolol bioavailability from buccal films, the therapeutic drug dosing or dosages can be markedly reduced, e.g., the extent of drug absorption, expressed as AUC, obtained from 30 mg PRH buccal films is in the same range as obtained from 80 mg PRH IR tablet (which is less than 10% difference). Future in vivo studies are encouraged to support our in silico prediction results.

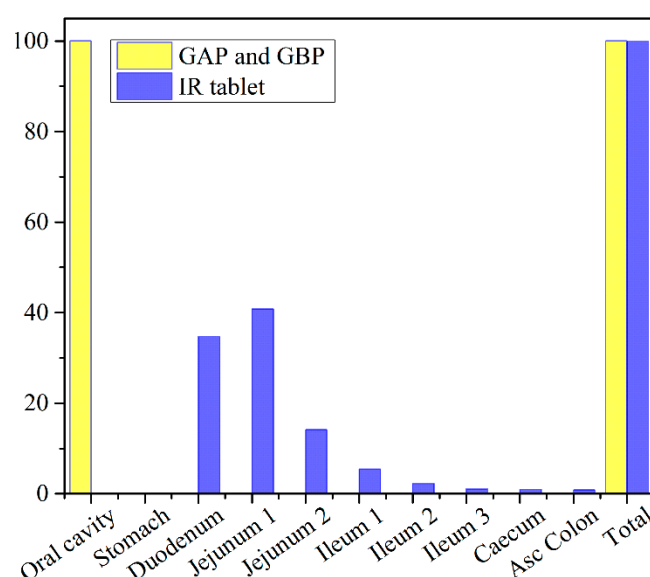


Figure 11. Predicted regional absorption profiles for the tested 30 mg PRH formulations and 80 mg PRH IR tablets.

4. Conclusions

In this work, two types of gelatin (gelatin from porcine skin, type A (GA), and gelatin from bovine skin (GB)), were compared as suitable candidates for PRH carriers in the form of mucoadhesive buccal films. A comprehensive characterization was implemented to gain insight into the phenomenology of processing, morphology, mechanical and mucoadhesive behavior, and the drug delivery as well. PRH was chosen for the model drug because in a buccal film, it has a possibility to avoid loss during extensive first-pass metabolism. Elucidation of pure gelatin films structure (GA and GB) and also ones loaded with PRH (GAP and GBP) were investigated and compared.

Both types of drug-loaded films have shown good morphological homogeneity and have contained the amorphous form of PRH, which leads to better bioavailability of the drug. This was also confirmed by *in silico* simulation of regional absorption profiles, and due to the improved PRH bioavailability from buccal films in comparison with the immediate-release tablets, the therapeutic drug dose can be markedly reduced. The obtained GAP film shows 2.5 times higher elastic modulus, more than four times higher tensile strength, and 33% higher hardness in comparison to GBP film. Statistical analysis of the mucoadhesion tests have shown that neither polymer type nor drug nor their interaction had a statistically significant influence the strength and work of adhesion. Both films, GAP and GBP, have a similar drug release profile, with a slightly slower dissolution rate for GBP. Some tests and analyses were conducted regarding the ionic nature of GA, GB, and the drug. The dissolution test, FTIR, and DSC analysis together indicate that in GBP, the ion-ion complex interaction was more pronounced, while GA formed a physical mixture with PRH. The results presented in this work allowed us to estimate that GAP film will have better processing availability and higher solubility followed by faster drug release. On the other hand, GBP showed slightly lower mechanical strength and slower drug release profile, but stronger mucoadhesion force. In conclusion, gelatin-based mucoadhesive films with PRH have good potential for drug delivery to the buccal mucosa, allowing targeted delivery and reduced drug dosing.

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4.2. 3D Printed Buccal Films for Prolonged-Release of Propranolol Hydrochloride: Development, Characterization and Bioavailability Prediction

Article

3D Printed Buccal Films for Prolonged-Release of Propranolol Hydrochloride: Development, Characterization and Bioavailability Prediction

Marija Jovanović ^{1,*}, Miloš Petrović ¹, Sandra Cvijić ², Nataša Tomić ³, Dušica Stojanović ¹,
Svetlana Ibrić ² and Petar Uskoković ¹

¹ Department of Materials Science and Engineering, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11120 Belgrade, Serbia; mpetrovic@tmf.bg.ac.rs (M.P.); duca@tmf.bg.ac.rs (D.S.); puskokovic@tmf.bg.ac.rs (P.U.)

² Department of Pharmaceutical Technology and Cosmetology, Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, 11221 Belgrade, Serbia; gsandra@pharmacy.bg.ac.rs (S.C.); svetlana.ibric@pharmacy.bg.ac.rs (S.I.)

³ Innovation Center, Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 111200 Belgrade, Serbia; ntomic@tmf.bg.ac.rs

* Correspondence: marija.jovanovic@tmf.bg.ac.rs; Tel.: +381-11-330-3616



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Abstract: Gelatin-polyvinylpyrrolidone (PVP) and gelatin-poly(vinyl alcohol) (PVA) mucoadhesive buccal films loaded with propranolol hydrochloride (PRH) were prepared by semi-solid extrusion 3D printing. The aim of this study was to evaluate the effects of the synthetic polymers PVP and PVA on thermal and mechanical properties and drug release profiles of gelatin-based films. The Fourier-transform infrared spectroscopy showed that hydrogen bonding between gelatin and PVP formed during printing. In the other blend, neither the esterification of PVA nor gelatin occurred. Differential scanning calorimetry revealed the presence of partial helical structures. In line with these results, the mechanical properties and drug release profiles were different for each blend. Formulation with gelatin-PVP and PRH showed higher tensile strength, hardness, and adhesive strength but slower drug release than formulation with gelatin-PVA and PRH. The in silico population simulations indicated increased drug bioavailability and decreased inter-individual variations in the resulting pharmacokinetic profiles compared to immediate-release tablets. Moreover, the simulation results suggested that reduced PRH daily dosing can be achieved with prolonged-release buccal films, which improves patient compliance.

Keywords: 3D printing; semi-solid extrusion; buccal films; gelatin; propranolol hydrochloride; prolonged-release; physiologically based simulations

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1. Introduction

Three-dimensional (3D) printing, an additive manufacturing technology, has been widely used to prepare different formulations that provide a personalized approach in drug delivery and tissue engineering [1–3]. The main principle of this method is based on the fabrication of different forms from digital models via layer-by-layer addition of printed materials [4,5]. The advantage of 3D printing is the manufacturing of high-quality formulations with different drug release profiles. Different 3D printing methods have found application in the pharmaceutical field, such as fused deposition modeling (FDM), stereolithography (SLA), inkjet-based 3D printing, selective laser sintering (SLS), and semi-solid extrusion (SSE) [6]. Moreover, additive manufacturing is considered a particularly attractive method for developing oral dosage forms as well as mucosal drug delivery systems [7,8]. Herein, the properties of the active pharmaceutical ingredients (APIs) and polymers, the designed geometry/structure, and targeted drug release profiles influence the choice of a suitable method for 3D printing of the desired dosage form [4].

Semi-solid extrusion (SSE) 3D printing uses a paste as a material loading system. A syringe filled with a paste is pushed under pressure during the process, and the paste is deposited through a nozzle. Preparation of the paste is a simple procedure consisting of mixing the API with polymers and other excipients [4,6]. There is also no need for special post-processing, except drying or cooling. Because this method does not require high temperature or UV curing, thermosensitive and UV sensitive APIs can be used [6]. The processing parameters (temperature, flow rate of the paste, and size and speed of the print nozzle) and formulation parameters (viscosity, polymer concentration, fluidity, and solvent type) affect the properties of the final product [6].

The blending of polymers provides many possibilities for modifying materials' characteristics and drug release profiles [9]. Natural or synthetic mucoadhesive polymers are usually used for buccal formulations because they increase the residence time of a dosage form on the application site and optimize drug delivery [7]. Gelatin is a natural polymer that is biocompatible, biodegradable, adhesive, non-toxic, and affordable. Because of these properties, gelatin is used in the pharmaceutical and biomedical fields for drug delivery and tissue engineering [10]. Gelatin is often blended with other polymers to obtain better material properties such as mechanical behavior and printability. Polyvinylpyrrolidone (PVP) and poly(vinyl alcohol) (PVA) are commonly employed polymers in pharmaceutical technology for manufacturing tablets, granules, hydrogels, nanofibers, as well as films and coatings [11,12]. They have a good film-forming ability and adhesiveness and can interact with hydrophilic materials like gelatin [7,11,12].

Drug delivery through buccal mucosa is an attractive route of drug administration because it enables the API to avoid the hepatic first-pass metabolism and degradation in the gastrointestinal (GI) tract [7,13]. Therefore, the bioavailability of some drugs can be increased by using the buccal dosage forms [13]. The oral mucosa has very good vascularization, and the API absorbed there directly enters systemic circulation [13,14]. This application route is convenient and accessible, and the buccal dosage form can be easily removed if needed [15]. In addition, patient compliance is improved, especially for the geriatric and pediatric population, as well as for patients with gastroesophageal disorders or patients having trouble swallowing [15]. In the process of developing buccal films, some important material properties have to be considered: biocompatibility, blending behavior, and mucoadhesion of the used polymers, as well as molecular weight, distribution coefficient (logD), lipophilicity (logP), solubility, and permeability of the API [7]. Buccal films can be developed as immediate-release (IR) or prolonged-release (PR) formulations. Buccal films can also be used for local diseases of the oral cavity [16] as part of a preventive and specific treatment strategy for gingivitis [17] and periodontitis [18] or to obtain systemic therapeutic effect [19–21]. These films should have the appropriate size and thickness to be used on the buccal mucosa [15]. Mechanical properties and mucoadhesive behavior should provide the flexibility, softness, elasticity, and resistance to breakage of films during manufacturing and application [15,22].

Propranolol hydrochloride (PRH), a model drug used in this study, is a non-selective β -adrenergic blocker. It has been used in the therapy of cardiovascular diseases such as hypertension [21], arrhythmias [23], and many others. It can also be used to treat anxiety [24], migraine [19], and tremor [25]. However, PRH is a drug that undergoes extensive first-pass metabolism that results in reduced bioavailability following peroral administration, so only approximately one-quarter of the dose reaches systemic circulation [19]. For this reason, the buccal route of administration is a suitable alternative for PRH formulations [26].

In this work, gelatin-based films for buccal delivery of PRH with different polymer blends were 3D printed using semi-solid extrusion to explore the effect of the synthetic polymers (PVP and PVA) on the films' mechanical and mucoadhesive properties, drug release profile, and the corresponding buccal drug absorption. The prepared buccal films were evaluated using various physicochemical, mechanical, and biopharmaceutical *in vitro* and *in silico* characterization tools.

2. Materials and Methods

2.1. Materials

Type A gelatin from porcine skin (~300 g Bloom) (GA), polyvinylpyrrolidone (K30) (PVP), and poly(vinyl alcohol) (Mw 85,000–124,000) (PVA) were obtained commercially from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Glycerol 85%, used as a plasticizer, was obtained from Zorka Pharma (Zorka Pharma HEMIJA d.o.o., Sabac, Serbia). Propranolol hydrochloride (PRH) (Galenika a.d., Belgrade, Serbia), which was used as a model substance, was Ph. Eur. 10 grade. Deionized water (DI) (resistance of 18 MW cm) was used to prepare the solutions. In vitro drug release and mucoadhesion studies were carried out in simulated saliva of the following composition: disodium hydrogen phosphate, potassium dihydrogen phosphate, and purified water (pH = 6.8 was adjusted with 0.1 M hydrochloric acid) [27]. The mucoadhesion studies used the mucin from a porcine stomach, Type II (Sigma-Aldrich Co., St. Louis, MO, USA).

2.2. Methods

2.2.1. Preparation of 3D Printed Films

The buccal films were prepared by semi-solid extrusion 3D printing. First, a 10% *w/w* gelatin solution was made by dissolving gelatin and 10% glycerol (on a gelatin dry basis) in distilled water. The solution was then stirred for 2 h at 50 °C. For PRH loaded paste, this procedure was followed by adding 25% PRH (on a gelatin dry basis) and stirring for 3 h at 50 °C to get a homogenous solution. For gelatin solutions blended with PVP or PVA, 10% PVP or PVA solutions were made separately; polymer and 10% glycerol (on a dry polymer basis) were added to distilled water and were stirred for 2 h at 50 °C. When homogenous polymer solutions were obtained, 10% gelatin and PVP or PVA solutions were mixed at a ratio of 1:1 and stirred again for 3 h at 50 °C to get a good blend. The procedure for PRH loaded paste was the same; after obtaining polymer blend solutions, 25% PRH (on a dry polymer basis) was added and stirred for 3 h at 50 °C to get a homogenous solution. Finally, each formulation solution (Table 1) was transferred into a syringe and kept at room temperature overnight for gelatination.

Table 1. Composition of paste formulation for mucoadhesive buccal films.

Formulations	F1	F2	F3	F4	F5	F6
Ingredients						
Gelatin, type A	6 g	6 g	3 g	3 g	3 g	3 g
PVP			3 g	3 g		
PVA					3 g	3 g
Glycerol 85%	0.6 g	0.6 g	0.6 g	0.6 g	0.6 g	0.6 g
PRH		1.5 g		1.5 g		1.5 g
Purified water	ad 60 g	ad 60 g	ad 60 g	ad 60 g	ad 60 g	ad 60 g

Before 3D printing, the syringe filled with paste was kept at 36 °C to obtain good printing properties [1,3]. Ultimaker 2+ printer (Ultimaker B.V., Utrecht, The Netherlands) adapted with Discov3ry paste extruder (Structur3d Printing, Kitchener-Waterloo, ON, Canada) was used for 3D printing. The pastes were extruded through a 0.84 mm internal diameter nozzle tip, moving at a 15 mm/min speed at room temperature. The films were printed in the shape of 60 mm × 15 mm × 0.5 mm strips. After printing, they were left to dry at room temperature for a day. The dried films were stored in a desiccator (NaBr saline saturated solution, relative humidity = 58%) at room temperature until analysis [26].

2.2.2. Fourier-Transform Infrared Spectroscopy (FTIR)

The formation of new chemical bonds between films' components during processing was observed by Fourier-transfer infrared spectroscopy (FTIR) using a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in the attenuated total reflectance

(ATR) mode. The equipment had a single bounce 45 °F Golden Gate ATR accessory with a diamond crystal and an electronically cooled DTGS detector. The spectra were recorded within 64 scans, with a resolution of 4 cm^{−1}, and processed by OMNIC software in the wavenumber range from 4000 cm^{−1} to 500 cm^{−1}.

2.2.3. Differential Scanning Calorimetry (DSC)

Thermal properties of starting materials, polymer blends, and printed films loaded with PRH were analyzed with differential scanning calorimetry (DSC) (Q10, TA Instruments, Crawley, UK). Measurements were performed in the temperature range from 30 °C to 200 °C with a heating rate of 10 °C/min. Samples of 6–9 mg were investigated under nitrogen flow of 50 mL/min. The characteristic temperatures were determined in the accompanying software [26,28].

2.2.4. Mechanical Properties

Tensile Test

The tensile test of films was performed on Texture Analyzer Shimadzu EZ Test LX (Shimadzu, Kyoto, Japan) equipped with a 500 N load cell, at room temperature, with a strain rate of 5 mm/min. The exact dimensions of the cross-section area and gauge length were accurately determined before each measurement. Tensile strength and modulus of elasticity were calculated by software Trapezium (Shimadzu, Kyoto, Japan). A minimum of three measurements was made for each film formulation, and average values were calculated.

Microindentation

The microindentation experiments were performed on Texture Analyzer Shimadzu EZ Test LX (Shimadzu, Kyoto, Japan) with a 500 N load cell and a spherical indenter with a $d = 4$ mm diameter. In each experiment, the load was set to continuously increase by 0.25 N/s until it reached 5 N. The maximum load was maintained for 20 s and then gradually decreased by 0.25 N/s until the full release. The values of force, time, and relative position of the indenter were measured. The microindentation was performed on a minimum of three positions on each film formulation to reduce the influence of possible inhomogeneity and to obtain more realistic results.

Microindentation is a promising method for evaluating the dynamic behavior of buccal and other drug-loaded thin films. From load (P) vs. depth (h) curves, it is possible to obtain values for hardness (H) and reduced elastic modulus (E_r) according to the Oliver–Pharr method [29,30]:

$$H = \frac{P_{\max}}{A} \quad (1)$$

$$E_r = \frac{S}{2} \cdot \sqrt{\frac{\pi}{A}} \quad (2)$$

where $P_{\max}$ represents the maximum load and S is the stiffness, i.e., the slope of the unloading curve, which was found at maximum displacement/load ($h_{\max}$, $P_{\max}$):

$$S = \frac{dP}{dh} \quad (3)$$

Additionally, A represents the projected area of the indenter impression and, given the spherical shape of the indenter, it was calculated as:

$$A = (dh_c - h_c^2) \cdot \pi \quad (4)$$

where h_c is the contact depth that can be determined as:

$$h_c = h_{\max} - \frac{P_{\max}}{S} \quad (5)$$

The corrective parameter ($\epsilon = 0.75$) for the spherical shape of the indenter was also used in the analysis [29].

2.2.5. Mucoadhesive Properties

Mucoadhesion tests were performed on Texture Analyzer Shimadzu EZ Test LX (Shimadzu Corporation, Kyoto, Japan) with a 500 N load cell, as in our previous research [26]. Obtained cylindrically cut films with a 10 mm diameter, fixed on a movable metal rod, were in contact with mucin disc with a 13 mm diameter, previously soaked in simulated saliva, for 60 s with the applied force of 1 N. The rod was then lifted with the constant velocity of 0.1 mm/s, and data for force, time, and stroke were recorded. The tests were stopped when the film got fully separated from the mucin disk. The area under the curve (AUC) was calculated from the force vs. stroke plot as the work of mucoadhesion [31]. Each experiment was carried out at least three times per film formulation.

2.2.6. Field Emission Scanning Electron Microscope (FESEM)

The structure and morphology of obtained films were observed by FESEM TESCAN MIRA 3 (Tescan Orsay Holding, a.s., Brno-Kohoutovice, Czech Republic). Fractured surfaces of the films were sputtered with gold.

2.2.7. Drug Content Uniformity

Three samples from different places of each film formulation were cut in 15 mm × 15 mm × 0.5 mm samples. The samples were then transferred into a 250 mL volumetric flask and dissolved in simulated saliva (pH = 6.8) [20]. After that, the solutions were filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA), and the absorbance of PRH was measured on UV-Vis spectrophotometer (LLG-uniSPEC 2 Spectrophotometer, Lab Logistics Group GmbH, Meckenheim, Germany) at 319 nm. To determine the drug content uniformity of films, we used the same method as in our previous work [26].

2.2.8. In Vitro Drug Release

The dissolution studies were performed using an orbital shaker (Orbital shaker PSU-10i, Grant-bio, Grant Instruments Ltd., Cambridge, UK) at 50 rpm in a thermostatic incubator (MBIN50 Microbiological Incubator, Colo Lab Experts, Novo Mesto, Slovenia) at 37 °C [32]. The dissolution medium was 100 mL of simulated saliva [32,33]. The dimensions of the buccal films were 15 × 15 × 0.5 mm. The buccal film samples were fixed at the bottom of the beaker with double-sided tape, enabling drug release solely from one side of the films. Aliquots were withdrawn at predetermined time points, and the same amount of fresh prewarmed medium was added. The samples were filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA, USA) and analyzed by a UV-Vis spectrophotometer (LLG-uniSPEC 2 Spectrophotometer, Lab Logistics Group GmbH, Meckenheim, Germany) at 319 nm. For every formulation, the experiment was carried out in triplicate.

2.2.9. Drug Release Kinetics

The release kinetics of the PRH from the buccal films were analyzed using the DD-Solver software (Microsoft Excel add-in program) developed by Zhang et al. [34]. The cumulative drug release data were fitted with different mathematical models, including zero-order, first-order, Higuchi, and Korsmeyer–Peppas models [35–37]. The highest values of the coefficient R^2 and the model selection criterion (MSC) were utilized to determine the best fitting model [37].

2.2.10. Physiologically Based Simulations

Commercially available software GastroPlus™ (v. 9.7.0009, Simulations Plus Inc., Lancaster, CA, USA) was used to simulate PRH absorption and disposition following intraoral administration of the tested buccal films. For comparison purposes, the simulations

were also performed for PRH IR tablets. Our previous publication [26] describes basic data about the software performance and the applied model, along with the drug-specific input data used for model construction. In brief, the selected parameters describing PRH physicochemical and pharmacokinetic properties, in conjunction with the software default physiological parameters values for human adults in the fasted state, were used to model drug transport and absorption through the oral cavity mucosa and/or intestinal membrane, and to estimate the corresponding plasma concentration-time profiles. Here, the software-integrated Advanced Compartmental Absorption and Transit (ACAT) model of the GI tract was used to simulate the bioperformance of IR tablets, whereas the adjunct Oral Cavity Compartmental Absorption and Transit (OCCAT) model was used to estimate drug absorption from the tested buccal films. As explained in our previous publication [26], the only adjusted physiological parameter referring to the OCCAT model was saliva production rate (Table 2). The additional OCCAT model parameters, specific for the tested buccal film formulations, are noted in Table 2. In order to describe drug release rates from the tested formulations, experimentally obtained dissolution data, fitted by a single-Weibull function, were used as inputs in the model. A Weibull function was used to obtain smoother profiles and allow for more accurate extrapolation of the data. In addition, the model assumed that the contact surface area of the film with buccal mucosa is constant (software default) and that drug release happens in one direction (which complied with the in vitro dissolution test settings). The simulations were performed for a single PRH dose (as buccal film or IR tablet) and for repeated drug dosing to estimate the expected plasma concentration in the steady state.

Table 2. The study-specific input parameters in the OCCAT model.

OCCAT Parameter	Value
Formulation type	Buccal patch ^a
Drug dose	20 mg ^a
Film surface area	2.25 cm ² ^a
Oral transit model	Normal swallowing ^a
Saliva production rate (base value)	0.6 mL/min ^b
Fraction unbound in oral tissue (F_{ut})	0.21 ^c
Drug diffusivity through oral mucosa	7.21×10^{-7} cm ² /s ^c

^a applies for the investigated buccal films; ^b optimized value [38]; ^c calculated using the software integrated equations.

In order to account for inter-individual variations in the physiological and drug pharmacokinetic parameters, the simulations were performed for a virtual group of 50 subjects, using the Population Simulations software module. The selected input parameters were varied randomly within the predefined range in each simulation. This range was defined for each parameter based on the initial (base) value (initial physiological parameters were software default values) and software default coefficient of variation (CV%), using a log-normal distribution. The default CVs% were 20% for transit times in different GI compartments, the volume of distribution, distribution rate constants, and hepatic first-pass extraction, 30% for hepatic blood flow, 40% for clearance, and 10% for all other variables.

2.2.11. Statistical Analysis of the Experimental Results

The data obtained by evaluating mechanical and mucoadhesive properties were also analyzed using a two-way analysis of variance (ANOVA) at a confidence level of $p < 0.05$. Polymer type and drug presence were used as independent variables (factors), whereas tensile strength, modulus of elasticity, hardness, reduced modulus, maximum force, and work of adhesion from respective tests were used as dependent variables. The statistical analysis was performed in the OriginPro software (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Fourier-Transform Infrared Spectroscopy (FTIR)

The FTIR spectrums of 3D printed films are presented in Figure 1. The FTIR spectrum of F1 showed intense bands at: 3320 cm^{-1} related to the OH stretching of internal hydrogen bonds overlapped with a band of N–H stretching, corresponding to Amide A; 1660 cm^{-1} which could be assigned to the vibration of the amide carbonyl group corresponding to Amide I; 1549 cm^{-1} associated with bending of the free amino groups coupled with CN stretching-Amide II; 1243 cm^{-1} related to NH bending-Amide III; 1045 cm^{-1} assigned to the characteristic C–O stretching band of glycerol [26,39,40].

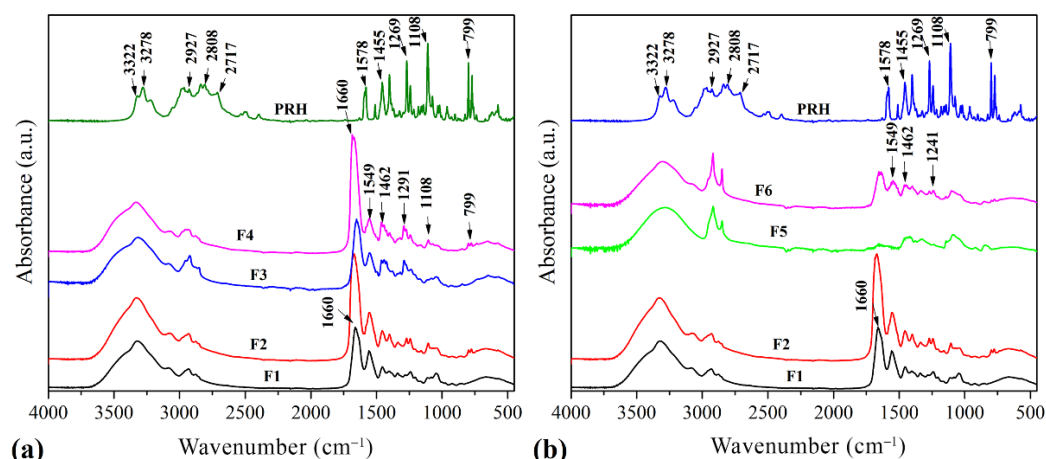


Figure 1. FTIR spectrum of 3D printed films (a) F1, F2, F3, F4, and PRH; (b) F1, F2, F5, F6, and PRH.

The spectrum of the F3 blend is very similar to the spectrum of F1 with emphasized bands at 2929 and 2869 cm^{-1} due to the asymmetric and symmetric C–H stretching vibrations, respectively [39]. There is also another prominent peak at 1653 cm^{-1} due to C=O stretching vibration.

Due to the addition of PVP, the peak at 1460 cm^{-1} shifted to 1438 cm^{-1} , which corresponds to in-plane bending vibration from C–H [41]. This shift could be attributed to hydrogen bonding between C=O in PVP and OH in gelatin [40]. A similar explanation could be given for the peak at 1243 cm^{-1} , which shifted to 1290 cm^{-1} after established hydrogen bonding coupled with NH bending [40]. In the case of the F3 spectrum, these peaks are positioned at 1680 cm^{-1} and 1460 cm^{-1} . Hence the peaks for functional groups of PRH and GA were unchanged as there was no interaction between the drug and gelatin [26]. Finally, the spectrum of F4 showed peaks at 1685 , 1549 , 1462 , and 1291 cm^{-1} that revealed established hydrogen bonds between GA and PVP, whereas peaks from PRH (the C–O–C stretching in aryl alkyl ether a stretching band at 1108 cm^{-1} , and the α -substituted naphthalene peak at 797 cm^{-1}) were unchanged. Therefore, there was no interaction of the blend with PRH [26].

The spectrum of F5 showed peaks at 3299 cm^{-1} due to the stretching vibration of O–H bonds; at 2929 and 2869 cm^{-1} due to the asymmetric and symmetric C–H stretching vibrations, respectively; and a peak of medium intensity at 1462 cm^{-1} due to the bending vibrations of C–H bonds. [42,43]. Further, in the spectrum of F6, the observed peak at 1653 cm^{-1} is assigned to C=O stretching vibration. Finally, it can be seen that, in this spectrum, there are not any peaks at 1715 – 1730 cm^{-1} , which are normally attributed to aromatic esters [44]. Therefore, this indicates that neither the esterification of PVA nor of the gelatin has taken place in formulation with PRH [43,44].

3.2. Differential Scanning Calorimetry (DSC)

DSC analysis was performed to observe the thermal behavior of films as a consequence of intermolecular structure changes during processing. The DSC curves for starting

materials Gelatin A, PVP, PVA, and films F1, F3 are presented in Figure 2a, whereas the curves for films F1, F2, and PRH are presented in Figure 2b. The curves for polymer blends loaded with PRH, F4 and F6, are presented in Figure 2c. Glycerol with a small hydrophilic molecule affects gelatin water absorption and also acts as a plasticizer. The molecules of glycerol are interspaced in a protein network and increase the distance between protein chains. In this work, glycerol was also added to establish better printability of films. In terms of thermal behavior, glycerol decreased the melting temperature (T_m) of gelatin. It also made gelatin more sensitive to moisture, and an endothermic denaturation peak was observed on the thermogram of F1 [45–48]. Within the presented DSC curves, in the case of F1, two areas can be distinguished: the first one in the 45–60 °C range, in which glass transition temperature (T_g) and triple helix denaturation of gelatin are observed, and the second one in the 160–180 °C range, in which peaks of cis and trans isomerism of glycine in gelatin are observed [26,48–51]. For formulations F3 and F5, it can be observed that the blending with PVP or PVA stabilizes gelatin, and then there is no cis-trans isomeric transformation.

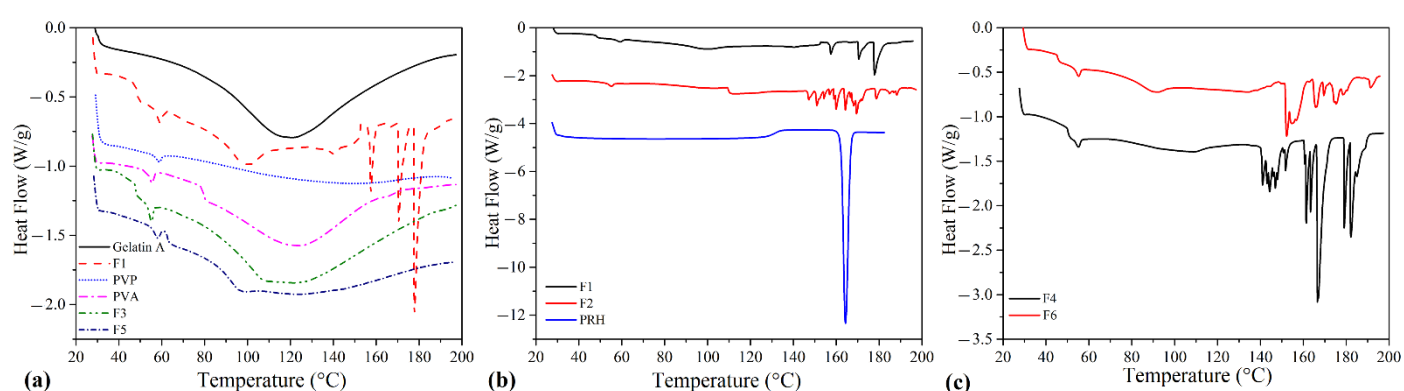


Figure 2. DSC thermograms of (a) Gelatin A, F1, PVP, PVA, F3, and F5; (b) F1, F2, and PRH; and (c) F4 and F6.

Endothermic peaks in the range 140–185 °C are present in thermograms of drug-loaded formulations (F2, F4, and F6). The DSC thermogram of pure PRH shows a melting point at 163 °C [26]. However, the thermograms of drug-loaded formulations did not show the melting peak of the PRH, indicating that the 3D semi-solid extrusion method induced the amorphization of propranolol [27,52,53]. The addition of glycerol as a plasticizer leads to the depression of characteristic temperatures of polymers and the melting temperature of PRH, causing further decomposition from 146 °C for F2, 140 °C for F4, and 151 °C for F6. As is noted in our previous research [26], the drug's amorphous form should be advantageous in terms of solubility and bioavailability.

3.3. Mechanical Properties

3.3.1. Tensile Test

Results of tensile tests of all formulations of 3D printed films are given in a form of histograms (mean values) and as a diagram (for one representative film) in Figure 3a,b, respectively. In addition, the results of the ANOVA analysis are shown in Table 3. It can be seen that F1 showed quite satisfactory tensile strength, modulus of elasticity, toughness (area under the tensile curve), and breaking elongation, in line with previous research [26]. With the addition of the drug or when blended with PVP or PVA, toughness and breaking elongation decrease. Obtained results for blended films F3 and F5 are in agreement with literature data [39,44,54].

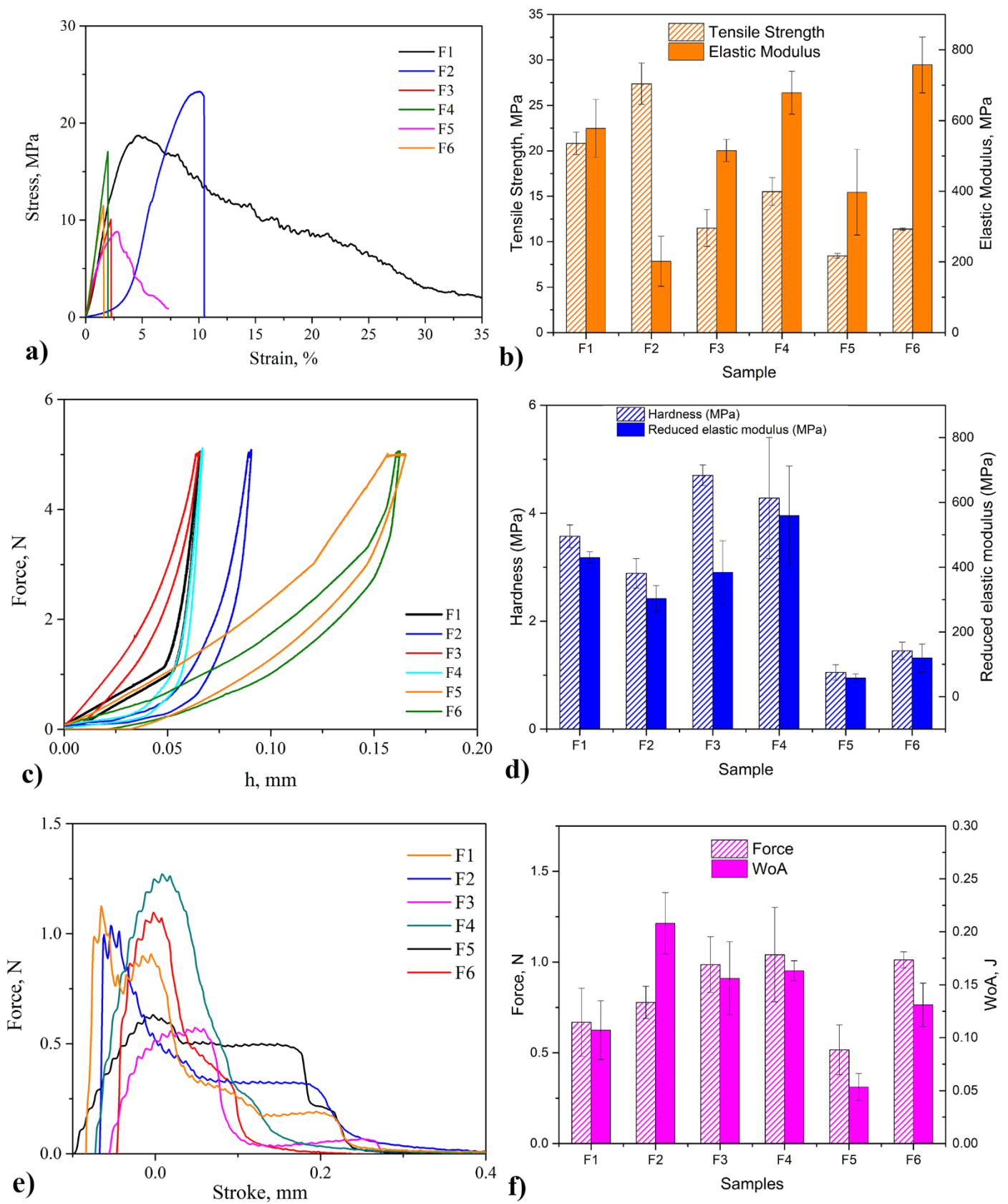


Figure 3. Results of: (a,b) tensile test; (c,d) microindentation; (e,f) mucoadhesion.

Table 3. The results of ANOVA tests for the obtained mechanical and mucoadhesive properties.

Factor	Tensile Strength		Hardness		Strength of Adhesion	
	F Value	p Value	F Value	p Value	F Value	p Value
Polymer	42.94	<0.001	21.93	<0.001	1.96	0.173
Drug	11.23	0.007	0.34	0.570	2.91	0.107
Interaction	0.69	0.525	0.66	0.535	1.12	0.350
Factor	Elastic Modulus		Reduced Modulus		Work of Adhesion	
	F Value	p Value	F Value	p Value	F Value	p Value
Polymer	3.92	0.055	12.71	0.001	4.27	0.032
Drug	0.50	0.494	0.33	0.575	8.56	0.010
Interaction	10.79	0.003	1.89	0.194	1.78	0.201

F value represents the ratio of between-groups variance and within-groups variance; p-value determines whether the observed test results are statistically significant.

The tensile tests revealed that F6 showed 30% lower tensile strength and 11% higher modulus of elasticity than F4. That could be explained by the formation of hydrogen bonds between GA and PVP, which leads to higher tensile strength and decreased energy absorption ability during deformation [55]. Additionally, the presence and higher content of triple-helical structures improve mechanical properties. The influence of both polymer and the presence of drug on tensile strength and their interaction on elastic modulus were shown to be statistically significant ($p < 0.05$), as shown in Table 3.

3.3.2. Microindentation

Microindentation is a suitable method for investigating visco-elastic properties of biopolymers, like gelatin [56–59]. The load-depth curves and obtained values for reduced modulus, E_r , and hardness, H are presented in Figure 3c,d, respectively. The reduced modulus of F4 is approximately four times higher than that of F6, whereas the hardness is approximately three times higher. A different structure of these films, the higher content of triple-helical structure, and cis-trans isomeric transformation in F4 could explain this, as is supported by the DSC analysis. Gelatin is a product of collagen degradation. The higher content of disordered, coiled, and shortened collagen chains leads to greater probabilities of chain tangling and formation of helical structure bonding. With the higher bonding, a greater force is necessary to deform the gelatin-based film, and the stiffness increases as well [60]. The influence of polymer on hardness and reduced modulus were shown to be statistically significant ($p < 0.05$), as shown in Table 3.

3.4. Mucoadhesive Properties

Mucoadhesion is a significant property of buccal films; it describes how close a film is attached to the buccal mucus layer. The results of this research were analyzed from the fracture theory perspective, but the adhesive behavior of different formulations could be explained by other theories, such as electronic or charge, diffusion, wetting, and adsorption theories [61,62]. Force-stroke curves and results of tests—maximum detachment force and work of adhesion—are shown in Figure 3e,f, respectively.

It can be seen that the highest value of the adhesion force was obtained for F4, which agrees with other good mechanical properties of this formulation. Better attachment of F4 on the mucin can be explained by the formation of hydrogen bonds, the higher content of triple-helical structures, and the cis-trans isomeric transition during processing. Additionally, the electrostatic interaction between positively charged gelatin and negatively charged mucin could be modified by adding PRH and PVP or PVP. These molecules, according to their polarity, enhance or suppress this interaction [15,63]. F5 showed a lower adhesion force than F1, whereas F2 and F3 showed a higher one. Finally, F4 achieved a higher force than F6 because of the similar polarity of PVA and PRH. Work of adhesion correlates with the crosslinking of polymers and the possibility of liquid permeation in a

polymer network. Among drug-loaded formulations, F2 has the highest value of the work, which implies the low density of crosslink [26]. The influence of polymer on the work of adhesion was shown to be statistically significant ($p < 0.05$), as shown in Table 3.

3.5. Field Emission Scanning Electron Microscope (FESEM)

SEM was used to examine the morphology of obtained films. SEM images are presented in Figure 4. It can be seen that all films have consistent structure without pores and show the surface's roughness. However, sharper creases can be observed in the images of formulations F4 and F6 (Figure 4d,f, respectively). The creases could be explained by the different stiffness of triple-helix gelatin molecules compared to PVP or PVA molecules [64,65]. The presence of the undissolved drug was not observed, as can be seen in Figure 4b,d,f [66].

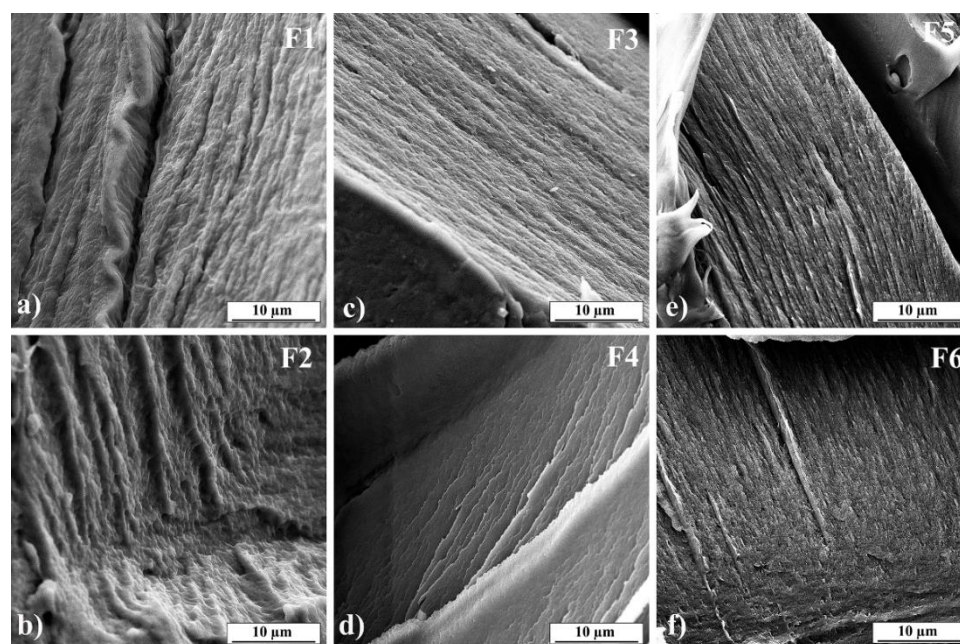


Figure 4. SEM images of 3D printed films: (a) F1, (b) F2, (c) F3, (d) F4, (e) F5, (f) F6.

3.6. Drug Content Uniformity

The results of drug content uniformity are given in Table 4. Because they are within the limit of drug content uniformity, i.e., 85–115% [67], the buccal films have a uniform drug distribution.

Table 4. Drug content uniformity.

Formulations	F2	F4	F5
Drug content percentage	98.27 ± 2.39	97.58 ± 3.65	95.74 ± 3.17

3.7. In Vitro Drug Release

Cumulative drug release profiles from all tested formulations are illustrated in Figure 5. During the test, all samples stayed fixed at the bottom of the beaker, and the sink conditions were maintained. It can be seen that complete PRH release from each formulation was achieved within 4 h. However, there were notable differences between the PRH release rates of different formulations. Formulation F2 demonstrated the fastest drug release (complete dissolution in 1 h), with initial burst PRH release (more than 50% in 15 min) followed by a somewhat slower dissolution rate. The other two formulations exhibited prolonged drug release, with complete PRH dissolution in 2.5 and 4 h for formulations F6 and F4, respectively.

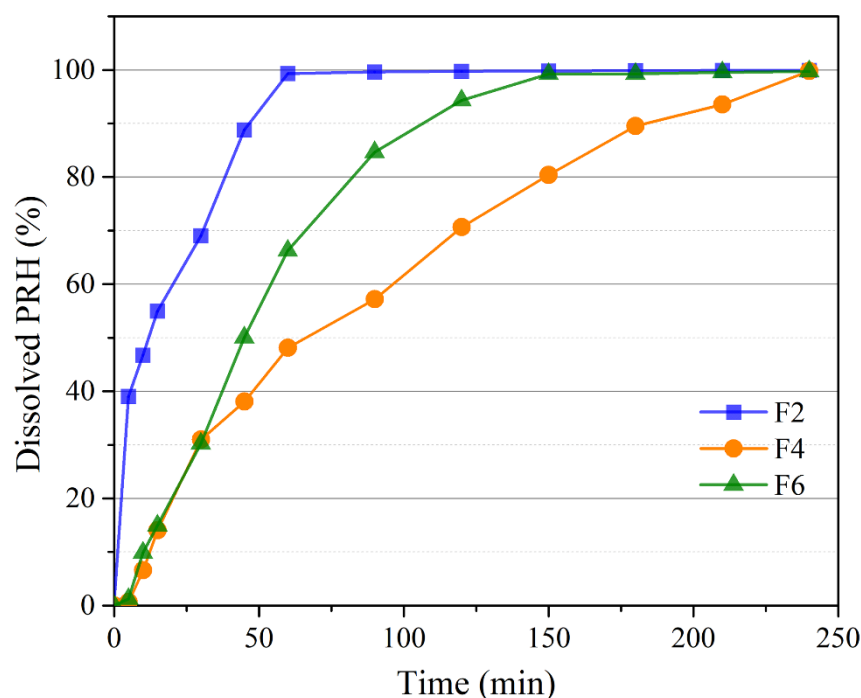


Figure 5. In vitro drug release.

In formulation F4, besides the formation of hydrogen bonds between molecules of the same polymer, there are also established hydrogen bonds between two different macromolecules (hydroxyl groups of the GA and carbonyl groups of PVP; amine groups of the GA and carbonyl groups of PVP). These hydrogen bonds cause the high cross-linked structure of the F4 formulation [40,64,68].

Due to the different polarity of molecules, this structure associated with PVP and PRH attraction slows down the liquid penetration in a polymer network and hence cause slower PRH release than in F2 and F6. As for formulation F6, a lower drug release rate when compared to formulation F2 can be explained by densely arranged molecules of gelatin and synthetic polymer PVA in this formulation [65]. On the contrary, in formulation F6, there are no new chemical bonds, and because molecules of PVA and PRH have similar polarity, this results in faster PRH release from F6 than from F4. The blending of GA with PVP or PVA makes it possible to prolong the PRH release time [44,69].

3.8. Drug Release Kinetics

The fitting results of the drug release profiles from 3D printed buccal films are shown in Table 5. The release model with the best fit is one with the highest values of R^2 and MSC (a value greater than 2 indicates a good fit [34]). In this study, the best mathematical model for all formulations was first-order kinetic, which means that the release rate shows dependence on PRH concentration, and it is proportional to the drug remaining in the matrix [36,70]. The Korsmeyer–Peppas model also resulted in a good fit, which is why we calculated the release exponent (n value) to understand the PRH release mechanism from buccal films better [37]. Formulation F2 has an n value lower than 0.5, suggesting that the PRH release followed the Fickian diffusion mechanism. Formulations F4 and F6 have an n value between 0.5 and 1, meaning that the PRH release mechanism is both swelling and diffusion and follows anomalous or non-Fickian transfer [37,38,71].

Table 5. Drug release kinetics.

Model	Zero Order		First Order		Higuchi Model		Korsmeyer–Peppas Model		
Criteria	R ²	MSC	R ²	MSC	R ²	MSC	R ²	MSC	<i>n</i> Value
Formulation									
F2	0.6777	1.2998	0.9682	2.6653	0.5167	0.0553	0.9259	2.1537	0.215
F4	0.8940	1.9315	0.9902	4.3081	0.9655	3.0539	0.9858	3.7843	0.630
F6	0.7281	0.9723	0.9797	3.5694	0.9256	2.2687	0.9268	2.7314	0.528

R²—coefficient of determination; MSC—model selection criterion; *n* value—release exponent, indicating the drug-release mechanism.

3.9. Physiologically Based Simulations

The simulation results for single-dosed 20 mg PRH buccal films, along with the data for PRH IR tablets containing different drug doses, are shown in Figure 6 and Table 6. The data in Figure 6 refer to the mean profiles for a virtual population of 50 subjects.

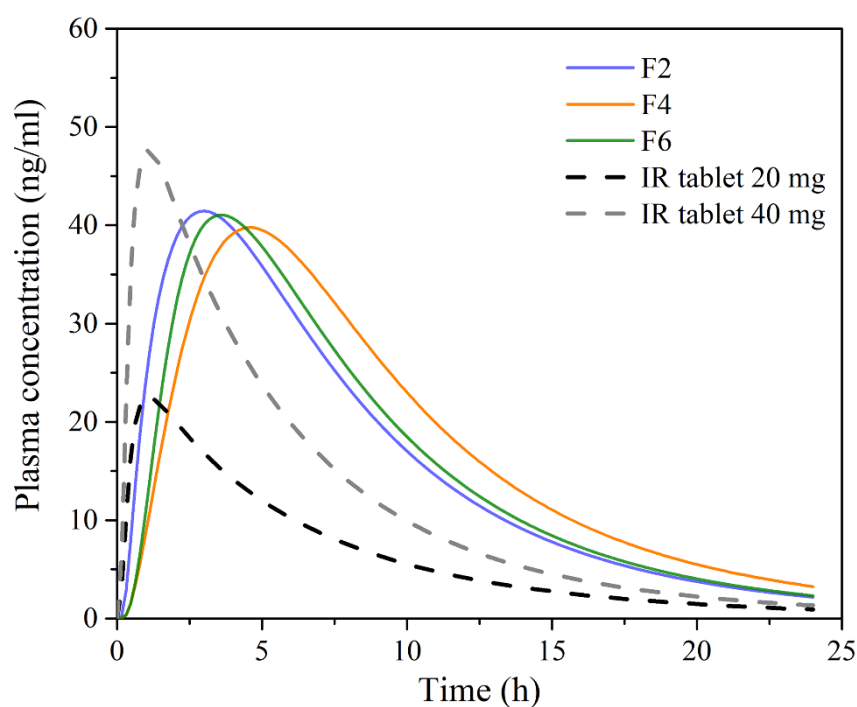


Figure 6. Mean predicted plasma concentration-time profiles following single dosing of the tested 20 mg PRH buccal films, and 20 mg and 40 mg IR tablets in 50 virtual subjects.

The obtained results demonstrate certain differences in PRH absorption profiles following administration of the tested buccal films, mostly in terms of t_{max} , whereas the differences in C_{max} and AUC values were not pronounced. The observed differences in the rate of drug absorption are caused by different PRH release rates from the tested formulations (Figure 5), i.e., slower drug release rate resulted in slower drug absorption. Following dissolution, the drug goes directly into the buccal epithelium through the contact surface area, resulting in a concomitant increase in the percent of drug absorbed into the buccal tissue (Figure 7). After the drug enters the buccal epithelium, it diffuses across the epithelium and lamina propria and eventually passes into the systemic circulation. The rate of these processes depends on the physiological characteristics of the buccal tissue and drug properties, such as diffusivity through the oral mucosa and F_{ut} value [72]. Therefore, the resulting cumulative percent of PRH that entered systemic circulation lags behind the percent of drug absorbed (Figure 7). However, drug uptake into the systemic circulation following administration of the tested buccal films is complete, in contrast to the limited

drug bioavailability from IR tablets (due to the first-pass metabolism in the liver) (Table 6). Moreover, simulation results indicated that the extent of drug absorption (expressed as AUC) from the tested formulations lies in the range predicted for IR tablets containing a twice higher drug dose (Table 6). These outcomes imply that the same therapeutic effect may be achieved with reduced PRH doses in buccal films while causing fewer side effects.

Table 6. Predicted pharmacokinetic parameters following single-dose administration of the tested 20 mg PRH buccal films and 20 mg and 40 mg IR tablets in 50 virtual subjects.

Formulation (Dose)	C_{max} (ng/mL)		t_{max} (h)		$AUC_{0-\infty}$ (ng h/mL)		F (%)	
	Mean (Range)	CV%	Mean (Range)	CV%	Mean (Range)	CV%	Mean (Range)	CV%
F2 (20 mg)	42.13 (23.16–71.36)	26.59	3.10 (1.86–4.58)	19.43	404.87 (197.69–803.03)	34.80	99.89 (99.88–99.90)	2.62×10^{-3}
F4 (20 mg)	40.72 (21.43–68.54)	24.26	4.78 (3.24–7.08)	16.98	451.74 (210.46–808.55)	31.49	99.99 (99.93–100.00)	1.08×10^{-2}
F6 (20 mg)	41.67 (23.08–70.61)	26.56	3.70 (2.56–5.16)	15.32	404.10 (197.32–801.51)	34.80	99.70 (99.69–99.71)	2.77×10^{-3}
IR tablet (20 mg)	22.81 (0.74–54.38)	45.42	1.12 (0.65–1.62)	19.35	168.02 (4.44–461.53)	53.45	35.24 (1.00–54.92)	33.82
IR tablet (40 mg)	48.14 (1.23–100.97)	44.76	1.08 (0.74–1.62)	16.99	319.63 (5.36–1088.80)	57.97	33.12 (1.32–57.57)	36.38

C_{max} —maximum plasma concentration; t_{max} —time to reach C_{max} ; AUC—area under the plasma concentration-time curve; F—bioavailability, CV%—coefficient of variation.

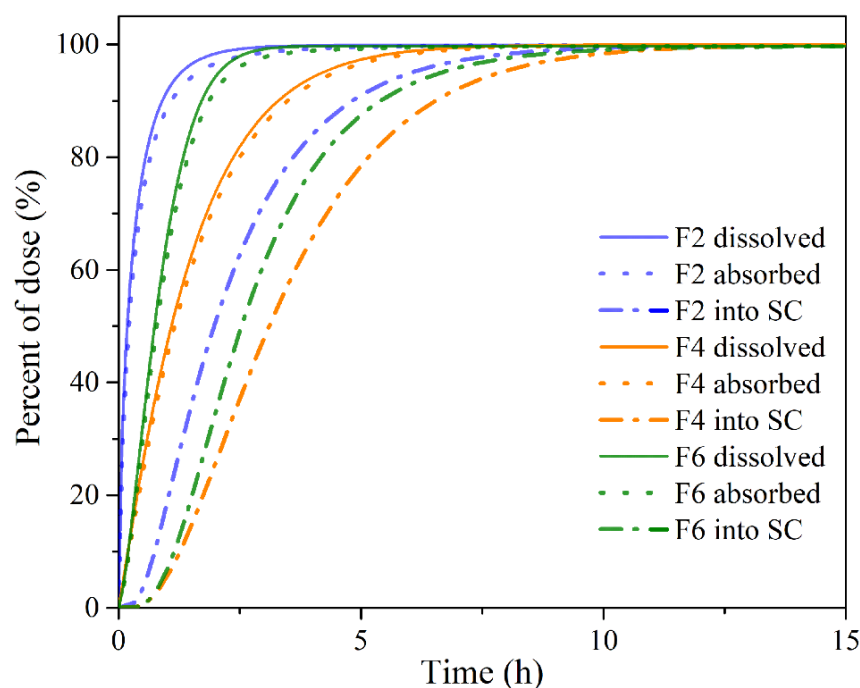


Figure 7. Predicted cumulative percent of PRH dissolved, absorbed, and entered into system circulation (SC) following single-dose administration of the tested buccal films.

In addition, simulations with repeated PRH dosing under different dosing regimens (Figure 8) revealed that buccal administration of the tested films might enable reduced PRH daily dosing compared to IR tablets, thus improving patient compliance. PRH therapeutic dose and dosing regimen depend on the indication, but the drug is most commonly given in divided doses 2–4 times per day [73,74]. For example, the recommended or the commercially available dose of PRH tablets for hypertrophic subaortic stenosis (thickened heart muscle) for adults is 20 to 40 mg three or four times per day [75]; for arrhythmias, hypertrophic obstructive cardiomyopathy and anxiety tachycardia, the dose for adults

is in the range of 10 to 40 mg three or four times per day [76]. However, according to the simulations, even 3–4 times daily administration of 20 mg PRH IR tablets may not be able to maintain drug plasma concentration above the minimum effective concentration (MEC) of 20 ng/mL (preferably more) required to achieve inhibition of beta-adrenergic receptors [73,77]. Figure 8 shows only the mean data for a virtual population, meaning that the risk of subtherapeutic dosing is expected to be even higher when inter-individual differences in PRH pharmacokinetics are considered. Opposite to IR tablets, the investigated buccal films dosed three times per day are expected to maintain PRH plasma concentration in a “safe space” above the MEC (Figure 8). Additionally, it was observed that formulation F4, dosed two times per day, should provide a greater extent of PRH absorption in comparison to an IR tablet containing the same drug dose but administered more frequently, i.e., four times per day.

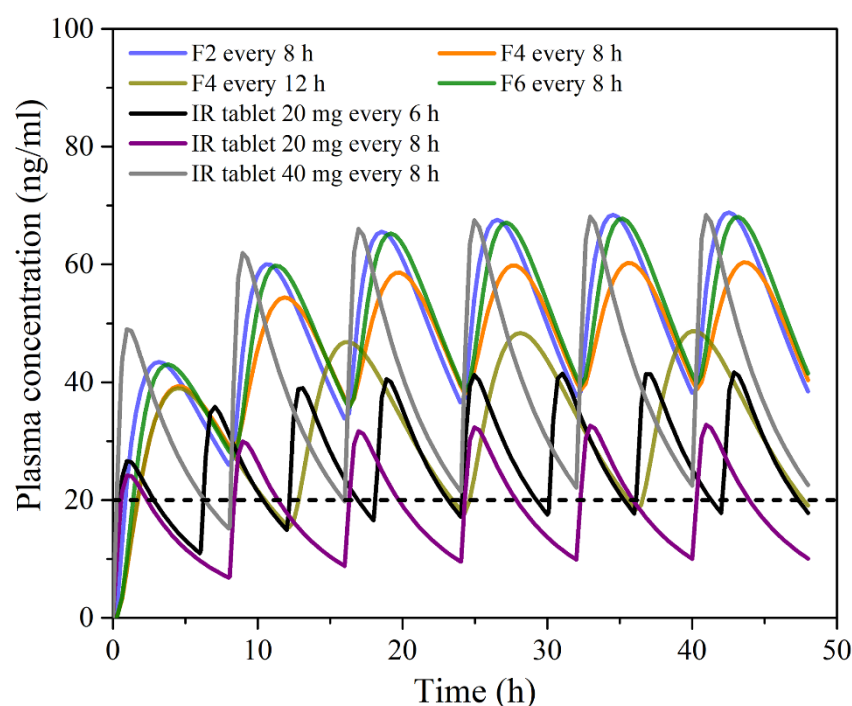


Figure 8. Mean predicted plasma concentration-time profiles following multiple dosing of the tested 20 mg PRH buccal films, and 20 mg and 40 mg IR tablets in 50 virtual subjects; the dotted line represents the minimum effective concentration.

Another observation regarding PRH absorption from the tested buccal films is that the predicted pharmacokinetic parameters (i.e., C_{max} , $AUC_{0-\infty}$ and drug bioavailability) for the virtual population are less prone to variations between subjects compared to the values predicted for IR tablets (Table 6). Namely, the simulated data suggest that inter-individual variability in C_{max} and AUC would be decreased by cc. 40% when switching from PRH IR tablets to the tested buccal film formulations.

4. Conclusions

Three-dimensional printing, a modern technique for advanced pharmaceutical formulations, was used to prepare buccal mucoadhesive gelatin-based films. In order to improve the mechanical properties of these films loaded with PRH and increase drug bioavailability, two blends of gelatin (with PVP and PVA) were prepared as the polymer matrix. The influence of polymer blend type on the films' printability, structure, thermal and mechanical properties of the films, and drug release profile was investigated. Critical properties of 3D printed films, such as tensile strength, modulus of elasticity, hardness, mucoadhesion, drug content, and drug release rate, have been discussed, as these properties are significant

determinants of the mucoadhesive buccal films' behavior during application and the drug bioavailability from these films.

The presence of PVP in the blend and consequently in formulation F4 improves thermal and mechanical properties, exhibits a good mucoadhesion, and enables the prolonged release of PRH with the lowest rate. A highly cross-linked structure caused by the established hydrogen bonds between two different macromolecules (gelatin and PVP) could explain this behavior. On the contrary, in the case of the F6 formulation, no additional chemical bonding between gelatin and PVA occurred. Therefore, the drug release rate in this formulation is higher than in F4 but lower than in F2. This difference could be ascribed to densely arranged molecules of gelatin and synthetic polymer PVA. The addition of PVA to the gelatin-based matrix leads to thermal stability, a higher modulus of elasticity, and better mucoadhesion.

The simulation results revealed that PRH bioavailability, following application of the tested buccal films, is expected to be approximately three times higher than from IR tablets because the drug absorbed from the oral cavity bypasses first-pass extraction in the liver. In addition, the simulations suggested reduced dosing frequency of buccal films with prolonged PRH release (F4 and F6) in comparison to conventional IR tablets, which may increase patient compliance. As another advantage, population simulations indicated decreased inter-individual variations in PRH pharmacokinetics following administration of the buccal films.

By blending gelatin with PVP or PVA, we obtained PRH loaded buccal mucoadhesive films with good mechanical and mucoadhesive properties and prolonged drug release. The presented results indicate that with proper choice of synthetic polymer and blend composition, it is possible to tune mechanical properties as well as drug release profiles, depending on the application requirements. Finally, good printability was achieved, and the semi-solid 3D printing method was successfully introduced in buccal film preparation.

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4.3. 3D-Printed Gelatin-Based Scaffold Crosslinked by Genipin: Evaluation of Mechanical Properties and Biological Effect

ARTICLE

3D-Printed Gelatin-Based Scaffold Crosslinked by Genipin: Evaluation of Mechanical Properties and Biological Effect

Marija Jovanović¹ | Miloš Petrović¹ | Dušica Stojanović¹ | Nataša Radulović² | Danijel Pantelić² | Ivana Stajčić³  | Petar Uskoković¹

¹University of Belgrade, Faculty of Technology and Metallurgy, Belgrade, Serbia | ²Institute for Biological Research “Siniša Stanković” - National Institute of Republic of Serbia, University of Belgrade, Belgrade, Serbia | ³Department of Physical Chemistry, “Vinča” Institute of Nuclear Sciences - National Institute of the Republic of Serbia, University of Belgrade, Belgrade, Serbia

Correspondence: Ivana Stajčić (ivana_r@vinca.rs)

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ABSTRACT

In this study, scaffolds based on natural polymer gelatin A, blended with polyvinylpyrrolidone were crosslinked by genipin (0.5 and 1 wt%), in order to investigate their mechanical performance and potential for biomedical application. Semi-solid extrusion (SSE) 3D printing technique was used, enabling in situ crosslinking of the blend during processing. Swelling test showed that the swelling ratio reduces with higher concentration of genipin due to an increased crosslinking. The FTIR analysis confirmed the crosslinking of scaffolds by genipin. DSC analysis and mechanical testing have shown improved thermal and mechanical properties. Morphological analysis of scaffolds by FESEM showed increased toughening of the material with the crosslinking. Tensile strength and microhardness showed a significant rise in scaffolds with the increase in genipin content, which was up to 93.8% and 125.3%, respectively. These findings were in accordance with morphological features present in samples. The biological effect of the scaffold matrix system was evaluated by qualitative and quantitative cytotoxicity assessment in vitro, demonstrating the absence of cytotoxicity in tested preparations in a direct test. The cytotoxicity index based on the metabolic activity of cells in an indirect test showed up to 20% reduction of viability compared with the control, confirming the absence of cytotoxicity, which was additionally verified by propidium iodine staining of the cells exposed to scaffolds. The presented gelatin-based crosslinked scaffolds obtained by 3D printing represent good candidates for biomedical application and future research that includes further in vitro and in vivo analysis.

1 | Introduction

Over the past two decades, biomaterials have found their application in tissue engineering due to their unique properties, such as bioactivity and high biocompatibility [1, 2]. Scaffolds are extensively used for tissue regeneration, providing mechanical and molecular signals that stimulate targeted tissue cells, thus promoting tissue regeneration [3]. Choosing biologically inspired materials and fabrication processes is important for designing

engineered constructs that replicate the complex structures and natural properties of native tissue [4]. Collagen, gelatin, and chitosan are the most commonly natural biomaterials used in tissue engineering [5, 6]. Gelatin has been found to be applicable in various industries due to its economic viability, absorption capacity, biodegradability, and nontoxicity. Furthermore, gelatin promotes cell proliferation, which makes it a valuable material in medical applications from bone to cardiac tissue regeneration [7, 8]. The major drawbacks of using gelatin are poor

mechanical properties and fast degradation, both of which can be improved with crosslinking [9]. Genipin, a natural derivative of geniposide found in fruits, has been proven to be a highly efficient crosslinker for gelatin, leading to enhanced mechanical performance [10–12]. Furthermore, gelatin is frequently used in blends with other polymers to enhance its mechanical properties. Polyvinylpyrrolidone (PVP) is an amorphous polymer known for its solubility in organic solvents and hydrophilic nature, widely used in pharmaceutical technology for making tablets, hydrogels, films, and coatings [13, 14]. It can form thin films, has good adhesiveness, is stable across various pH levels, resists high temperatures, crosslinks well and has good mechanical strength [15]. Mishra et al. have investigated the potential of gelatin/PVP blend scaffold for application in bone tissue engineering, establishing mechanical properties similar to bone, as well as high biocompatibility [16]. Recently, Nasiri et al. incorporated hydroxyapatite (HA) in gelatin-PVP crosslinked with glutaraldehyde in order to increase swelling ratio, biocompatibility, and mechanical properties [17]. They determined that the addition of HA increased swelling, tensile strength and cell viability. Jana et al. prepared dissolving microneedles based on gelatin and PVP with the aim of obtaining patches with stable drug release and enhanced mechanical strength [18]. The potential of these materials in biomedical applications is currently the focus of researchers, with challenges in processing, efficient crosslinking, and biocompatibility. Additive manufacturing, also known as 3D printing, creates three-dimensional objects by adding material layer-by-layer [19]. This method offers several advantages, such as design freedom, customization, rapid prototyping, and reduced material waste. It has emerged as a promising technique for the application in pharmaceutical industry and medicine [20]. Using a direct ink writing system 3D printing technique, Zafeiris et al. prepared HA–chitosan composite scaffolds crosslinked with genipin, which showed very high biocompatibility [21]. Montalbano et al. used a bioprinter for the production of a bioactive collagen-based bone scaffold that showed a favorable release of strontium ions [22]. Izgordu et al. have used PVP for the printing of polycaprolactone-based articular cartilage, with optimized parameters for the most suitable mechanical and thermal properties, where PVP contributed to the thermal stability of the samples [23]. Among the numerous 3D printing techniques, semi-solid extrusion (SSE) has proven valuable for the production of various biomedical materials [24, 25]. This technique can be performed at room temperature, enabling the processing of thermosensitive materials. In situ crosslinking of gelatin via SSE has not been investigated before, although it could provide uniform distribution of the crosslinking agent. Furthermore, this technique could be used for the 3D printing of gelatin-PVP scaffolds crosslinked with genipin, which would enable the desired shape design for the intended application.

The objective of this study was to process and evaluate a suitable scaffold matrix system for tissue regeneration. The scaffold based on natural polymer gelatin, blended with polyvinylpyrrolidone and crosslinking agent genipin, was used as directly 3D-printed ink by SSE. Crosslinking also took place during 3D printing and after that. Investigation of 3D-printed gelatin-PVP blend crosslinked with genipin has not been reported before. The influence of PVP and genipin, a natural crosslinking agent, on the mechanical and thermal properties and the cytotoxicity of obtained scaffolds were investigated.

2 | Materials and Methods

2.1 | Materials

Type A gelatin from porcine skin (~300 g Bloom) (GA), polyvinylpyrrolidone (K30) (PVP), and genipin (GEN) were purchased from Sigma-Aldrich (Sigma-Aldrich Co. St. Louis, MO, USA). Glycerol (G) 85% was used for good printability and was purchased from Zorka Pharma (Zorka Pharma HEMIJA d.o.o., Sabac, Serbia).

2.2 | Processing of Scaffolds

Solution of gelatin 10%w/w in water with 5% glycerol (in relation to the mass of the polymer) was mixed for 2 h at 50°C. Solution of PVP 10% w/w in water was also mixed for 2 h at 50°C.

After merging these two solutions at a ratio of 1:1, they were stirred for another 3 h at 50°C for homogeneity. For the crosslinking, the appropriate amount of genipin was added to solution of polymers 30 min before printing. Three series of samples were processed: pure polymer blend GAG-PVP with 0.5% w/w GEN and with 1.0% w/w GEN, marked as S0, S0.5, and S1, respectively.

3D printing was performed by Ultimaker 2+ printer (Ultimaker B.V., Utrecht, Netherlands) adapted with Discov3ry paste extruder (Structur3d Printing, Kitchener-Waterloo, ON, Canada) at 32°C. Process of 3D printing is presented in Figure 1a and obtained in Figure 1b.

2.3 | Characterization of Scaffolds

2.3.1 | Swelling

The swelling potential of the scaffolds was measured after immersing the scaffolds into PBS for 24, 48, and 168 h at 37°C. Thereafter, the surface water was removed using filter paper, and samples were weighed. Measurements were performed on three samples. The swelling ratio was calculated using the following formula:

$$\text{swelling ratio} = \frac{m_w - m_d}{m_d}$$

where m_w represents the weight of wet samples, and m_d stands for the weight of dry samples.

2.3.2 | Fourier-Transform Infrared Spectroscopy (FT-IR)

Processing of obtained scaffolds was followed by FTIR analysis, ATR mode (Nicolet 6700 spectrometer, Thermo Fisher Scientific, Waltham, MA, USA) with the aim to detect the establishment of new chemical bonding. The spectrums were recorded within with a resolution of 4 cm⁻¹ in the wavenumber range from 4000 to 500 cm⁻¹.

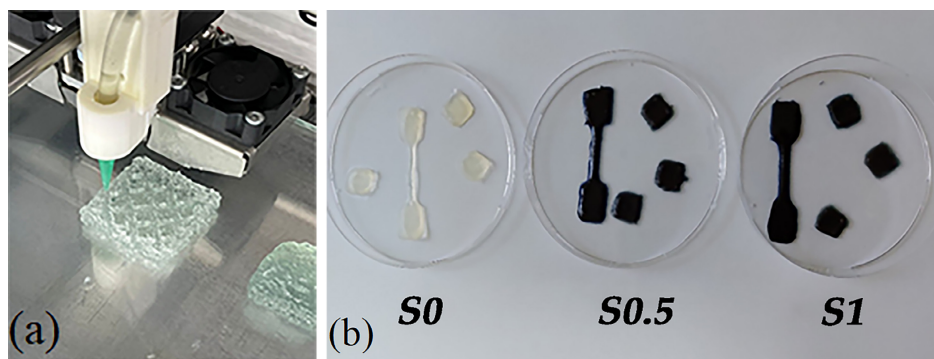


FIGURE 1 | (a) 3D printing of scaffolds and (b) scaffolds processed by 3D printing for mechanical testing.

2.3.3 | Differential Scanning Calorimetry (DSC)

Thermal properties of obtained scaffolds were determined by DSC (Q10, TA Instruments, Crawley, United Kingdom). Measurements were performed in the temperature range from 40°C to 200°C with a heating rate of 10°C/min. The samples of the initial weight of about 10 mg were placed in a hermetically sealed aluminum pan and measured three times. The glass transition temperature (T_g), denaturation temperature (T_d), and isomerization temperature (T_i) were obtained by the DSC.

2.3.4 | Field Emission Scanning Microscopy (FESEM)

The morphological changes of the scaffolds were examined using a FESEM TESCAN MIRA 3 (Tescan Orsay Holding, a.s., Brno-Kohoutovice, Czech Republic); scaffold fractured surfaces were sputter-coated with gold prior to imaging.

2.3.5 | Mechanical Testing

Tensile test and microindentation were performed on Texture Analyzer Shimadzu EZ Test LX (Shimadzu, Kyoto, Japan). The tensile test was performed with a 500 N load cell at room temperature, with a strain rate of 5 mm/min. Printed samples were in bone shape with a gauge length of approximately 22 mm. The microindentation was performed with a spherical indenter with a diameter of 4 mm. In each indentation, the load was set to continuously increase/decrease by 0.25 N/s, with an indentation time of 20 s and maximum loading of 5 N. The microindentation was performed in three positions on each sample.

The statistical significance of the tensile and microindentation test results was evaluated through one-way ANOVA, which was performed in Origin 9 software (OriginLab Corporation, Northampton, MA, USA). The analysis aimed to affirm the influence of genipin on mechanical properties.

2.3.6 | Cytotoxicity Test

Cytotoxicity analysis was performed according to ISO 10993-5. The principle of the cytotoxicity test is based on examining the effect of the test material in direct contact with the target L929 cells in culture, as well as examining the effect of the material

extract, that is, the material in liquid form, in indirect contact with the target cells. Based on the appearance and viability of the tested cells after culture in direct contact with the test material (direct test) or the metabolic activity of the cells in indirect contact (indirect test), the degree of cytotoxicity is evaluated and expressed in the form of indices and/or numerical values.

L929 cell line was originally purchased from European Collection of Animal Cell Cultures (Salisbury, UK). Cells are kept in the culture no longer than 3 weeks continuously and their morphology is monitored thoroughly. No changes in cell morphology were observed, and locally performed tests did not raise a doubt; therefore, line was not sent for authentication. L929 cells were cultivated in 6-well and flat bottom 96-well microtiter plates (Thermo Fisher Scientific, Massachusetts, USA), in complete cultivation media—RPMI 1640 supplemented with 10% FCS and penicillin/streptomycin (all from Capricorn Scientific GmbH, Germany), starting in density 3×10^5 and 1×10^4 cells/well respectively. When they became semi-confluent (70%–80% confluency), the treatment was added to cells instead of the cultivation medium.

For both the direct and indirect cytotoxicity test, the treatment was prepared as conditioned media—test sample extracts from solid material (S0 and S1) and negative control (aluminum oxide stick) were prepared by placing the material samples in sterile polypropylene test tubes in the complete RPMI medium (the ratio of the surface area of the material to the volume of the medium should be $3 \text{ cm}^2/\text{mL}$) for 24 h. As a positive control, the 5% phenol solution (Merck, New Jersey, USA) was used. In the direct test, conditioned medium, as well as positive control, was dripped onto the filter paper, dimensions $5 \times 5 \text{ mm}$, in the volume of $20 \mu\text{L}$. Samples for testing, including positive and negative controls, were placed in the center of wells in a 6-well plate on the adherent layer of L929 cells, all in triplicates. After 24 h of incubation, at 37°C, 5% CO_2 , each well was evaluated under the phase contrast inverted microscope (Carl Zeiss, Germany), and the qualitative assessment was performed according to all observed changes in the cell morphology (malformations, degenerations, detachment from the surface, presence of inclusions, fragmentations, or cell lyses) under and around the tested preparation. After that, the samples were carefully removed, and cells were washed with phosphate buffer saline (PBS) and treated with 0.1% Trypsine (Merck) diluted in PBS supplemented with 0.02% NaEDTA (Merck) in order to detach the adherent cells from the surface. All collected cells were washed in a complete cultivation medium and counted in Tripzan blue, at least 500 cells per sample. Quantitative assessment of the

cytotoxic effect was calculated by measuring the percentage of nonviable (lysed cells), that is, the percentage of cytotoxicity after staining the cells with a trypan blue solution, based on which the indexes of cytotoxicity were assigned. Range of reference values: 0—absence of cytotoxicity (undetectable or basal cytotoxicity which is detected in the negative control); 1—discrete cytotoxicity (less than 20% of lysed [nonviable] cells); 2—mild cytotoxicity (20%–40% of lysed [nonviable] cells); 3—moderate cytotoxicity (>40% to <60% of lysed [nonviable] cells); 4—serious cytotoxicity (>60% to <80% of lysed [nonviable] cells); 5—pronounced cytotoxicity (>80% of lysed [nonviable] cells).

Conditioned medium was used in an indirect assay at different dilutions (100%, 50%, 25%) in the complete medium. Each dilution of conditioned medium, as well as negative and positive control, were tested in six wells with L929 cells. After 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to cells in the concentration of 0.5 mg/mL. After 1 h of incubation at 37°C, the reaction was stopped with dimethyl sulfoxide (both from Thermo Fisher Scientific).

The absorbance of the solution is read at 570 nm wavelength, subtracting the reference wavelength at 690 nm (optical correction) ($OD_{570} = \text{abs}_{570} - \text{abs}_{690}$). Cell viability is calculated based on the following formula:

$$\text{Viability (\%)} = \frac{100 \times (\text{OD}_{570} \text{ sample} - \sum \text{OD}_{570} \text{ blank} / 6)}{\frac{\sum \text{OD}_{570} \text{ control}}{6} - \frac{\sum \text{OD}_{570} \text{ blank}}{6}}$$

To show a cytotoxic effect, a measure of 30% reduction in viability compared with the control for 100% extract is taken. Range of reference values: 0—the absence of cytotoxicity (0%–30% decrease in viability compared with control [100%]); 1—discrete cytotoxicity (30%–50% decrease in viability compared with control); 2—moderate cytotoxicity (50%–75% decrease in viability compared with control); 3—pronounced cytotoxicity (75%–100% decrease in viability compared with the control).

In order to stain the treated cells with propidium iodide and confirm results obtained by the MTT test, L929 cells were cultivated on coverslips in 24-well plates (Thermo Fisher Scientific), in a density of 5×10^4 cells/well. When they became semi-confluent (70%–80% confluency), the treatment was added to cells instead of the cultivation medium in the same manner as described above. After 24 h treatment, the cells on coverslips were washed in PBS, pH 6.5, and fixed with 4% paraformaldehyde (Merck), upon which they were washed with PBS three times. Propidium iodide (Merck) was added to each coverslip (1 mg/mL). Coverslips were mounted with fluorescent medium (G Fluoromount, Thermo Fisher Scientific) on microscope slides, and analysis was performed on the Axio Imager light microscope (Carl Zeiss).

3 | Results and Discussion

3.1 | Swelling Ratio

After 24 h, S0 completely dissolved in PBS. S0.5 showed a swelling ratio of 602.4%, while it was reduced in S1, with 444.5%,

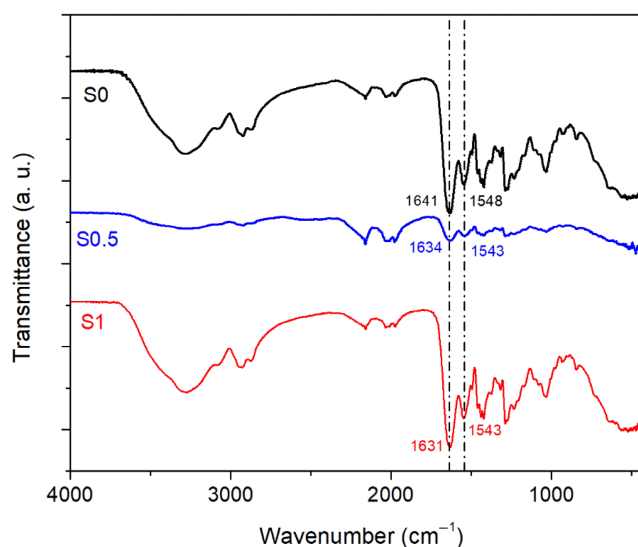


FIGURE 2 | FTIR spectrums of scaffolds.

which is a consequence of higher genipin concentration. After 48 h, S0.5 was partly dissolved, so the overall swelling was 249.4%, but this value could not be compared with S1. On the other hand, in S1, there was no degradation. Instead, deswelling to 380.7% occurred, which is consistent with higher elastic restorative forces expected in crosslinked gelatin [26]. After 168 h, both samples were partly degraded, losing their shape and transforming into small plate-like crystallites. This indicates that slow degradation occurs, with acceptable swelling at a low concentration of genipin.

The high swelling ratio could be explained by the high hydrophilicity of gelatin and PVP. Crosslinking density affects the swelling behavior of scaffolds. With an increased content of GEN, the crosslink density increased, and consecutively, the swelling of scaffolds decreased.

3.2 | FTIR Analysis

The FTIR spectrum (Figure 2) of S0 exhibits characteristic peaks at 1641 cm^{-1} (amide I), 1548 cm^{-1} (amide II), and 1227 and 2920 cm^{-1} (amide III) for gelatin and 1438 cm^{-1} , which corresponds to in-plane bending vibration from C–H, which could be attributed to hydrogen bonding between C=O in PVP and OH in gelatin [24, 27].

A possible way of crosslinking is a ring-opening reaction in genipin by an amine group of gelatins, followed by radical-induced polymerization of genipin. The FTIR spectrums on the S0.5 and S1 revealed the variation of the amide I bond (–CONH–) shifted to 1634 – 1631 cm^{-1} (amide I), respectively, and 1543 cm^{-1} (amide II). This indicates that the crosslinking is strongly related to genipin in accordance with the abovementioned reaction [28–30]. The band at 1543 cm^{-1} can be associated with the reaction between ester groups of GEN and primary amine groups on gelatin. The dark blue pigment was formed through the reaction of GEN with primary amine groups in the presence of oxygen [31].

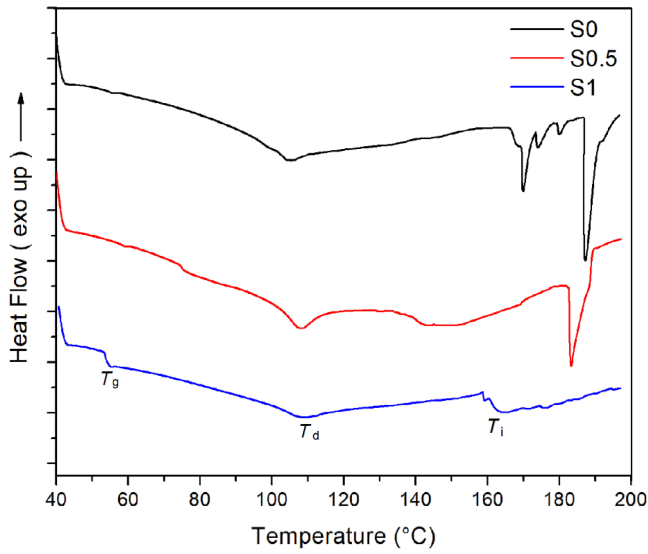


FIGURE 3 | DSC thermogram of scaffolds crosslinked with genipin in different contents.

3.3 | DSC Analysis

The DSC analysis results of noncrosslinked and crosslinked scaffolds with varying GEN contents are shown in Figure 3. The thermograms of S0, S0.5, and S1 displayed the first endothermic peaks at 55.07°C, 57.62°C, and 55.75°C, respectively, which correspond to their glass transition temperature (T_g). The addition of glycerol and PVP enhances the flexibility and printability of the scaffolds. Glycerol acts as a plasticizer, reducing denaturation temperature of gelatin, while PVP improves the melting temperature of the 3D-printed scaffolds [24]. The second endothermic melting peak associated with the denaturation temperature (T_d) of gelatin and corresponds to the helix coil transition of collagen parts [7]. In the scaffold's thermal behavior, three characteristic temperatures can be identified: glass transition temperature (T_g), denaturation temperature (T_d), and isomerization temperature (T_i) related to the stereoisomerism of the peptide bonds in gelatin [32]. The *trans-cis* isomerization region occurs at around 160°C–190°C. For samples S0.5 and S1, it is observed that blending PVP with gelatin and crosslinking with GEN shifts the temperature of *cis-trans* isomeric transformation toward higher values, and it almost disappears for sample S1.

The T_d values of the cross-linked scaffolds S 0.5 and S 1 are shifted toward higher temperatures compared with the value of noncrosslinked scaffold S0. Also, as content of GEN increased, the T_d was slightly raised [33–35]. For S0 obtained T_d was 104.46°C, while for crosslinked S0.5 and S1 were 107.72°C and 109.58°C, respectively. Increasing T_d is associated with breaking of hydrogen bonds and rearrangement of the oriented peptide chains [36, 37] and covalent crosslinking, which lead to better thermal stability and mechanical properties of crosslinked scaffolds [38]. In agreement with previous data [39], crosslinking leads to a decrease in the area of the endothermic peak, which is directly related to the denaturation enthalpy (Table 1), due to an increase in the degree of covalent crosslinking.

TABLE 1 | Glass transition temperature T_g , denaturation temperature, T_d and denaturation enthalpy, ΔH_d of scaffolds as a function of crosslinker contents.

Sample	DSC		
	T_g	T_d (°C)	ΔH_d (J/g)
S0	55.07	104.46	6.04
S0.5	57.62	107.72	5.50
S1	55.75	109.58	4.60

3.4 | FESEM Analysis

Figure 4 shows the FESEM images of cross-section morphologies of scaffolds S0 without genipin, and crosslinked scaffolds with different contents of genipin, S0.5 and S1.

It can be observed that the cross-section of untreated scaffold S0 showed smooth fracture surface with weak river-like pattern in the 3D-printing direction. Fracture behavior of polymer blends is influenced by interfacial entanglements and fracture energy as a measure of resistance to crack propagation [40]. Polymer blends amplified toughness, so the blending of gelatin with PVP results in smooth, tough fracture surface. In S0.5, genipin crosslinking contributed to the strengthening of the blend due to the creation of a 3D network, which was observed through rough surface where crack dissipation and strong plastic deformations were observed. FESEM of S1 showed a network structure with crack bridging toughening mechanism, along with the high surface roughness, as a consequence of synergic effect of blending and crosslinking [40, 41] due to reaction of residual amine groups in gelatin chains with genipin [34]. These differences in fracture surface morphology are indicative for their mechanical behavior.

3.5 | Mechanical Testing

Results of mechanical testing, tensile test, and microindentation are presented in Figure 5. It could be seen that tensile strength (TS), modulus of elasticity (E), hardness (H), and reduced modulus of elasticity (E_r) increased with crosslinking and with the increase in genipin content. Tensile strength rose by 68.3% and 93.8%, while modulus showed an increase of 53.4% and 57.3% in S0.5 and S1, respectively. Microhardness showed increase by 96% in S0.5 and 125.3% in S1, suggesting successful in situ crosslinking of gelatin in blend with PVP, which resulted in more rigid polymer network. Furthermore, the breaking strain of S0, S0.5, and S1 was $2.65\% \pm 0.99\%$, $3.67\% \pm 0.47\%$, and $4.11\% \pm 0.8\%$, respectively, which confirms that toughness and plastic deformation raised with crosslinking and with genipin content. The increase is relatively low because of the crosslinking-blending synergy, observed through fracture morphology differences examined by FESEM.

These results are also compatible with other results from the literature for scaffolds without crosslinking [16]. The mechanical properties of a scaffold should match the properties of the host

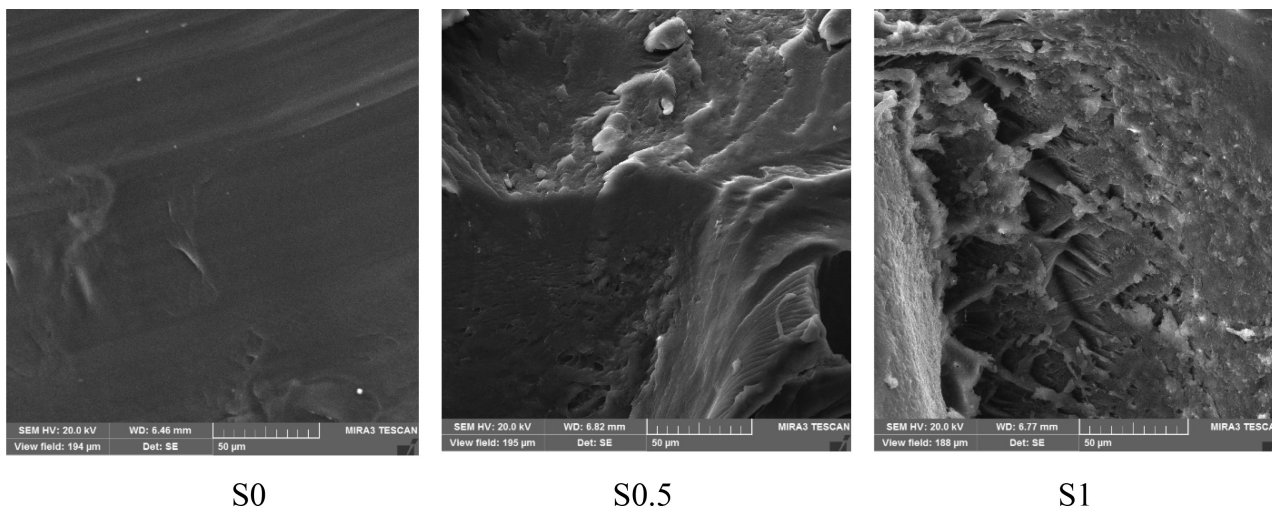


FIGURE 4 | FESEM images of scaffolds.

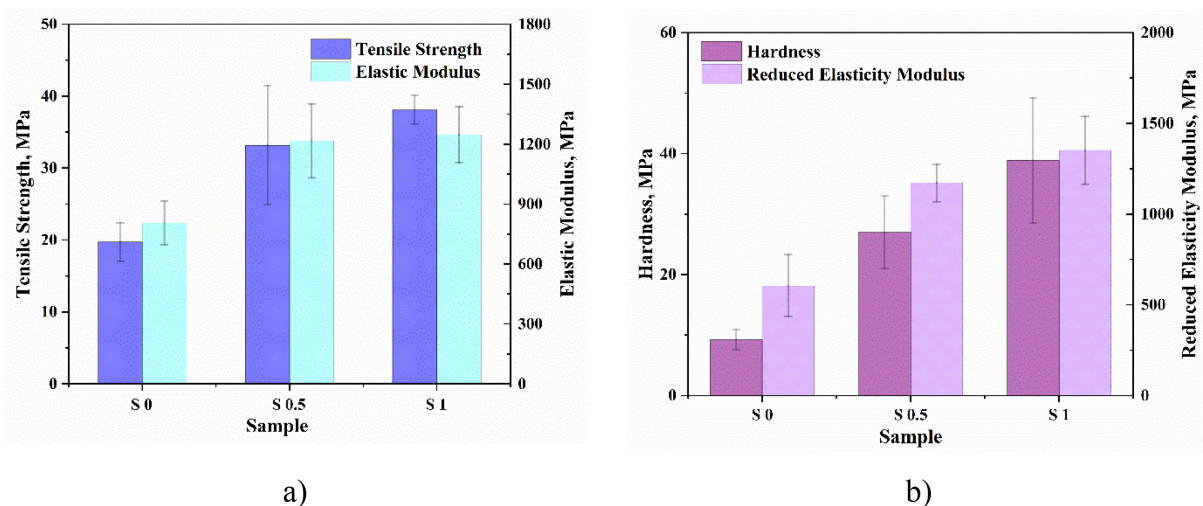


FIGURE 5 | Results of mechanical testing: (a) tensile test; (b) microindentation.

tissue, providing load transfer. The obtained crosslinked scaffolds have shown proper mechanical properties for bone tissue application at lower loading because the reference data show that the elastic modulus of cortical bone and cancellous bone is 15–20 and 0.1–2 GPa, respectively [42], while the hardness of cortical bone and cancellous bone is about 40 and 35 MPa, respectively [43, 44].

The statistical analysis of the influence of genipin content has shown that the factor significantly affects all the observed mechanical properties: hardness (at the confidence level of $p < 0.01$) and reduced modulus of elasticity, tensile strength, and elastic modulus (at the confidence level of $p < 0.001$).

According to the Tukey post hoc test, there is a statistical difference between the hardness of the samples that contain genipin (S0.5 and S1) compared with the samples without the crosslinking agent (S0) ($p < 0.05$ and $p < 0.01$, respectively). Likewise, this test confirms the statistical difference ($p < 0.01$) between the corresponding pairs of samples in terms of the remaining mechanical properties.

3.6 | Impact of S0 and S1 on Cellular Morphology and Confluence

The cytotoxic potential of S0 and S1 was assessed by exploring their impact on the viability of L929 cells upon 24 h treatment in the direct assay. L-929 cells are a fibroblast-like cell line derived from the subcutaneous connective tissue of a 100-day-old male C3H/An mouse. They are characterized by their spindle-shaped, fibroblastic morphology, and adherent growth. This cell line is frequently applied in various biological and medical research fields due to its robustness, ease of culture, and versatility in applications. Furthermore, L-929 cells serve as a standard model to assess the biocompatibility of materials and the toxic effects of various substances, which is particularly relevant in the fields of biomaterials and tissue engineering. S1 was chosen for the investigation due to a higher genipin concentration and better mechanical and swelling properties. Upon direct contact with the material containing S0 and S1 conditioned medium, the cell morphology was evaluated compared with the negative control. It was observed that cells treated either with S0 or S1 exhibited unaltered morphology and confluence; they were adherent and without signs of

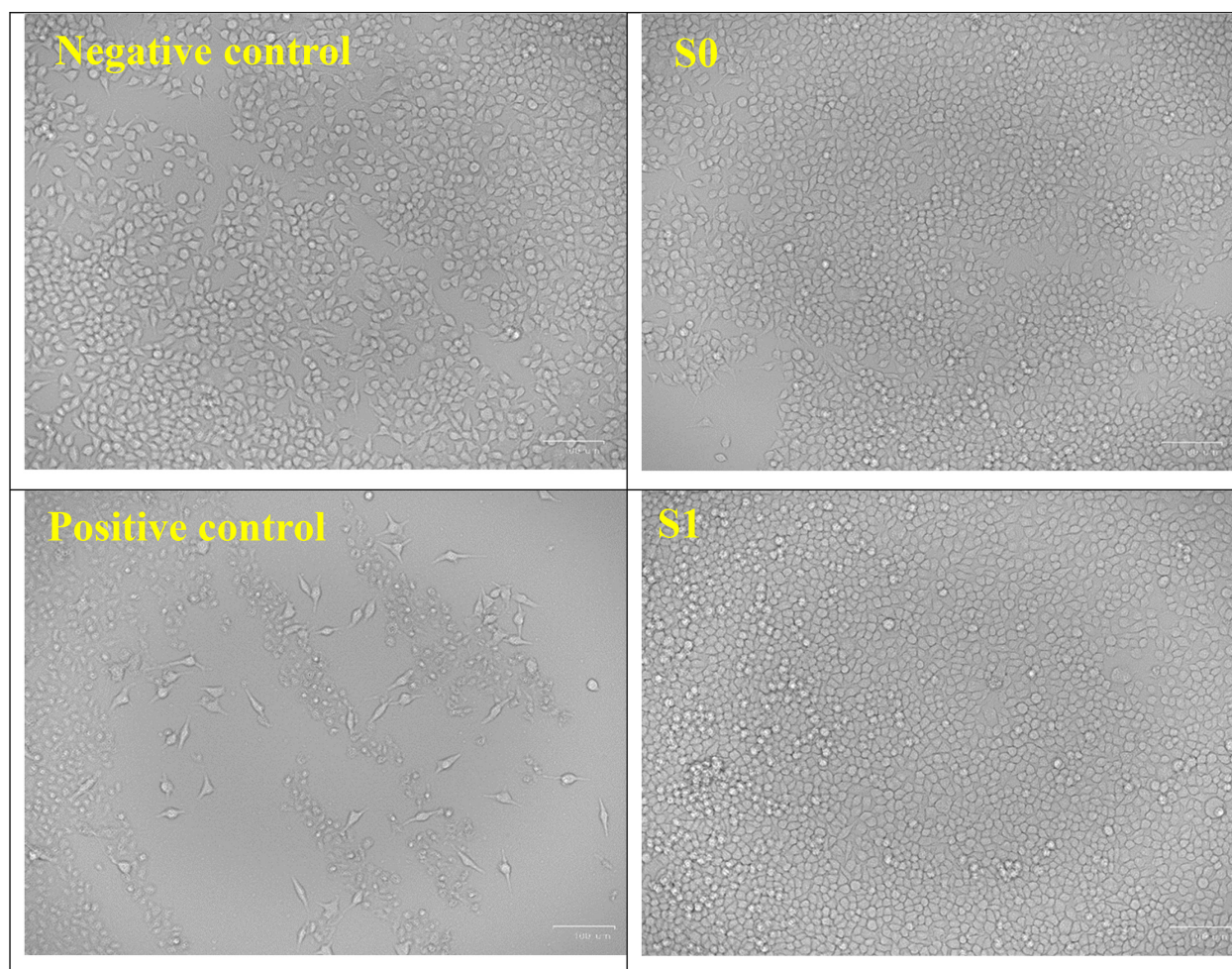


FIGURE 6 | The effect of S0 and S1 on the morphology and phenotype of L929 cells. Morphological and intracellular changes of cells treated with the S0 and S1 for 24 h were determined without staining on live cells in the culture (Zoe Fluorescent Cell Imaging System [Bio-Rad, CA, United States], 200× magnification).

lyses or degeneration, highly resembling negative control, unlike positive control, which caused the complete destruction of the cell layer (Figure 6). Quantitative assessment of cytotoxicity in the direct assay was performed by calculating the percentage of nonviable cells treated with S0 and S1, and both materials showed the absence of cytotoxic effect, that is, the basal level of lysed cells of <2%, which could be observed in negative control, while positive control induced the lyses on <80% of treated cells (Table 2).

3.7 | Impact of S0 and S1 on Cellular Viability

Cytotoxicity of S0 and S1 preparations was also evaluated in an indirect MTT test (treatment with different concentrations of conditioned medium) based on the metabolic activity of L929 cells. Cells treated with S0 showed unaltered viability compared with the control ($95.3\% \pm 1.5\%$ vs. 100%), while cells treated with S1 exhibited decreased viability compared with the control ($79.3\% \pm 3.2\%$ vs. 100%) in all three tested concentrations of conditioned medium (Figure 7a). However, this decrease in viability, although statistically significant, has no biological significance since the relative viability decrease by up to 30% compared with the control is considered as the absence of cytotoxicity according to the ISO 10993-5 (a sample is considered cytotoxic if the percentage viability value

TABLE 2 | The cytotoxic effect of S0 and S1 on L929 cells upon direct contact—quantitative assessment of the cytotoxic effect calculated by measuring the percentage of nonviable, based on which the indexes of cytotoxicity were assigned.

	Negative control	Positive control	S0	S1
Concentration of medium	Index of cytotoxicity in direct assay			
100%	0	5	0	0
50%	0	5	0	0
25%	0	5	0	0

Note: Range of reference values: 0—absence of cytotoxicity (undetectable or basal cytotoxicity which is detected in the negative control); 1—discrete cytotoxicity (less than 20% of lysed [nonviable] cells); 2—mild cytotoxicity (20%–40% of lysed [nonviable] cells); 3—moderate cytotoxicity (>40% to <60% of lysed [nonviable] cells); 4—serious cytotoxicity (>60% to <80% of lysed [nonviable] cells); 5—pronounced cytotoxicity (>80% of lysed [nonviable] cells).

obtained in MTT test (indirect viability test) is <70% and noncytotoxic if the percentage viability value is >70%) [45–47]. Hence, the index of cytotoxicity of both S0 and S1 preparations is 0, as the index assigned to the negative control (Figure 7b).

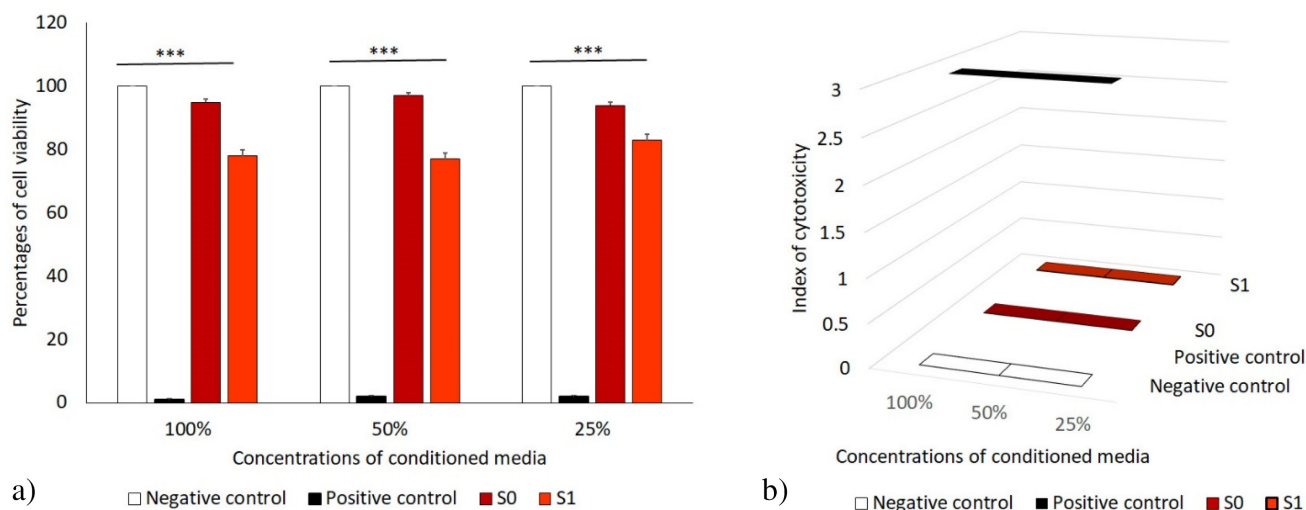


FIGURE 7 | The cytotoxic effect of media conditioned with S0 and S1 on L929 cells based on the metabolic activity of cells in an indirect assay (MTT). (a) Cell viability is represented as a percentage of the absorbance value of the negative control culture, which was arbitrarily assigned a viability value of 100%. Results are presented as mean $\pm$ SD of three independent experiments, and statistical significance was determined using the Student *t*-test. *** $p < 0.001$ compared with the control. (b) Index of cytotoxicity was defined according to the reference range: 0—the absence of cytotoxicity (0%–30% decrease in viability compared with control [100%]); 1—discrete cytotoxicity (30%–50% decrease in viability compared with control); 2—moderate cytotoxicity (50%–75% decrease in viability compared with control); 3—pronounced cytotoxicity (75%–100% decrease in viability compared with the control).

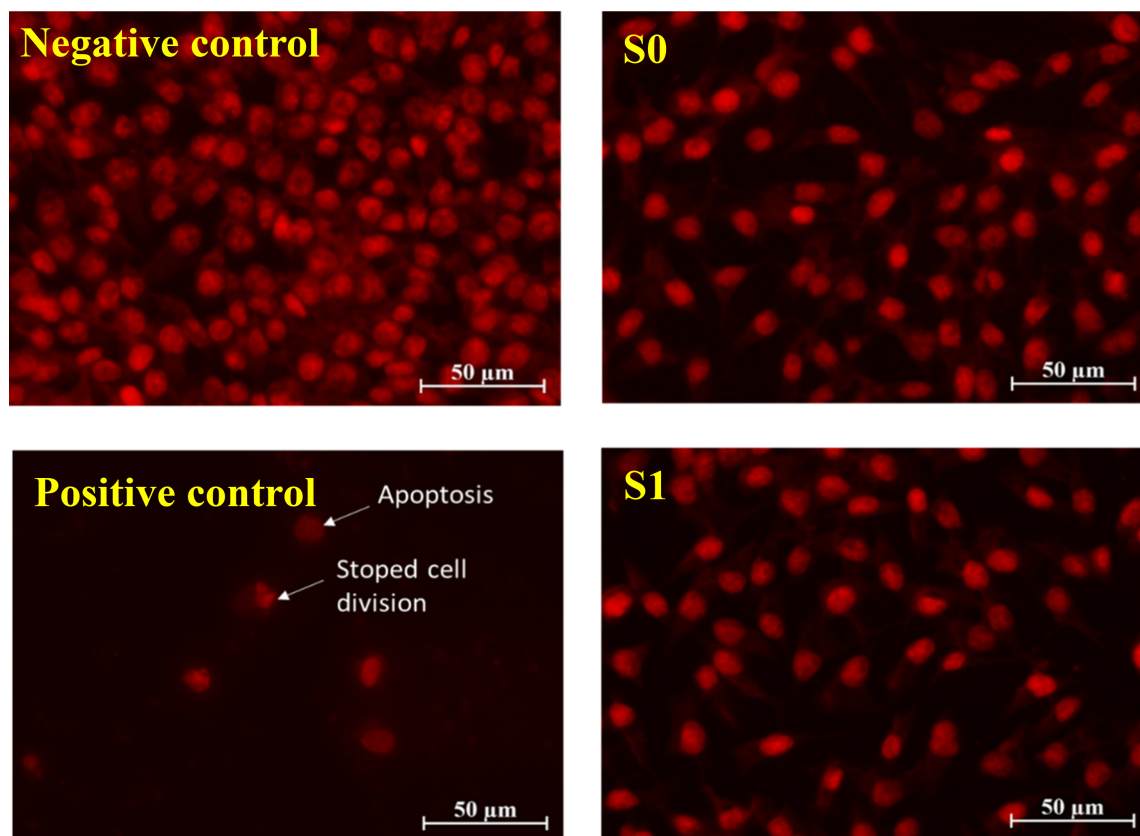


FIGURE 8 | The effect of S0 and S1 on the morphology and phenotype of L929 cells. Morphological and intracellular changes of cells treated with GAG-PVP w/o GEN for 24h were determined by propidium iodide staining and subsequent fluorescent microscopy (Axio Imager, Carl Zeiss microscope, 400 $\times$ magnification) (apoptotic nuclei and stopped cell division are indicated by white arrows on positive controls). Representative micrographs are shown.

The obtained results indicate that, although both examined materials are not cytotoxic, they could have a different in vitro behavior on cell proliferation in sense of promotion or inhibition. In this study in vivo effect were not considered because the primary focus is on printability and mechanical performance of the cross-linked gelatin-PVP system.

To verify the potential cytotoxicity of S0 and S1 observed in the indirect test, cells treated for 24 h with these preparations, along with negative and positive control, were stained with propidium iodide, and their morphology, including the nuclear morphology, was analyzed under the fluorescent microscope. S0 and S1-treated cells showed normal phenotypes without the change in nuclei dimensions, chromatin condensation, or apoptosis. At the same time, the positive control destructed the cell layer, caused massive apoptosis, and stopped cell division (Figure 8). The obtained results confirmed the absence of S0 and S1 cytotoxicity, which was demonstrated via the metabolic activity of treated cells in the MTT test.

The MTT assay results were consistent with those observed in the direct assay, indicating the preserved viability of target cells. This test measures cell viability through the increased metabolism of a tetrazolium salt [48], and in some cases, certain compounds with reducing activity could convert the MTT into the colored formazan and give a false positive result in this test. For that reason, it is desirable to perform the staining of cells in parallel with the MTT assay in order to confirm the obtained results [49]. Herein, GAG-PVP, as well as GAG-PVP containing 1% w/w GEN, did not show any cytotoxic effect and the viability, as well as morphology of target cells, corresponded to the level observed in the control.

Single type of cell line (L929) with following parameters was used to prove biocompatibility of 3D-printed samples that showed significant mechanical improvements. Therefore, this study shows the suitability of easy and rapid cell-based methods to assess the biocompatibility and the in vitro effects of S0 and S1 and also predicts a possible in vivo effect in terms of safety and efficacy.

4 | Conclusions

In this work, 3D printing by SSE of gelatin-polyvinylpyrrolidone scaffolds with almost in situ crosslinking by genipin was performed to obtain systems with improved mechanical properties and low cytotoxicity. Concentration of genipin was 0.5 and 1 wt%. The FTIR analysis confirmed the crosslinking process, while the DSC analysis revealed the achievement of better thermal stability of crosslinked scaffolds. Swelling ratio was reduced in the sample with the higher genipin concentration, as a consequence of better crosslinking. Furthermore, samples with 1 wt% of genipin showed slower degradation. Mechanical investigation showed that genipin contributed to an increase in tensile strength and microhardness, which rose up to 93.8% and 125.3%, respectively. The obtained scaffolds have improved mechanical strength; they are biodegradable and biocompatible, that is, without cytotoxicity. Due to these properties, it has been shown that by applying SSE 3D-printing technique, gelatin-genipin-polyvinylpyrrolidone universal scaffolds could

be obtained, with a potential for a wide application, depending on the incorporation of additional components, making them also good candidates for further investigations in regards to their biological influence on the surrounding tissue.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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4.4. Synergistic Effects of Genipin and Alendronate in 3D-Bioprinted Gelatin-Polyvinylpyrrolidone Scaffolds



Synergistic Effects of Genipin and Alendronate in 3D-Bioprinted Gelatin-Polyvinylpyrrolidone Scaffolds

Marija Jovanović¹ · Ivana Stajčić² · Miloš Petrović¹ · Dušica Stojanović¹ · Nataša Radulović³ · Svetlana Ibrić⁴ · Petar Uskoković¹

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Abstract

In this study, we introduce a one-step semi-solid extrusion 3D printing strategy to fabricate gelatin-polyvinylpyrrolidone (GAG-PVP) scaffolds loaded with a low dose (0.5 wt%) of alendronate (ALN) and crosslinked in situ with 1 wt% genipin. The genipin-crosslinked ALN scaffold (GAG-PVP-GEN-ALN) demonstrated enhanced functional performance compared to both non-crosslinked GAG-PVP and GAG-PVP-ALN controls. Its peak swelling reached 462% at 5 h, surpassing the 362% of the unmodified scaffold and preventing the rapid dissolution observed for GAG-PVP-ALN, before gradually deswelling for 96 h. Water contact angle measurements confirmed that genipin fully restored surface hydrophobicity (101.9°), counteracting the pronounced wettability induced by ALN (47.8°) and exceeding the 78.2° of the GAG-PVP matrix, which is consistent with swelling ratio. Differential scanning calorimetry (DSC) indicated enhanced thermal stability of the crosslinked gelatin, with shifts in both glass transition and denaturation temperatures reflecting greater molecular rigidity despite the presence of glycerol as a plasticizer. Mechanical testing showed that while alendronate alone reduced mechanical performance, the combined inclusion of alendronate and genipin significantly enhanced scaffold properties compared to gelatin-polyvinyl pyrrolidone blend: tensile strength increased from 19.7 MPa to 39.8 MPa, elastic modulus rose from 805 MPa to 1174 MPa, and microhardness improved from 9.24 MPa to 22.3 MPa, values nearing those of native cancellous bone. The sustained ALN release profile extended from an abrupt 3 h burst in GAG-PVP-ALN to a controlled 48 h delivery in GAG-PVP-GEN-ALN, following first-order kinetics. Both direct and indirect cytotoxicity assays confirmed high cell viability (>85%) without morphological abnormalities. These results highlight that embedding low-dose ALN within a genipin-crosslinked gelatin-PVP network results in a mechanically robust, biocompatible scaffold with tunable swelling and prolonged drug release, offering a versatile platform for localized bone tissue engineering.

Keywords Alendronate · Semi-solid extrusion · Hybrid scaffold · Biocompatibility

Introduction

Bone defects arising from trauma and degenerative conditions such as osteoporosis or cancer are increasingly common and frequently exceed the natural capacity of bones for spontaneous healing in larger area defects [1–3]. Traditional treatments like autografts allografts and bone cements remain the dominant methods for hard tissue reconstruction [4, 5]. However, there are certain drawbacks, such as surgical complications, immune rejection risks and potential disease transmission [6]. Consequently, tissue engineering has emerged as a promising strategy to address these challenges, focusing on the design of scaffolds that can mimic native tissue or serve as drug delivery systems [7–11].

✉ Ivana Stajčić
ivana_r@vinca.rs

¹ University of Belgrade, Faculty of Technology and Metallurgy, Karnegijeva 4, Belgrade 11120, Serbia

² Department of Physical Chemistry, “Vinča” Institute of Nuclear Sciences, National Institute of the Republic of Serbia, University of Belgrade, Mike Petrovića Alasa 12–14, P.O. Box 522, Belgrade 11001, Serbia

³ Institute for Biological Research “Siniša Stanković”, National Institute of Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, Belgrade 11108, Serbia

⁴ Faculty of Pharmacy, University of Belgrade, Vojvode Stepe 450, Belgrade 11221, Serbia

Three-dimensional (3D) printing has found wide application in bone tissue engineering, enabling the fabrication of scaffolds with high biocompatibility, controlled drug release, architecture and mechanical performance [12–14]. Among various strategies, extrusion-based bioprinting is particularly attractive due to its compatibility with highly viscous bioinks and its ability to produce structurally robust scaffolds suitable for osteogenic applications [15, 16]. However, the development of bioinks that simultaneously ensure high printability, biocompatibility and mechanical integrity remains a central challenge [17]. Gelatin, a partially hydrolyzed form of collagen, has been widely employed as a base material in bioinks due to its biocompatibility, biodegradability and the presence of glycine-proline-4-hydroxyproline acids, which provide structural integrity [18–20]. However, gelatin alone suffers from inadequate mechanical strength and rapid degradation in physiological environments [21]. To address these limitations, gelatin is often combined with synthetic polymers such as polycaprolactone (PCL), or reinforced with bioactive fillers such as hydroxyapatite (HA) [22–24]. However, polycaprolactone is a hydrophobic, semi-crystalline polyester with relatively poor water solubility and low swelling behavior [25]. Achieving sustained release of a hydrophilic drug from a hydrophobic polymer matrix can be challenging, as it may lead to phase separation and expulsion of the drug from the carrier, resulting in an abrupt or uncontrolled release of the active compound [26]. In contrast, polyvinylpyrrolidone (PVP) is a hydrophilic polymer known for its excellent biocompatibility, processability, and ability to form clear blends with gelatin [27–29]. Highly hygroscopic nature of PVP supports uniform dispersion of small-molecule therapeutics and increases solubility of poorly soluble drugs in water, making it particularly suitable for applications requiring in situ drug embedding [30]. Therefore, PVP offers a promising alternative to PCL in scaffold systems aimed at combining drug delivery with enhanced printability and biointegration. In order to improve mechanical stability and achieve controlled drug release by reducing solubility of gelatin, crosslinking is often employed [31, 32]. Recently, Skopinska-Wisniewska et al. summarized various crosslinking agents and printing conditions, revealing that dual-crosslinked or solvent-assisted systems can yield mechanically robust and cytocompatible scaffolds [33]. Along with synthetic crosslinkers, such as glutaraldehyde, genipin has attracted attention as a biocompatible natural crosslinker that improves gelatin's mechanical strength and resistance to degradation [15]. Crosslinking of gelatin with genipin could result in a scaffold with controlled drug release and solubility that could be tailored with the addition of hydrophilic polymer into the system.

Alendronate (ALN) is a medication commonly employed in the treatment of osteoporosis; however, oral

administration is often associated with various side effects, highlighting the need for localized delivery systems [34, 35]. Incorporating ALN into scaffolds offers a targeted alternative that can minimize systemic complications. Chitosan/polycaprolactone (CS/PCL) scaffolds loaded with alendronate demonstrated improved osteogenic differentiation, with release rate dependent on the PCL presence [36]. Alendronate-loaded biphasic calcium phosphate (BCP) scaffolds have been shown to enhance osteogenic differentiation in vitro and improve bone regeneration in vivo [37]. Alendronate can be combined with other osteoinductive agents, such as bone morphogenetic proteins (BMPs), in dual drug delivery systems to synergistically promote bone regeneration [38]. However, most of the reported delivery systems employ relatively high concentration of ALN, or rely on inorganic carriers, such as hydroxyapatite or tricalcium phosphate phases, where even at low ALN content, the drug is still sequestered in a ceramic matrix rather than directly blended into the polymer network [39]. Drug release in such systems is predominantly governed by inorganic phase degradation, limiting tunability. No prior studies have directly blended ALN into a gelatin/PVP matrix while also employing genipin crosslinking to enhance mechanical properties and contribute to a controlled release of the drug. Hydrophilic PVP contributes to drug availability, while crosslinking ensures slower degradation of gelatin.

In this study, hybrid scaffolds composed of gelatin and PVP using semi-solid extrusion (SSE) 3D printing, with direct incorporation of a low ALN dose into the polymer matrix, with subsequent in situ crosslinking with genipin. Processing of scaffolds resulted in enhanced mechanical reinforcement and the absence of cytotoxicity, with controlled ALN delivery, making it a promising candidate for future further in vitro, as well as in vivo research in bone tissue engineering applications.

Materials and Methods

Materials

Type A gelatin from porcine skin (~300 g Bloom) (GA), polyvinylpyrrolidone (K30) (PVP), sodium alendronate trihydrate (ALN), genipin (GEN), phosphate buffered saline (PBS) and perchloric acid were purchased from Sigma-Aldrich (Sigma-Aldrich Co., St. Louis, MO, USA). Iron (III) chloride hexahydrate (99+%) was purchased from Thermo Scientific. Glycerol (G) 85%, was used for good printability and was purchased from Zorka Pharma (Zorka Pharma HEMIJA d.o.o., Sabac, Serbia).

Preparation of Hybrid Scaffolds

Solution of gelatin A (GA) 10%w/w in water with 5% glycerol (G) (in relation to the mass of the polymer) was mixed for 2 h at 50 °C. Solution of poly(vinyl pyrrolidone) (PVP) 10% w/w in water, was also mixed for 2 h at 50 °C. After merging these two solutions at a ratio of 1:1, they were stirred for another 3 h at 50 °C for homogeneity. For the crosslinking, the 1 wt% of genipin (GEN) and 0.5 wt% of alendronate (ALN) were added to solution of polymers 30 min before printing. Three series of samples were processed: pure polymer blend GAG-PVP with 1% w/w GEN, with 0.5 wt% of alendronate and with both. Samples were labelled, GAG-PVP, GAG-PVP-ALN and GAG-PVP-GEN-ALN, in accordance with their composition. 3D printing was performed by Ultimaker 2+ printer (Ultimaker B.V., Utrecht, Netherlands) adapted with Discov3ry paste extruder (Structur3d Printing, Kitchener-Waterloo, ON, Canada) at 32 °C. The pastes were extruded using a nozzle with an internal diameter of 0.84 mm at a printing speed of 10 mm/min. For mechanical testing purposes, the scaffolds were printed as dog-bone-shaped films, measuring 60 mm × 15 mm × 3.5 mm, with a narrowed section, as per tensile testing standards, measuring 25 mm in length. The printing was performed in four layers, with 100% infill density and a 45°–135° grid infill pattern. The scaffolds for cell viability tests were printed as rectangular prisms measuring 15 mm x 15 mm x 3.5 mm, with four layers, 50% infill density, and a 0°–90° grid infill pattern. Following extrusion, the printed samples were left to dry at ambient room temperature for 24 h. Figure 1 presents the obtained samples for mechanical testing and printing for cell viability tests.

The blue coloration is a consequence of gelatin crosslinking with genipin.

Swelling and Degradation of 3D Printed Hybrid Scaffolds

The swelling behavior of the scaffolds was evaluated by immersing them in phosphate-buffered saline (PBS) at 37 °C for 96 h within different intervals. After each time point, excess surface water was gently blotted with filter paper, and the samples were weighed. Each measurement was conducted in triplicate. The swelling ratio was determined using the following equation:

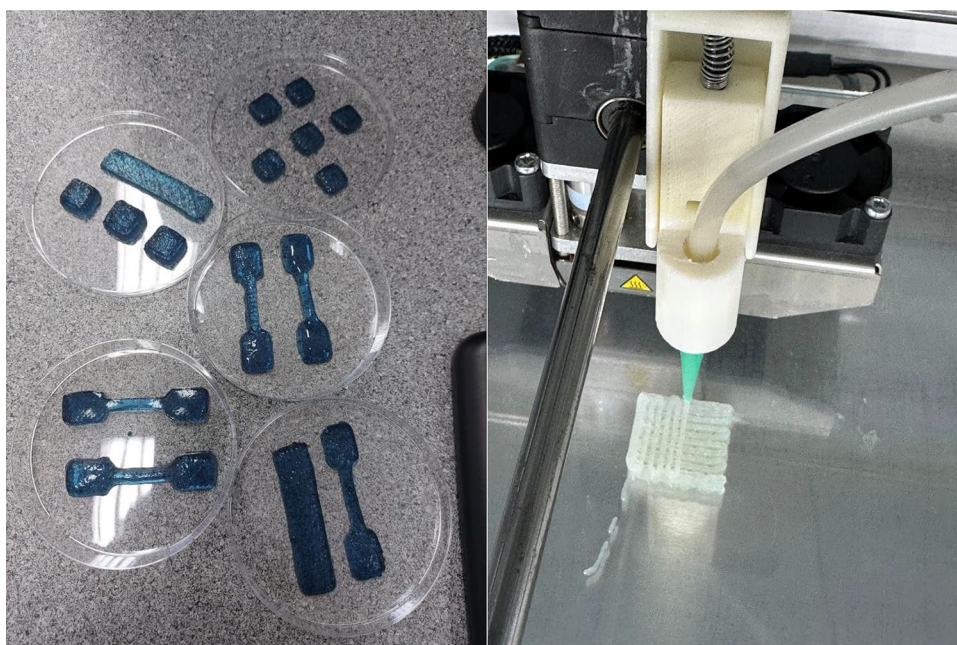
$$\text{swelling ratio} = \frac{m_w - m_d}{m_d}$$

where m_w is the mass of the swollen (wet) scaffold, and m_d is the mass of the initial dry scaffold.

Water Contact Angle (WCA) Analysis

The water droplet needs 3 s to reach steady state and the static contact angles at this time were measured using an optical USB microscope (5.0 MP PRO Delta Optical Smart, Warsaw, Poland). To conduct this measurement, 5 µL of deionized water was placed on different positions of each scaffold, with each position tested in triplicate. The images of the droplets were analyzed using Fiji ImageJ software (version 1.54p, NIH, Bethesda, MD, USA), specifically utilizing the DropSnake plugin to determine the contact angle.

Fig. 1 3D printing of scaffolds



Morphology and Composition of 3D Printed Hybrid Scaffolds

Surface morphology and composition by energy dispersive X-ray spectroscopy (EDS) of the scaffolds were investigated using a TESCAN MIRA 3 field emission scanning electron microscope (Tescan Orsay Holding, a.s., Brno-Kohoutovice, Czech Republic). Prior to imaging, the fractured surfaces of the samples were sputter-coated with a thin gold layer to ensure conductivity.

Structure of 3D Printed Hybrid Scaffolds

The chemical structure of the fabricated scaffolds was examined using Fourier-transform infrared spectroscopy in attenuated total reflectance (ATR) mode, employing a Nicolet 6700 spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Spectra were recorded across the wavenumber range of 4000–500 cm^{-1} with a resolution of 4 cm^{-1} , aiming to identify the formation of new chemical bonds indicative of material interactions or modifications.

Thermal Analysis

Thermal behavior of the scaffolds was assessed by differential scanning calorimetry (DSC) using a Q10 instrument (TA Instruments, Crawley, UK). Approximately 10 mg of each sample was hermetically sealed in aluminum pans and analyzed over a temperature range of 40 °C to 200 °C at a heating rate of 10 °C/min. Each measurement was repeated three times. Key thermal transition points, glass transition (T_g), denaturation (T_d), and isomerization (T_i) temperatures, were determined from the DSC thermograms.

Mechanical Properties

Mechanical performance was characterized via tensile testing and microindentation using a Shimadzu EZ Test LX Texture Analyzer (Shimadzu, Kyoto, Japan) equipped with a 500 N load cell. Tensile tests were conducted at ambient temperature with a strain rate of 5 mm/min on bone-shaped specimens with an approximate gauge length of 22 mm. Microindentation was performed using a 4 mm diameter spherical indenter. The load was ramped up and down at a rate of 0.25 N/s over a 20-second test cycle, reaching a peak load of 5 N. Indentations were made at three different locations on each sample.

Statistical Analysis

Statistical analysis of the mechanical testing results was carried out using one-way ANOVA in Origin 9 software

(OriginLab Corporation, Northampton, MA, USA) to evaluate the effect of genipin on scaffold properties.

In Vitro Drug Release

The ALN release studies were carried out on orbital shaker (Orbital shaker PSU-10i, Grant-bio, Grant Instruments Ltd., Cambridge, UK) at 50 rpm in a thermostatic incubator (MBIN50 Microbiological Incubator, Colo Lab Experts, Novo Mesto, Slovenia) at 37 °C [40]. The medium for dissolution was 100 mL of phosphate buffered saline (PBS). At preplanned time points, fractions were withdrawn and replaced with same amount of fresh prewarmed PBS. The samples were filtered through a 0.45 μm membrane filter (Millipore, Bedford, MA, USA) and tested with UV-Vis spectrophotometer (LLG-uniSPEC 2 Spectrophotometer, Lab Logistics Group GmbH, Meckenheim, Germany) at 306 nm, based on the chromophoric complex formation with Fe (III) ion in perchloric acid solution [41]. Each sample was performed in triplicate. The DDSolver software (Microsoft Excel add-in program) was applied to analyze the ALN release kinetics [42]. Several mathematical models, including zero-order, first-order and Higuchi, were used to fit the cumulative drug release data [43, 44]. For determination of the best fitting model, the highest correlation coefficient (R^2) and the model selection criterion (MSC) values were utilized for evaluation.

Cell Viability Tests

Cytotoxicity was evaluated in accordance with ISO 10993-5 guidelines using both direct and indirect methods. The murine fibroblast cell line L929, obtained from the European Collection of Animal Cell Cultures (Salisbury, UK), was cultured for no longer than three weeks, with routine monitoring of cell morphology. Cells were seeded in 6-well and 96-well plates (Thermo Fisher Scientific, MA, USA) at initial densities of 3×10^5 and 1×10^4 cells/well, respectively, in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (Capricorn Scientific GmbH, Germany). Conditioned media (test extracts) were prepared by incubating scaffold samples (GAG-PVP-ALN and GAG-PVP-GEN-ALN) and a negative control (aluminum oxide stick) in complete RPMI at a material surface area-to-volume ratio of 3 cm^2/mL for 24 h. A 5% phenol solution (Merck, NJ, USA) served as the positive control. For the direct test, 20 μL of extract or control was pipetted onto 5×5 mm filter papers, which were then placed on semi-confluent L929 cell layers in 6-well plates. After 24 h of incubation at 37 °C with 5% CO_2 , morphological changes such as cell detachment, deformation, or lysis were examined under a phase-contrast inverted microscope (Carl

Zeiss, Germany). Cells were then detached using 0.1% trypsin in PBS with 0.02% NaEDTA (Merck), washed, stained with trypan blue, and counted (minimum of 500 cells/sample). Index of cytotoxicity for indirect test was defined based on the percentage of non-viable cells:

- 0 absence of cytotoxicity (0–30% decrease in viability compared to control (100%));
- 1 discrete cytotoxicity (30–50% decrease in viability compared to control);
- 2 moderate cytotoxicity (50–75% decrease in viability compared to control);
- 3 pronounced cytotoxicity (75–100% decrease in viability compared to the control).

In the indirect assay, cells in 96-well plates were treated with conditioned media at 100%, 50%, and 25% dilutions. After 24 h, 0.5 mg/mL of MTT reagent was added, followed by 1-hour incubation at 37 °C. The reaction was halted using dimethyl sulfoxide (DMSO), and absorbance was read at 570 nm with background correction at 690 nm. Viability was calculated as:

$$\text{Viability (\%)} = \frac{100 \times (OD_{570\text{sample}} - \sum OD_{570\text{blank}}/6)}{\sum OD_{570\text{control}} - \sum OD_{570\text{blank}}}$$

To confirm MTT results, L929 cells seeded on coverslips in 24-well plates were treated as above, fixed with 4% paraformaldehyde (Merck), and stained with Mayer's Hematoxylin Solution (Merck). Coverslips were mounted with H&E mounting medium (Dako) and observed using an Axio Imager light microscope (Carl Zeiss) to visualize nuclear staining indicative of cytotoxicity.

Indexes of cytotoxicity in direct contact were assigned based on percentage of non-viable cells. Range of reference values:

- 0 absence of cytotoxicity (undetectable or basal cytotoxicity which is detected in the negative control);
- 1 discrete cytotoxicity (less than 20% of lysed (non-viable) cells);
- 2 mild cytotoxicity (20–40% of lysed (non-viable) cells);
- 3 moderate cytotoxicity (>40% - <60% of lysed (non-viable) cells);
- 4 serious cytotoxicity (>60% - <80% of lysed (non-viable) cells);
- 5 pronounced cytotoxicity (>80% of lysed (non-viable) cells).

Results and Discussion

Swelling and Degradation of 3D Printed Hybrid Scaffolds

Swelling ratio and degradation in PBS are presented in Fig. 2. GAG-PVP-ALN scaffolds were fully dissolved in PBS after 3 h, reaching their peak swelling ratio at approximately 75 min. In comparison, GAG-PVP scaffolds remained stable for up to 4 h, with a maximum swelling ratio of 362% recorded after 3 h. The difference between the two scaffolds could be the consequence of hydrophilic ALN presence. The GAG-PVP-GEN-ALN formulation exhibited the highest swelling capacity, reaching a maximum of 462% after 5 h, followed by a gradual deswelling phase. By 48 h, the swelling ratio had decreased to 388%, then to 368% at 72 h and further to 198% at 96 h, which aligns with the increased elastic recovery typically associated with genipin-crosslinked gelatin networks [45].

These observations suggest a slow degradation profile and sustained swelling behaviour, where crosslinking compensated hydrophilic nature of ALN and PVP.

Water Contact Angle (WCA)

The water contact angles (WCAs) for 3D-printed scaffolds are presented in Fig. 3. The average WCAs for GAG-PVP (Fig. 3a), GAG-PVP-ALN (Fig. 3b) and GAG-PVP-GEN-ALN (Fig. 3c) were determined to be $98.11 \pm 4.82^\circ$, $47.76 \pm 7.50^\circ$ and $101.90 \pm 6.62^\circ$, respectively. It is important to note that the WCA of these scaffolds can vary based on the concentration of glycerol plasticizers and the polyvinylpyrrolidone polymer used. PVP is a hydrophilic polymer with a contact angle of approximately 70° [46].

Gelatin, a protein-based material, typically has a water contact angle greater than 90° due to the presence of hydrophobic amino acids [47]. These amino acids, such as leucine, valine, phenylalanine, isoleucine, and methionine, can interact with glycerol plasticizers, prompting them to realign towards the air interface and contribute to a more hydrophobic surface. The incorporation of sodium alendronate trihydrate (ALN) significantly decreases hydrophobicity, as indicated by a lower contact angle. This reduction in contact angle suggests improved surface wettability, which is likely due to the presence of polar hydroxyl (-OH) groups. However, when genipin, a crosslinker, is added to the GAG-PVP-ALN combination, it restores hydrophobicity to levels similar to those of neat GAG-PVP, which agrees with the swelling test results.

Fig. 2 Swelling ratio of 3D printed scaffolds

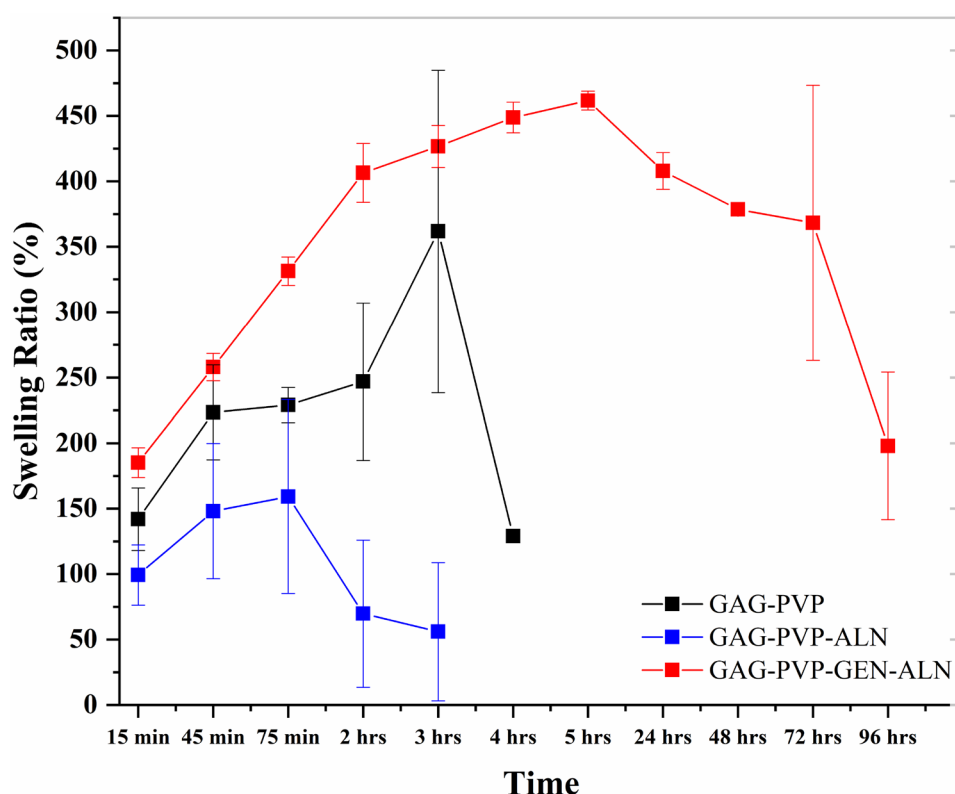


Fig. 3 Water contact angle for: (a) GAGP-PVP, (b) GAG-PVP-ALN and (c) GAG-PVP-GEN-ALN

FESEM Analysis

FESEM micrographs of fracture sites after the tensile test revealed clear distinctions in the cross-section morphology of the different scaffold formulations. The unmodified GAG-PVP (Fig. 4a) scaffold exhibited relatively smooth fracture surfaces with minor river-like patterns aligned along the 3D printing direction, indicative of limited interfacial bonding and poor energy dissipation during crack propagation [48]. GAG-PVP-ALN (Fig. 4b) scaffold displayed a slightly rougher surface topology and fracture path, but with obvious separation between printed layers.

GAG-PVP-GEN-ALN sample (Fig. 4c) showed rough surface, which could indicate higher mechanical performance [49]. Plastic deformation zones, microcrack deflection and regions suggestive of crack arrest mechanisms were observed [50]. These features are characteristic of

improved resistance to crack propagation, often attributed to covalent bonding and increased chain entanglement within crosslinked polymers. In addition, separation between 3D printed layers was not observed, which is a consequence of covalent bonds formation during crosslinking. EDS of GAG-PVP-GEN-ALN fracture surface confirmed presence of Na and P elements from ALN (Fig. 4d).

FTIR Analysis

Fourier-transform infrared spectroscopy (FTIR) provided essential evidence of molecular interactions and structural transformations within the developed 3D-printed hybrid scaffolds. Figure 5a presents the whole region, while Fig. 5b shows emphasized region $1400\text{--}1000\text{ cm}^{-1}$. A broad absorption band centered around 3300 cm^{-1} , corresponding to O–H and N–H stretching vibrations, was evident in

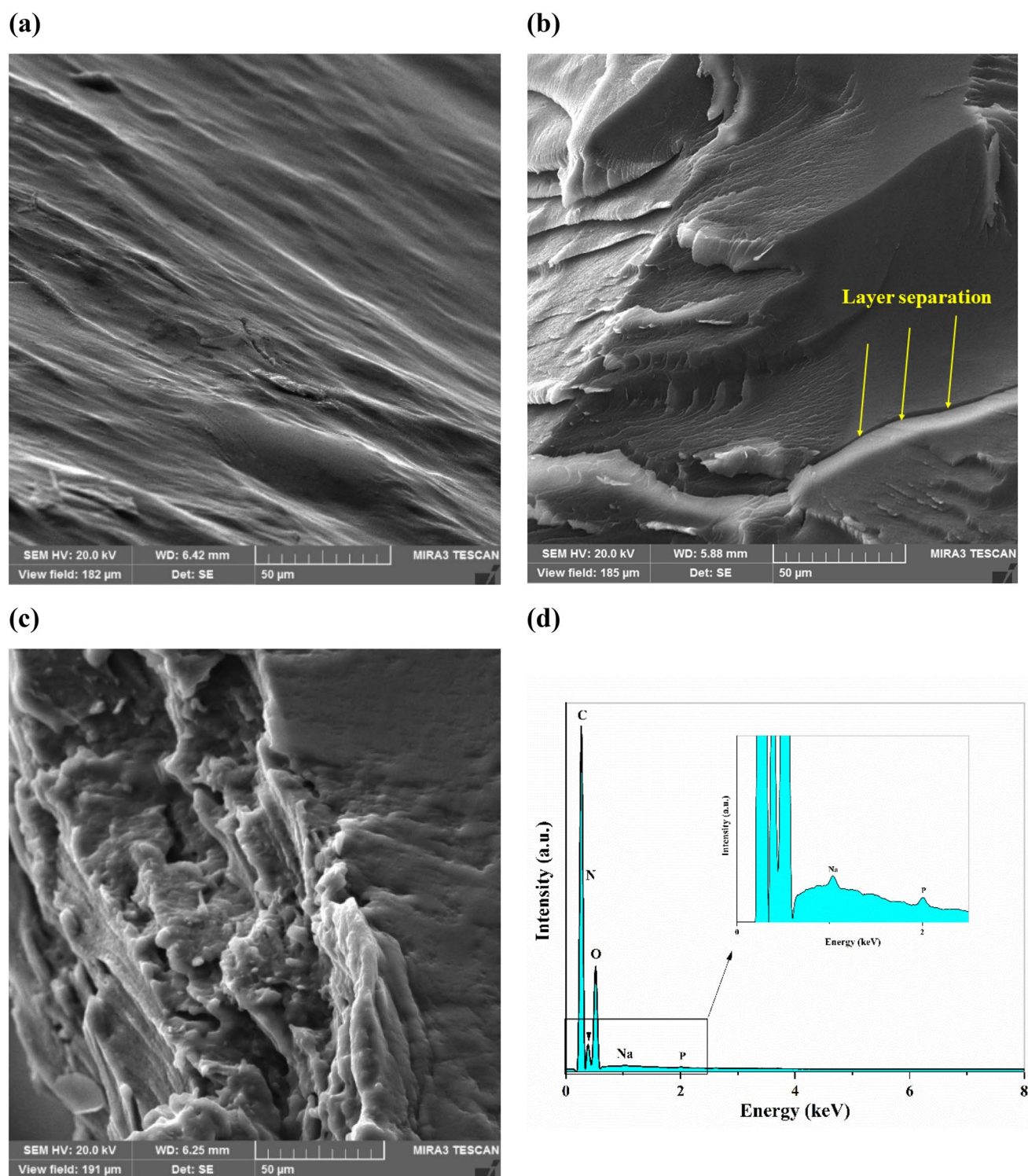


Fig. 4 FESEM of: (a) GAG-PVP, (b) GAG-PVP-ALN, (c) GAG-PVP-GEN-ALN cross-section and (d) EDS of GAG-PVP-GEN-ALN

all three scaffolds. However, in GAG-PVP-GEN-ALN, this band displayed reduced width and intensity, which is consistent with the known mechanism of genipin crosslinking that involves the consumption of free amine groups. The resulting restriction in polymer chain mobility leads to decreased

spectral broadness, a behavior also reported by Ng et al. in their work on genipin-modified biopolymers [51].

The characteristic amide bands, such as amide I ($1640\text{--}1650\text{ cm}^{-1}$) ($\text{C}=\text{O}$) and amide II ($1540\text{--}1550\text{ cm}^{-1}$) (N-H), were prominent in all formulations, underscoring the

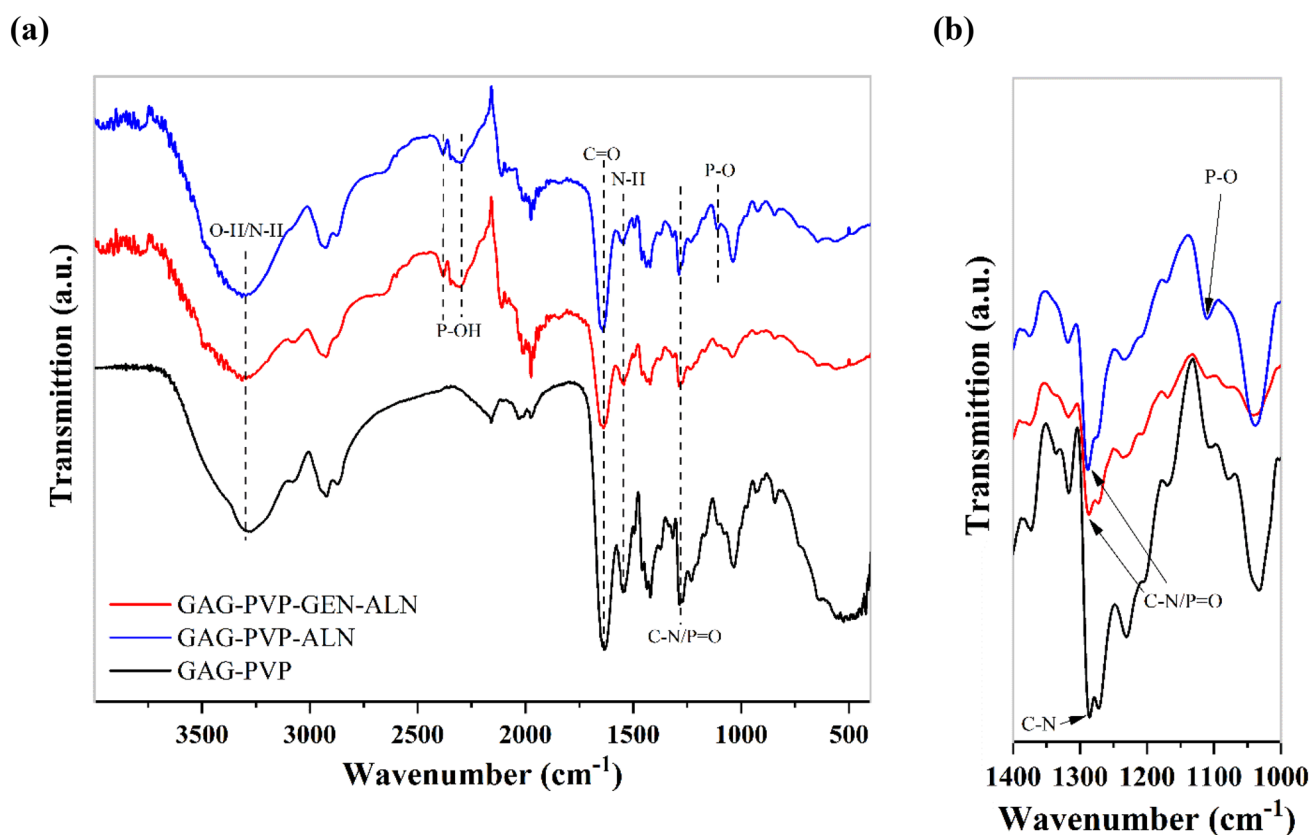


Fig. 5 FTIR spectra of GAG-PVP, GAG-PVP-ALN and GAG-PVP-GEN-ALN: (a) whole region 4000–400 cm^{-1} ; (b) region 1400–1100 cm^{-1}

presence of gelatin's peptide backbone. GAG-PVP-GEN-ALN exhibited a shift in the $-\text{CONH}-$ functional group, moving to 1641 cm^{-1} from 1631 cm^{-1} in GAG-PVP-ALN, suggesting that genipin was involved in the crosslinking process, consistent with the previously described reaction mechanism [52]. Additionally, the formation of a dark blue pigment confirms the reaction of genipin with primary amines in the presence of oxygen, which further corroborates crosslinking assumption [53]. Polyvinylpyrrolidone (PVP) $\text{C}=\text{O}$ stretching vibrations overlapped with the amide I band [54]. Furthermore, the CH_2 bending vibrations around 1420 cm^{-1} and $\text{C}-\text{N}$ stretching modes near 1280 cm^{-1} were also evident, corresponding to pyrrolidone ring deformation [55]. Another PVP-specific feature, the CH_2 rocking band at 1018 cm^{-1} , appeared slightly upshifted to 1040 cm^{-1} , which has been associated with hydrogen bonding or polar interactions in PVP-containing systems [56]. The incorporation of alendronate was indicated across multiple regions of the FTIR spectrum. Bands associated with $\text{P}-\text{OH}$ stretching vibrations were detected at 2382 and 2297 cm^{-1} in the GAG-PVP-ALN and at 2382 and 2306 cm^{-1} in GAG-PVP-GEN-ALN [57]. Further confirmation was found in the region around 1280 cm^{-1} . In the unmodified GAG-PVP scaffold, this band appeared as a symmetric doublet centered at 1287 cm^{-1} (Fig. 4b). Upon addition

of alendronate, the band became broader, more asymmetric and shifted to approximately 1282 cm^{-1} in both GAG-PVP-ALN and GAG-PVP-GEN-ALN, suggesting overlap between $\text{C}-\text{N}$ and $\text{P}=\text{O}$ [58]. Furthermore, a distinct band at 1113 cm^{-1} was detected in the GAG-PVP-ALN, corresponding to symmetric $\text{P}-\text{O}$ stretching [59]. Although a similar band was also present in the GAG-PVP-GEN-ALN scaffold, its intensity and shape were slightly altered, which may reflect partial entrapment of ALN within the crosslinked network. Finally, the broad region between 1050 and 950 cm^{-1} , typically attributed to $\text{P}-\text{O}$ stretching, was present in all spectra but appeared diminished and altered in the GAG-PVP-GEN-ALN formulation [60]. The reduced intensity and subtle shape variations likely result from restricted vibrational freedom of phosphate groups due to physical confinement within the crosslinked matrix. This interpretation aligns with findings by Panzavolta et al., who reported comparable spectral attenuation in genipin-crosslinked gelatin systems [61]. However, since peaks in this region overlap with characteristic PVP bonds, this identification is ambiguous. Presence of phosphorus-related groups is in accordance with EDS, where P was identified. Furthermore, presence of additional hydroxyl groups from ALN is in accordance with the increased hydrophilic nature of

the GAG-PVP-ALN, observed through lower water contact angle and higher degradation rate.

DSC Analysis

The DSC analysis reveals how thermal transitions of the 3D-printed scaffolds are influenced by chemical modifications (Fig. 6). The glass transition temperatures (T_g) of GAG-PVP, GAG-PVP-ALN and GAG-PVP-GEN-ALN remain in a narrow 53–55 °C range, indicating that adding alendronate or genipin has a negligible effect on the low-temperature chain mobility. This is likely due to the high content of glycerol plasticizer in the matrix, which significantly lowers T_g of the polymer blend [62, 63]. Glycerol can penetrate polymer chains, increasing free volume and flexibility, determining the T_g regardless of minor compositional changes [64]. The second endothermic event, mostly for dry gelatin conditions, associated with gelatin denaturation (T_d), revealed a distinct trend: T_d for GAG-PVP was 104.46 °C, while for GAG-PVP-ALN and GAG-PVP-GEN-ALN it decreased to 89.42 °C and 94.19 °C, respectively [65–67]. The observed reduction in T_d is attributed to the interference of alendronate with triple-helix stabilization in gelatin. However, partial recovery of T_d in GAG-PVP-GEN-ALN indicates that genipin-induced crosslinking reintroduces thermal stability, possibly by reinforcing the peptide backbone through covalent bonding. This interpretation is supported by the concurrent reduction in denaturation enthalpy (ΔH_d), which dropped from 6.04 J/g (GAG-PVP) to 5.11 J/g (GAG-PVP-ALN) and 3.79 J/g (GAG-PVP-GEN-ALN), consistent with higher crosslinking density and lower heat

requirement for structural disruption. No endothermic peak was observed near 125–129 °C, typically associated with the melting of crystalline sodium alendronate trihydrate, suggesting that ALN is present in an amorphous or molecularly dispersed state within the scaffold [67, 68]. This is consistent with the absence of crystalline water loss and indicates that alendronate is homogeneously embedded in the polymer matrix, which may also contribute to reduced ΔH_d . A third transition, denoted as T_i , was detected between 140 and 190 °C and corresponds to the isomerization of peptide bonds within the gelatin structure, specifically the transformation from *trans*- to *cis*- configuration in polyproline-II helices [69].

This transition is pronounced in GAG-PVP and GAG-PVP-ALN, while it was significantly reduced in GAG-PVP-GEN-ALN. The disappearance of a well-defined isomerization peak in the GAG-PVP-GEN-ALN thermogram suggests a suppression of peptide bond *trans*-*cis* transition, reinforcing the hypothesis of genipin-mediated structural rigidification.

Mechanical Testing

Mechanical characterization showed a substantial enhancement in mechanical performance upon crosslinking with genipin and the inclusion of alendronate (Fig. 7). Incorporating 0.5 wt% alendronate into the GAG-PVP scaffold (GAG-PVP-ALN) resulted in a noticeable reduction in mechanical performance relative to the unmodified GAG-PVP system. Tensile strength declined from 19.7 ± 2.6 MPa (GAG-PVP) to 15.2 ± 4.2 MPa (GAG-PVP-ALN), and the elastic modulus dropped from 805 ± 108 MPa to 627 ± 333 MPa (Fig. 7a). This reduction may be attributed to interference of ALN with gelatin native hydrogen bonding and polymer chain entanglement, resulting in a more brittle and less cohesive matrix. However, when 1 wt % genipin was introduced (GAG-PVP-GEN-ALN), mechanical properties not only recovered but significantly exceeded those of both preceding formulations. Tensile strength rose to 39.8 ± 7.7 MPa, which corresponds to a 102% increase over GAG-PVP and a 162% increase over GAG-PVP-ALN. The elastic modulus rose to 1174 ± 82 MPa, representing a 46% increase over GAG-PVP and an 87% increase over GAG-PVP-ALN. The most remarkable improvement was observed in microhardness (Fig. 7b): GAG-PVP-GEN-ALN reached 22.3 ± 6.9 MPa, marking a 141% increase over GAG-PVP (9.24 ± 1.7 MPa) and a 576% increase compared to GAG-PVP-ALN (3.28 ± 0.9 MPa). The improved breaking strain observed previously (2.65%, 3.67% and 4.11% for GAG-PVP, GAG-PVP-ALN, and GAG-PVP-GEN-ALN, respectively) confirms enhanced toughness and plastic deformation, in line with the FESEM findings. Statistical

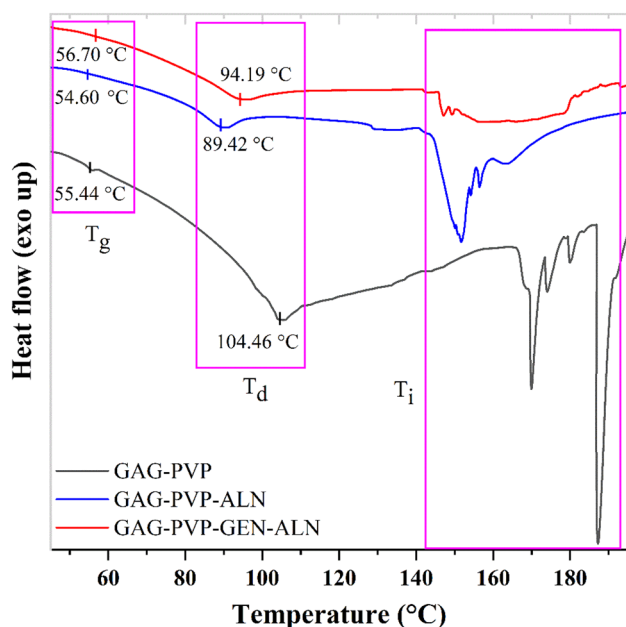
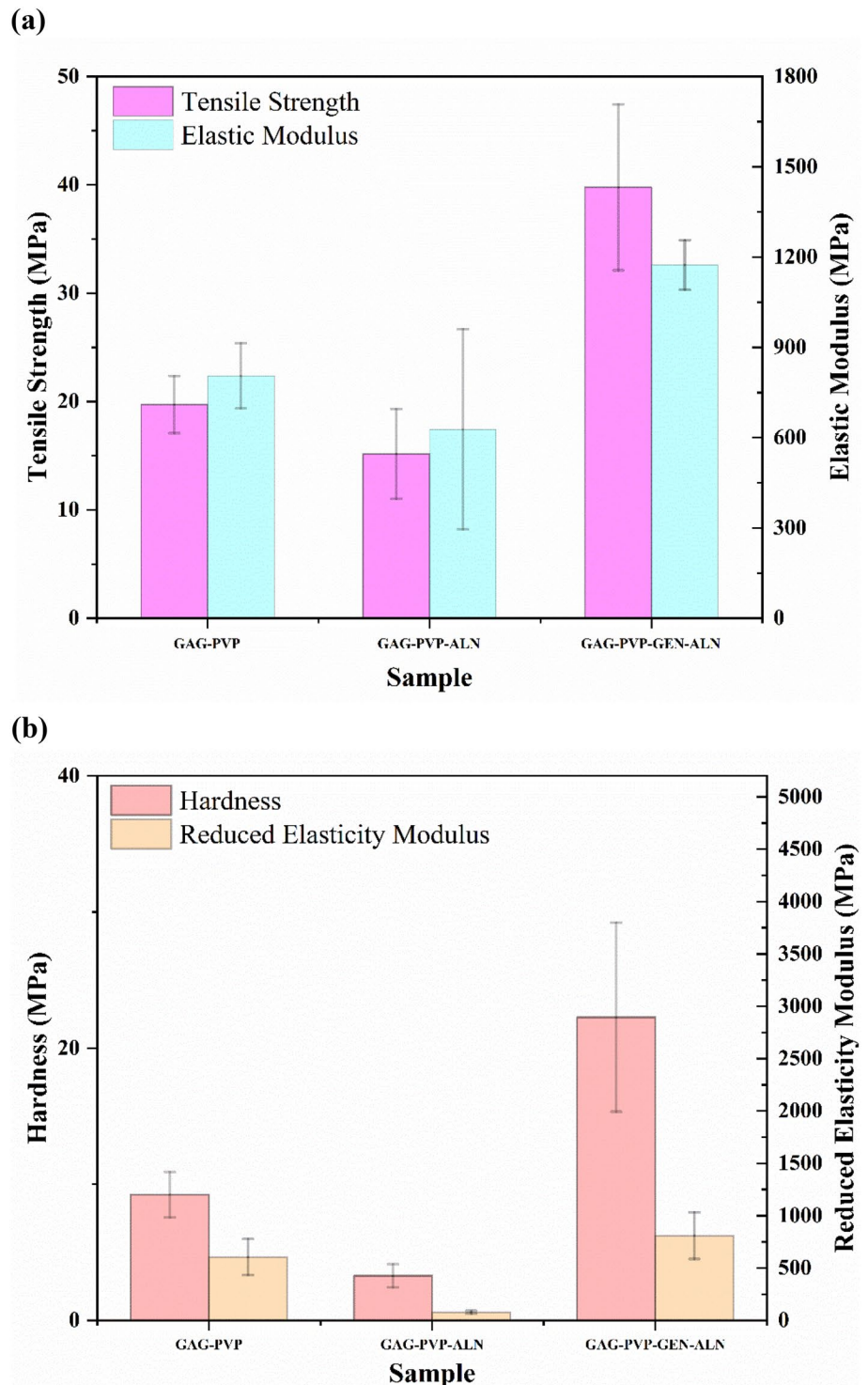


Fig. 6 DSC analysis of hybrid scaffolds

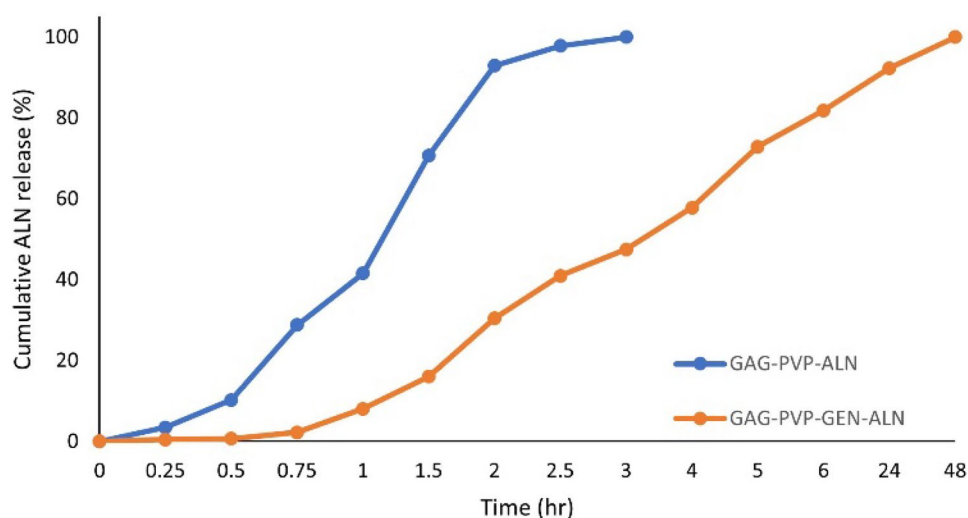
Fig. 7 Mechanical tests results for GAG-PVP, GAG-PVP-ALN and GAG-PVP-GEN-ALN : (a) tensile test; (b) microindentation



analysis confirmed the significance of genipin content in affecting all tested mechanical parameters. The one-way ANOVA showed that the mean values of tensile strength, hardness and reduced elasticity modulus in all three groups of samples were statistically different ($p < 0.001$), as well as the mean values of elastic modulus ($p < 0.01$). According to the Tukey post-hoc test, there is a statistical difference

between the hardness, tensile strength and elasticity modulus of the GAG-PVP-GEN-ALN scaffolds compared to the other two groups ($p < 0.01$), as well as between the reduced elasticity modulus of GAG-PVP-GEN-ALN and GAG-PVP-ALN ($p < 0.001$).

These results demonstrate that genipin does not merely offset the performance loss induced by ALN, it enhances

Fig. 8 In vitro drug release profiles for hybrid scaffolds**Table 1** Drug release kinetics

Model	Zero Order		First Order		Higuchi Model	
Criteria	R^2	MSC	R^2	MSC	R^2	MSC
Scaffold						
GAG-PVP-ALN	0.9428	2.4445	0.8938	1.8255	0.8334	1.3750
GAG-PVP-GEN-ALN	0.1273	0.1003	0.9580	2.9325	0.7395	1.1089

R^2 —coefficient of determination; MSC—model selection criterion

scaffold strength and rigidity well beyond the original matrix, yielding a biomaterial with mechanical characteristics approaching those of cancellous bone. This mechanical synergy highlights the novelty and practical value of the dual-component strategy for structural reinforcement in bone tissue scaffolding.

Furthermore, phosphonate groups of ALN appear to engage in secondary crosslinking-like interactions, disrupting gelatin triple helices ($T_d\downarrow$) yet creating additional H-bond clusters that genipin subsequently locks in, allowing it to act as an additional reinforcement.

In Vitro Drug Release

The cumulative ALN release profiles from scaffolds are presented in Fig. 8. Scaffold GAG-PVP-ALN exhibits faster drug release in comparison to scaffold GAG-PVP-GEN-ALN. GAG-PVP-ALN shows complete release of ALN within 3 h, with slightly slower initial release and significant increase from 30 min to 2 h. GAG-PVP-GEN-ALN shows gradual and prolonged release rate of the drug. The scaffold crosslinked with genipin has low initial release, only 10% of the ALN released in the first hour, with the continuous increase to reach total ALN release over 48 h.

The results of kinetic analysis using drug release profiles from 3D printed scaffolds are shown in Table 1. The best mathematical model of drug release has the highest values of R^2 and MSC, where a value higher than 2 indicates a

good fit [3]. The GAG-PVP-ALN scaffold has the best fit for the zero-order kinetic, meaning that the drug release is independent of ALN concentration [6]. The best fit for the GAG-PVP-GEN-ALN scaffold is first-order kinetics with concentration-dependent release [70].

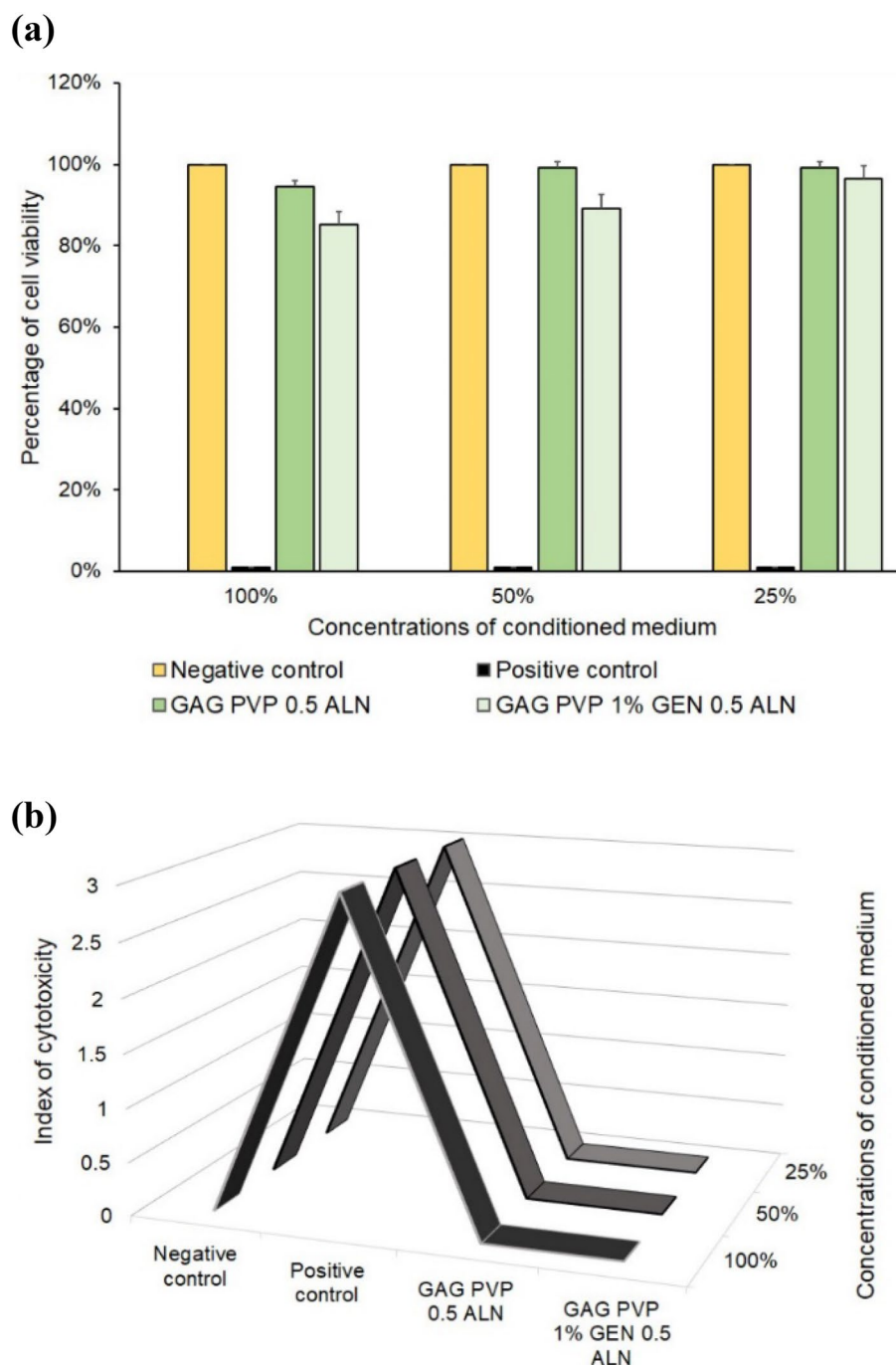
These results confirm that genipin crosslinking transforms an initially burst-dominated profile into a controlled, concentration-modulated release, a feature that could enhance both efficacy and safety in localized bone-regeneration therapies.

Cell Viability Tests

Indirect Assay (MTT)

Cytotoxicity of GAG-PVP-ALN and GAG-PVP-GEN-ALN scaffolds was evaluated in indirect MTT test based on the metabolic activity of L929 cells. In this assay, L929 fibroblast cells exposed to conditioned media from GAG-PVP-ALN and GAG-PVP-GEN-ALN scaffolds exhibited high viability across all tested dilutions (100%, 50% and 25%), with viability percentages ranging from 85.2% to 99.1% (Fig. 9a) without statistically significant difference compared to the negative control. These values correspond to an index of cytotoxicity of 0 (Fig. 9b), indicating no cytotoxic effect according to ISO 10993-5 standards. Such results indicate the biocompatibility of the scaffolds at the utilized ALN concentration.

Fig. 9 Cytotoxic effect of media conditioned with GAG-PVP-ALN and GAG-PVP-GEN-ALN on L929 cells based on metabolic activity of cells in an indirect assay (MTT): **(a)** Cell viability is represented as a percentage of the absorbance value of the negative control culture, which was arbitrarily assigned a viability value of 100%; **(b)** Index of cytotoxicity defined according to reference range presented in Materials and Methods section



Percentages of cell viability in 9a were calculated using the average values of obtained optical densities at 570 nm of different dilutions of conditioned media and negative control, each in hexaplicates. Results are presented as mean $\pm$ SD of three independent experiments and statistical significance was determined using the Student t-test. Results were considered statistically significant ($p < 0.05$) compared to the control.

Direct Assay

Morphological and intracellular changes of cells treated with the GAG-PVP-ALN and GAG-PVP-GEN-ALN for 24 h was determined without staining on live cells in the culture (Fig. 10). This analysis was undertaken in order to further corroborate findings from indirect assay by morphological analysis of cells in direct contact. The obtained results revealed that cells in direct contact with GAG-PVP-ALN and GAG-PVP-GEN-ALN changed neither the

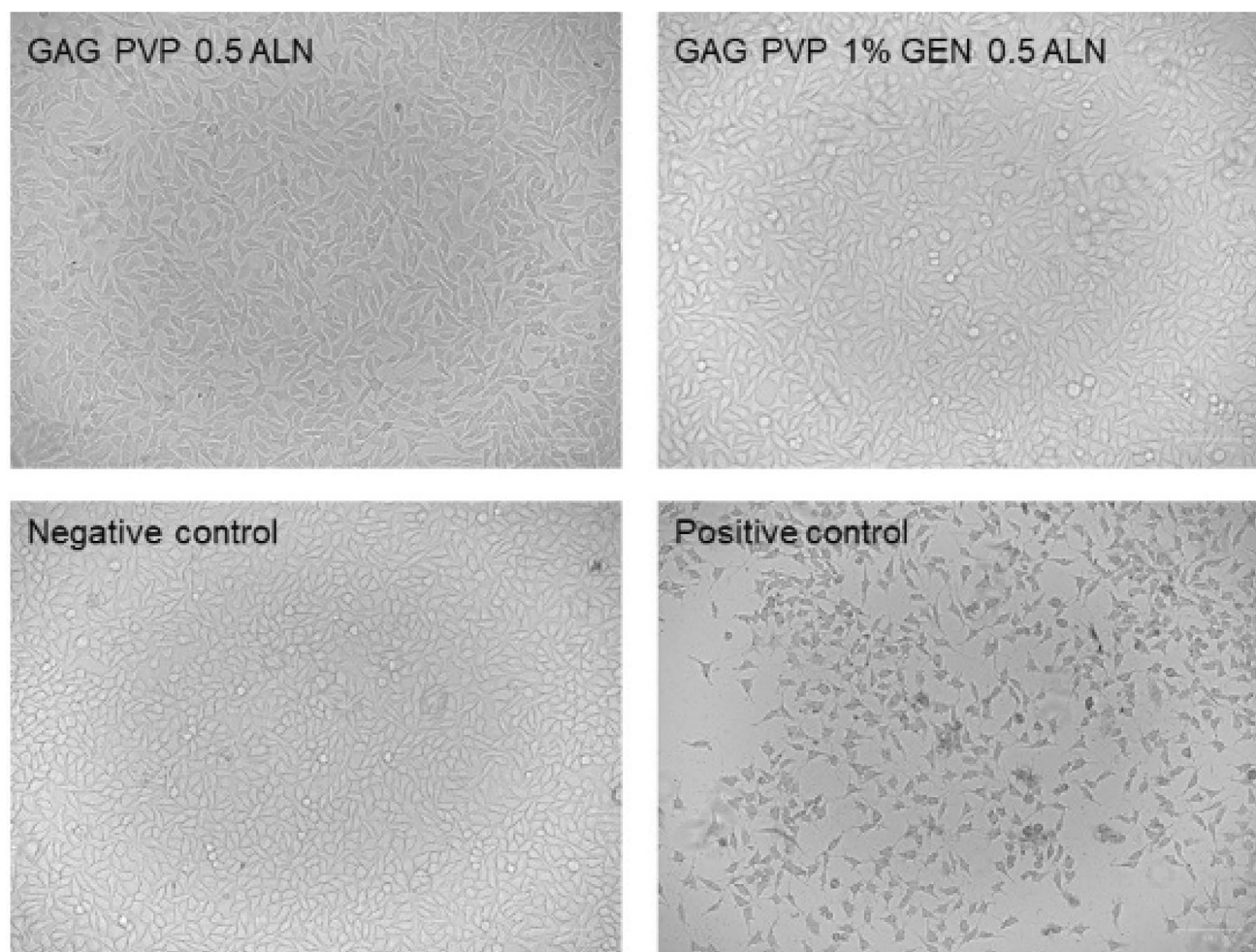


Fig. 10 The effect of GAG-PVP-ALN and GAG-PVP-GEN-ALN on the morphology of L929 cells. Morphological and intracellular changes of cells treated for 24 h was determined without staining on

live cells in the culture (Zoe Fluorescent Cell Imaging System (Bio-Rad), 200x magnification)

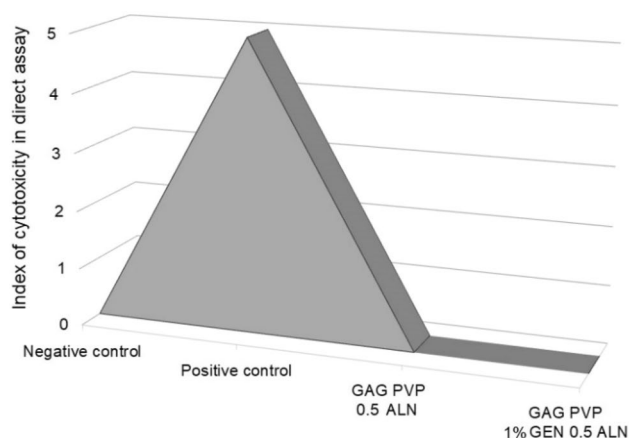


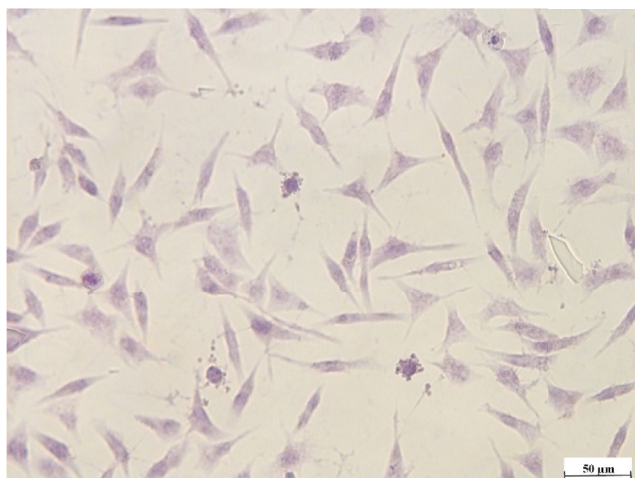
Fig. 11 Cytotoxic effect of GAG-PVP-ALN and GAG-PVP-GEN-ALN on L929 cells upon the direct contact – quantitative assessment. Indexes of cytotoxicity were defined according to reference range presented in Materials and Methods section

confluence nor the morphology i.e. there was no evidence of cell lysis, detachment or nuclear deformation, even after 24-hour exposure. While the cells in direct contact with positive control were destructed, those in contact with tested materials exhibited high morphological resemblance to negative control.

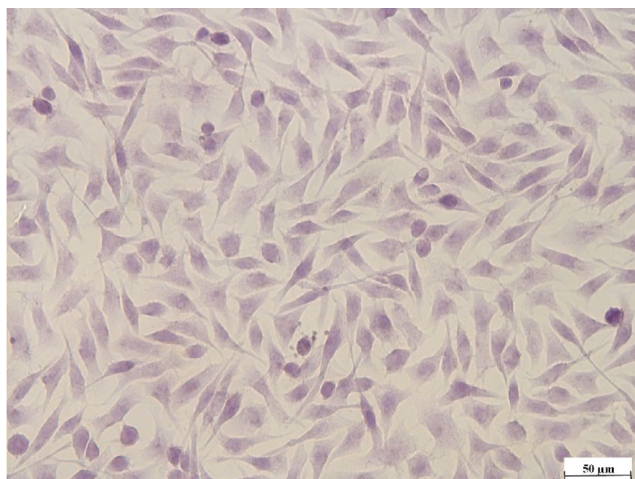
Quantitative assessment of the cytotoxic effect in direct contact was calculated by measuring the percentage of non-viable cells, based on which the indexes of cytotoxicity were assigned (Fig. 11). The percentage of non-viable cells remained well below the 20% threshold upon the direct contact with GAG-PVP-ALN and GAG-PVP-GEN-ALN, qualifying the scaffolds under the *absence of cytotoxicity* category. The use of 0.5 wt% alendronate appears to be a contributing factor in achieving this balance, since alendronate is known to exhibit dose-dependent cytotoxicity [71, 72].

To confirm the results obtained in direct and indirect tests, cells treated for 24 h with tested materials were stained

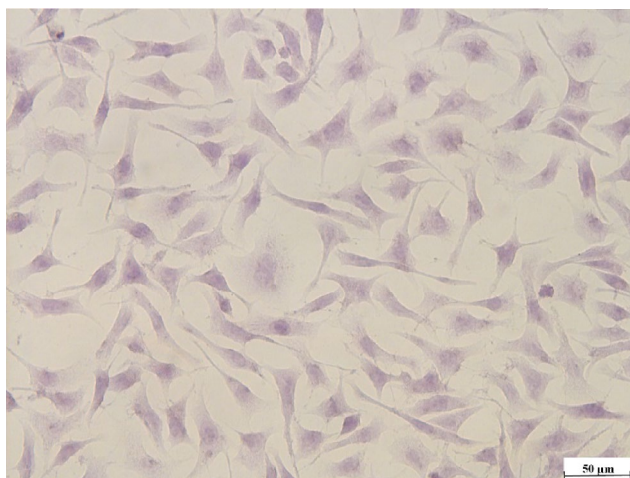
GAG-PVP-ALN



Negative control



GAG-PVP-GEN-ALN



Positive control

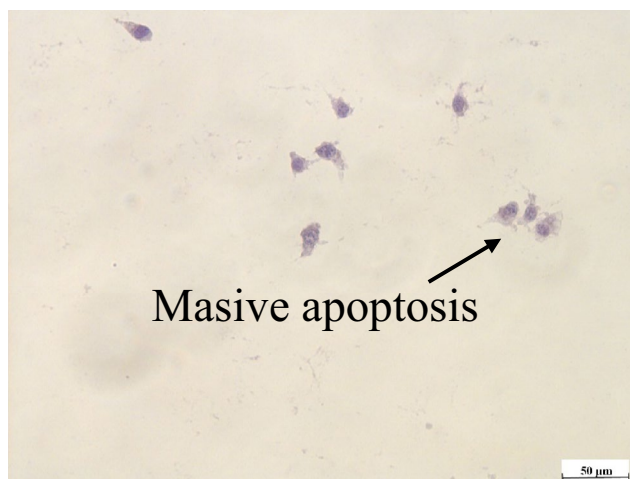


Fig. 12 The effect of GAG-PVP-ALN and GAG-PVP-GEN-ALN on the morphology and phenotype of L929 cells. Cells were cultivated on cover slips until they reached the confluency, fixed in 4% paraformaldehyde and stained with Mayer's Hematoxylin followed by the

analyses under the light microscope (Axio Imager, Carl Zeiss, 200x magnification) (apoptotic nuclei are indicated by white arrows on positive controls)

with Mayer's Hematoxylin and their morphology, including the nuclear morphology was analyzed under the fluorescent microscope. GAG-PVP-ALN and GAG-PVP-GEN-ALN treated cells preserved normal phenotype, while positive control induced massive apoptosis (Fig. 12). The obtained results confirmed the absence of both scaffold cytotoxicity that was demonstrated via the metabolic activity of treated cells in indirect test.

Genipin crosslinking may contribute to enhanced biocompatibility of the scaffolds. Unlike synthetic crosslinkers such as glutaraldehyde, genipin is known for its low toxicity and has even been reported to promote cellular adhesion some biopolymeric systems [73, 74]. The presence of a stabilized and less soluble gelatin-PVP network likely reduces

scaffold degradation byproducts in the medium, contributing to the observed high cell viability. These findings confirm that the GAG-PVP-GEN-ALN scaffold exhibits an optimal combination of structural integrity and biological safety, with high viability (>85%) and no morphological indicators of cytotoxic damage. Future research will include further in vitro and in vivo studies, as well as bioactivity of the scaffold prototype material.

Conclusions

The present study establishes that embedding a low dose (0.5 wt%) of alendronate directly into a gelatin-PVP bioink, coupled with genipin crosslinking, yields 3D-printed scaffolds exhibiting a balanced profile of swelling, mechanical strength, thermal stability and biocompatibility. Genipin restores hydrophobicity of gelatin disrupted by the addition of alendronate, and reinforces the polymer network, enabling a gradual deswelling over four days. Furthermore, in crosslinked scaffold, prolonged alendronate release for 48 h was achieved, following the first order kinetics. Mechanical evaluations revealed that although alendronate alone reduced mechanical performance of hybrid scaffolds, addition of alendronate in combination with genipin led to a 102% increase in tensile strength, 46% increase of elastic modulus and 141% rise in hardness, compared to GAG-PVP, revealing synergistic effect of genipin and alendronate in gelatin-polyvinyl pyrrolidone blend. Indirect and direct cytotoxicity assays confirmed that the low ALN dose maintains cell viability above 85%, proving high biocompatibility. These findings emphasize the promise of GAG-PVP-GEN-ALN hybrid scaffolds for bone tissue regeneration and future research involving deeper in vitro studies, in vivo validation and bioactivity investigation.

Author Contributions MJ - wrote the main manuscript text, performed investigation, formal analysis; IS - methodology, validation, formal analysis; MP - methodology, formal analysis; DS - formal analysis; NR - formal analysis; SI - conceptualization, methodology, supervision; PU - conceptualization, methodology, supervision. All authors reviewed the manuscript.

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Data Availability No datasets were generated or analysed during the current study.

Declarations

Competing interests The authors declare no competing interests.

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5. Diskusija

Cilj istraživanja ove doktorske disertacije obuhvata razvoj i karakterizaciju novih adhezivnih bukalnih filmova i implantata na bazi želatina za dostavu aktivnih supstanci i regeneraciju tkiva.

Istraživanja u okviru ove disertacije razvijala su se u dva pravca: razvoj mukoadhezivnih bukalnih filmova sa propanolol hidrohloridom (PRH) i razvoj implantata sa natrijum alendronat trihidratom (ALN). Dobijeni rezultati su prikazani kroz četiri faze koje su rezultirale i odgovarajućim brojem objavljenih radova. U prvoj fazi je izvršen izbor vrste želatina koji odgovara izabranoj aktivnoj supstanci radi dobijanja optimalnog profila otpuštanja i fizičko-mehaničkih svojstava. Izvedeno je procesiranje bukalnih filmova na bazi želatina A i B sa dodatkom PRH metodom izlivanja iz rastvora i ispitivana je morfologija, fizičko-hemijska i mehanička svojstva dobijenih filmova. U drugoj fazi je optimizovan sastav blende želatina A i sintetičkih polimera polivinilpirolidona (PVP) i polivinil alkohola (PVA) za 3D štampu. Izvedena je 3D štampa ekstruzijom polučvrstih materijala u dve serije uzoraka primenom različitih blendi polimera: želatin A-PVP i želatin A-PVA i ispitivan je uticaj sastava na strukturu, fizičko-mehanička svojstva i profile otpuštanja PRH iz dobijenih bukalnih filmova. U trećoj fazi, nakon izbora blende želatin A-glicerol-PVP (GAG-PVP) za dobijanje implantata za regeneraciju tkiva, ispitivan je uticaj agensa za umrežavanje, genipina, na fizičko-mehanička svojstva 3D štampanih implantata. Konačno, u četvrtoj fazi je razvijen implantat sa dodatkom ALN za primenu u regeneraciji tkiva kosti.

Sve faze ovog istraživanja i procesiranja praćene su karakterizacijom polaznih komponenti i dobijenih filmova i implantata. Mogućnost hemijskog vezivanja kao i potvrde umrežavanja i očuvanja aktivnih supstanci praćene su FTIR analizom. Morfologija dobijenih struktura ispitivana je FESEM analizom. Termička svojstva ispitivana su DSC analizom; mehanička svojstva ispitivanjem zatezanjem i mikroindentacijom; mukoadhezija metodom odvajanja (engl. *pull-off*) određivanjem sile razdvajanja i rada mukoadhezije. Takođe su analizirani profili otpuštanja iz bukalnih filmova i implantata, biodostupnost bukalnih filmova kroz *in silico* model, kao i testiranje citotoksičnosti dobijenih implantata.

Kao što je napomenuto, u prvoj fazi [144] su upoređene dve vrste želatina kao mogućih nosača PRH i kandidata za 3D štampu bukalnih filmova: želatin tipa A, koji se dobija kiselom hidrolizom kolagena i želatin tipa B koji se dobija baznom hidrolizom iz kolagena. Procesirane su četiri serije filmova: želatin A (GA), želatin B (GB), želatin A i PRH (GAP) i želatin B i PRH (GBP) sa dozama od 30 mg PRH po bukalnom filmu (15 mm x 15 mm).

Rezultati FTIR i DSC analize pokazali su da želatin A sa PRH gradi fizičku smešu u kojoj se PRH nalazi u amorfnom obliku, dok želatin B sa PRH gradi jonski ili polielektrolitski kompleks (PEK). Ovi rezultati su u skladu s istraživanjima izvedenim sa primenom želatina u sistemima-formulacijama za otpuštanje aktivne supstance [145-147] koji su pokazali da formiranje jonskih kompleksa želatina zavisi od pH sredine, odnosa prisutnih jona kao i od vrste aktivne supstance. U slučaju želatina B i PRH dolazi do jonske interakcije između karboksilnih grupa želatina i protonovanih amino grupa PRH. Posledica formiranja PEK između želatina B i PRH je razlika u mehaničkim i mukoadhezivnim svojstvima, kao i u profilima otpuštanja za GAP i GBP bukalne filmove. Film GAP pokazuje veću zateznu čvrstoću, modul elastičnosti, mikrotvrdoću kao i bolji rad mukoadhezije od GBP filma. To znači da molekuli PRH predstavljaju fizičku prepreku pomeranja molekula želatina tokom opterećenja i time ojačavaju strukturu. Pored toga, veći broj amino grupa u želatinu A dovode do manje gustine umrežavanja u GAP filmu što onda rezultira većim radom mukoadhezije. Takođe se i tokom ispitivanja *in vitro* otpuštanja PRH pokazalo da je iz GAP bilo brže otpuštanje leka i koje se završilo u okviru 60 min dok je iz GBP otpuštanje bilo nešto sporije. Svi prikazani rezultati potvrđuju da su želatin A i PRH u filmu GAP u fizičkoj smeši, dok je kod filma

GBP došlo do stvaranja jonskog kompleksa. Ovaj rezultat je u skladu sa objavljenim istraživanjima [148,149] koja su pokazala da stvaranje jonskog kompleksa utiče na profil otpuštanja, usled razlike u disocijaciji želatina i aktivne supstance.

Na osnovu rezultata *in vitro* ispitivanja otpuštanja urađena je *in silico* simulacija fiziološki zasnovanog modela za predviđanje apsorpcije aktivne supstance iz bukalnih filmova, odnosno predviđanje apsorpcije PRH i dispozicije u organizmu. Rezultati su pokazali da doza leka može značajno da se smanji primenom dobijenih bukalnih filmova sa dozom od 30 mg, jer je stepen apsorpcije PRH iz bukalnog filma u istom opsegu kao i iz oralne tablete sa dozom od 80 mg PRH (manje od 10 % razlike). Ovi rezultati *in silico* modela se slažu sa istraživanjima u literaturi [150] gde je sličan rezultat dobijen ispitivanjima *in vivo* za bukalne filmove sa PRH na bazi polietilen oksida, hidroksipropil metilceluloze, polivinil alkohola i propilen glikola. Mohamed i saradnici [151] su sličnu studiju izveli sa bukalnim filmovima na bazi mucina, polivinil alkohola i karbopola.

Nakon prve faze istraživanja izabran je želatin A za 3D štampu ekstruzijom polučvrstih materijala. Formirane su blende želatina A sa sintetičkim polimerima polivinilpirolidonom (PVP) i polivinil alkoholom (PVA) uz dodatak glicerola za bolju mogućnost štampanja. Izvedena je 3D štampa šest formulacija bukalnih filmova: 1. želatin A sa glicerolom (GAG); 2. želatin A, glicerol i PRH (GAG-PRH); 3. želatin A, glicerol i PVP (GAG-PVP); 4. želatin A, glicerol, PVP i PRH (GAG-PVP-PRH); 5. želatin A, glicerol, PVA (GAG-PVA); 6. želatin, glicerol, PVA i PRH (GAG-PVA-PRH). Ispitivan je uticaj sastava blende na fizičko-mehanička svojstva bukalnih filmova, kao i na profil otpuštanja, a *in silico* simulacijom izvedeno je predviđanje biodostupnosti dobijenih filmova i njihove pogodnosti za pacijente.

Primenom FTIR i DSC analize pokazano je da je tokom procesiranja došlo do formiranja vodoničnih veza između želatina i PVP dok se u ostalim formulacijama nije detektovala pojava novih hemijskih veza. Glicerol koji je dodat radi poboljšanja procesa štampanja, delovao je kao plastifikator i snizio karakteristične temperature polimera što je i očekivano. Ovo je takođe uočeno i u istraživanjima u literaturi [152] bukalnih filmova na bazi želatina i skroba, zatim istraživanja 3D štampanih filmova PVP i polisorbata 20 sa vitaminom B [153], kao i 3D štampanih filmova na bazi hidroksipropil metilceluloze sa aktivnom supstancom sakvinavir [154].

Profili otpuštanja PRH iz 3D štampanih filmova pokazali su da je najveća brzina otpuštanja kod filma na bazi čistog želatina (GAG-PRH). Nešto sporije otpuštanje pokazao je film blende želatina i PVA (GAG-PVA-PRH). Najsporije otpuštanje je postignuto iz filma blende želatina i PVP (GAG-PVP-PRH). Ovo je i očekivano, s obzirom na vodonične veze koje su se formirale između želatina i PVP, čime je postignuta i veća gustina umrežavanja. Usled veće gustine umrežavanja i zbog različite polarnosti molekula PVP i PRH i privlačnih sila, fluid teže prolazi kroz strukturu i dolazi do usporenog otpuštanja PRH [155-157].

Za 3D štampane filmove sa dozom od 20 mg PRH po bukalnom filmu (15 mm x 15 mm) izvedena je *in silico* simulacija i upoređena sa tabletama sa dozama od 20 mg i 40 mg. Dobijeno je da je stepen apsorpcije leka iz testiranih bukalnih formulacija u rang sa simuliranim oralnim tabletama koje imaju duplo veću dozu leka nego bukalni film. Znači, isti terapijski efekat može da se postigne sa duplo manjim dozama PRH u bukalnim filmovima što bi dovelo i do smanjenja neželjenih efekata kod pacijenta. Dodatno su urađene simulacije ponovljenog doziranja sa različitim vremenskim intervalima testiranih bukalnih filmova i oralnih tableta sa trenutnim otpuštanjem PRH. Rezultati su pokazali da bi testirane formulacije bukalnih filmova mogle da omoguće proređen režim doziranja u odnosu na tablete što bi bilo pogodnije za pacijente, što je i glavna prednost bukalnih filmova [150,158,159].

Pored biokompatibilnosti i profila otpuštanja aktivne supstance, za bukalne filmove su veoma važna mehanička i mukoadhezivna svojstva. U toku ovih istraživanja i razvoja bukalnih filmova sa PRH praćen je razvoj mikrostrukture filmova u korelaciji sa mehaničkim i mukoadhezivnim svojstvima. Mehanička svojstva filmova ispitivana su na zatezanje sa određivanjem zatezne čvrstoće, modula elastičnosti i prekidnog izduženja, kao i ispitivanjem mikrotvrdoće. Kod izlivenih filmova, čisti želatin A i želatin B pokazali su zateznu čvrstoću i modul elastičnosti koji odgovara vrednostima objavljenim u literaturi [160,161]. U filmovima sa lekom GAP i GBP, vrednosti za zateznu čvrstoću i modul elastičnosti opadaju, dok se prekidno izduženje značajno povećava. Dodatkom PRH omogućava se lakše kretanje polimernih lanaca, čime se povećava mogućnost plastične deformacije filma. S druge strane 3D štampani filmovi pokazuju slična mehanička svojstva, uz efekat glicerola kao plastifikatora. Filmovi blendi GAG-PVP i GAG-PVA pokazuju mehanička svojstva u skladu sa literaturnim podacima [162,163].

Ispitivanje mukoadhezije je sprovedeno sa ciljem da se proverí kvalitet vezivanja filmova za bukalnu mukozu, što značajno utiče i na otpuštanje aktivne supstance. Rezultati testa mukoadhezije kvantitativno su prikazani preko adhezivne čvrstoće, kao i rada adhezije. Kod izlivenih filmova, GAP je pokazao najvišu vrednost rada adhezije usled čega je mnogo bolja penetracija fluida u film i bolja adhezija za mukozu. Sa druge strane, GBP ima najveću vrednost sile adhezije, ali najmanji rad adhezije jer je postignuta veća gustina umrežavanja i na taj način je penetracija fluida sporija, stoga je i adhezija slabija.

3D štampani film GAG-PVP-PRH je pokazao najveću vrednost sile adhezije, što je u saglasnosti sa ostalim dobijenim dobrim mehaničkim svojstvima. Bolja adhezija se može objasniti formiranjem vodoničnih veza, većim sadržajem trostrukog heliksa i cis-trans izomerne tranzicije tokom procesiranja filma. Prema elektrostatičkoj teoriji film GAG-PVP-PRH pokazuje veću adhezivnu čvrstoću zbog razlike u polarnosti molekula PVP i PRH, dok je kod filma GAG-PVA-PRH slična polarnost molekula PVA i PRH pa je i adhezivna čvrstoća manja [164,165].

Tabela 5.1. prikazuje neke od formulacija bukalnih filmova sa postignutim mehaničkim i mukoadhezivnim svojstvima. Bukalnih filmovi razvijeni u ovom istraživanju su u rangu s filmovima drugih formulacija i dobijenih drugim metodama. Prednost pristupa u ovom istraživanju je što su filmovi dobijeni *in situ* SSE metodom tako da nije bilo potrebe ni za prethodnom ni za naknadnom obradom što je naročito pogodno za rad sa aktivnim supstancama osetljivim na npr. UV zračenje ili termički tretman.

Prezentovani rezultati drugih istraživanja su u skladu sa rezultatima dobijenim u ovoj disertaciji [92] i pokazuju da je proces 3D štampe ekstruzijom polučvrstih materijala potpuno optimizovan tokom istraživanja u okviru ove disertacije. Razvijeni su filmovi želatina sa PVP i PVA sa poboljšanim mehaničkim i mukoadhezivnim svojstvima i produženim otpuštanjem PRH u odnosu na bukalne filmove na bazi čistog želatina.

Tabela 5.1. Neke od formulacija bukalnih filmova dobijenih različitim metodama i postignuta mehanička i mukoadhezivna svojstva (*naučni rad proizašao iz ove doktorske disertacije)

Polimer	Ekscipijens - pomoćna supstanca	Aktivna supstanca	Metoda	Svojstvo	Referenca
Želatin A - PVP	Glicerol	PRH	3D štampa SSE	Zatezna čvrstoća 15 ± 2 Mpa, Modul elastičnosti 680 ± 70 Mpa Tvrdoća $4,3 \pm 2,9$ Mpa Sila mukoadhezije $1,04 \pm 0,45$ N	[92]*
Želatin A - PVA	Glicerol	PRH	3D štampa SSE	Zatezna čvrstoća $11 \pm 0,16$ Mpa, Modul elastičnosti 767 ± 90 Mma Tvrdoća $1,5 \pm 0,28$ Sila mukoadhezije $1,01 \pm 0,08$ N	[92]*
Želatin A (Bloom 100-270)	-	-	Izliivanje iz rastvora	Zatezna čvrstoća 35-90 Mpa, Modul elastičnosti 2,5-3 Gpa	[160]
Želatin A (Bloom 60-240)	-	-	Izliivanje iz rastvora	Zatezna čvrstoća 45-80 Mpa, Modul elastičnosti 4-5 Gpa	[161]
PVA - Hitozan	-	Natrijum diklofenak	3D štampa FDM	Zatezna čvrstoća 4.2-14 Mpa, Modul elastičnosti 35-68 Gpa Sila razdvajanja mukoadhezije $0,25-0,52$ N	[130]
Želatin B - karboksimetilceluloza	-	<i>Crescentia cujete</i> L. Extracts	Izliivanje iz rastvora	Zatezna čvrstoća 57 Mpa, Modul elastičnosti 2700 Mpa	[164]

PVA	Glicerol	Aripripazol	Izlivanje iz rastvora Elektropredenje 3D štampa	Zatezna čvrstoća 6,1 Mpa, Modul elastičnosti 30-240 Mpa	[134]
Karagenan, Natrijum alginat	Polietilen glikol (PEG), L-arginin	Omeprazol	Izlivanje iz rastvora	Zatezna čvrstoća 10-5 Mpa, Modul elastičnosti 8-10 Mpa	[166]
PVA, hidroksipropil celuloza (HPC)	-	Varfarin-natrijum	3D-SSE	Zatezna čvrstoća 4,1 Mpa,	[167]
Soluplus - Hidroksipropil metilceluloza (HPMC)	Glicerol	Furosemid	Izlivanje iz rastvora	Zatezna čvrstoća 0,21-1,14 Mpa Rad mukoadhezije WoA, 10-240 gs	[168]
HPMC, Hitozan, Želatin	Natrijum hijaluronat	Ondansetron hidrohlorid	Izlivanje iz rastvora	Vreme zadržavanja kao mera mukoadhezije 1200 min	[169]
Hitozan, Želatin	-	PRH	Izlivanje iz rastvora	Sila razdvajanja mukoadhezije (10-18)x10 ⁻⁵ N	[170]
Želatin, karboksi metilceluloza (CMC)	Glicerol	Lisozim	Izlivanje iz rastvora	Adhezion čvrstoća 4380 Pa	[171]
Hitozan, Želatin PVP	Umreživač gluteraldehid	Progesteron	Izlivanje iz rastvora	Sila mukoadhezije (0,15-0,45) x10 ⁻⁵ N	[172]

Hidroksi etil celuloza (HEC)	Glicerol	Canabidiol (CBD)	3D štampa	Zatezna čvrstoća 0.67 ± 0.04 Mpa Sila mukoadhezije 0.14 ± 0.02 N	[135]
Polietilen oksid	fenil-bis (2,4,6-tri metil benzoil) fosfin oksid (TPO) Polietilen glikol (PEG) Poli etilen glikol diakrilat (PEGDA)	Bupropion hidrohlorid	3D štampa pomoću LCD ekrana	Sila mukoadhezije 0.3 ± 0.05 N	[132]
PVA	-	Aripripazol	3D štampa FDM Izlivanje iz rastora	Zatezna čvrstoća 9.90 ± 0.78 Mpa, Modul elastičnosti 70.45 ± 9.36 Mpa Zatezna čvrstoća 19.57 ± 3.48 Mpa, Modul elastičnosti 320.08 ± 21.90 Mpa	[133]

Pošto je pokazan dobar potencijal blende GAG-PVP u primeni za bukalne filmove, dalja istraživanja su krenula u pravcu razvoja implantata na bazi ovog materijala za primenu u regenerativnoj medicini, odnosno implantata za otpuštanje aktivne supstance. Zbog toga se u trećoj fazi eksperimentalnog rada ispitivanja mogućnost umrežavanja GAG-PVP genipinom kao najprihvatljivijim agensom za umrežavanje zbog minimalne citotoksičnosti. Ispitivan je uticaj sadržaja genipina na fizičko-mehanička svojstva implantata. Sa tim ciljem su procesirani implantati 3D štampom ekstruzijom polučvrstih materijala sa direktnom inkorporacijom genipina u mastilo za 3D štampu. Ovaj metod je inventivan u odnosu na klasične metode u kojima umrežavanje obično izvodi nakon procesiranja potapanjem uzoraka u rastvor genipina. Procesirane su i ispitivane dve serije uzoraka: sa 0,5 mas% i 1 mas% genipina.

FTIR analiza je potvrdila proces umrežavanja kroz reakciju estarske grupe genipina i primarnih amino grupa želatina, što je praćeno pojavom tamnoplave boje [173-175]. Sa druge strane, DSC analiza je pokazala da umrežavanje genipinom popravља termičku stabilnost želatina, a veći sadržaj genipina (1 mas%) dovodi do izraženog ukrućenja polimernih lanaca, usled čega je cis-trans izomerizacija peptidnih lanaca želatina gotovo potpuno sprečena [176,177]. Očekivano je da umrežavanje vodi i ka boljim mehaničkim svojstvima što je i pokazano ispitivanjem na zatezanje i mikroindentacijom, pri čemu su analizirani zatezna čvrstoća, modul elastičnosti, tvrdoća i redukovani modul elastičnosti. Rezultati su pokazali da umrežavanje genipinom poboljšava otpornost materijala, a povećanjem koncentracije genipina dolazi do daljeg rasta svih ispitivanih parametara. Ovakvo ponašanje potvrđuje uspešno umrežavanje želatina u blendi sa PVP, što dovodi do formiranja rigidnije polimerne strukture. Istovremeno, prekidno izduženje se povećava sa porastom sadržaja genipina, što ukazuje da umrežavanje ne samo da povećava čvrstoću materijala već doprinosi i većoj žilavosti. Zahvaljujući tome, implantati pokazuju širi potencijal za primenu. U ovom istraživanju predviđeno je da se implatati koriste za regeneraciju koštanog tkiva, pa je bitno da i mehanička svojstva implatata budu što bliža mehaničkim svojstvima koštanog tkiva. Dobijene vrednosti za zateznu čvrstoću i modul elastičnosti umreženih implatata odgovaraju literaturnim podacima za trabekularno koštano tkivo [178, 179].

Pri ispitivanju citotoksičnosti (prema standardu ISO 10993-5) neumreženog i umreženog implantata, i u direktnom i u indirektnom testu, pokazalo se da je indeks citotoksičnosti 0. Kvantitativno određivanje citotoksičnosti u direktnom testu izvedeno je proračunom procenta nevijabilnih ćelija u kontaktu sa implantatima GAG-PVP i GAG-PVP-GEN. Oba materijala su pokazala odsustvo citotoksičnosti u odnosu na bazni nivo liziranih ćelija za negativnu kontrolu (<2 %) i pozitivnu kontrolu koja je pokazala prisustvo liziranih ćelija (>80 %). Rezultati indirektnog testa pokazali su da je smanjenje vijabilnosti ćelija kod tretmana ekstraktom implantata GAG-PVP bilo 5 %, a kod implantata GAG-PVP-GEN je iznosio 20 % u poređenju sa negativnom kontrolnom grupom koja ne pokazuje nikakvo smanjenje vijabilnosti. Prema standardu ISO 10993-5 relativna smanjenja ispod 30 % u odnosu na kontrolnu grupu smatraju se odustvom citotoksičnosti.

S obzirom na to da su dobijene umrežene strukture pokazale dobra mehanička svojstva, biodegradabilnost kao i odsustvo citotoksičnosti, pokazan je potencijal da se u njih ugradi i aktivna supstanca koja će pomoći u regeneraciji koštanog tkiva.

Zbog boljih mehaničkih svojstava, za dalji rad izabrana je struktura sa 1 mas% genipina i u poslednjoj fazi istraživanja razvijan je implantat sa inkorporacijom natrijum alendronat trihidrata (ALN). Izvedena je 3D štampa ekstruzijom polučvrstih materijala sa direktno dodatim ALN u mastilo. Prvi korak je bio da se odredi sadržaj ALN s obzirom na citotoksičnost ovog leka. U literaturi ima vrlo malo studija o citotoksičnosti alendronata u čvrstim implantatima. Uglavnom se radilo o studijama sadržaja ALN u rastvorima gde se došlo do zaključka da koncentracija veća od 10^{-6} M izaziva citotoksičnost [180-182]. U literaturi je pokazana visoka citotoksičnost dentalnog cementa sa ALN u polietilenglikolu (PEG) u koncentraciji od 2 g/ml (0.6×10^{-5} M) [183,184]. Na osnovu

objavljenih rezultata u okviru ovih istraživanja određena je koncentracija ALN od 0,5 mas% sa kojom je izvedena 3D štampa. Procesirane su tri serije uzoraka: GAG-PVP, GAG-PVP-ALN (0,5 mas% ALN) i GAG-PVP-GEN-ALN (1 mas% genipin i 0,5 mas% ALN).

FTIR i DSC analize potvrdile su umrežavanje genipinom koje doprinosi ukrućivanju peptidnih veza i smanjenju cis-trans tranzicije kod želatina [177,178]. Odsustvo kristalizacionog pika za ALN u DSC termogramu pokazalo je da je aktivna supstanca u amorfnom, rastvorenom obliku što je povoljno za proces efikasnog otpuštanja.

Mehanička ispitivanja zatezanjem i mikroindentacijom implantata sa sadržanim ALN pokazali su da dodatkom ALN dolazi do smanjenja zatezne čvrstoće, modula elastičnosti, tvrdoće i redukovanog modula elastičnosti u odnosu na implantat GAG-PVP. Umrežavanjem genipinom implantata sa sadržanim ALN postignute su najveće vrednosti ovih mehaničkih svojstava. Takođe, i prekidno izduženje raste od 2,65 % za GAG-PVP do 4,11 % za GAG-PVP-GEN-ALN, što ukazuje na poboljšanu žilavost i mogućnost plastične deformacije. Umrežavanje genipinom ne samo da je popravilo mehanička svojstva implantata sa ALN, već je dovelo do znatnog povećanja ovih vrednosti i u odnosu na osnovni implantat GAG-PVP. Ovaj rezultat se može objasniti sinergijskim efektom u okviru kog se fosfonatne grupe ALN pojavljuju kao dodatni umreživač poremećajem strukture trostruke spirale (engl. *triple helix*) u želatinu. Na taj način je postignuto dodatno ojačanje pored osnovnog koje je postignuto dodavanjem genipina za umrežavanje.

Pregled tabele 5.2. pokazuje da su mehanička svojstva razvijenog implantata GAG-PVP-GEN-ALN u okviru vrednosti postignutih u drugim istraživanjima i da odgovaraju zahtevima za implantate za regeneraciju koštanog tkiva. Takođe, prikazana su i mehanička svojstva za kortikalnu i trabekularnu kost i vidi se da bi razvijeni implantat mogao da se koristi za tretman osteoporoze koja najčešće pogađa trabekularnu kost [192,193]

Ispitivanje bubrenja je pokazalo da umreženi implantat GAG-PVP-GEN-ALN ima maksimalni stepen bubrenja od 462 % nakon 5 h i zadržao je stabilnost do 96 h sa stepenom bubrenja od 196 %, što je u skladu sa rezultatima istraživanja objavljenim u literaturi gde je pokazan uticaj sadržaja i molekulske mase želatina na postignute stepene bubrenja od 200-380 % [194]. Dobijeni rezultati ukazuju da ALN povećava hidrofilnost sistema i ubrzava degradaciju, dok umrežavanje genipinom stabilizuje strukturu implantata i usporava procese bubrenja i razgradnje [194,195]. Ovakvo ponašanje je potvrđeno i ispitivanjem ugla kvašenja, koje pokazuje afinitet površine implantata prema fluidu oko njega. Prisustvo ALN u blendi GAG-PVP-ALN povećava hidrofilnost implantata u odnosu na čistu blendu GAG-PVP koja se po uglu kvašenja može smatrati relativno hidrofobnom. Umrežavanje genipinom je ponovo vratilo hidrofobna svojstva implantata povećanjem ugla kvašenja što je u skladu sa testom na bubrenje.

Pri ispitivanju citotoksičnosti *in vitro*, citotoksični efekat GAG-PVP-ALN i GAG-PVP-GEN-ALN na L929 ćelije pri direktnom kontaktu je definisan kao 0, tj. Ukazuje na odsustvo citotoksičnosti (nedetektovana ili bazalna citotoksičnost koja je detektovana u negativnoj kontroli). Vijabilnost ćelija nakon tretmana u kondicioniranim medijima u indirektnom testu za GAG-PVP-ALN i GAG-PVP-GEN-ALN pokazuje visoke vrednosti u svim testiranim razblaženjima, što odgovara odsustvu citotoksičnosti prema standardu ISO 10993-5. Prikazani rezultati ukazuju da implantati sa koncentracijom ALN 0,5 mas% ne pokazuju citotoksičnost. To ukazuje da su oba implantata sa aktivnom supstancom dobri kandidati za dalja ispitivanja biokompatibilnosti.

Tokom *in vitro* testa otpuštanja sadržanog leka, neumreženi implantat GAG-PVP-ALN pokazao je brže otpuštanje leka u poređenju sa implantatom umreženim genipinom (GAG-PVP-GEN-ALN). ALN se iz implantata GAG-PVP-ALN potpuno oslobodio za 3 h, uz sporije početno otpuštanje. Nasuprot tome, GAG-PVP-GEN-ALN je pokazao postepeno i produženo oslobađanje

leka. Umreženi implantat je otpustio samo 10 % leka u prvom satu, nakon čega sledi kontinuirani porast, sve do potpunog oslobađanja ALN u periodu od 48 h.

Iako je potpuno *in vitro* otpuštanje ALN iz umreženog GAG-PVP-GEN-ALN implantata ostvareno tokom 48 h, ovaj rezultat ne treba poistovećivati sa trajanjem biološkog dejstva ALN nakon implantacije. Bisfosfonati, uključujući ALN, pokazuju izražen afinitet prema mineralnoj fazi kosti i adsorbuju se na hidroksiapatit, što značajno doprinosi njihovom lokalnom zadržavanju i farmakološkom dejstvu [196,197]. To potvrđuju ispitivanja na humanom koštanom tkivu u kojima je ALN zadržao antiresorptivnu efikasnost i nakon ispiranja slobodnog leka sa koštane površine [198]. Stoga se može pretpostaviti da ALN oslobođen iz implantata tokom inicijalnog perioda može i nakon toga biti vezan za okolni koštani mineral i ostati dostupan osteoklastima tokom naknadne resorpcije kosti [199,200]. U tom smislu, produženje oslobađanja sa približno 3 h kod neumreženog sistema na 48 h nakon umrežavanja genipinom predstavlja značajnu modifikaciju profila oslobađanja.

U cilju određivanja kinetike otpuštanja ALN iz implantata, na rezultate *in vitro* ispitivanja oslobađanja primenjeni su kinetički modeli: nultog i prvog reda i Higuči. Dobijeni rezultati ukazuju na to da umrežavanje sa genipinom menja početno i brzo oslobađanje ALN (kinetika nultog reda) u kontrolisano otpuštanje koje je zavisno od koncentracije ALN (kinetika prvog reda). Ovo svojstvo umreženog implantata može da poboljša i efikasnost i da obezbedi bezbednu lokalnu terapiju za regeneraciju koštanog tkiva.

Sagledavši ukupne rezultate razvoja implantata za tretman osteoporoze i regeneraciju koštanog tkiva može se zaključiti da je razvijeni implantat GAG-PVP-GEN-ALN optimizovan u pogledu mehaničkih svojstava koja odgovaraju svojstvima trabekularne kosti, zatim u pogledu kinetike oslobađanja ALN koje se odvija u toku 48h uz odsustvo citotoksičnosti. Stoga je ova formulacija spremna za dalje faze pretkliničkih i kliničkih istraživanja koja bi potencijalno mogla dovesti do primene ovog farmaceutskog oblika u kliničkoj praksi.

Tabela 5.2. Primeri implantata za regeneraciju tkiva dobijenih različitim metodama i postignuta mehanička svojstva (*naučni rad proizašao iz ove doktorske disertacije)

Polimer	Agens za umrežavanje	Aktivna supstanca	Metoda	Svojstvo	Referenca
Želatin A-PVP	Genipin	-	3D štampa ekstruzijom polučvrstih materijala	Zatezna čvrstoća 38 MPa Modul elastičnosti 1,25 GPa Tvrdoća 39 MPa Red. Modul Elastičnosti 1,35 GPa	[185]*
Želatin A-PVP	Genipin	Natrijum alendronat trihidrat	3D štampa ekstruzijom polučvrstih materijala	Zatezna čvrstoća 39 MPa Modul elastičnosti 1,17 GPa Tvrdoća 22 MPa Red. Modul Elastičnosti 820 MPa	[186]*
Želatin B-PVP	Gluteraldehid (GTA)	-	<i>In situ</i> stvaranje pene gasom	Zatezna čvrstoća 2 MPa Pritisna čvrstoća 2.08 ± 0.02 Mpa	[187]
PLA-HAP	-	-	3D štampa PLA ekstruzijom iz rastopa a zatim nanošenje HAP	Pritisna čvrstoća 16-53 MPa U zavisnosti od ugla štampanja	[141]

PLA-HAP	-	-	3D štampa PLA ekstruzijom iz rastopa uz prethodnu ekstruziju filamenata	Modul elastičnosti 1164-1614 MPa	[142]
Želatin riblji-Na alginat	CaCl ₂ za alginat i EDC- NHS (N-hidroksisukinimid)	-	3D štampa ekstruzijom polučvrstih materijala	Pritisna čvrstoća 2-9 MPa Pritisni Modul 10-22 MPa	[137]
poli(ε-kaprolakton) (PCL) sa Pkering PCL faznom emulzijom (HIPEs)	-	-	3D štampa ekstruzijom polučvrstih materijala	Modul elastičnosti G' 26-69 MPa	[139]
PCL-Zeolit	Cisplatin	-	Metoda ispiranja porogena i liofilizacije	Pritisna čvrstoća 5-8 MPa Pritisni Modul 2-5 MPa	[188]
PLA-HAP	-	Ciprofloksacin hidrohlorid	3D štampa ekstruzijom iz rastopa i ekstruzijom polučvrstih materijala	Pritisna čvrstoća 11.30 (SSE) do 17.82 (FDM) MPa	[189]
Fibrin i poli(mlečna-kogilkolna) kiselina	Genipin za fibrin = FibGen	-	Brizganje u kalup	Modul elastičnosti 230-300 MPa Pritisni Modul 80-170 MPa	[190]

Kortikalna kost	-	-	-	Zatezna čvrstoća 135MPa Modul elastičnosti oko 18 000 MPa	[191]
Trabekularna kost	-	-	-	Zatezna čvrstoća 0,1–30 Mpa Modul elastičnosti 10-3000 MPa	
Vertebralna trabekularna kost	-	-	-	Pritisna čvrstoća 10 MPa Pritisni Modul 356 MPa zdrava 189 MPa osteopor. Zatezna čvrstoća 1,33-3,53MPa Modul elastičnosti 190-620 MPa	[179]

6. Zaključak

Cilj ove doktorske disertacije bio je razvoj multifunkcionalnog materijala na bazi želatina za primenu u mukoadhezivnim bukalnim filmovima i implantatima za dostavu aktivnih supstanci i regeneraciju koštanog tkiva. Procesirane su i karakterisane različite formulacije blendi želatina, sintetičkih polimera i aktivnih supstanci, kao i umreženi oblici implantata. U prvom delu su razvijeni bukalni filmovi sa željenim profilom otpuštanja propranolol hidrohlorida (PRH) i poboljšane bioraspoloživosti. Na osnovu razvijenog optimalnog sastava bukalnih filmova, u drugom delu razvijani su implantati za regeneraciju kostiju, umrežavanjem sa genipinom i inkorporiranjem natrijum alendronat trihidrata (ALN).

U prvoj fazi istraživanja, izvedena je uporedna analiza dve vrste želatina kao mogućih nosača PRH: želatina A, koji se dobija kiselom hidrolizom kolagena i želatina B koji se dobija baznom hidrolizom. Pokazalo se da su sa oba želatina dobijeni homogeni bukalni filmovi sa prisutnim PRH u amorfnom obliku čime je poboljšana biodostupnosti aktivne supstance. FTIR i DSC analize su pokazale da želatin A (GA) sa lekom formira fizičku smešu, dok želatin B (GB) gradi jonski kompleks. Posledica ovoga je da je kod bukalnih filmova želatina A sa PRH (GAP) postignuto brže otpuštanje aktivne supstance. Pored toga, GAP bukalni filmovi su pokazali bolja mehanička svojstva od GBP. *In silico* simulacija je pokazala da ovi bukalni filmovi na bazi želatina pokazuju bolju biodostupnost PRH od komercijalno dostupnih oralnih tableta, dok je terapijska doza značajno smanjena.

U drugoj fazi istraživanja, dobijeni su bukalni filmovi sa sadržanim PRH metodom 3D štampe ekstruzijom polučvrstih materijala na bazi želatina A. Da bi se poboljšala mehanička svojstva i dobilo produženo oslobađanje PRH, procesirane su blende želatina A sa dve vrste sintetičkih polimera, sa PVP i sa PVA. Ispitivan je uticaj dodatog polimera na kvalitet štampe, strukturu materijala, termička i mehanička svojstva, mukoadheziju, kao i na profile otpuštanja aktivne supstance. FTIR i DSC analize su pokazale da se i u 3D štampanim bukalnim filmovima, PRH nalazi u amorfnom obliku. Bukalni film GAG-PVP-PRH je pokazao bolja mehanička svojstva (veće vrednosti zatezne čvrstoće, tvrdoće i redukovanog modula elastičnosti), kao i bolju mukoadheziju od filma GAG-PVA-PRH. Ovaj rezultat se može objasniti formiranjem vodoničnih veza između GA i PVP čime se učvršćuje struktura. Bukalni film GAG-PVP-PRH pokazao je najduže vreme oslobađanja PRH u odnosu na GAG-PRH i GAG-PVA-PRH. *In silico* simulacija je pokazala da je biodostupnost u ovim filmovima 3 puta veća od tableta PRH sa trenutnim otpuštanjem leka. Pored toga, rezultati simulacije ukazuju da primena bukalnih filmova omogućava smanjenje učestalosti doziranja čime se poboljšava aderenza pacijenata. Predviđanjem bioraspoloživosti *in silico* simulacijom omogućeno je efikasnije istraživanje, razvoj i optimizacija proizvoda, uz smanjenje eksperimenata na životinjama i ljudima.

Na osnovu dobrih fizičko-mehaničkih svojstava koje je pokazala blenda GAG-PVP, ovaj materijal je primenjen za razvoj implantata za dostavu aktivne supstance i regeneraciju koštanog tkiva. Za dalje poboljšanje mehaničkih svojstava koje zahteva koštano tkivo, struktura implantata je umrežena pri čemu je kao umreživač izabran genipin zbog svojih netoksičnih i biokompatibilnih svojstava.

U trećoj fazi istraživanja optimizovan je sadržaj genipina (GEN) u pogledu rezultujućih fizičko-mehaničkih svojstava i odsustva citotoksičnosti implantata. 3D štamptom metodom ekstruzije polučvrstog materijala, dobijeni su implantati sa 0,5 mas% i 1 mas% genipina. Treba napomenuti da je genipin direktno inkorporiran u mastilo, za razliku od objavljenih istraživanja u kojima se obično implantat naknadno umrežavao potapanjem u rastvor genipina. U procesu 3D štampe optimizovan je

odnos brzine štampe i brzine umrežavanja što je izuzetno važno za strukturna i mehanička svojstva dobijenog implantata. FTIR i DSC analize su potvrdile proces umrežavanja blende GAG-PVP genipinom; dobijen je materijal krute strukture i povećane termičke stabilnosti. Dobijeni rezultati su pokazali da implantat sa 1 mas% genipina ima bolja mehanička svojstva, bolju termičku stabilnost, i sporiju degradaciju. Ovaj implantat je takođe pokazao odsustvo citotoksičnosti, i u direktnom i u indirektnom testu, prema standardu ISO 10993-5.

U poslednjem delu istraživanja, razvijen je implantat za otpuštanje bisfosfonata natrijum alendronat trihidrata, aktivne supstance koja se koristi u lečenju osteoporoze i za regeneraciju tkiva kosti. Izvedena je 3D štampa implantata primenom mastila GAG-PVP sa inkorporiranim i ALN i GEN uz prateću karakterizaciju neumreženih i umreženih implantata sa sadržanim ALN. FTIR i DSC analize su potvrdile umrežavanje strukture primenom genipina. FTIR analiza je pokazala da nije došlo do hemijske interakcije između ALN i GEN, dok je DSC analizom potvrđena amorfna struktura ALN u implantatu. Ispitivanja mehaničkih svojstava su pokazala da prisustvo ALN smanjuje zateznu čvrstoću, modul elastičnosti i tvrdoću u odnosu na čist GAG-PVP, dok se umrežavanjem genipinom ovaj efekat poništava, pa implantat GAG-PVP-GEN-ALN ima najveće vrednosti zatezne čvrstoće, modula elastičnosti i tvrdoće odnosno pokazao je najbolja mehanička svojstva. Rezultati *in vitro* testa otpuštanja pokazali su da je oslobađanje ALN iz umreženog implantata značajno produženo u odnosu na neumreženi implantat. Oba implantata su pokazala biokompatibilnost odnosno odsustvo citotoksičnih efekata u kulturama ćelija L929 u monosloju u direktnom i indirektnom testu.

Rezultati istraživanja potvrđuju da inkorporacija 0,5 mas% ALN u blendu želatina i PVP, uz umrežavanje genipinom, omogućava dobijanje 3D štampanih implantata sa uravnoteženim svojstvima bubrenja, unapređenom mehaničkom čvrstoćom, povećanom termičkom stabilnošću i zadovoljavajućom biokompatibilnošću. Ovakva svojstva su u skladu sa navodima iz relevantne literature i ukazuju na sinergijski efekat dodatka ALN i procesa umrežavanja genipinom. Dobijeni rezultati pokazuju značajan potencijal ovih materijala za primenu u regeneraciji tkiva kosti.

Rezultati ove doktorske disertacije potvrđuju da želatin, u kombinaciji sa odgovarajućim polimerima i kontrolisanim umrežavanjem pomoću genipina, predstavlja perspektivnu platformu za razvoj savremenih sistema za dostavu aktivnih supstanci i regeneraciju koštanog tkiva. Primenom savremene metode 3D štampe značajno je unapređen proces dobijanja naprednih multifunkcionalnih adhezivnih filmova i implantata sa unapred dizajniranim svojstvima.

Razvijeni 3D štampani bukalni filmovi i implantati pokazali su poboljšana fizičko-mehanička svojstva, odgovarajuće profile oslobađanja aktivnih supstanci, odsustvo citotoksičnosti i značajan potencijal za povećanje efikasnosti terapije i poboljšanje adherence pacijenata. Dobijeni rezultati predstavljaju dobru osnovu za dalja istraživanja kao i za razvoj personalizovanih biomaterijala namenjenih primeni u farmaciji i regenerativnoj medicini.

7. Literatura

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Biografija autora

Marija N. Jovanović, rođ. Radojević, magistar farmacije, rođena je 28.12.1991. godine u Beogradu. Osnovnu školu i gimnaziju završila je u Beogradu. Integrisane akademske studije na Farmaceutskom fakultetu Univerziteta u Beogradu, studijski program farmacija, završila je 2015. godine sa prosečnom ocenom 8,57. Završni rad pod naslovom „Formulacija, izrada i karakterizacija nanovlakana i oralnih filmova” odbranila je na Katedri za Farmaceutsku tehnologiju i kozmetologiju sa ocenom 10.

Stručni ispit za dobijanje licence Farmaceutske komore Srbije položila je oktobra 2016. nakon obavljenog pripravničkog staža za farmaceute u Zdravstvenoj ustanovi „Romanija”, Apoteka Beograd i Kliničko bolničkom centru Zvezdara u Beogradu.

Doktorske studije na Tehnološko-metalurškom fakultetu Univerziteta u Beogradu, upisala je školske 2017/2018. godine na studijskom programu Inženjerstvo materijala, pod mentorstvom prof. dr Petra Uskokovića. U okviru doktorskih studija položila je sve ispite predviđene studijskim programom sa prosečnom ocenom 9,83 i školske 2018/2019. godine odbranila Završni ispit pod nazivom „Mukoadhezivni filmovi za bukalnu primenu“ ocenom 10. Od novembra 2017. godine angažovana je kao doktorand na projektu Ministarstva prosvete, nauke i tehnološkog razvoja Republike Srbije TR 34011 „Razvoj opreme i procesa dobijanja polimernih kompozitnih materijala sa unapred definisanim funkcionalnim svojstvima”. Zaposlena je na Tehnološko-metalurškom fakultetu Univerziteta u Beogradu, najpre kao istraživač-pripravnik, a potom kao istraživač-saradnik.

Marija N. Jovanović je autor 8 radova objavljenih u međunarodnim naučnim časopisima, 2 rada objavljenih u nacionalnim naučnim časopisima i 9 saopštenja na međunarodnim konferencijama.

Изјава о ауторству

Име и презиме аутора Марија Н. Јовановић
Број индекса 4010/2017

Изјављујем

да је докторска дисертација под насловом

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Наслов рада „Адхезивни филмови и имплантати на бази желатина за букалну примену и регенерацију ткива“

Ментор Др Петар Ускоковић, ред. проф. Универзитета у Београду, Технолошко-металуршки факултет

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду**.

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис аутора

У Београду, 23.09.2026.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

„Адхезивни филмови и имплантати на бази желатина за букалну примену и регенерацију ткива“

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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