Gdański Uniwersytet Medyczny
Wydział Lekarski

Mgr Justyna Hajtuch

„Ocena wpływu funkcjonalizowanych nanocząstek srebra na układ hemostazy oraz aktywność przeciwbakteryjną w badaniach in vitro i ex vivo”

„Evaluation of the impact of functionalized silver nanoparticles on the hemostasis system and antibacterial activity in in vitro and ex vivo studies”

Rozprawa doktorska

Praca została wykonana w Katedrze i Zakładzie Patofizjologii Farmaceutycznej
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SPIS TREŚCI

WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ / LIST OF MANUSCRIPTS INCLUDED IN THE DOCTORAL DISSERTATION

Hajtuch J, Iwicka E, Szczoczarz E, Flis, D, Megiel E, Cieciórski P, Radomski MW, Santos-Martinez MJ, Inkielewicz-Stępniak I.

The pharmacological effects of silver nanoparticles functionalized with eptifibatide on platelets and endothelial cells

Int. J. Nanomed. 2022; 17

IF_{2022}: 7,033 | MNiSW: 140 pkt

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IF_{2022}: 5,988 | MNiSW: 100 pkt

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Curr. Med.Chem. 2021; 28(27)

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Effects of functionalized silver nanoparticles on aggregation of human blood platelets

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STRESZCZENIE W JĘZYKU POLSKIM

I. WYKAZ STOSOWANYCH SKRÓTÓW

6-keto-PGF1-alpha	produkt hydrolizy prostacykliny PGI_2
AgNPs	nanocząstki srebra
AgNPs-EPI	nanocząstki srebra funkcjonalizowane eptyfibatydem
AgNPs-GSH	nanocząstki srebra funkcjonazliowane glutationem
AgNPs-LA	nanocząstki srebra funkcjonazliowane kwasem liponowym
AgNPs-PEG	nanocząstki srebra funkcjonazliowane glikolem polietylenowym
APTT	czas częściowej tromboplastyny po aktywacji
ATCC	(*ang. American Type Cell Culture)*, Amerykańska Kolekcja Hodowli Komórkowych
cGMP	cykliczny guanozyno-3′,5′-monofosforan
CT	czas okluzji
CVD	choroby układu krążenia
DCF-DA	dioctan 2,7'-dichlorodihydrofluoresceiny
DNA	kwas deoksyrybonukleinowy
EPI	eptyfibatyd
ESKAPE	szczepy bakteryjne powszechnie kojarzone z opornością na środki przeciwdrobnoustrojowe
GPIIb/IIIa	glikoproteina IIb/IIIa
GSH	glutation
HGF-1	linia komórkowa ludzkich fibroblastów dziąseł
HMEC-1	linia komórkowa naczyń włosowatych skóry
HUVEC	linia komórkowa śródbłonka żyły pępowinowej
INR	stosunek czasu protrombinowego do kontrolnego standaryzowanego czasu protrombinowego
LDH	dehydrogenaza mleczanowa
MIC	*(ang. minimal inhibitory concentration*), minimalne stężenie hamujące
MMP-1	metaloproteinaza 1
MMP-2	Metaloproteinaza 2
MTT	bromek 3-(4,5-dimetyltiazol-2-ilo)-2,5-difenyloterazoliowy
NPs	nanocząstki
PEG	(*ang. poli (ethylene glycol*)), poli (tlenek etylenu)

PI	jodek propidyny
PT	czas protrombinowy
QCM-D	mikrowaga z kryształem kwarcu z monitorowaniem rozpraszania
ROS	(*ang. reactive oxygen species*), reaktywne formy tlenu
TEM	transmisyjny mikroskop elektronowy
tPa	tkankowy aktywator plazminogenu
TT	czas trombinowy
vWF	czynnik von Willebranda
WHO	światowa organizacja zdrowia
WP	(*ang. washed platelets)* myte płytki krwi
ŻChZZ	żylna choroba zakrzepowo-zatorowa

II. WPROWADZENIE

Według Światowej Organizacji Zdrowia (WHO) choroby układu krążenia (CVD) stanowią jedną z głównych przyczyn zgonów na świecie. CVD charakteryzują się zaburzeniami pracy serca i naczyń krwionośnych, obejmując zawał mięśnia sercowego, udar oraz zakrzepicę żył głębokich [1]. Patogeneza zawału mięśnia sercowego, żylnej choroby zakrzepowo-zatorowej (ŻChZZ) oraz udaru niedokrwiennego polega na powstawaniu w naczyniu krwionośnym zakrzepu, który może prowadzić do ograniczenia lub całkowitego zablokowania jego światła, a tym samym przepływu krwi [2]. W przebiegu tego schorzenia istnieje również możliwość oderwania się fragmentu skrzepu od ściany żyły. Skrzep wraz z krwią przedostając się do narządów wewnętrznych może doprowadzić do niedokrwienia i niedotlenienia lokalnych tkanek, prowadząc do bólu w klatce piersiowej lub objawów neurologicznych. W każdym przypadku nieleczenie tych sytuacji może prowadzić do poważnych powikłań, które mogą zakończyć się śmiercią pacjenta [3].

W trakcie uszkodzenia naczynia system hemostatyczny inicjuje zdarzenia naczyniowe, przywraca równowagę hemostatyczną i zapobiega patologicznemu tworzeniu zakrzepu [4]. Hemostaza to ściśle regulowany proces, który zapewnia integralność naczyń krwionośnych. Polega na oddziaływaniach między płytkami krwi, białkami krzepnięcia i fibrynolizy oraz komórkami śródbłonka. Początkowo uważano, że śródbłonek stanowi prostą pasywną barierę między przedziałem wewnątrznaczyniowym i pozanaczyniowym, ale obecnie jego funkcja ma kluczowe znaczenie dla utrzymania naczyń. Odpowiednie napięcie naczyń krwionośnych jest możliwe za pośrednictwem wydzielanych przez śródbłonek przekaźników zarówno o działaniu wazodylatacyjnym jak i wazokonstrykcyjnym. Ważną rolę w tym procesie odgrywa tlenek azotu, który odpowiada za utrzymanie stanu wazorelaksacyjnego w naczyniu. Głównym mechanizmem działania tlenku azotu jest aktywacja cyklazy guanylowej, która za jego pomocą pobudza cykliczny guanozyno 4’5’monofosforan (cGMP). Wzrost cGMP, pełniącego funkcję przekaźnika II rzędu, prowadzi do zwiększenia aktywności fosfatazy w komórkach mięśni gładkich, która następnie hydrolizuje miozynę. Proces defosforylacji miozyny skutkuje inaktywacją łańcuchów regulatorowych oraz zahamowaniem ich oddziaływania z aktyną, co w konsekwencji prowadzi do rozkurczu naczynia krwionośnego [5]. Tlenek azotu wraz z innymi

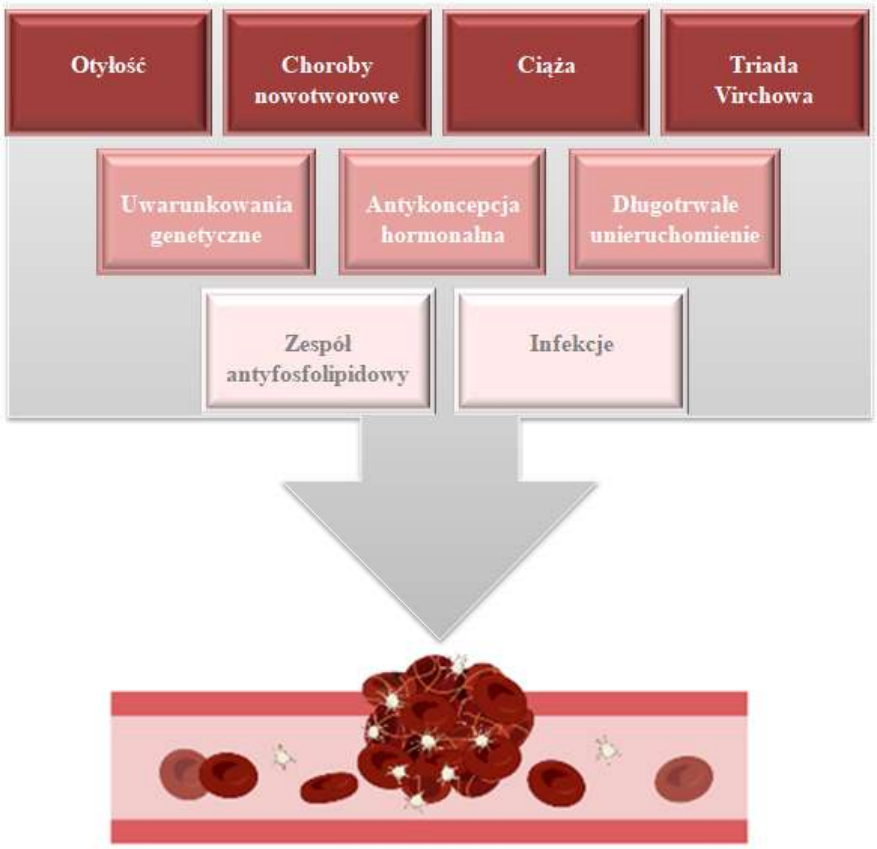
mediatorami wydzielanymi przez komórki śródbłonka przyczynia się również do zapobiegania agregacji płytek krwi i tworzenia fibryny.

Komórki śródbłonka wydzielają czynniki przeciwpłytkowe i przeciwzakrzepowe, które zapobiegają agregacji płytek i tworzeniu się fibryny [6]. Dysfunkcja śródbłonka występuje w wyniku procesu patologicznego, który może prowadzić do zwiększonej adhezji płytek, agregacji, wytwarzania fibryny i tworzenia skrzepliny. Ponadto, w trakcie jego uszkodzenia dochodzi do zwężenia naczyń krwionośnych, uwalniania czynnika von Willebranda (vWF) i wzrostu fibrynogenu oraz zmniejszenia aktywności tkankowego aktywatora plazminogenu (tPA). Nienaruszony śródbłonek hamuje adhezję płytek poprzez uwalnianie tlenku azotu i prostaglandyny I_2, podczas gdy aktywowane komórki śródbłonka wydzielają różne cząsteczki, które zwiększają adhezję i agregację płytek krwi do miejsca uszkodzenia [7].

Płytki krwi odgrywają znaczącą rolę w procesie hemostazy pierwotnej, podczas której dochodzi do wytworzenia czopu płytkowego. Proces ten rozpoczyna adhezja płytek krwi do miejsca, w którym przerwana została ciągłość naczynia krwionośnego. W wyniku urazu odsłonięte zostają włókna kolagenu, z którymi wiąże się kompleks receptorów Ib/V/IX obecny na powierzchni płytek krwi [8]. Adhezja płytek krwi do uszkodzonego śródbłonka jest wzmacniana przez vWF, który tworzy wiązanie z glikoproteiną Ib. Płytki krwi przylegające w miejscu uszkodzeniu ulegają jednocześnie aktywacji, która prowadzi do degranulacji i uwolnienia substancji biologicznie aktywnych, magazynowanych w ziarnistościach [9]. Formowanie czopów płytkowych z dyskoidalnych płytek jest zależne od prędkości ścinania krwi. Podczas początkowego etapu kluczową rolę w tym procesie odgrywają wypustki błonowe, które umożliwiają przyleganie do siebie płytek. Są to odcinki dwuwarstwy lipidowej w kształcie cylindrów, które powstają na powierzchni płytek w wyniku działania oporu hemodynamicznego, natomiast II faza polega na zmianie kształtu i uwolnieniu ziarnistości z płytek krwi przy udziale odpowiednich agonistów tj. ADP, trombiny i tromboksanu TXA_2 [10]. Agregacja płytek krwi, która ma na celu wytworzenie czopu płytkowego zamykającego uszkodzone naczynie krwionośne, jest również głównym czynnikiem patofizjologicznym w rozwoju zatorów tętniczych, w tym choroby wieńcowej, incydentów naczyniowo-mózgowych, choroby tętnic obwodowych w cukrzycy, chorobach nerek, nowotworach, chorobie Alzheimera i CVD. Zawartość ziarnistości mogą stanowić: białka adhezyjne (vWF, fibrynogen, fibronektyna, trombospondyna), czynniki krzepnięcia V i XI, inhibitory fibrynolizy (inhibitor

aktywatora plazminogenu, inhibitor α-plazminy), chemokiny (czynnik płytkowy 4 i β-tromboglobulina), P-selektyna, receptory dla glikoproteiny (GP) GPIb (CD42b-c) i GPIIb/IIa (CD41/CD61) oraz inne składniki, takie jak: czynniki leukotaktyczne, mitogeny oraz czynniki naczynioruchowe warunkujące przepuszczalność naczyń [11].

W połowie XIX wieku Rudolf Virchow postulował, że uszkodzenie ściany naczynia, zastój żylny oraz zmiany w składzie krwi sprzyjają powstawaniu skrzepów. Zespół tych trzech czynników został nazwany Triadą Virchowa, które leżą u podłoża rozwoju zakrzepicy [12]. Większości stanów klinicznych obarczonych ryzykiem rozwoju zakrzepicy żył głębokich można przypisać jeden lub więcej elementów tej triady. Do innych czynników ryzyka należą: uwarunkowania genetyczne, choroby nowotworowe, zespół antyfosfolipidowy, ciąża, długotrwałe unieruchomienie, otyłość, stosowanie antykoncepcji hormonalnej lub zakażenia (Rycina 1) [5–8, 13].



Rycina 1. *Czynniki wpływające na rozwój zakrzepicy żył głębokich*

Zastosowanie długotrwałych cewników i portów dożylnych znacznie ułatwia leczenie pacjentów przewlekle chorych, ponieważ umożliwiaj opiekę paliatywną, chemioterapie, transfuzje, żywienie pozajelitowe i pobieranie próbek krwi [14,15]. Według badań przeprowadzonych na oddziałach intensywnej terapii w Stanach Zjednoczonych

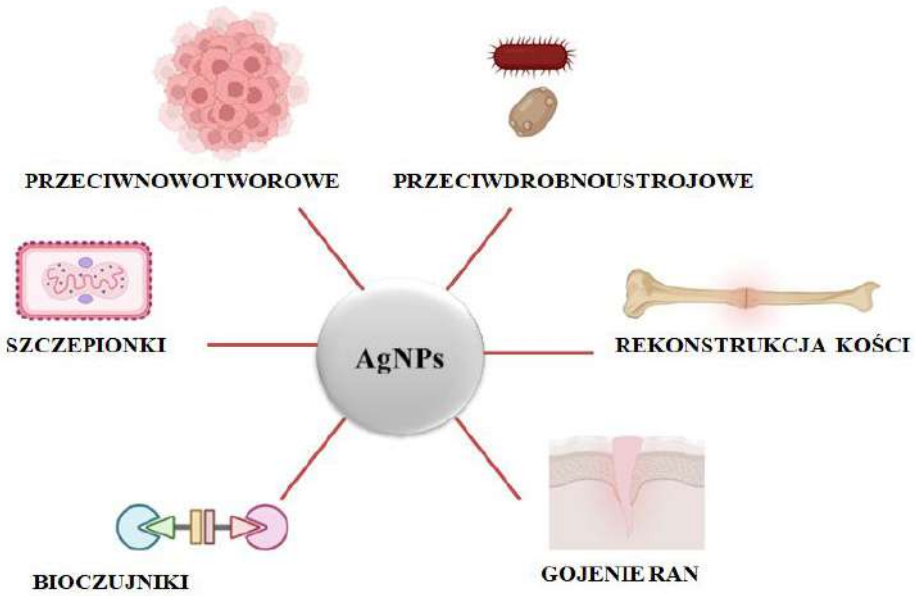
wykazano, że 87% pierwotnych infekcji krwi było związanych z założeniem cewników dożylnych. Ponadto, ich długotrwałe stosowanie, zwłaszcza w przebiegu chemioterapii może wiązać się z występowaniem zakrzepicy żylnej kończyny górnej [16]. Należy również podkreślić, iż zakażenia dróg oddechowych, dróg moczowych, skóry, jamy brzusznej i zakażenia bakteryjne rozpoznane w szpitalu lub leczone poza szpitalem wiązały się z ponad dwukrotnym wzrostem ryzyka ŻChZZ [17]. Nie ulega zatem wątpliwości, że opracowanie nowych metod zapobiegania powstawaniu zakrzepów krwi związanych z chorobami zakaźnymi ma ogromne znaczenie, ponieważ może znacznie zmniejszyć zachorowalność, śmiertelność i koszty opieki zdrowotnej.

Postęp w poszukiwaniu środków przeciwkrzepliwych to ogólnoświatowe wyzwanie medyczne. Wyróżnia się trzy grupy leków: leki przeciwpłytkowe, antykoagulanty i fibrynolityczne. W wielu zastosowaniach klinicznych istnieje potrzeba optymalizacji skuteczności i bezpieczeństwa tych leków, głównie u pacjentów z wysokim ryzykiem krwawienia i zakrzepicy. Wykazano, że ryzyko krwawienia może być większe u pacjentów przyjmujących leki hamujące hemostazę osoczową i płytkową. Ograniczeniem dla wszystkich leków przeciwzakrzepowych jest brak zdolności do zapobiegania lub odwracania zakrzepicy bez zakłócania hemostazy. Innym problemem jest gromadzenie się w tkankach i krótki okres półtrwania w wyniku ich enzymatycznej degradacji, co zmniejsza ich stężenie we krwi, a następnie efekt farmakologiczny. W związku z tym, istotne jest poszukiwanie nowych metod terapeutycznych w celu poprawy działania dostępnych substancji leczniczych [18–21].

Nanobiomateriały dzięki unikalnym właściwościom nanocząstek (NPs) mogą mieć zastosowanie w profilaktyce i leczeniu chorób człowieka. Do końca 2021 roku w bazie PubMed indeksowano ponad 320 000 artykułów o tej tematyce [22]. Nanomedycyna powstała poprzez zastosowanie nanotechnologii w aplikacjach medycznych dzięki użyciu wszelkiego rodzaju materiałów w skali nano [23]. NPs to cząstki o wielkości w zakresie od 1 do 100 nm. Właściwości NPs można modyfikować, zmieniając ich właściwości fizykochemiczne i powierzchniowe, rozmiary, kształty, strukturę i morfologie [24]. Ich korzyści wynikają z wysokiego stosunku pola powierzchni do objętości, zdolności penetrowania przez błony komórkowe i bariery biologiczne oraz wysokiej reaktywności. Funkcjonalizacja NPs to modyfikacja ich powierzchni, funkcyjnych, połączonych wiązaniami kowalencyjnymi. Proces ten wpływa na zwiększenie biokompatybilności, zmniejszenie cytotoksyczności i zwiększenie aktywności przeciwdrobnoustrojowej. Do powierzchni NPs można

przyłączyć leki, przeciwciała, kwasy nukleinowe, białka, czy substancje organiczne takie jak poli(tlenek etylenu), (PEG) lub zredukowany glutation (GSH) [25–28].

Dostępne dane literaturowe szeroko opisują właściwości przeciwdrobnoustrojowe i przeciwnowotworowe AgNPs. Mechanizm tego działania związany jest między innymi z uszkodzeniem struktur komórkowych, generowaniem ROS (Reaktywnych Form Tlenu), uszkodzeniem DNA (kwasu deoksyrybonukleinowego), inaktywacją enzymów i denaturacją białek [22, 29–32]. Oprócz właściwości przeciwdrobnoustrojowych i przeciwnowotworowych, AgNPs mogą być również wykorzystane w innych zastosowaniach medycznych. Według danych literaturowych wykazują aktywności, które pozwalają na ich potencjalne wykorzystanie w rekonstrukcji kości, gojeniu ran, jako adiuwant w szczepionce lub bioczujniki do wykrywania glukozy we krwi, enzymów, markerów molekularnych komórek nowotworowych oraz patogenów (Ryc. 2)[28–32].



Rycina 2. *Zastosowania oraz potencjalne wykorzystanie medyczne właściwości nanocząstek srebra*

Dotychczasowe dane literaturowe nie pozwalają w sposób jednoznaczny określić wpływu funkcjonalizowanych AgNPs na układ hemostazy. NPs po dostaniu się do krążenia, natychmiast trafiają na komórki krwi, białka i komórki śródbłonka, a także kluczowe elementy układu krzepnięcia, takie jak: płytki krwi i czynniki krzepnięcia osocza. Niepożądane właściwości pro- i antykoagulacyjne NPs stanowią obawy

w dziedzinie nanomedycyny. AgNPs powlekane i niepowlekane PEGiem mogą wykazywać właściwości przeciwpłytkowe w zależności od użytego agonisty [33, 34]. Z drugiej strony, dostępne wyniki potwierdzają indukowanie agregacji płytek krwi w sposób zależny od stężenia i wielkości [35]. Rageem i wsp. wykazali również, że AgNPs nie wpływają na ekspresję czynników trombogennych [36]. Dane literaturowe są niejednoznaczne, gdyż dowodzą, że AgNPs przy wysokich stężeniach mogą zwiększać ekspresję vWF i zmniejszać ekspresję tPA, co sugeruje działanie protrombogenne [37–39].

Połączenie AgNPs z innymi związkami (antybiotykami i związkami przeciwnowotworowymi lub przeciwwirusowymi) prowadzi do efektu synergicznego, który może potencjalnie pomóc w opracowaniu nowych bardziej skutecznych i bezpiecznych opcji terapeutycznych. W związku z tym, interesujące są szczegółowe badania na temat wpływu AgNPs na elementy układu hemostazy w celu ustalenia ich potencjału farmakologicznego i terapeutycznego.

III. CELE PRACY

Cel główny: Synteza funkcjonalizowanych nanocząstek srebra oraz określenie ich interakcji z układem hemostazy i działania przeciwdrobnoustrojowego na modelu *in vitro* i *ex vivo*.

Cele szczegółowe:

1) Podsumowanie najnowszych badań *in vitro* i *in vivo* związanych z lekami opartymi na nanocząstkach oraz korzyści terapeutycznych nanoleków stosowanych w chorobach układu krążenia oraz chorobach nowotworowych w kontekście angiogenezy.
2) Ocena wpływu funkcjonalizacji nanocząstek srebra kwasem liponowym na ich biokompatybilność względem komórek naczyń śródbłonka, płytek krwi i erytrocytów.
3) Ocena wpływu funkcjonalizacji nanocząstek srebra kwasem liponowym, gluationem oraz glikolem polietylenowym na agregację płytek krwi.
4) Ocena wpływu nanocząstek srebra połączonych z eptyfibatydem na agregacje płytek krwi oraz czynniki aktywacji naczyń śródbłonka związane z układem krzepnięcia.

IV. MATERIAŁY I METODY

Nanocząstki

Nanocząstki srebra funkcjonalizowane kwasem liponowym, glutationem oraz glikolem polietylenowym zostały zsyntezowane przez zespół z Uniwersytetu Warszawskiego pod kierunkiem dr. Michała Wójcika.

Niepowlekane nanoczastki srebra zostały zakupione z US Research Nanomaterials, Inc. (Houston, Teksas).

Nanocząstki srebra funkcjonalizowane eptyfibatydem zostały zsyntetyzowane przez zespół z Uniwersytetu Warszawskiego pod kierunkiem dr hab. Elżbiety Megiel, prof. UW.

Hodowle komórkowe

W badaniach wykorzystano linie komórkowe: ludzkie komórki śródbłonka żyły pępowinowej (HUVEC), ludzkie komórki fibroblastów dziąseł (HGF-1), unieśmiertelnioną ludzką linie komórek śródbłonka mikronaczyniowego (HMEC-1). Komórki pochodziły z Merck (Sigma Aldrich) i American Type Culture Collection (ATCC). Hodowla komórkowa była prowadzona w standardowych warunkach. [37, 38].

Izolacja płytek krwi i erytrocytów

Materiał badawczy stanowiły płytki krwi oraz erytrocyty wyizolowane z krwi obwodowej pobranej od zdrowych ochotników, którzy w okresie 2 tygodni przed badaniem nie przyjmowali leków, mających udowodnione działanie wpływające na funkcję płytek. Wszyscy ochotnicy wyrazili świadomą zgodę przed pobraniem, badanie zostało zatwierdzone przez Niezależną Komisję Bioetyczną ds. Badań Naukowych Gdańskiego Uniwersytetu Medycznego (NKBBN/552/2018-2022) oraz przez Komisję ds. Etyki - Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, The University of Dublin, Irlandia (MS 2016-03-01 i 2020-06-01). Badania przeprowadzano również zgodnie z Kodeksem Etyki Światowego Towarzystwa Lekarskiego, normami etycznymi właściwej komisji ds. Eksperymentów na ludziach (instytucjonalnymi i krajowymi) oraz Deklaracją Helsińską z 1975 r. z późniejszymi zmianami w 2000 r. Do oznaczenia testu dehydrogenazy mleczanowej (LDH), agregometrii świetlnej,

mikrowagi z kryształem kwarcu z rozpraszaniem energii (QCM-D) oraz cytometrii przepływowej krew pobrano na 3,15% cytrynian trisodowy (9:1, v:v). Zawiesinę mytych płytek krwi (WP) izolowano z krwi, i zawieszano w buforze Tyrode'a w końcowym stężeniu 250 000 płytek/μL [39]. Do badań układu koagulologii, pobrano 3 mL krwi do probówki zawierającej 3,8% cytrynian sodu i przygotowywano zgodnie z zaleceniami referencyjnymi dla każdej metody.

Ocena biokompatybilności nanocząstek sreba

Do oceny potencjalnej cytotoksycznego NPs wobec ludzkich komórek naczyń śródbłonka, płytek krwi oraz erytrocytów użyto odpowiednio testu opartego na pomiarze aktywności metabolicznej komórek MTT oraz integralności błony komórkowej LDH. Absorbancje została mierzona za pomocą czytnika płytek (FLUO star, OPTIMA).

Ocena indukowania zmian przez nanocząstki na poziomie ultrastrukturalnym komórki

Zdolność przenikania przez błonę komórkową, lokalizację na poziomie komórkowym oraz indukowanie zmian na poziomie ultrastrukturalnym przez NPs określono za pomocą Transmisyjnej Mikroskopii Elektronowej (TEM, Tecnai G2Spirit BioTWIN).

Ocena poziomu wewnątrzkomórkowych reaktywnych form tlenu

Poziom wewnątrzkomórkowych reaktywnych form tlenu (ROS) oceniono przy użyciu cytometrii przepływowej (BD FACSArray, Biosciences, San Jose, CA) z dwuoctananem 2,7 – dichlorofluoresceiny (DCF-DA) i analizowano przy użyciu programu CellQuest Pro.

Ocena apoptozy/nekrozy

Do pomiaru apoptozy/nekrozy użyto cytometrii przepływowej z zastosowaniem aneksyny V oraz jodku propidyny (PI) i analizowano przy użyciu programu CellQuest Pro.

Pomiar destabilizacji potencjału mitochondrialnego

Pomiar destabilizacji potencjału mitochondrialnego wykonano za pomocą cytometrii przepływowej z wykorzystaniem barwnika fluoroscencyjnego JC-1. Uzyskane dane analizowano przy użyciu oprogramowania CellQuest Pro. Do analizy mikroskopowej użyto mikroskopu fluorescencyjnego (Olympus Life Science).

Ocena ekspresji P-selektyny i glikoproteiny GPIIb/IIIa

Do analizy ekspresji receptorów P-selektyny i glikoproteiny GPIIb/IIIa wykorzystano cytometrię przepływową z zastosowaniem odpowiednich przeciwciał. Wyniki analizowano przy użyciu oprogramowania CellQuest Pro.

Pomiar agregacji płytek krwi za pomocą agregometrii świetlnej

Pomiar agregacji płytek krwi indukowany kolagenem mierzono w dwukanałowym agregometrze świetlnym (CHRONO-LOG-700).

Pomiar agregacji płytek krwi za pomocą mikrowagi z kryształem kwarcu z monitorowaniem rozpraszania energii

Do pomiaru agregacji płytek krwi w warunkach imitujących mikrośrodowisko naczyniowe wykorzystano mikrowagę z kryształem kwarcu z monitorowaniem rozpraszania energii QCM-D (Q-Sense AB, E4 QCM-D system).

Ocena TXB2, MMP-1, MMP-2, 6-keto-PGF1alpha, tPa, vWf, and cGMP

Za pomocą komerycjnie dostępnych testów ELISA (Cayman, Abcam and BioFine) określono poziom parametrów z zakresu układu hemostazy: przeciwzakrzepowych (6-keto-PGF1alpha cGMP, przeciwpłytkowych (TXB, MMP-1 i MMP-2), fibrynolitycznych (tPa) oraz marker uszkodzenia naczyń komórek śródbłonka (vWF).

Analiza parametrów z zakresu koagulologii

Parametry związane z układem koagulologii: PT, APTT, TT INR, fibrynogen, D-dimery oraz CT ocenione zostały w akredytowanym laboratorium szpitalnym (Centralne Laboratorium Kliniczne Uniwersyteckiego Centrum Klinicznego w Gdańsku) przy użyciu analizatora BCS-XP (Siemens Healthcare) oraz Innovate PFA-200 system (Siemens Healthcare).

Analiza statystyczna

Analiza statystyczna została przeprowadzona przy użyciu jednoczynnikowej analizy wariancji (one-way ANOVA) i testu post-hoc Tukey'a. Analizę statystyczną przeprowadzono w programie GraphPad Prism (przedział ufności = 95%, α=0,05).

V. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

W ramach mojej rozprawy doktorskiej przygotowałam jako współautor 4 prace (trzy oryginalne i jedną poglądową) opublikowane w międzynarodowych czasopismach indeksowanych na Liście Filadelfijskiej. Artykuły skupiają się na tematyce wpływu funkcjonalizacji AgNPs na ich właściwości przeciwbakteryjne, cytotoksyczne, działanie przeciwpłytkowe, przeciwzakrzepowe i fibrynolityczne w kierunku ich potencjalnego wykorzystania jako platform dostarczania leków w chorobach układu krążenia.

Publikacja 1

Hajtuch J, Niska K, Inkielewicz-Stępniak I. *Nanodrugs as a new approach in therapy of cardiovascular diseases and cancer with tumor-associated angiogenesis*. Curr. Med.Chem. 2021; 28(27)):5527-5550. doi: 10.2174/0929867328666201231121704

Choroby nowotworowe wraz z CVD definiowane są jako główna przyczyna zgonów na całym świecie [40]. Badania wskazują, że terapie onkologiczne, a zwłaszcza chemioterapia wiąże się ze zwiększonym ryzykiem wystąpienia CVD [16]. Co ważne, niektóre czynniki ryzyka, jak nadciśnienie, cukrzyca lub palenie tytoniu są wspólne dla CVD i chorób nowotworowych [41]. Istotną rolę w progresji nowotworowej odgrywa proces angiogenezy i agregacji płytek [42].

W niniejszej publikacji przeglądowej przedstawiono najbardziej aktualne badania *in vitro* i *in vivo* związane z lekami opartymi na NPs oraz korzyści terapeutyczne potencjalnych nanoleków stosowanych w chorobach układu krążenia oraz chorobach nowotworowych w kontekście angiogenezy. Przeprowadzona analiza dostępnych wyników potwierdza ich ulepszone właściwości farmakologiczne i biologiczne w porównaniu do klasycznych leków, dające obiecujące działanie przeciwpłytkowe, przeciwkrzepliwe i antyangiogenne. Ponadto, w przeglądzie uwzględniono NPs z chemioterapeutykami zatwierdzonymi przez Agencję Żywności i Leków (FDA- Food and Drug Administration).

Mając na uwadze optymalizację wielkości NPs i ich właściwości, nanotechnologia stwarza możliwość dostarczenia leków o zwiększonej biodostępności i skuteczności terapeutycznej w leczeniu CVD, nowotworów i angiogenezy.

Publikacja 2

Hajtuch J, Hante N, Tomczyk E, Wójcik M, Radomski MW, Santos Martinez MJ, Inkielewicz-Stępniak I. *Effects of functionalized silver nanoparticles on aggregation of human blood platelets.* Int. J. Nanomed. 2019; 14. 7399–7417. https://doi.org/10.2147%2FIJN.S213499

Bazując na danych literaturowych oraz naszych wcześniejszych wynikach, dotyczących wpływu funkcjonalizacji na cytotoksyczność NPs oraz ich działania przeciwpłytkowego postanowiliśmy określić interakcję pomiędzy AgNPs i układem hemostazy w zależności od ich rozmiaru, właściwości fizykochemicznych i funkcjonalizacji w aspekcie działania przeciwzakrzepowego [27, 34, 36, 43].

Zsyntetyzowano AgNPs funkcjonalizowane glikolem polietylenowym (AgNPs-PEG), kwasem liponowym (AgNPs-LA) oraz zredukowanym glutationem (AgNPs-GSH), następnie oceniliśmy ich wpływ na agregację płytek krwi w warunkach przepływu, które odzwierciedlają mikrośrodowisko naczyniowe *in vivo*. Do pomiaru agregacji wykorzystano mikrowagę kryształu kwarcu z rozpraszaniem energii (QCM-D). Oceniono również potencjał bezpieczeństwa zsyntetyzowanych AgNPs względem komórek HUVEC, HGF-1 i płytek krwi za pomocą testów MTT i LDH. Ponadto, skupiono się na poznaniu molekularnych mechanizmów oddziaływań pomiędzy płytkami krwi a NPs, w tym określeniu receptorów płytkowych wykorzystując cytometrię przepływową. Do pomiaru poziomu tromboksanu B2 (TXB2) i metaloproteinaz (MMP-1, MMP-2) uwalnianych przez płytki krwi jako markerów aktywacji płytek wykorzystano dostępne na rynku testy ELISA.

Wyniki przedstawione w publikacji potwierdzają, że 2 nm AgNPs-GSH, 3,7 nm AgNPs-PEG zarówno w stężeniu 50 i 100 μg/ml, jak i 2,5 nm AgNPs-LA w stężeniu 100 μg/ml zmniejszały agregację płytek krwi, hamowały wzrost ekspresji P-selektyny i glikoproteiny GPIIb/IIIa indukowanych kolagenem, tworzenie TXB_2 oraz uwalnianie MMP-1 i MMP-2. Funkcjonalizowane AgNPs, w badanych stężeniach, nie wpływały na żywotność płytek krwi, komórek śródbłonka i fibroblastów.

Podsumowując, funkcjonalizowane AgNPs hamowały agregację płytek krwi w niecytotoksycznych stężeniach. Zsyntetyzowane przez nas AgNPs mogą mieć zastosowanie jako środek przeciwpłytkowy lub materiał przy projektowaniu

i wytwarzaniu urządzeń medycznych mających kontakt z krwią, takich jak cewniki naczyniowe, stenty i zastawki serca.

Publikacja 3

Hajtuch J, Santos-Martinez MJ, Wójcik M, Tomczyk E, Jaśkiewicz M, Kamysz W, Narajczyk M, Inkielewicz-Stępniak I. *Lipoic acid-coated silver nanoparticles: biosafety potential on the vascular microenvironment and antibacterial properties*; Front. Pharmacol. 2022; 12:733743 https://doi.org/10.3389/fphar.2021.733743

Poszukiwanie nowych środków leczniczych w chorobach układu krążenia to ogólnoświatowe wyzwanie medyczne. Szacuje się, że około 30% wszystkich bakteriemii szpitalnych związanych jest ze stosowaniem urządzeń wewnątrznaczyniowych, takich jak cewniki dożylne, które również przyczyniają się do powstania zakrzepów. Modyfikacja powierzchni NPs obejmuje procesy, które mogą skutkować bardziej pożądanymi właściwościami NPs, w tym zmniejszeniu cytotoksyczności [44]. Z uwagi na aktywność przeciwdrobnoustrojową oraz przeciwpłytkową AgNPs, mogą być wykorzystane do powlekania urządzeń medycznych [22, 37].

W związku z tym postanowiono porównać właściwości przeciwbakteryjne powlekanych kwasem liponowym nanocząstek srebra (AgNPsLA) i niepowlekanych nanocząstek srebra (AgNPs) oraz ocenić potencjalne efekty cytotoksyczne w mikrośrodowisku naczyń krwionośnych. W badaniach wykorzystano dostępne komercyjnie AgNPs (2,6 nm) i AgNPsLA (2,5 nm) zsyntetyzowane i scharakteryzowane w sposób opisany wcześniej przez nasz zespół [45]. Aktywność przeciwdrobnoustrojowa została oceniona na referencyjnych szczepach bakterii należących do patogenów bakteryjnych powszechnie kojarzonych z opornością na dostępne środki przeciwdrobnoustrojowe (ESKAPE) - *Enterococcus faecium* (ATCC 700221), *Staphylococcus aureus* (ATCC 33591), *Klebsiella pneumoniae* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella aerogenes* (ATCC 13048) oraz *Enterobacter aerogenes* (ATCC 130480) i wyrażona przez minimalne stężenia hamujące (MIC). Śródbłonek naczyniowy jest zaangażowany w procesy fizjologiczne i patologiczne, takie jak hemostaza i zakrzepica oraz zapalenie i przebudowa ściany naczynia[4]. Dlatego, ocenę cytotoksyczności przeprowadzono na ludzkich komórkach śródbłonka żyły

pępowinowej (HUVEC) za pomocą testu MTT. Poziom RFT, apoptozy i nekrozy komórek HUVEC oraz pomiar destabilizacji mitochondriów w HUVEC i płytkach krwi przeprowadzono metodą cytometrii przepływowej. Potencjalny cytotoksyczny wpływ nanocząstek na erytrocyty zbadano, mierząc poziom hemoglobiny i LDH uwalnianych po ekspozycji na AgNPs oraz AgNPsLA. Za pomocą TEM określono, czy AgNPs i AgNPsLA mogą indukować zmiany ultrastrukturalne w komórkach HUVEC i bakteriach *Staphylococcus aureus*.

Uzyskane wyniki potwierdziły, że AgNPs i AgNPsLA mają właściwości przeciwdrobnoustrojowe przeciwko patogenom związanym z zakażeniami krwi towarzyszącymi umieszczeniu cewników lub portów w naczyniu. AgNPsLA, w przeciwieństwie do AgNPs nie indukowały wewnątrzkomórkowej produkcji ROS i apoptozy w HUVEC, zmian ultrastrukturalnych w HUVE depolaryzację błony mitochondrialnej w HUVEC i płytkach krwi oraz hemolizy. Co ważne, wykazano że funkcjonalizacja AgNP za pomocą LA utrzymuje znaną aktywność przeciwbakteryjną dla AgNP.

Podsumowując, AgNPsLA zsyntetyzowane przez naszą grupę mają aktywność przeciwdrobnoustrojową i lepszy profil bezpieczeństwa biologicznego w porównaniu do niepowlekanych AgNPs o podobnej wielkości. Obserwacje te są istotne dla przyszłych badań *in vivo* i potencjalnego zastosowania AgNPsLA w wyrobach medycznych stosowanych u pacjentów.

Publikacja 4

Hajtuch J, Iwicka E, Szczoczarz E, Flis, D, Megiel E, Cieciórski P, Radomski MW, Santos-Martinez MJ, Inkielewicz-Stępniak I. *The pharmacological effects of silver nanoparticles functionalized with eptifibatide on platelets and endothelial cells*. Int. J. Nanomed. 2022. 17:4383-4400. doi: 10.2147/IJN.S373691

W skład mojej rozprawy doktorskiej wchodzą badania dotyczące połączenia AgNPs z eptyfibatydem, syntetycznym cyklicznym heptapeptydem, który w sposób odwracalny wiąże się z płytkowym receptorem GPIIb/IIIa, powodując krótkotrwałą blokadę receptora i hamowanie agregacji płytek.

Zsyntetyzowano i scharakteryzowano sferyczne AgNPs połączone z eptifibatydem (EPI). Oceniliśmy biokompatybilność AgNPs-EPI w komórkach śródbłonka mikronaczyniowego (HMEC-1), płytkach krwi i erytrocytach. Wpływ AgNPs-EPI na agregację płytek krwi mierzono metodą agregometrii świetlnej,

parametry z zakresu koagulologii *ex vivo* określono referencyjnymi metodami laboratoryjnymi. Za pomocą cytometrii przepływowej określono wpływ AgNPs-EPI na poziom receptorów - selektyny P i GPIIb/IIIa na powierzchni płytek krwi. Poziom 6-keto-PGF1alfa (stabilnego metabolitu prostacykliny), tkankowego aktywatora plazminogenu (tPa), czynnika von Willebranda (vWF) i cyklicznego monofosforanu guanozyny (cGMP) mierzono za pomocą testów ELISA.

Zsyntetyzowane AgNPs-EPI nie wpływały na żywotność płytek krwi, komórek śródbłonka i erytrocytów. AgNPs-EPI hamowały indukowaną kolagenem agregacją płytek krwi oraz ekspresję selektyny P i zmiany konformacyjne GPIIb/IIIa w stężeniach niższych niż sam lek. AgNPs-EPI powodował wydłużenie czasu okluzji w obecności kolagenu/ADP i kolagenu/adrenaliny, nie wpływając znacznie na pozostałe czynniki z zakresu koagulologii: PT, APTT, TT INR, fibrynogen, D-dimery oraz CT. AgNPs-EPI regulowały poziom 6-keto-PGF1alfa, tPa, vWf i cGMP w stymulowanych trombiną komórkach HMEC-1.

Podsumowując, AgNPs-EPI wykazują aktywność przeciwagregacyjną w stężeniach niższych niż sam lek, działając poprzez regulację agregacji płytek krwi, krzepnięcia krwi i aktywność komórek śródbłonka. Wyniki potwierdzają, że AgNPs stanowią potencjał do wykorzystywania jako skuteczna platforma dostarczania leków przeciwpłytkowych.

VI. PODSUMOWANIE

Osiągnięcia z dziedziny nanotechnologii znalazły szerokie zastosowanie w medycynie jako urządzenia medyczne, substancje terapeutyczne, materiały opatrunkowe czy platformy dostarczania leków [46]. Ze względu na wszechstronne właściwości AgNPs są wykorzystywane w leczeniu, profilaktyce i diagnostyce chorób.

Badania przeprowadzone w ramach mojej pracy doktorskiej wykazały, że funkcjonalizacja AgNPs wpływa na ich biokompatybilność względem komórek naczyń śródbłonka, płytek krwi i erytrocytów. Zsyntetyzowane przez nasz zespół AgNPs mają zdolność hamowania agregacji płytek krwi i właściwości przeciwbakteryjne.

Określiliśmy mechanizmy tych właściwości, co jest istotne dla zrozumienia interakcji między AgNPs i płytkami krwi. W artykule przeglądowym, uwzględniając najnowsze osiągnięcia naukowe, omówiliśmy informacje na temat najbardziej aktualnych badań *in vitro* i *in vivo* związanych z lekami opartymi na NPs, korzyści terapeutycznych potencjalnych nanoleków stosowanych w chorobach układu krążenia oraz chorobach nowotworowych.

W naszych badaniach po raz pierwszy, zsyntetyzowaliśmy nowy koniugat AgNPs z eptifibatydem. Analiza profilu farmakologicznego *in vitro* wykazała, że jest on skuteczniejszy w hamowaniu agregacji płytek krwi i aktywacji śródbłonka indukowanej trombiną w stężeniach niższych niż sam lek. AgNPs w połączeniu z eptyfibatydem nie wpływają na żywotność komórek, co ma kluczowe znaczenie w zapobieganiu uszkodzeniom naczyń i utrzymaniu płynności krwi.

Wykazaliśmy, że dzięki unikalnym cechom AgNPs mogą być potencjalnymi substancjami terapeutycznymi lub służyć do opracowania biomateriałów. Uzyskane przez nas wyniki oraz zebrane dane literaturowe mogą okazać się pomocne w zaprojektowaniu modyfikowanych AgNPs, tak aby zmaksymalizować ich korzystne działania biologiczne i jednocześnie zachować profil bezpieczeństwa.

Dlatego, przyszłe badania powinny skupić się przede wszystkim na ich modyfikacji. AgNPs zasługują na dalsze badania w celu poszerzenia ich zastosowań w nanomedycynie, zatem zrównoważone i zaawansowane podejście do syntezy oraz badań biologicznych może stanowić realną alternatywę w medycynie.

SUMMARY IN ENGLISH

I. LIST OF ABBREVIATIONS

6-keto-PGF1-alpha	hydrolyses product of prostacyclin PGI_2
AgNPs	silver nanoparticles
AgNPs-EPI	silver nanoparticles functionalized with eptifibatide
AgNPs-GSH	silver nanoparticles functionalized with glutathione
AgNPs-LA	silver nanoparticles functionalized with lipoic acid
AgNPs-PEG	silver nanoparticles functionalized with polyethylene glycol
aPTT	activated partial thromboplastin time
ATCC	American Type Cell Culture
cGMP	cyclic guanosine-3 ′, 5′-monophosphate
CT	closure time
CVD	cardiovascular diseases
DCF-DA	2,7'-dichlorodihydrofluorescein diacetate
DNA	deoxyribonucleic acid
EPI	eptifibatide
ESKAPE	bacterial strains commonly associated with antimicrobial resistance
GPIIb/IIIa	glycoprotein IIb/IIIa
GSH	glutathione
HGF-1	human gingival fibroblast cells
HMEC-1	human microvascular endothelial cells
HUVEC	human umbilical vein endothelial cells
INR	international normalized ratio
LDH	lactate dehydrogenase
MIC	minimal inhibitory concentration
MMP-1	matrix metalloproteinase 1
MMP-2	matrix metalloproteinase 2
MTT	3- (4,5-dimethylthiazol-2-yl) -2,5-diphenylterazolium bromide
NPs	nanoparticles
PEG	poli (ethylene glycol)
PI	propidium iodide
PT	prothrombin time

QCM-D	quartz crystal microbalance with dissipation monitoring
RBCs	red blood cells
ROS	reactive oxygen species
TEM	transmission electron microscope
tPA	tissue plasminogen activator
WP	washed platelets
TT	thrombin time
VTE	venous thromboembolism
vWF	von Willebrand factor
WHO	World Health Organization

II. INTRODUCTION

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are one of the leading causes of death worldwide. By definition, CVDs are characterized by heart and/or blood vessel dysfunction, and include among others, myocardial infarction, stroke, and deep vein thrombosis [1]. Pathogenesis of myocardial infarction, venous thromboembolism (VTE), and ischemic stroke involves the formation of a thrombus in the blood vessel that can lead to a limitation or complete blockage of its lumen and therefore of the blood flow [2]. In fact, arterial thrombosis, such as for example during the development of coronary heart disease (angina, myocardial infaction) or ischemic stroke, can result in the development of ischemia and hypoxia in the local tissues leading to chest pain or neurological symptoms. On the other hand, when the thrombus is localized in a deep vein, as it happens during DVT, the thrombus can travel and lead to a pulmonary embolism. In any case, failure to treat those situations can derive in serious complications that may result in patient death [3].

During vessel damage, the hemostatic system initiates a series of vascular events, with the aim to restore the hemostatic balance and to prevent pathological thrombus formation [4]. Hemostasis involves interactions between platelets, endothelial cells, coagulation and fibrinolysis proteins, in a tightly regulated process that ensures the integrity of the blood vessels and preservation of adequate blood flow. Although the endothelium was originally thought to be a simple passive barrier between the intravascular and extravascular compartments, its function today is considered crucial for vascular maintenance. In fact, appropriate tone of the blood vessel is possible, among other factors, to the effect of mediators secreted by the endothelium with vasodilating and vasoconstrictive effects. An important player during this process is nitric oxide, which is responsible for inducing vasorelaxation. Nitric oxide mechanism of action depends on the activation of guanylate cyclase that increases cyclic guanosine 4'5' monophosphate (cGMP) and acting as a second transmitter, leads to an increase in the activity of phosphatase in smooth muscle cells, which then hydrolyzes myosin. The process of myosin dephosphorylation results in the inactivation of regulatory chains and the inhibition of their interaction with actin, which in turn leads to the relaxation of the blood vessel [5]. Nitric oxide, together with other mediators secreted by endothelial cells, also contributes to the prevention of platelet aggregation and fibrin formation [6]. Indeed, intact endothelium inhibits platelet adhesion by releasing nitric oxide and

prostaglandin I_2, while activated endothelial cells secrete molecules that promote adhesion and aggregation of platelets to the site of damage [7]. When endothelial dysfunction occurs as a result of a pathological process, increased platelet adhesion and aggregation, fibrin production, and thrombus formation takes place in the blood vessel. Indeed, platelets are the main players in the process of primary hemostasis, during which, together with the coagulation system (secondary hemostasis), the platelet plug is formed. This process begins with the adhesion of platelets to the site where the continuity of the blood vessel has been interrupted. As a result of the injury, collagen fibers are exposed, to which the glycoprotein (GP) Ib/V/IX receptor complex present on the surface of platelets binds [8]. The adhesion of platelets to the damaged endothelium is enhanced by von Willebrand factor (vWF), which binds to the platelet receptor GPIb. Platelets adhering to the damaged site are simultaneously activated, leading to degranulation and the release of biologically active substances stored in the cytoplasm. During the initial stages of platelet activation, membrane protrusions, that allow the platelets to adhere to each other, play a key role in the process of platelet plug formation. These are sections of the lipid bilayer in the shape of cylinders, which are formed on the platelet surface as a result of of the reorganization of the platelet cytoskeleton that contribute to platelet adhesion and spreading and in the presence of platelet agonists, i.e. ADP, thrombin and thromboxane (TX) A2, platelets not only change their shape but release their granules content [9]. Platelet aggregation, which aims to form a platelet plug at the damaged blood vessel, is also a major pathophysiological factor in the development of thrombus during coronary artery disease, ischemic cerebrovascular accidents, peripheral arterial disease in diabetes, kidney disease or cancer [10]. Granule's content includes adhesion proteins (vWF, fibrinogen, fibronectin, thrombospondin); coagulation factors V and XI; fibrinolysis inhibitors (plasminogen activator inhibitor, α-plasmin inhibitor); chemokines (platelet factor 4 and β-thromboglobulin); P-selectin, GPIb (CD42b-c) and GPIIb/IIa (CD41/CD61) receptors and other molecules such as leukotactic factors, mitogens and vasomotor factors that affect vascular permeability [11].

In the mid-nineteenth century, Rudolf Virchow postulated that damage to the vessel wall, venous stasis, and changes in the composition of the blood (coagulation) promote the formation of blood clots. The combination of these three factors has been called the Virchow Triad, which underlies the development of thrombosis [12]. In fact, most of the clinical conditions at risk of developing deep vein thrombosis can be attributed to

one or more elements of this triad. Other risk factors may include genetic predisposition, cancer, antiphospholipid syndrome, pregnancy, prolonged immobilization, obesity, use of hormonal contraception, or infections (Figure 1) [5–8, 13].

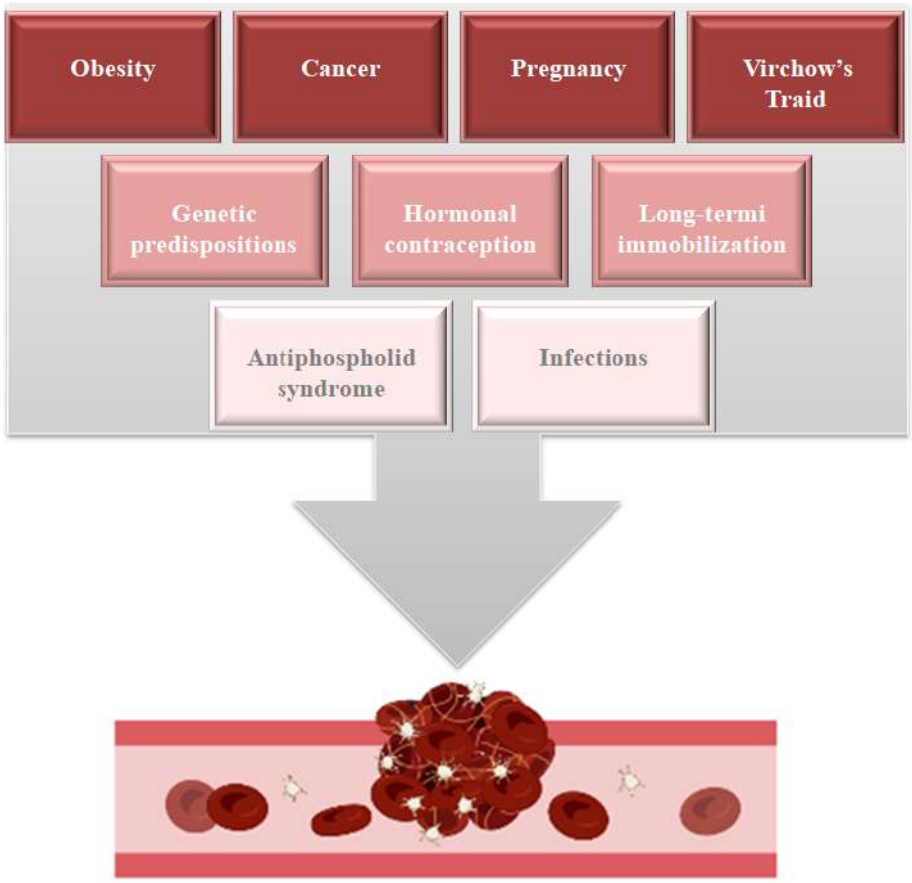


Figure 1. *Factors influencing the development of deep vein thrombosis*

The use of long-term catheters and intravenous ports significantly facilitates the treatment of chronically ill patients because they enable palliative care, chemotherapy, transfusions, parenteral nutrition and blood sampling [14, 15]. According to a study conducted in intensive care units in the United States, 87% of nosocomial infections are related to the use of intravenous catheter. In addition, their long-term use, especially during chemotherapy, may be associated with the occurrence of venous thrombosis of the upper limb [16]. It should also be emphasized that infections of the respiratory tract, urinary tract, skin and abdominal cavity have been also associated with a more than two fold increase in the risk of VTE [17]. Therefore, there is no doubt that the development

of new methods for preventing blood clot formation associated to infectious diseases are of great importance as they could significantly reduce morbidity, mortality and health care costs.

Advances in the treatment of pathological thrombosis are a global medical challenge. There are three classes of drugs available in the market for the treatment of thrombosis: antiplatelet drugs, anticoagulants and fibrinolytic agents. One of the main limitations associated to the use of those drugs is the inability to prevent or reverse thrombosis without interfering with physiological hemostasis. In fact, in many clinical applications, there is a need to optimize the efficacy and safety of these drugs, mainly due to the increased risk of bleeding that the patients are exposed to. Another problem associated to some of those drugs is their potential accumulation in tissues and their short half-life, because of their enzymatic degradation, which reduces their blood concentration and therefore jeopardize their potential pharmacological effect [18–21].

Nanoparticles (NPs) are defined as particles with sizes ranging from 1 to 100 nm. Their physicochemical and biological properties can be modified by changing features such as their surface charge, size, shape, chemical structure and morphology. Some of their beneficial attributes result from their high surface area to volume ratio, their ability to penetrate cell membranes and biological barriers and to their high reactivity [22].

In view of the unique properties of NPs, nanomaterials have been proposed as promising tools for the prevention and treatment of human diseases [23]. In fact, the application of nanotechnology in medical applications (nanomedicine) is a research topic that has led to a continuous and significant increase in the number of publications over the past years. Just by the end of 2021, over 320,000 articles on this subject were indexed in the PubMed database [22].

Nanoparticles can be functionalized with the aim of increasing their biocompatibility, reducing their cytotoxicity, increasing their efficiency and/or even providing antimicrobial activity [24]. Indeed, drugs, antibodies, nucleic acids, proteins, or organic substances such as poli (ethylene glycol) (PEG) or reduced glutathione (GSH) have been attached to the surface of NPs [25–28].

The potential applications of silver nanoparticles (AgNPs) to nanomedicine have been widely investigated. The data available in the literature extensively describe the antimicrobial and anticancer properties of AgNPs. The mechanism of action of AgNPs has been related to, among others, damage to cellular structures, generation of reactive

oxygen species (ROS), damage to deoxyribonucleic acid (DNA), inactivation of enzymes and denaturation of proteins [22, 29–32]. In addition to their use due to their antimicrobial and anticancer properties, AgNPs can also be used in other medical applications and their potential use in bone reconstruction, wound healing, as an adjuvant in a vaccine or biosensors for detecting blood glucose, enzymes, molecular markers of cancer cells and pathogens have been also demonstrated (Fig. 2) [28–32].

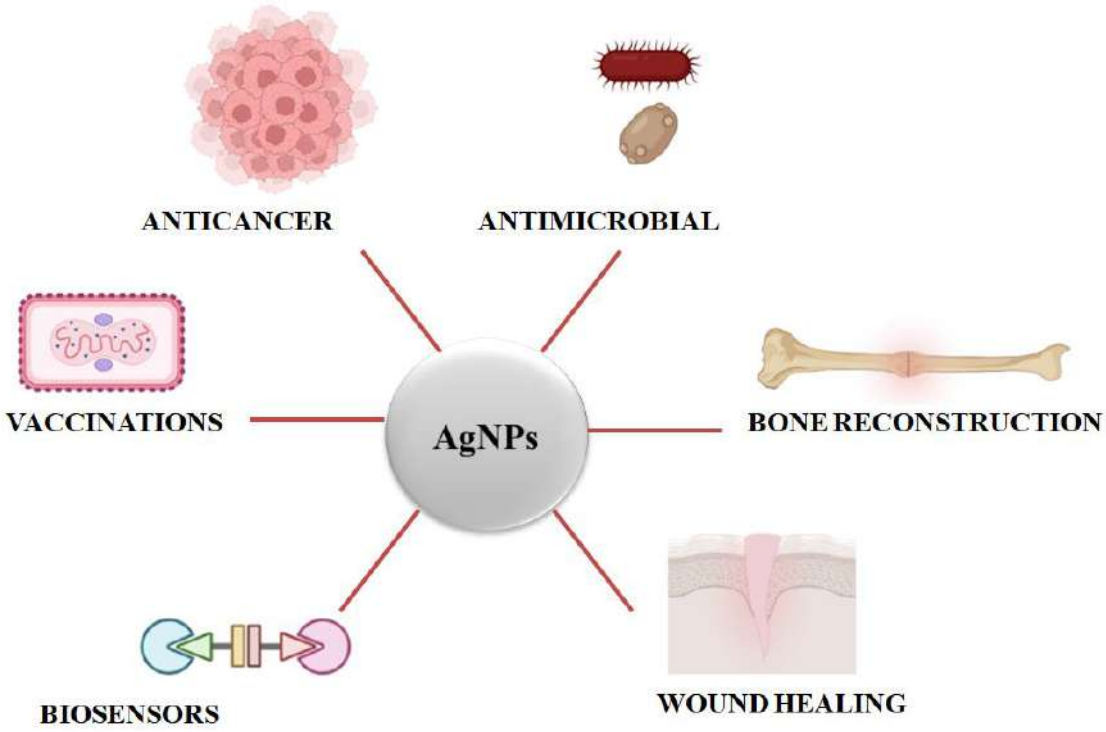


***Figure 2**. Applications and potential medical use of silver nanoparticles*

The existing literature data do not allow to unambiguously determine the influence that functionalized AgNPs exert on the hemostatic system. After reaching the blood stream, NPs immediately interact with blood cells, proteins and endothelial cells, as well as key elements of the hemostatic system, such as platelets and plasma coagulation factors. The undesirable pro- and anti-coagulant/aggregatory properties of NPs are of concern in the field of nanomedicine as there are contradictory results available in the literature. It has been previously demonstrated that AgNPs coated and uncoated with PEG may exhibit antiplatelet properties depending on the agonist used [33, 34]. On the other hand, others have observed, with the use of AgNPs, induction of platelet aggregation in a concentration and size-dependent manner [35]. Ragaseema et al. have showed that AgNPs do not affect the expression of thrombogenic factors [36]. However, others have proven that AgNPs at high concentrations may increase the

expression of vWF and decrease the expression of tPA, which suggests a prothrombogenic effect [37–39].

The combination of AgNPs with other compounds (antibiotics and anticancer or antiviral compounds) could potentially help in the development of new more effective and safer therapeutic options. However, detailed studies on the interaction of AgNPs with the hemostatic system are of crucial importance to determine their pharmacological and therapeutic potential.

III. AIMS OF THE STUDY

Main aim: Synthesis and functionalization of silver nanoparticles and determination of their potential interactions with the hemostatic system by combining in vitro and ex vivo experiments.

Specific aims:

1) Literature review and summary of the latest *in vitro* and *in vivo* studies related to drugs-based nanoparticles and the therapeutic benefits of nanodrugs used in cardiovascular diseases and angiogenesis-related cancers.

2) Evaluation of the effect of silver nanoparticles functionalized with lipoic acid on endothelial cells, blood platelets and erythrocytes.

3) Evaluation of the effect of silver nanoparticles functionalized with lipoic acid, glutathione and polyethylene glycol on platelet aggregation.

4) Evaluation of the effect of silver nanoparticles functionalized with the antiplatelet agent eptifibatide on platelet aggregation and endothelial vascular activation factors associated with the hemostatic system.

IV. MATERIALS AND METHODS

Nanoparticles

Silver nanoparticles (AgNPs) functionalized with lipoic acid (LA), glutathione (GSH) and polyethylene glycol (PEG) were synthesized by a team from the University of Warsaw under the supervision of dr Michał Wójcik.

Uncoated silver nanoparticles were purchased from US Research Nanomaterials, Inc. (Houston, Texas).

Silver nanoparticles functionalized with eptifibatide were synthesized by a team from the University of Warsaw under the supervision of dr hab. Elżbieta Megiel, prof. UW.

Cell culture

Human umbilical vein endothelial cells (HUVEC), human gingival fibroblast cells (HGF-1), and immortalized human microvascular endothelial cell lines (HMEC-1) were obtained from Merck (Sigma Aldrich) and the American Type Culture Collection (ATCC). Cell culture was performed following the manufacturer's recommendations [37, 38].

Isolation of platelets and RBCs

Blood platelets and red blood cells (RBCs) were isolated from peripheral blood collected from healthy volunteers who, 2 weeks before the study, did not take drugs that have proven effects on platelet function. All volunteers gave their consent before blood collection. The study was approved by the Bioethics Committee of the Medical University of Gdańsk (NKBBN / 552 / 2018-2022) and by the School of Pharmacy and Pharmaceutical Sciences Ethics Committee, Trinity College Dublin, The University of Dublin, Ireland (MS 2016-03-01 and 2020-06-01). The research was conducted in accordance with the Code of Ethics of the World Medical Association, the ethical standards of the competent commission for human experiments (institutional and national) and the Helsinki Declaration of 1975, as amended in 2000.

For the determination of lactate dehydrogenase (LDH), and experiments involving light aggregometry, quartz crystal microbalance with dissipation technology (QCM-D) and flow cytometry, blood was collected with 3.15% trisodium citrate (9: 1,

v: v). Platelet rich plasma (PRP) was prepared from whole blood by differential centrifugation and washed platelet suspensions (WP) as previously described by Radomski et al. and resuspended in Tyrode's solution at a final concentration of 250,000 platelets / μL [39].

For coagulation studies, 3 mL of blood was collected using 3.8% buffered sodium citrate and prepared according to the reference recommendations for each method.

Assessment of the biocompatibility of silver nanoparticles

To evaluate the potential cytotoxic effect of AgNPs on endothelial vascular cells, a colorimetric test based on the measurement of the metabolic activity of cells (MTT) was used. The cell membrane integrity test (LDH) was used for investigating the effect of AgNPs on platelets and RBCs. Absorbance was recorded using a microplate reader (FLUOstar, OPTIMA).

Assessment of changes induced by nanoparticles at the ultrastructural level of the cell

The ability to penetrate the cell membrane, localization at the cellular level and potential changes induced at the ultrastructural level by AgNPs were determined by Transmission Electron Microscopy (TEM) using a Tecnai G2Spirit BioTWIN TEM.

Measurement of reactive oxygen species

The level of intracellular reactive oxygen species (ROS) was assessed using flow cytometry (BD FACSArray, Biosciences, San Jose, CA) with 2,7-dichlorofluorescein diacetate (DCF-DA) and analyzed using the CellQuest Pro software.

Assessment of apoptosis / necrosis

Flow cytometry with annexin V and propidium iodide (PI) was used to measure apoptosis / necrosis and results analyzed using CellQuest Pro software.

Measurement of the destabilization of the mitochondrial potential

Measurement of the destabilization of the mitochondrial potential was performed by flow cytometry with the use of the JC-1 fluorescent dye. A fluorescence

microscope (Olympus Life Science) was used for microscopic analysis. The obtained data was analyzed using the CellQuest Pro software**.**

Assessment of P-selectin and GPIIb / IIIa expression

Flow cytometry was used to measure the expression of P-selectin and GPIIb / IIIa receptors. The results were analyzed using the CellQuest Pro software.

Measurement of platelet aggregation using light aggregometry

Collagen-induced platelet aggregation was measured in a two-channel light aggregometer (CHRONO-LOG-700).

Measurement of platelet aggregation using a quartz crystal microbalance with dissipation monitoring

A quartz crystal microbalance with dissipation monitoring (Q-Sense AB, E4 QCM-D system) was used to measure platelet aggregation under flow conditions that mimic the vascular microenvironment.

Assessment of TXB2, MMP-1, MMP-2, 6-keto-PGF1alpha, tPA, vWF, and cGMP

Commercially available ELISA kits (Cayman, Abcam and BioFine), were used for measuring potential endothelial cell damage (vWF), platelet's (6-keto-PGF1alpha cGMP, TXB2, MMP-1 and MMP-2), and fibrinolytic (tPA) effect of the AgNPs tested

Analysis of coagulation parameters

Prothrombin time (PT), activated partial thromboplastin time (aPTT), thrombin time (TT); international normalized ratio (INR), fibrinogen, D-dimers and closure time (CT) were assessed in an accredited hospital laboratory (Central Clinical Laboratory of the University Clinical Center in Gdańsk) using the BCS-XP analyzer (Siemens Healthcare) and the Innovate PFA-200 system (Siemens Healthcare).

Statistical analysis

Statistical analysis was performed in GraphPad Prism (confidence interval = 95%, $\alpha = 0.05$) and One-way ANOVA and Tukey's post-hoc test were used when appropriated. The IC_{50} value was determined by log(inhibitor) vs normalized response nonlinear regression analysis.

V. DESCRIPTION OF THE PUBLICATIONS INCLUDED IN THE DOCTORAL THESIS

My doctoral thesis consists of four papers (three original and one review) published in international journals indexed on the ISI Master List. Those papers focus on the influence of nanoparticle functionalization on the antimicrobial, cytotoxic, antiplatelet, antithrombotic and fibrinolytic properties of silver nanoparticles and on their potential to be used as a platform for drug delivery in cardiovascular diseases.

Publication 1

Hajtuch J, Niska K, Inkielewicz-Stępniak I. *Nanodrugs as a new approach in therapy of cardiovascular diseases and cancer with tumor-associated angiogenesis*. Curr. Med.Chem. 2021; 28(27):5527-5550. doi: 10.2174/0929867328666201231121704.

Cancer, together with CVD, is defined as the leading cause of death worldwide [40]. Studies indicate that oncological therapies, especially chemotherapy, are associated with an increased risk of CVD [16]. Importantly, some risk factors, such as hypertension, diabetes or smoking, are common to CVD and cancer [41]. The processes of angiogenesis and platelet aggregation play an important role in neoplastic progression [42].

This review publication presents the most up-to-date research in order to provide comprehensive information on the latest *in vitro* and *in vivo* studies related to NPs-based drugs and the therapeutic benefits of potential nanodrugs in cardiovascular diseases and angiogenesis-related cancers. The analysis of the available results confirms their improved pharmacological and biological properties, giving promising antiplatelet, anticoagulant and antiangiogenic effects. In addition, NPs with FDA-approved chemotherapeutic agents were included in the review.

By optimizing the size of NPs and their surface properties, nanotechnology is able to deliver drugs with increased bioavailability in the treatment of CVD, cancer and angiogenesis.

Publication 2

Hajtuch J, Hante N, Tomczyk E, Wójcik M, Radomski MW, Santos-Martinez MJ, Inkielewicz-Stępniak I. *Effects of functionalized silver nanoparticles on aggregation of human blood platelets.* Int. J. Nanomed. 2019; 14: 7399–7417. https://doi.org/10.2147%2FIJN.S213499

Based on the data available in the literature and previous results from our group on the effect of commercially available AgNPs on platelet function, the interaction between functionalized AgNPs and the hemostatic system was investigated [27, 34, 36, 43].

For this purpose, AgNPs functionalized with polyethylene glycol (AgNPs-PEG), lipoic acid (AgNPs-LA) and reduced glutathione (AgNPs-GSH) were synthesized and their effect on platelet function measured under flow conditions using a QCM-D. The biocompatibility of the synthesized AgNPs was assessed on HUVEC, HGF-1 cells and platelets using MTT and LDH assays. In addition, determination of platelet receptors and levels of thromboxane B2 (TXB2) and metalloproteinases (MMP-1, MMP-2) released by platelets as markers of platelet activation were measured using flow cytometry and commercially available ELISA assays respectively.

The results presented in this publication confirm that 2 nm AgNPs-GSH, 3.7 nm AgNPs-PEG at 50 and 100 μg/ml, and 2.5 nm AgNPs-LA at 100 μg/ml reduced collagen-induced platelet aggregation, downregulate P-selectin and GPIIb/IIIa receptors, TXB2 formation and release of MMP-1 and MMP-2. Functionalized AgNPs, did not affect the viability of platelets, endothelial cells and fibroblasts at the concentrations tested.

In conclusion, functionalized AgNPs inhibited platelet aggregation at non-cytotoxic concentrations. The AgNPs synthesized in this study could be used as an antiplatelet agent or as a material for manufacturing blood contact medical devices such as vascular catheters, stents and heart valves.

Publication 3

Hajtuch J, Santos-Martinez MJ, Wójcik M, Tomczyk E, Jaśkiewicz M, Kamysz W, Narajczyk M, Inkielewicz-Stępniak I. *Lipoic acid-coated silver nanoparticles: biosafety potential on the vascular microenvironment and antibacterial properties*; Front. Pharmacol. 2022; 12:733743 https://doi.org/10.3389/fphar.2021.733743

The search for new therapeutic agents in cardiovascular diseases is a global medical challenge. It is estimated that about 30% of all nosocomial bacteremias are related to the use of endovascular devices, such as intravenous catheters, which also contribute to thrombus formation. Surface modification of NPs involves processes that may result in more desirable properties of NPs, including reduced cytotoxicity. [44] and due to their recognized antimicrobial and antiplatelet activity they could be potentially used for coating medical devices [22, 37].

In this study, the antibacterial properties of lipoic acid-coated silver nanoparticles (2.5 nm AgNPsLA) synthesized and characterized as previously described by our team [45] and commercially available uncoated silver nanoparticles (2.6 nm AgNPs) and their potential cytotoxic effects in the microenvironment of blood vessels were evaluated. Antimicrobial activity was evaluated using reference bacterial strains of bacterial pathogens commonly associated with resistance to available antimicrobial agents (ESKAPE) - *Enterococcus faecium* (ATCC 700221), *Staphylococcus aureus* (ATCC 33591), *Klebsiella pneumoniae* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 9027), *Klebsiella aerogenes* (ATCC 13048) and *Enterobacter aerogenes* (ATCC 130480) and expressed by minimum inhibitory concentrations (MIC). As the vascular endothelium is involved in physiological and pathological processes such as hemostasis and thrombosis as well as inflammation and remodeling of the vessel wall, cytotoxicity evaluation on human umbilical vein endothelial cells (HUVEC) was also performed using the MTT assay [4]. The level of ROS, apoptosis and necrosis of HUVEC cells and the measurement of mitochondrial destabilization in HUVECs and platelets were performed by flow cytometry. The potential cytotoxic effect of NPs on erythrocytes was investigated by measuring hemoglobin levels and LDH released after exposure to AgNPs and AgNPsLA. Using TEM, it was determined whether AgNPs and AgNPsLA could induce ultrastructural changes in HUVEC cells and *Staphylococcus aureus* bacteria.

The obtained results confirmed that both, AgNPs and AgNPsLA, have antimicrobial properties against pathogens associated with bloodstream infections and related to the use of ports and catheters. AgNPsLA, unlike AgNPs, did not induce intracellular ROS production and apoptosis in HUVEC cells, ultrastructural changes in HUVEC cells, depolarization of the mitochondrial membrane in HUVEC cells and platelets or hemolysis. Importantly, functionalization of AgNPs with LA was shown to maintain the known antibacterial activity for AgNPs.

In conclusion, the AgNPsLA synthesized by our group have antimicrobial activity and a better biosafety profile than uncoated AgNPs of similar size. These observations are important for future *in vivo* studies and the potential use of AgNPsLA in medical devices in patients.

Publication 4

Hajtuch J, Iwicka E, Szczoczarz E, Flis, D, Megiel E, Cieciórski P, Radomski MW, Santos-Martinez MJ, Inkielewicz-Stępniak I. *The pharmacological effects of silver nanoparticles functionalized with eptifibatide on platelets and endothelial cells*. Int. J. Nanomed. 2022;17:4383-4400. doi: 10.2147/IJN.S373691.

In this research, AgNPs were functionalized with eptifibatide, a synthetic cyclic heptapeptide that reversibly binds to the platelet GPIIb/IIIa receptor, causing a short-term receptor blockade and inhibition of platelet aggregation.

Spherical fused AgNPs were synthesized and characterized with eptifibatide (EPI). The biocompatibility of AgNPs-EPI in microvascular endothelial cells (HMEC-1), platelets and erythrocytes was evaluated. The effect of AgNPs-EPI on platelet aggregation was measured by light aggregometry and parameters of coagulation were determined using reference laboratory methods. Flow cytometry was used to determine the effect of AgNPs-EPI on - P-selectin and GPIIb/IIIa receptors on the platelet surface. Levels of 6-keto-PGF1alpha (stable prostacyclin metabolite), tissue plasminogen activator (tPA), von Willebrand factor (vWF) and cyclic guanosine monophosphate (cGMP) were measured by ELISA.

The synthesized AgNPs-EPI did not affect platelets, endothelial cells and erythrocytes viability. AgNPs-EPI inhibited collagen-induced platelet aggregation as measured by light transmission aggregometry and downregulation of P-selectin expression and GPIIb/IIIa conformational changes in platelets were observed at lower concentrations of AgNPs-EPI than of the drug alone. AgNPs-EPI increased the

occlusion time in the presence of collagen/ADP and collagen/adrenaline, without significantly affecting the other parameters of coagulation: PT, APTT, TT INR, fibrinogen, D-dimers and CT. AgNPs-EPI also modulated the level of 6-keto-PGF1alpha, tPA, vWf and cGMP in thrombin-stimulated HMEC-1 cells.

In summary, the AgNPs-EPI synthesized by our team showed pharmacological activity at lower concentrations than the drug alone modulating platelet aggregation, blood coagulation and endothelial cell activity. The results of this work confirm, once again, that AgNPs have the potential to be used as an effective platform for the delivery of antiplatelet drugs.

VI. CONCLUSION

Nanotechnology has found wide applications in medicine as medical devices, as therapeutic substances, as dressing materials or as drug delivery platforms [46]. Due to their versatile properties, AgNPs can be used in the treatment, prevention and diagnosis of diseases.

The research carried out as part of my doctoral thesis has shown that the developed functionalized AgNPs by our research team are biocompatible with endothelial cells, platelets and erythrocytes. In addition, the synthesized AgNPs have demonstrated their ability to inhibit platelet aggregation while retaining their antibacterial properties. We have investigated and unravel some of the mechanisms for understanding the interactions between AgNPs and platelets. Our work confirms once again the importance of conducting research in the field of biocompatibility while assessing the potential application of nanotechnology in medicine.

In the review article and taking into account the latest scientific developments, we discussed the latest *in vitro* and *in vivo* research related to NPs-based drugs and the therapeutic benefits of potential nanodrugs used in cardiovascular diseases and angiogenesis-related cancers.

In our research, and for the first time, a new conjugate of AgNPs with eptifibatide (AgNPs-EPI) was developed. The *in vitro* pharmacological assays performed showed that the AgNPs-EPI were more effective than the free drug for inhibiting platelet aggregation and thrombin-induced endothelial activation. In addition, AgNPs-EPI did not affect cell viability, which is crucial for preventing vascular damage and for maintaining blood fluidity.

We have shown that, due to the unique features of AgNPs, they can be potentially used as therapeutic substances or for the development of biomaterials. The results obtained in this research may be extremely helpful for the future design of modified AgNPs in order to maximize their potential beneficial effects and at the same time of maintaining their safety profile.

From the results obtained from this work it can be concluded that AgNPs deserve further research to expand their applications in nanomedicine, a sustainable and advanced approach to synthesis and biological research that may be a viable alternative in medicine.

WYKAZ CYTOWANEGO PIŚMIENNICTWA

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REVIEW ARTICLE

Nanodrugs as a New Approach in the Therapy of Cardiovascular Diseases and Cancer with Tumor-associated Angiogenesis

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Abstract: ***Background*:** Cancer, along with cardiovascular diseases, is globally defined as the leading cause of death. Importantly, some risk factors are common to these diseases. The process of angiogenesis and platelet aggregation is observed in cancer development and progression. In recent years, studies have been conducted on nanodrugs for these diseases that have provided important information on the biological and physicochemical properties of nanoparticles. Their attractive features are that they are made of biocompatible, well-characterized, and easily functionalized materials. Unlike conventional drug delivery, sustained and controlled drug release can be obtained by using nanomaterials.

***Methods*:** In this article, we review the latest research to provide comprehensive information on nanoparticle-based drugs for the treatment of cancer, cardiovascular disease associated with abnormal haemostasis, and the inhibition of tumor-associated angiogenesis.

***Results*:** The results of the analysis of data based on drugs with nanoparticles confirm their improved pharmaceutical and biological properties, which give promising antiplatelet, anticoagulant, and antiangiogenic effects. Moreover, the review included *in vitro*, *in vivo* research and presented nanodrugs with chemotherapeutics approved by Food and Drug Administration.

***Conclusion*:** By the optimization of nanoparticle size and surface properties, nanotechnology is able to deliver drugs with enhanced bioavailability in treatment of cardiovascular disease, cancer and inhibition of cancer-related angiogenesis. Thus, nanotechnology can improve the therapeutic efficacy of the drug, but there is a need for a better understanding of the nanodrugs interaction in the human body because this is a key factor in the success of potential nanotherapeutics.

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1. INTRODUCTION

The relationship between cancer and venous thromboembolism (VTE) has been recognised for almost two centuries [1]. It has been accepted that Bouillard established the first description of deep vein thrombosis (DVT) in patients with cancer in 1823 [2]. Cancer is responsible for 18% of all cases of VTE. Across all patients with diagnosed cancer, a 7-fold increase of the risk for VTE is observed, and in certain malignancies, the risk may be elevated up to 28-fold [3]. Although more individuals are cured or are living longer with these diseases, evidence has been emerging that cancer survivors show an increased risk of death from cardiovascular disorders related to homeostasis disturbances [4]. Additionally, risk factors for the development of cardiovascular diseases are similar to those of cancer. In patients diagnosed with cancer prior to the initiation of anticancer therapy, cardiovascular risk factors such as hypertension, advanced age, smoking, and diabetes are also visible [5]. The study shows that cancer therapies, particularly chemotherapy, play a prominent role in cardiovascular risk [6]. Importantly, some risk fac-

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tors are found common to both cardiovascular diseases and cancer (Fig. **1**). Thus, in multiple dimensions, there is a unique interplay between cardiology and oncology, and this is known as "cardio-oncology." The development of a supportive vasculature is essential for tumor progression. It has been shown that tumor angiogenesis played a significant role in its growth, invasion, metastasis, and recurrence [7, 8]. An increasing number of evidence supports the idea that tumor growth and metastatic potential are depended on the ability of a tumor to induce a neovascular response [9, 10]. Currently, drugs used in the treatment of both cancer and cardiovascular diseases have various limitations. These problems are related to bioavailability, drug resistance, the lack of tumor-selective cytotoxicity, low tumor uptake and retention, and short-half life. Moreover, long-term uses may lead to secondary cancers and even increase side effects [11-15]. Considerable amounts of marketed drugs and many new chemical drug candidates (as much as 70%) can not be absorbed in the gastrointestinal, and if giving them as intravenous injections, they present low water solubility. The solubilizing agents, such as dimethyl sulfoxide or surfactants, are typically added for systemic administration. However, these agents can still lead to neurotoxicity, even when used in very low doses, thereby restricting the applications of free drugs [16].

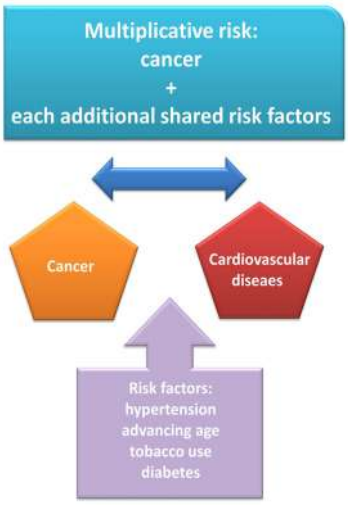

Fig. (1). Bidirectional relationships between cancer and cardiovascular diseases. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Within the past few decades, nanotechnology, mainly the manufacturing of nanoparticles (NPs), has gained unparalleled attention in most fields of science [17]. A PubMed search "nanoparticles" reveals that in 2019, there were "22,030" articles published related to various aspects of NPs technology [18]. Nanotechnology is a multidisciplinary scientific field that applies engineering principles at the molecular level. Smart NPs have revolutionized the way of drug formulation and delivery. Due to the use of nanotechnology in medicine, NPs have been designed to mimic or change biological processes [19]. NPs with ~100 nm have been widely used to improve drug accumulation, internalization, and therapeutic efficacy. The physicochemical and biological properties of NPs can be modified by adjusting their chemical and surface properties, sizes, shapes, structures, and morphologies [20]. According to nanotechnology, nanocarriers from organic and inorganic materials are synthesized to improve drug efficacy, reduce systemic side effects and improve the pharmacological profile. A drug may be adsorbed on, encapsulated, or attached to the nanocarrier. The targeting of drugs can be passive or active; the first exploits the characteristic features of target tissue biology; instead, in active approaches, nanocarriers are conjugated with molecules able to bind overexpressed antigens or receptors present on the target cell surface (Fig. **2**). In addition, active targeting can also be achieved through the manipulation of physical stimuli (*e.g.*, temperature, pH, magnetism). The drug is released in a controlled manner by factors such as changes in the physiological environment (temperature, pH, osmolality) or enzymatic activity at a target site in the diseased tissue [21]. In recent years, delivery systems based on NPs have been used in a variety of medical applications. Their main advantage is that they are made of well-characterized and easily functionalized materials, and they are biocompatible [22]. In contrast to conventional drug delivery, using nanomaterials, a prolonged and controlled drug release can be achieved. Nanomaterials exhibit a highly differential targeting and uptake efficiency in a cell- or tissue-specific manner. Moreover, the drug molecule on the nanocarrier is protected from harsh conditions before it can reach the target [23, 24]. Thus, nanomedicine is an innovative field with great potential for treatment by combining NPs with particles such as drugs.

The purpose of this review is to summarize the latest research in NPs-based drugs used to treat cancer, cardiovascular diseases and inhibit tumor-associated angiogenesis in cancer therapy. In addition, in this chapter, we want to consolidate the benefits of potential NPs-based therapy, which reduces the problems related to currently used clinical drugs. This information also sheds light on scaling-up research and the use of nanoproducts in treatment.

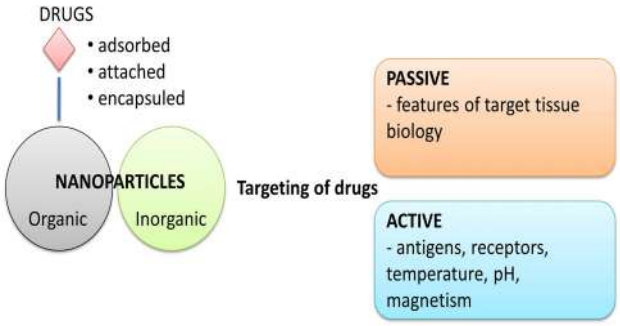

Fig. (2). Attaching of drugs to the NPs and targeting of drugs. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2. GENERAL EPIDEMIOLOGY OF CANCER

It is currently observed, in some high-income countries and upper-middle-income countries, that the mortality rate due to cancer can be higher than deaths from cardiovascular diseases. According to the Prospective Urban Rural Epidemiology study, if the effectiveness of prevention and treatment of heart disease is not improved, in a few decades, cancer will become the leading cause of death in the world [25]. The increasing cancer rate is associated with population ageing, as well as increased exposure to risk factors, including sunlight, radiation, infectious agents, chemicals, and other substances.In addition, genetic predisposition and negative lifestyle behaviors like unbalanced diet, lack of physical activity, smoking, or obesity conduce to cancer [26]. In 2018, approximately 18.1 million new cancer cases and an estimated 9.6 million deaths caused by cancer were documented [27, 28]. The five predominant cancer types in 2018 were lung (2,093,876), breast (2,088,849), contributing 11.6% of the total cancer incidence burden each; colorectum (1,849,518, 10.2% of the total), prostate (1,276,106, 7.1%), and stomach (1,033,701, 5.7%) (Fig. **3**). The highest mortality rate in 2018 was observed for lung cancer (1,761,007 deaths, 18, 4% of total), especially within men (1,184,947 deaths, 22% of total). The second cause of cancer deaths was colorectal cancer (880,792), followed by stomach cancer (782,685), liver cancer (781,631), and breast cancer (626,679), which is the most commonly diagnosed cancer within women (24, 2% of total) (https://www.who.int) [29]. According to a recent prediction of the World Health Organization (WHO), the global cancer burden will rise to 21.6 million cases and more than 13 million deaths in 2030 (http://gco.iarc.fr) [28]. The above data revealed that cancer poses a serious problem in modern medicine. Difficultness in cancer treatment is associated with several factors. Carcinogenesis is a complex multistage cellular and molecular process, which involves various genetic or epigenetic factors. Understanding the cancer biology, interactions between cancer cells and their surrounding environment, known as the tumor microenvironment, is very important to inhibit the progression of the diseases effectively. However, still, there is a lot of outstanding questions about the pathophysiology of cancer. Another problem is the lack of a sufficient amount of reliable biomarkers for different cancer types, which are useful not only for diagnostic purposes or monitoring therapeutic response but also as significant prognostic tools [30]. Furthermore, many cancers are diagnosed at a later stage due to a non-specific nature of cancer symptoms as oesophageal, prostate, and pancreatic cancer [31]. Most cancers tend to spread locally or to different parts of the human body from their original location through the bloodstream and *via* the lymphatic system [32]. Some certain metastatic events smaller than 2 mm are called micrometastases might be difficult to detect because of the size [33]. Treatment for advanced cancer is usually more complicated and often has a poor prognosis. Nowadays, the most common and widely used types of cancer treatments are surgery, radiotherapy, chemotherapy, and radiation-based surgical knives. Additionally, new methods like hormone-based therapy, anti-angiogenic modalities, stem cell therapies, immunotherapy, and dendritic cell-based immunotherapy are being applied [34]. The choice of combination regimen is based on the type of cancer, its locality, and stage of progression. The major limiting problems to achieve satisfying results of treatment are drug resistance and non-specific actions. Causes of drug resistance, responsible for up to 90% of cancer-related deaths, are multipronged. The critical biological determinants of drug resistance are tumor size and growth kinetics, tumor heterogeneity, physical barriers created by cancer cells, and the tumor microenvironment consisting of immune cells, stroma, and vasculature. Moreover, undruggable oncogenes and tumor suppressor genes, as well as changes in tumor and its ecosystem, as a result of conventional therapy, can contribute to enhanced resistance [35]. Besides, currently used chemotherapeutics are toxic for both normal and cancer cells, resulting in serious and unpredictable side effects [36]. For these reasons, the development of new cancer drugs or modification of already being used drugs is needed. New chemotherapeutics should exhibit selective cytotoxicity and improved pharmacokinetic properties.

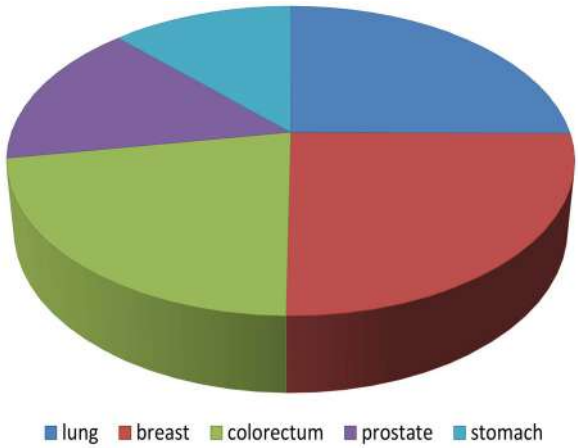

Fig. (3). Predominant cancer types in 2018. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

2.1. Nano-Chemotherapeutics

Based on their mechanism of action, conventional chemotherapeutics are categorized as alkylating agents, intercalating agents, antimetabolites, anthracyclines, mitotic inhibitors, and hormone chemotherapeutic drugs [35, 36].

The alkylating drugs are the earliest developed class of anticancer agents, which are still being used in several types of cancers, mainly leukemia and solid tumors, but are also applied in the treatment of cancers like breast, lung, ovarian, and prostate, as well as lymphomas, myelomas, sarcomas and Hodgkin's disease [37]. The alkylating compounds are directly involved in DNA damage by attaching methyl- or alkyl group to DNA bases, the formation of cross-links between atoms in DNA, and mispairing of the nucleotides causing mutations, structural changes in DNA, leading to inhibition of DNA synthesis and cell division, which result in cells death [12]. The five groups of alkylating chemotherapeutics can be distinguished, including nitrogen mustards, nitrosoureas, alkyl sulfonates, ethylenimine, and triazines [34]. However, the application of alkylating agents is limited in clinical uses due to drug resistance, gastrointestinal side effects, and dose-limiting toxicity to bone marrow. Moreover, long-term uses can contribute to permanent infertility and secondary cancers [12].Therefore, in order to minimalize side effects and to enhance the sensitivity of alkylating agents, for some of them, modification by nanostructures was formulated. One of the commonly used alkylating antitumor prodrugs, used especially in the treatment of sarcoma, is ifosfamide (IFS) which is characterized by high neurotoxicity, nephrotoxicity, and instability in various ranges of pH. IFS-loaded-lipid-core nanocapsules (IFS-LNC) and ifosfamide-loaded poly (lactic-co-glycolic acid) PLGA-dextran polymeric NPs were proposed to improve the anticancer effects against osteosarcoma [38, 39]. Nanoformulations demonstrated a high drug loading capacity and exhibited higher cytotoxicity than unmodified IFS in both cases. Moreover, Velmurugan and Nair showed that nanostructured lipid carriers containing IFS decreased nephrotoxicity in the Wistar albino rats model and could be administrated orally [40]. Chlorambucil (CLB) is an unstable, poorly soluble in water, antineoplastic drug, and is used to treat chronic lymphocytic leukemia, Waldenström macroglobulinaemia, indolent non-Hodgkin lymphoma, Hodgkin lymphoma, and advanced ovarian and breast cancer [41]. Sharma *et al.* presented stearic acid-CLB-DDA nanocomplex (CLB encapsulated in lipid stearic acid nanocomplex covered with dimethyl dioctadecyl ammonium bromide (DDAB)). *In vivo* studies on obtained complex demonstrated enhanced biodistribution and pharmacokinetic properties of CLB as well as low tissue toxicity and greater ability to accumulate in tumors [13]. CLB loaded into PLGA NPs showed higher cytotoxicity on breast cancer cells and non-Hodgkin's lymphoma compared to free CBL [42, 43]. Melphalan (MLN) is another bifunctional alkylating agent widely used in cancer treatment, mainly multiple myeloma, advanced ovarian adenocarcinoma, breast cancer, ovarian cancer, melanoma, sarcoma, retinoblastoma, and childhood neuroblastoma [44]. The major problems in therapy with MLN are insolubility in an aqueous solution, tendency to precipitate in water, short-half life, rapid elimination, and high liver, lung, and kidney toxicity. Surface-modified MLN NPs by peptides, MPG (3177 Da), PEG (5000 Da), and TET1 (1873 Da) decreased the viability of Y79 retinoblastoma cells in comparison to unmodified MLN NPs [14]. Encapsulated MLN in 171 nm lipid NPs (LNP) significantly reduced the viability of Y79 cells and decrease the number of retinoblastoma cells in *in vivo* Rb model in rats. Additionally, the cytotoxic effect was intensified by the addition of miR-181a. The proposed drug delivery system could minimalize side effects due to the application of a lower concentration of melphalan [45]. Rudhrabatla *et al.* showed that MLN was released in a controlled manner and improved pharmacokinetic properties. It could be possible due to the use of solid lipid NPs containing MLN (MLN-SSLNs). The *in vivo* study confirmed that MLN-SSLNs were characterized by long circulation, low elimination rate, and better resistance time in comparison to free MLN [46]. Altretamine (ALT) is alkylating hexamethylmelamine with low oral bioavailability and sparingly solubility in water and is used in the

treatment of ovarian cancer. Gidwani and Vyas studied 151.5 nm solid-lipid-NPs (SLNs) of ALT complexed epichlorohydrin-β-cyclodextrin, which demonstrated improved pharmacokinetic properties of the drug. Oral administration of SLNs nanosuspension formulation in Wistar rats revealed 2.75 times higher relative bioavailability compared to pure ALT. The highest concentration of a nanoformulation in the plasma C_{max} was 0.94 μg/ml (0.38 μg/mL for pure ALT), time of maximum concentration (T_{max}) was 22 min (45 min for pure ALT) and 3 times higher area under the curve was observed for nanosuspension in comparison to the free drug [47]. Cisplatin and carboplatin represent intercalating agents based on platinum, which are widely used in cancer therapy, including gynecological cancers, germ cell tumors, head and neck cancer, thoracic cancers, and bladder cancer. These compounds are the most active cytotoxic drugs. They form DNA crosslinks, affecting DNA synthesis and transcription, and thus contribute to apoptosis induction. Moreover, DNA adducts with proteins, lipids, and RNA and mitochondrial DNA are formed. Both drugs exhibit strong side effects. Cisplatin has shown high nephrotoxicity and ototoxicity, while for carboplatin, myelotoxicity (particularly thrombocytopenia) and neurotoxicity are often observed [48]. The penetration rates of cisplatin and carboplatin in the central nervous system (CNS) are limited (3.7% and 2.6%, respectively) [49]. Cisplatin loaded on poly (Butylcyanoacrylate) NPs increases apoptosis in human glioblastoma in comparison to pure cisplatin. Additionally, it is assumed that penetration through the blood-brain barrier can be improved due to the nanosize of the cisplatin delivery system [50]. The new drug candidates for the treatment of lung cancer are cisplatin-loaded poly butyl cyanoacrylate NPs (355-386 nm in size). These formulations are also modified by temperature and the various polyethylene glycol (PEG) concentrations. A study showed that modulation enhanced drug loading efficiency and *in vivo* antitumor properties of cisplatin by extending the survival time of lung-cancer-bearing mice by 20% compared to the control group [51]. Gotov *et al.* evaluated hyaluronic acid-coated cisplatin conjugated gold NPs, which were tested against the potential application in the treatment of breast cancer both in *in vitro* and *in vivo* models. The proposed NPs showed a higher cytotoxic effect than the free cisplatin and could be applied for cancer therapy in the future [52]. To improve the properties of carboplatin, Karanam and Marslin proposed poly (ε-caprolactone) NPs of carboplatin in size ranging between 23.77 to 96.73 nm. Obtained NPs were uptaken by U-87 MG cells (human glioblastoma astrocytoma) with high effectiveness, which was associated with intense cytotoxic effect. Importantly, these NPs did not induce hemolysis in rat erythrocytes, which is a frequent complication of chemotherapy [53]. Magnetic NPs also present a huge potential to modify anticancer agents. Song *et al.* proposed carboplatin prodrug loading Fe_3O_4 NPs (NPs@carboplatin) with size 7.88 nm for ovarian cancer treatment. A study showed that NPs@carboplatin exhibit a much higher ability to carboplatin internalization and accumulation in ovarian cancer cells compared to free carboplatin, resulting in reduced cancer cell proliferation. Moreover, examined NPs inhibited tumor growth and did not display a cytotoxic effect on normal tissue in *in vivo* conditions. Due to using magnetic-based-NPs, it could be possible to target a tumor by determining tumor location and monitoring antitumor efficiency [54]. Satisfactory results for ovarian cancer therapy were also obtained for carboplatin-complexed cyclo (Arg-Gly-Asp-D-Phe-Cys) peptide-conjugated NPs [55]. Antimetabolites, structural analogs of nitrogen-containing bases: purines and pyrimidines, as well as folic acid antagonists, are characterized by high toxicity. For this reason, compounds belonging to this group are widely used as chemotherapeutic drugs. The mechanism of cytotoxic action of antimetabolites is to damage DNA indirectly through erroneous incorporation into DNA followed by disturbed timing or progression of DNA synthesis or altered function of enzymes involved in pyrimidine and purine synthesis. Cell death after treatment with antimetabolites is mostly induced during the S-phase of the cell cycle [56]. However, antimetabolites, like a vast majority of chemotherapeutic, also suppress the normal cell proliferation, primarily in the bone marrow and gastrointestinal tract, causing unwanted side-effects [57]. 6-mercaptopurine (6-MP) is a hypoxanthine derivative and is used in anticancer applications, especially in human leukemia. 6-MP is converted by specific hypoxanthine-guanine phosphoribosyl transferase (HGPRT) to 6-thioinosine monophosphate (T-IMP) and 6-thioguanine triphosphate (6-TGTP). 6-MP metabolites can be incorporated into DNA or RNA and also inhibit *de novo* purine synthesis, leading to disruption of DNA replication and synthesis [58]. Myelotoxicity, liver and kidney injury, often occurs as a side effect of the action of 6-MP in log-term treatment; therefore, its usage is limited. To improve bioavailability and short plasma half-life, 6-MP was entrapped into a chitosan NPs (6-MP-CNPs), which resulted in improvement of pharmacologic properties, increased anti-cancerous effects, and reduced toxic profile on blood, liver, and kidneys [59, 60]. Moustafa *et al.* proposed Fe_3O_4 NPs coated with PVA to load 6-MP. The results showed that the reduction of the therapeutic dose of 6-MP did not affect

the anti-tumor properties. Moreover, obtained nanoformulations exhibited a higher cytotoxic effect against human leukemia cells [61]. 5-fluorouracyl (5-FU) is one of the representatives of pyrimidines analogues, and is targeted to liver, breast, colorectum, and brain cancer treatment. 5-FU acts mainly as a thymidylate synthase inhibitor, impending DNA replication. 5-FU is rapidly converted to inactive metabolites, causing multiple adverse effects, including neurotoxicity, hemolytic activity, or bone marrow suppression [62]. 5- FU-NPs, modified by galactosylated chitosan, induced higher apoptosis of hepatic cancer cells, compared to pure 5-FU, by arresting the cell cycle in the G0/G1 phase. Studies *in vivo,* conducted on the orthotropic liver cancer mouse model, demonstrated the higher rate of NPs accumulation in hepatic cancer tissue, changes in tumor size, and improved survival time than 5-FU [63]. Jiang *et al.* developed the hyaluronic acid (HA)-conjugated mesoporous silica NPs loaded with 5-FU to target colon cancer. The hyaluronic acid in the structure of proposed NPs, specifically targeted CD44 receptors overexpressed in colon cancer cells, which improved the anticancer efficiency of the 5-FU [64]. Another type of system delivery of 5-FU for colon cancer was liposomes conjugated with folic acid [65]. Gemcitabine (GEM) is a structural analogue of deoxycytidine and is used for pancreases, ovarian, breast, bladder, and non-small cell lung cancers in the first-line chemotherapy and the second-line for mesothelioma. A well-known mechanism of action of gemcitabine is incorporation of gemcitabine metabolites: gemcitabine diphosphate (dFdCDP) or gemcitabine triphosphate (dFdCTP) into the DNA [66]. Moreover, the activity of enzymes responsible for nucleotide biosynthesis, such as deoxycytidylate deaminase, can be inhibited. Gemcitabine induces apoptosis of cancerous cells specifically by activation of p38 mitogen-activated protein kinase (MAPK) [67]. Long-term administration results in gemcitabine resistance due to disturbance of the functioning of nucleoside transporters that are needed for the cellular uptake of GEM or dysfunction of intracellular deoxycytidine kinase [68]. Due to the short blood circulation time of GEM (less than 30 min) and drug resistance, Zhang *et al.* proposed a 30 nm lipid-coated calcium phosphate NPs loaded with gemcitabine monophosphate. Nanoformulations showed a higher ability to induce apoptosis in the syngeneic mouse model of B16F10 melanoma, decreased rate of immunosuppression in the tumor microenvironment, and promoted depleting of MDSC (myeloid-derived suppressor cells), causing increased antitumor response [69]. Mozar and Chowdhury obtained gemcitabine-encapsulated carbonate apatite NPs modified by polyethylene glycol (PEG), which were tested on a syngeneic mouse model of breast cancer. The results showed that the concentration of chemotherapeutic agents in the tumor was successfully increased, and prolonged half-life of the GEM and decreased liver and spleen retention were also observed [70]. Similarly, fucoidan/chitosan NPs pose a good alternative to a delivery system for GEM against breast cancer. GEM loaded into NPs showed increased toxicity against human breast cancer cells (MDA-MB-231) compared to human umbilical vein endothelial (EA.hy926) [71]. Methotrexate (MTX), also known as amethopterin, is a folate antagonist used to treat childhood acute lymphoblastic leukemia and other hematologic malignancies, as well as osteosarcoma, breast and lung cancer [72]. The cytotoxic effect of MTX is mainly based on the inhibition of dihydrofolate reductase (DHFR), which blocks the synthesis of purine and pyrimidine precursors, and finally, DNA synthesis [73]. The most common side effects associated with MTX chemotherapy are nephro-, hepato- and neurotoxicity, pulmonary toxicity, and bone marrow suppression [74]. In order to improve the effectiveness of the treatment of MTX in lymphatic cancers, MTX is loaded into 163.7 nm poly-(lactide- co-glycolide) (PLGA) NPs (MTX-PLGA NPs). *In vivo* studies demonstrated a higher lymphatic antiproliferative effect, improved pharmacological profile (including prolonged blood circulation), and reduced renal and hepatic uptake. Attari *et al.* synthesized 27 nm magnetic iron oxide NPs, which were modified by arginine and conjugated with MTX (Fe-Arg-MTX). *In vitro* studies demonstrated higher cytotoxicity against breast cancer cells (MCF- 7 and 4T1 cell lines) for obtained NPs, while no toxic effect was observed for normal human foreskin fibroblasts (HF-F-1 cell line) and human red blood cells, which provide high biocompatibility of NPs. Moreover, the release of MTX from NPs can be increased in the presence of enzymes like proteinase K at acidic pH due to enzymatic cleavage of peptide bond [75]. Doxorubicin (DOX) and daunorubicin (DAUN) are representative drugs of anthracycline antibiotics, which pose a widely used group of chemotherapeutics in various types of cancer. Anthracyclines are able to modify DNA in different ways, including intercalating, forming covalent bonds as well as oxidative alteration of nitrogenous bases. Furthermore, they can inhibit DNA replication enzymes like the topoisomerase II enzyme, which plays a critical role in DNA replication and survival of the cells [76]. However, the application of anthracyclines in cancer therapy is limited due to high cardiotoxicity. In order to minimalize systematic side effects of DOX, enhance pharmacological properties, and

DOX efficacy of biological action in breast cancer cells, a few nanocomplexes were proposed, including DOX-conjugated lentinan NPs, anti-HER2-targeted DOX core-shell chitosan NPs and doxorubicin encapsulated in transferrin-conjugated polymeric NPs [77-79]. According to conducted studies, all of the proposed formulations are able to boost breast cancer chemotherapy, which is associated with higher anticancer properties of obtained nanostructures. Bi *et al.* developed DOX-loaded NPs coating with PDA (polydopamine) conjugated with folate (FA) and a peptide (Arg-Gly-Asp, RGD). Both of them exhibited good stability in physiological media, pH sensitivity, and compared to pure DOX, higher anticancer and antitumor activity against Hela cells and in the HeLa tumor-bearing mice, respectively [80]. To improve oral bioavailability of DAUN, Ahmad *et al.* developed 198.3 nm PLGA NPs with DAUN, whose surface was functionalized by natural biodegradable chitosan. Results demonstrated a higher cytotoxic effect of CS-DAUN-PLGA-NPs in human breast adenocarcinoma cell lines. Moreover, the bioavailability in plasma of Wistar rats was increased 10 times, compared to pure DAUN [81]. Bao *et al.* developed DAUN-loaded poly-(lactic-co-glycolic acid)-poly-l-lysine-polyethylene glycol-transferrin (Tf) NPs, which were tested against hematologic malignancies. *In vitro* and *in vivo* studies were performed. Obtained results showed an enhanced intracellular distribution of DAUN in primary leukemia cells which were associated with slow drug-releasing, higher tumor inhibition rate after 18 days of incubation, and upregulated expression of caspase-3 [82]. Paclitaxel (PTX) is a natural member of the taxanes family of anticancer agents, which is used in many types of cancers like ovarian, breast, bladder, non-small cell lung, malignant brain tumors, and other malignancies [83]. The cytotoxic effect of PTX is associated with disruption of normal microtubule dynamics by enhanced polymerization of tubulin. Microtubule disruption leads to the inhibition of spindle formation, resulting in G2/M phases of the cell cycle arrest and ultimately stimulating apoptosis cell death [84]. The most common adverse effects of PTX are myelosuppression and peripheral neuropathy [83]. To improve the pharmacological properties of PTX against breast cancer, 102 nm tannic acid-PTX NPs (TAP NPs) were designed. Proposed NPs demonstrated a significant reduction in proliferation of MDA-MB-231 and MCF-7 breast cancer cells and decreased ability of migration and invasion of metastatic MDA-MB-231 cells, compared to native PTX [85]. PTX resistance is associated with overexpression of P-glycoprotein (P-gp), an ATP-dependent drug efflux pump [86]. TAP NPs decreased P-gp function by partly inhibiting ATPase due to the presence of tannic acid in structure, resulting in achieving a higher concentration of PTX in breast cancer cells [85]. For osteosarcoma therapy, Zhao *et al.* developed stable 290.6 nm PTX NPs, modified by polydopamine (PDA) and targeted by alendronate (ALN). In comparison to nontargeted NPs, the inhibitory cell rate of K_7M_2 osteosarcoma cells was significantly higher for targeted PTX-PDA-ALN-NPs. Moreover, PTX-PDA-ALN-NPs easily penetrated the tumor site in K_7M_2 tumor-bearing mice, resulting in improved biodistribution and attenuated growth rate of the tumor. Furthermore, PTX-PDA-ALN-NPs did not impact the liver and spleen and reduce the normal tissue toxicity of PTX [87]. Zhai *et al.* proposed PTX system delivery, based on cubic phase lipid NPs, composed of lipid monoolein, stabilized by both Pluronic F127 and PEG (PTX-CB) [88]. Both *in vitro* and *in vivo* studies obtained promising results for the treatment of aggressive ovarian that proved strong anticancer and antitumor properties of PTX-PDA-ALN-NP. Table **1** contains a summary of the selected nanodrugs with anticancer activity, including *in vitro* and *in vivo* research.

Importantly, nanoformulations of drugs were approved by Food and Drug Administration (FDA) for the treatment of various types of human cancers (Table **2**). The first developed drug based on nanostructures was DOX, which was encapsulated in PEGylated liposomes. Nanodrug delivery system boosts the effectiveness of conventional chemotherapeutics and reduces side effects associated with their use [89-96].

3. CARDIOVASCULAR DISEASES LINKED TO HEMOSTASIS SYSTEM IMPAIRMENT

Due to advances in medical science and increased life expectancy, cancer and cardiovascular diseases are the leading causes of death in developed countries. Many cardiovascular diseases are caused by blood clots, *e.g.,* myocardial infarct, stroke, deep vein thrombosis. Cancer also leads to coagulopathy and increases the risk of thrombotic complications. The relationship between cancer and thromboembolism is well known, but the mechanisms involved in thromboembolic events in cancer patients are still unclear. Cancer patients are generally in a hypercoagulable or pre-thrombotic state as they tend to have pathologies in every component of the Virchow triad, thus contributing to thrombosis. The three components of the Virchow triad are blood flow congestion, endothelial damage, and hypercoagulability, the latter including abnormalities in the coagulation and fibrinolytic pathway and platelet activation [2, 3]. Additionally, the risk of cancer-related thrombosis increases with hospitalization, infection,

Table 1. Potential nanodrugs with improved anticancer activity *compared* to *conventional* drugs.

Nanodrug	Size	*In vitro* study	Dose	*In Vivo* Study	Dose	Improved Biological Effect	References
lipid-core-nanocapsules (ifosfamide)	-	MG63	10 μg/mL	-	-	Sustained release of IFS; Induction of apoptotic death	[38]
CHB-PLGA (chlorambucil)	240 - 320 nm	MCF-7 fibroblast NIH3T3	-	-	-	Reduction in cell viability	[42]
LNP/miR-181a (melphalan)	200 nm	human Rb Rb Y-79	150 μM	rats	-	Antiapoptotic; Reduction in cell viability	[45]
PBCA (cisplatin)	451.2±11 nm	A172	25 μM	-	-	Sustained release of cisplatin Reduction of tumor size	[50]
PCL (carboplatin)	100 nm	U-87 MG	0.519 μg/mL	-	-	Higher cytotoxicity; Reduction of carboplatin- induced haemolysis	[53]
6-MP-CNPs (6-mercaptopurine)	137.9 nm	MCF-7 HT-1080	6.85 μM	-	-	Sustained release and improved bioavailability of 6-MP; Higher cytotoxicity	[59]
GC/5-FU (5-fluorouracil)	35.19±9 nm	SMMC-7721 LO2	1.6 mg/L	mice	-	Sustained release of GMP, Reduction of tumor size	[63]
LCP-GMP (gemcitabine)	~30 nm	B16F10	-	mice	50.4 μmol/kg	Induction of apoptosis; Reduced immuno-suppression	[69]
PLGA (methotrexate)	117±9 nm	CWR22Rv1 MCF-7	0.01 mg/mL	rats	5 mg/kg	Sustained and controlled release of methotrexate	[74]
anti-HER2-DOX-NPs (doxorubicin)	34.92±1.80 nm	MCF-7	14.74 ng/mL	-	-	Sustained release of doxorubicin; Higher cytotoxicity	[78]
(CS)-coated-DAUN-PLGA-poly (daunorubicin)	198.3±9.21 nm	MCF-7 Caco-2	-	-	-	Improved bioavailability; Higher cytotoxicity	[81]
TAP (paclitaxel)	102.22±14.0 nm	MDA-MB-231 MCF-7	50 nmol/L	-	-	Sustained release of PTX; Higher cytotoxicity	[85]

chemotherapy, blood transfusions, comorbidities, and central venous catheters [4, 5]. With regards to the above information and the statement mentioned in the introduction regarding the bilateral relationship between cancer and cardiovascular diseases, *e.g.* common risk factors and applied therapies, we decided to discuss this topic.

WHO reports that approximately 18 million people died each year from CVDs, accounting for 31% of all deaths worldwide. CVD is characterized by disorders of the heart and blood vessels and includes, *e.g.* vascular thrombosis (https://www.who.int) [97]. The pathogenesis of myocardial infarction, venous thromboembolism (VTE), stroke, and other ischemic diseases includes thrombosis [98]. Thrombosis describes the formation of a clot within a blood vessel that reduces blood flow and may cause infarction of tissues supplied by that vessel. The formation of a thrombus begins when the vessel wall or endothelium is broken, and collagen and tissue factor are exposed to the blood flowing. The collagen leads to the accumulation and activation of platelets, and the tissue factor facilitates the formation of thrombin, which converts fibrinogen into fibrin and activates platelets [99]. Typical risk factors for vascular thrombosis include cancer, fractures, surgery, pregnancy, childbirth, paralysis, and the use of estrogens [100-102]. These factors increase the risk of thrombosis in people and can also cause episodes in patients with a tendency to prothrombotic [103]. These factors still remain inconclusive in up to 30% of patients, although most patients have acquired or heritable risk factors that could potentially be responsible for the disorder [100-102, 104-106].

It has been shown that people with atherosclerosis maybe at a higher risk for venous thrombosis. Several case studies have found an association between venous thromboembolic disorders and chronic arterial diseases of the legs, hyperlipidemia, or hypertension [107-110]. Moreover, thrombotic complications can cause atherosclerosis, which is associated with the activation of platelets and blood coagulation and an increased fibrin level [111-115].

Table 2. FDA-Approved Nanosystem-based chemotherapeutic drugs available for clinical use.

Chemo-Therapeutic	Drug Name	Type of Nanocarrier	Dose of Drug	Indication(s)	Approved (year)	Advantages
Daunorubicin	DaunoXome®	Liposomes composed of a lipid bilayer of distearoylphosphatidylcholine and cholesterol.	50 mg	Advanced HIV-related Kaposi's Sarcoma	1996	Extended circulation time; Enhanced tumor accumulation; Reduced toxicity to normal tissue.
Daunorubicin and cytarabine	Vyxeos® (CPX-351)	Liposomes composed of bilayers of distearoylphosphatidylcholine, distearoylphosphatidylglycerol, and cholesterol.	44 mg DAUN, 100 mg cytarabine	Acute myeloid leukemia	2017	Extended circulation time; Selective uptake by leukemia cells; Decreased toxicity to normal cells; Enhanced efficiency through synergistic anti-tumor activity of co-encapsulated drugs.
Cytarabine	DepoCyt®	Liposomes composed of bilayers of distearoylphosphatidylcholine, distearoylphosphatidylglycerol, triolein, and cholesterol.	50 mg	Lymphomatous meningitis	1999	Lower systemic toxicity; Sustained tumor exposure.
Doxorubicin	Doxil®/ Caelyx™	PEGylated nano-liposomes composed of hydrogenated phosphatidylcholine, cholesterol, and N-1,2- distearoyl-sn-glycero-3-phosphoethanolamine sodium salt.	20/50 mg	AIDS-Related Kaposi's Sarcoma, Ovarian Cancer, Multiple Myeloma	1995 2005 2007	Decreased systemic toxicity; Improved delivery to diseases site; Extended circulation time.
	Myocet®	Non-PEGylated liposomes composed of acidic egg phosphatidylcholine and cholesterol.	50 mg DOX	Metastatic breast cancer in combination with cyclophosphamide	2000 (in Europe and Canada)	Extended circulation time; Decreased systemic toxicity.
Paclitaxel	Abraxane®	Albumin-bound paclitaxel nanoparticles.	100/ 250 mg	Metastatic breast cancer, Advanced Non-Small Cell Lung Cancer, Pancreatic cancer	2005 2012 2013	Enhanced solubility; Improved delivery to tumor.
Vincristine	Marqibo® (VSLI)	Sphingomyelin and cholesterol-based liposomes.	0.16 mg/mL	Acute lymphoblastic leukemia	2012	Extended circulation time; Decreased systemic toxicity; Improved delivery to tumor.
Irinotecan	Onivyde®	PEGylated liposomes composed of distearoyl phosphatidylcholine, cholesterol, and methoxy-terminated polyethylene glycol- distearoylphosphatidyl ethanolamine.	4.3 mg/mL	Metastatic pancreatic cancer	2015	Extended circulation time; Decreased systemic toxicity; Improved delivery to tumor.
Mifamurtide	Mepact®	Liposomes composed of dioleoyl-phosphatidylserine and 1-palmitoyl-2-oleoyl-phosphatidylcholine.	4 mg	Nonmetastatic, resectableosteosarcoma	2009 (in Europe)	Extended circulation time.

3.1. General Treatment of Cardiovascular Diseases

The search for antithrombotic agents (ATAs) is a worldwide medical challenge. Theoretically, three groups of drugs from the ATA group can be distinguished: antiplatelet agents, anticoagulants, and fibrinolytic. Each of these categories differs with respect to the mechanism of action, time to onset, route of administration, duration of effect, and ease of monitoring. The antiplatelet and anticoagulant agents, which are commonly used to prevent clotting, instead of fibrinolytic agents, are a rescue therapy in acute cases [98]. Anticoagulants inhibit the coagulation and fibrin formation; they have been used mainly for primary or secondary prevention of embolic events (and their causes), for example, cardiogenic cerebral embolism (and atrial fibrillation), pulmonary embolism (and DVP), and following heart valve replacement [116-119]. When it comes to the antiplatelet agents, they demonstrate the ability to inhibit the formation of clots, thus preventing the activation and aggregation of platelets [120]. They are used for primary or secondary prevention of arterial thrombosis in areas where arteriosclerotic changes occur, such as in non-cardiogenic ischemic stroke [121, 122]. Additionally, in some acute conditions, where there is a repeated cycle of clot growth, maturation, and dissolution, many ATAs are preventive and therapeutic. However, in many clinical applications, there is a great need to optimize efficacy and safety, mainly in patients with a high risk of bleeding and thrombosis [98]. It has been shown that the risk of bleeding may be greater in patients who are taking anticoagulants. For example, there is an approximately 2-fold increase in bleeding in patients taking warfarin [122]. It should be noted that anticoagulants, concomitantly administered with antiplatelet drugs, have a higher risk of hemorrhage. Thus, the addition of aspirin to vitamin K antagonists increases the risk of hemorrhage. With triple therapy with anticoagulants (warfarin, aspirin, and clopidogrel), the risk of non-clotting and fatal bleeding was more than 3-fold compared to warfarin monotherapy. Importantly, also non-steroidal anti-inflammatory drugs may contribute to an increased risk of bleeding, so they should not be used in patients taking chronic anticoagulants [123]. The use of newer anticoagulants, such as thrombin inhibitors and factor Xa inhibitors, show a lower rate of major bleeding and therefore reduce the risk of fatal bleeding [124]. Dabigatran has also been shown to promote cranial bleeding in elderly patients and patients with renal insufficiency [125]. Currently available clinical strategies for clot lysis rely on the intravenous administration of a fibrinolytic agent such as urokinase, streptokinase, and tissue plasminogen activators (tPA), ultimately inducing fibrin lysis to break down clots [126-129]. One limitation for all ATAs is the inability to prevent or reverse thrombosis without interfering with hemostasis. Another problem is that they accumulate in non-targeted tissues and have a short half-life due to their enzymatic degradation, which reduces their concentration in the blood and then reduces efficiency [130]. Fibrinolytics are not used for prophylaxis due to the fact that they quickly become inactivated and then removed from the blood, which requires high and dangerous doses to stay active [131]. Thrombolytic drugs, *e.g*. tPA, have a short circulation period with a 2 to 6-minute half-life, leading to the use of high doses of drugs to induce thrombolysis. The results of the studies also indicate that the increased administration and systemic distribution of fibrinolytic drugs are detrimental due to the disruption of normal hemostatic abilities, which may consequently lead to hemorrhagic complications [132-134]. Due to its size and negative charge, heparin should be parenterally administered as it is not orally bioavailable and requires careful monitoring of the patient. It has been observed that a large amount of heparin can cause an allergic reaction in the human body when blood is transfused [135]. One of the oldest painkillers and anti-inflammatory drugs is aspirin and is still widely used today. It is used in diseases of the joints to prevent chronic deformation that occurs as a result of inflammation, synovial hyperplasia, and bone erosion. The dose of aspirin used in these cases is as low as 3-5 g/day, and in most cases, the treatment time is also long [136].

3.2. Nanodrugs in Some Cardiovascular Diseases

Advanced drug delivery systems can help overcome many limitations and further widen the therapeutic window of antithrombotic interference through two interrelated functions: lengthen the circulation time and reduction of the undesirable drug interactions end route to the therapeutic side and spatiotemporally localizing drug action [137, 138]. Most carriers are composed of biocompatible elements artificially assembled supramolecular structures, with sizes ranging from tens of nanometers to a few microns. The antiplatelet agents block various pathways in platelet activation and aggregation. For example, eptifibatide is one of the antagonists of the platelet glycoprotein IIb/IIIa (GP IIb/IIIa), which inhibits the binding of fibrinogen to the activated GP IIb/IIIa site, which prevents platelet interaction and clot formation [139]. Due to the inactivation and elimination by renal filtration and enzymatic degradation, which reduce its concentration in the blood, it has a short half-life. Moreover, free eptifibatide has some side effects such as severe bleeding

and accumulation in nontargeted tissues [140]. The main objective of the Bardania *et al.* study was to prepare the RGD-modified nanoliposomes (RGD-MNL) as a vehicle for the targeted delivery of the eptifibatide. It showed good stability of the RGD- MNL nanoliposomes, no significant cytotoxic effect on HUVEC cells, and the *ex vivo* activity for encapsulated eptifibatide was higher than the free drug [141]. The next platelet GP IIb/IIIa receptor antagonist has been demonstrated to be promising for liposomal formulations. For example, Haller *et al.* showed that the liposomal delivery of CD39 (the mechanism of CD39 is the physiological antithrombotic action, blocking platelet aggregation) had the ability to restore enzyme activity and assess the thrombo-regulatory potential [142]. Another strategy for liposomes may include the study of TS liposomes conjugated with RGD (Arg-Gly-Asp) peptide and encapsulated streptokinase.It was observed that TS liposomes are specific to the site of thrombus and released the drug following their binding with the activated platelets more successfully than that with resting platelets. RGD conjugated TS liposomes could not only reduce the clot lysis time but also increase the total clot dissolution during the clot-dissolving study [143]. Tirofiban, a glycoprotein IIb/IIIa receptor inhibitor, is also an effective antiplatelet agent in the treatment of acute coronary syndromes. Regarding the physicochemical and biological characteristics of thrombi, such as an abundance of fibrin and an elevated level of hydrogen peroxide (H_2O_2), Kang *et al.* synthesized a fibrin-targeted imaging and antithrombotic nanomaterial, defined as a FTIAN theranostic system for obstructive thrombosis. FTIAN showed reduced production of H_2O_2 and inhibited the expression of tumor necrosis factor-alpha (TNF-α) and soluble CD40 ligand in activated platelets, indicating antioxidant, anti-inflammatory, and antiplatelet activity effects. Using a mouse model of ferric chloride ($FeCl_3$)-induced carotid thrombosis, FTIAN specifically targeted the obstructive thrombus resulting in an enhancement of the fluorescence/ photoacoustic signal. Then, loaded with the antiplatelet drug tirofiban, FTIAN significantly abated thrombus formation [144]. PEGylation or encapsulation was used to improve the thrombolytic stability of the drug in sustained blood circulation, resulting in efficient drug accumulation at the clot site to enable targeted thrombolysis [145, 146]. Korin *et al.* designed "smart" carriers sensitive to hemodynamic changes to avoid the need for an external mechanism to release ATAs. These nanotherapeutics (SA-NTs) represent micron-size (~4 μm diameter) aggregates of tPA-coated polymeric NPs (~200 nm diameter) that are relatively stable under normal flow (10-30 dyne/cm^2) but dissociate into NPs at the elevated shear stress levels encountered in areas of arterial narrowing(>100 dyne/cm^2). To deliver ATA, NPs showed less resistance forces than their parental micron particles and accumulated in affected vessels in a mouse model of thrombotic arterial stenosis; SA-NTs prolonged the time to vessel occlusion from ~10 to ~30 minutes *in vivo* and reduced pulmonary emboli in an *ex vivo* model at a 100-fold lower dose than free tPA. It uses a biophysical drug targeting strategy that lowers the required doses and minimizes side effects while maximizing drug's efficacy, representing a new potential opportunity for the treatment of life-threatening diseases that result from acute vessel occlusion [147]. Another targeted delivery system has been developed by utilizing magnetically-activated nanomaterials to revise the transport of tPA at the blood clot surface for more effective local ischemic stroke therapy [148]. Nevertheless, the dependence on luminal high shear stress or an externally rotating magnetic nanomaterial may prevent the use of such a system in a general clinical setting [149, 150]. Also nanocarriers have been used to increase the targeted delivery of tPA to a thrombus *in vitro* and *in vivo* using peptide sequences of fibrinogen, such as arginine-glycine-aspartic acid (RGD) motifs situated in each of its two Aα chains and the sequence (CQQHHLGGAKQAGDV) located within its γ chains that selectively bind to αIIbβ3 integrins on the surface of activated platelets [151-153]. Huang *et al.* successfully developed an activated-platelet-sensitive nanoliposome - tPA-PEGcRGD-lip. This nanocarrier is characterized for selective thrombolysis through targeted delivery and controlled release of tPA to the blood clot. The tPA-loaded, cRGD-coated, PEGylated liposome had a small size (~160 nm in diameter) and good stability. It demonstrated highly specific binding of tPA-PEG-cRGD-lip to activated platelets, and productive tPA release from tPA-PEG-cRGD-lip was induced through liposomal membrane destabilization engaging membrane fusion upon selective binding of the liposome to activated platelets. Furthermore, tPA-PEG-cRGD-lip had distinctly higher thrombolytic activity and significantly diminished the time needed for clot lysis compared to noncRGD-coated liposomes [154]. Heparin is an anticoagulant consisting of a highly sulfated, anionic polysaccharide that can prevent thrombus formation by interacting with antithrombin III (AT-III) after contacting blood. It is used mainly in the prevention of venous thrombosis and pulmonary embolism in patients undergoing surgery [155]. There have been several attempts to develop new oral forms of heparin, but these have had limited success. Jiao *et al.* have reported that heparin-loaded NPs prepared with blends of

Table 3. Potential nanodrugs with improved effectiveness in cardiovascular diseases *compared* to *conventional* drugs.

Nano-drugs	Mean Size	*In vitro* Study	Dose	*In Vivo* Study	Dose	Improved Biological Effect	References
Liposomes (RGD-MNL eptyfipatyd)	9 ±10 nm	HUVEC	50-200 μg/mL	rats	800 μg/mL	Antiplatelet	[141]
Liposomes (CD39)	-	-	-	mice	563.6 ± 42.2 platelets/ microm^3	Antiplatelet	[142]
Liposomes (streptokinase)	100-120 nm	human blood clots	7000 IU/mL	-	-	Antithrombotic	[143]
FTIAN (tirofiban)	±200 nm	murine macrophage, arterial endothelial	20-200 μg/mL	mice	5 mg/kg	Antioxidant; Antinflammatory; Anitplatelet; Antithrombotic	[144]
SA-NT (tPA)	200 nm	endothelial	100 μg/mL	mice	50 ng/mL	Antithrombotic	[147]
Magnetic Nanorods (t-PA)	-	human blood clots	±7 mg/mL	mice	100 mg/kg	Antithrombotic	[148]
Polymeric (heparin)	260-300 nm	-	-	rabbits	600 IU/kg	Antithrombotic	[156]
RS/PLGA (heparin)	100 nm	Caco-2	-	-	-	Antithrombotic	[157]
Albumin (aspirin)	46-190.8 nm	human platelet	0.06 mg/mL	-	-	Antiplatelet	[159]

biodegradable polymers and no biodegradable positively charged polymers facilitated the gastrointestinal absorption of heparin in rabbits model, with doses similar to those administered by the parenteral route in humans. In addition, they showed an increase and a prolongation of the anti-factor Xa activity and fragmentary thromboplastin time (aPTT) [156]. Others have observed that NPs increase the bioavailability of heparin when administered orally, but the reason for this is unknown. The authors used a resonant mirror system and Caco-2 cells to study the mechanism of how heparin NPs interact with mucin and epithelial cells, respectively [157]. Owing to the magnetic Fe_3O_4 core and the immobilization of heparin on PMNPs (polyvinyl alcohol-shell magnetic NPs), these could be used as recyclable anticoagulants by magnetic fields to prevent heparin from getting into the body to cause allergic reaction [158]. Aspirin has anti-inflammatory properties and is widely used in a variety of conditions because inhibition of platelets by aspirin is irreversible and is the basis of its use in diseases like diabetic retinopathy and thromboembolic disorders. It has been shown that aspirin-loaded albumin NPs can be useful in these situations because they are small in size (46 to 190.8 nm in diameter); therefore, they can potentially be applied topically and exhibit slow and sustained drug release over several days [159]. Other authors have prepared and characterized ASA/CS copolymer (chitosan (C-S)-acetylsalicylic acid (ASA) NPs). The prepared ASA/CS NPs showed high stability against environmental changes. Moreover, *in vitro* release studies showed that ASA could be easily released in slightly alkaline conditions [136]. Table **3** contains a summary of the selected drugs with antiplatelet and antithrombotic effect, including *in vitro* and *in vivo* research.

4. ANGIOGENESIS IN CANCER TREATMENT

Angiogenesis is a normal and complex process controlled by certain biomolecules produced in the body. This process relates to creating new blood vessels from a pre-existing one [160, 161]. New blood vessels are made from preexisting blood cells by the “sprouting” of endothelial cells, which leads to the expansion of the vascular tree. The next steps towards angiogenesis include protease production, endothelial cell migration and proliferation, vascular tube formation, anastomosis of newly formed tubes, synthesis of a new basement membrane, and incorporation of pericytes and smooth muscle cells. Proteolytic enzymes activate endothelial cells by angiogenic stimuli, which degrade the perivascular extracellular matrix and the basement membrane. Then, endothelial cells proliferate and migrate into the perivascular area, forming “primary shoots.” Capillary loops then form, and the synthesis of a new basement membrane and maturation of blood vessels begin to

complete tube-like structures through which blood can flow. Angiogenesis plays a crucial role in different physiological states like embryo development, ovulation, wound healing, and collateral formation for improved organ perfusion [162]. Nevertheless, abnormal accelerated angiogenesis processes sometimes occur, or pathological angiogenesis is associated with a variety of disorders, including ocular neovascularization, leading to a loss of vision [163]. Moreover, it is important in the progression of many diseases such as diabetic retinopathy, arthritis, and metastasis [164]. In a pathological condition such as cancer, angiogenesis is required for tumor survival, proliferation, and proper nourishment and removal of metabolic wastes from tumor sites during tumor growth [165]. For the first time in 1968, it was emphasized that the tumor secretes a substance that stimulates angiogenesis [166]. Subsequently, various proangiogenic factors were investigated that disperse the tumor cells when their mass reaches a limited size of an early tumor [167-169]. Beginning studies conducted by Folkman in 1971 suggested an insightful anticancer therapy by starvation of blood supply [170]. Through these studies, the idea was developed that blocking tumor nutrition may be one way to avoid tumor propagation, but the multiplicity of angiogenetic signals from tumor cells is unlikely to be effective as an approach to inhibit angiogenic stimuli directly for anti-cancer therapy [171]. There are two groups of angiogenesis inhibitors: direct inhibitors that target endothelial cells in the growing vasculature and indirect inhibitors that target either tumor cells or the other tumor associated stromal cells. Direct or indirect anti-angiogenic therapy restores the balance between pro-angiogenic and anti-angiogenic factors by reducing vascular permeability and hypoxia and increasing uniformity of blood flow and perivascular cell coverage [172]. For instance, bevacizumab blocks tumor cell-derived vascular endothelial growth factor A (VEGF-A), impairing the development of new vessels and leading to tumor starvation and, consequently, growth inhibition [15]. It has been proved that the side effects of bevacizumab increased when it was combined with chemotherapy. The bleeding complications were observed during the treatment of colorectal cancer treated with IFL (a chemotherapy regimen consisting of concurrent treatment with irinotecan, leucovorin (folic acid), and fluorouracil in combination with bevacizumab [173]. Also, combination therapy with bevacizumab, carboplatin, and paclitaxel improved the overall response and time to progression in patients with advanced or recurrent non-small cell lung cancer but ended in severe or fatal pulmonary hemorrhage [174]. The treatment when thromboembolism occurs or antithrombotic prophylaxis remains problematic in patients treated with antiangiogenic agents. In fact, all these agents have been associated with an increased risk of venous (VTE) and arterial (ATE) thromboembolism [175]. The meta-analysis of 1,745 patients treated with chemotherapy with or without bevacizumab showed a two-fold higher incidence of ATE in those patients treated with the combination of anti-VEGF drugs plus chemotherapy; VTE was observed in many studies but was not statistically significant [176]. A lower frequency of bleeding complications, but still higher than in the control group, occurred during treatment. Other small-molecule tyrosine kinase inhibitors like sunitinib or sorafenib showed an overall lower rate of bleeding complications. The main hypothesis on the mechanisms of bleeding induced by anti-VEGF agents is complex and not yet fully elucidated that VEGF may promote survival and integrity of endothelial cells in adult blood vessels, while inhibiting it may reduce the ability to renew damaged endothelial cells [177]. Moreover, the acquired resistance of cancer cells to these agents is considered to be one of the most difficult challenges, limiting their effectiveness. Additionally, angiogenesis inhibitors do not have important and standardized biomarkers to assess their effectiveness and/or tolerance, which significantly increases the limitations of therapy [178, 179]. It is still far from a complete understanding of the mechanisms that could determine the expectation that an efficacious inhibitor of one key step in this cascade would be able to stop angiogenesis and thus tumor growth.

4.1. Nanoparticles as a New Tool for Inhibition of Cancer Angiogenesis

It has been shown that NPs can effectively target the tumor vasculature through specific and non-specific biochemical and biophysical mechanisms. NPs (~ 50-200 nm) can accumulate in tumors if they have a long circulation time due to a leaky vascular system and the lack of a draining lymphatic system present in the tumor bed [180-183]. To increase circulation time, anti-angiogenic therapies have used cationic liposomes that can target new blood vessels to deliver the anti-cancer drugs oxaliplatin or PTX, with or without PEGylation [184, 185]. In studies on glioblastoma multiforme, αvβ3 and αvβ5 are overexpressed on brain tumor cells and tumor vessels in the brain, leading to interactions between glial cells and the extracellular matrix. These integrins are characterized by specific targeting of the arginine-glycine-aspartic acid (RGD) peptide. Cyclic forms of RGD and DOX loaded double- modified liposomes of peptide-22 have been shown to cross the blood-brain barrier while inhibiting the growth of glioblastoma multiforme [186].

Table 4. Potential nanodrugs with improved anticancer activity *compared* to *conventional* drugs.

Nano Drugs	*In Vitro* Study	Dose	*In Vivo* Study	Dose	Improved Biological Effect	References
PEG Liposomes (oxaliplatin, paclitaxel)	mouse melanoma	0.176 mg/mL	mice	5 mg/kg	Antiangiogenic; Anticancer.	[180]
Liposomes (paclitaxel)	HUVEC, human melanoma	0.01 ng/mL	mice	12.5 mg/kg	Antiangiogenic; Anticancer.	[185]
RGDfK Liposomes (RGD, doxorubicin)	HUVEC, U87MG, BCEC	-	mice	5mg/kg	Overcome BBB/BBTB barriers; Anticancer.	[184]
RGDyK -PEG-PLA-PTX Liposomes (RGD, paclitaxel)	U87MG	0.1-250 nM	mice	7.5 mg/kg	Antiangiogenic; Anticancer.	[187]
eBev-DPPN PLGA (Bevacizumab, Dexamethasone)	HUVEC	-	rabbits	-	Antiangiogenic; Reduced the number of blood vessels formed.	[189]

Cilengitide (cyclo [RGDfV]) is a cyclic peptide that selectively binds αv integrins. Micelle c (RGDyK) -PEG-block-poly (lactic acid) (c [RGDyK]-PEG- PLA-PTX) loaded with PTX was constructed and then tested in U87MG cells. c (RGDyK) -PEG-PLA after PTX loading inhibited the tumor growth more strongly compared to the tested PTX preparations and increased the mean survival time of the treated mice to 48 days [187]. Similarly, in recent studies, the pharmacological compound characterized by a platinum anticancer drug also containing a polymeric cyclic RGD micelle has allowed high permeability and storage capacity in tumor tissue [55]. A structural relationship of nano-DDS targeted Pep-1 and CREKA GBM peptides (PC-NP) was used to overcome the BBTB tumor blood-brain barrier, inhibit glioma cells *via* IL-13rα2 mediated endocytosis, and bind to fibrin complexes - fibronectin expressed in the tumor microenvironment. The results obtained showed that the PC-NP cellular uptake by U87MG cells was significantly increased compared to non-target NP. Additionally, PC-NPs significantly increased the cytotoxicity of their PTX charge against U87MG cells [188]. Other nanostructures were electrostatically coupled poly (D, L-lactide-co-glycolide)/polyethyleneimine (eBev-DPPN) molecules containing bevacizumab, containing dexamethasone, with a diameter of about 200 nm, with a neutral surface charge (0.85 ± 0.37 mV), which ensured the stability of eBev-DPPN under physiological conditions. The authors' research on apoptosis, migration, invasion, and tube formation has shown that eBev-DPPN has a good anti-angiogenic effect on HUVEC. EBev-DPPN also provided a strong inhibitory effect on VEGF secretion from HUVEC [189]. Table **4** contains a summary of the selected drugs with antiangiogenic and anticancer effects, including *in vitro* and *in vivo* research.

5. CHALLENGES IN USING NANOPARTICLES WITH DRUGS

In view of pharmaceuticals, which are specific drugs for drug delivery, they can be used to increase the therapeutic index or the ratio between the dose needed for clinical efficacy and the dose causing undesirable side effects (toxicity). This is especially true of NPs drug delivery applications [190]. Current tests and procedures for NPs-based drug and device evaluation may be suitable for detecting many of the risks associated with the use of NPs, but it cannot be assumed that these tests will detect all potential risks. Due to different physicochemical properties, NPs can redistribute themselves in the body by crossing the blood-brain barrier and triggering blood clotting pathways. Given these features, particular emphasis should be placed on the (pharmacological) kinetics and distribution of NPs. According to experts, the current knowledge about the biological behavior of NPs in terms of *in vivo* distribution, both at the organ and cell level, is insufficient. In the safety assessment, the toxicity of the entire formulation is tested, while the results of the NPs themselves are not described. This does not distinguish the toxicity of drugs and NPs, so the focus should also be on the toxicity of non-drug particles. This is a priority when slow or non-degradable particles are used for drug delivery, which may persist and accumulate at the injection site, ultimately leading to chronic inflammatory responses [191].

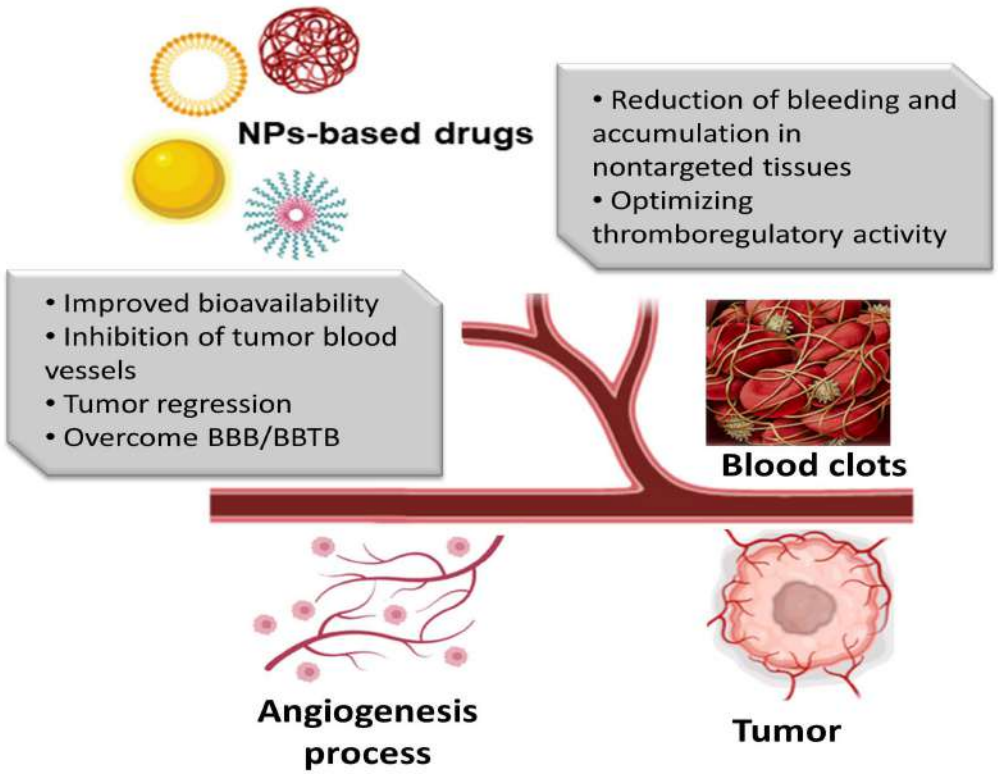

Fig. (4). This scheme shows the most important benefits of nanodrugs in cardiovascular diseases and cancer with tumor-associated angiogenesis. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

CONCLUSION

Ultimately, by the optimization of NPs size and surface properties, nanotechnology is able to deliver drugs with enhanced bioavailability in the treatment of cardiovascular diseases, cancer, and inhibition of cancer-related angiogenesis. Thus, nanotechnology can improve the therapeutic efficacy of the drug by targeting the thrombolytic agent to the activated platelets in the thrombus, and even oral heparin could avoid parenteral injections and consequently be highly desirable for patients. Huge advantages of nanoformulations also result from restoring enzyme activity and optimizing thromboregulatory potential. Having regard to clinical oncology, nanomedicine has the potential to substantially improve the treatment of aggressive diseases. NPs-based treatments show promising results for overcoming drug resistance and achieving site-specific delivery. For antiangiogenic cancer therapy, it is imperative to look for alternative modern approaches, such as nanomedicine, that can effectively target and inhibit tumor angiogenesis. NPs are providing innovative, controlled, and targeted techniques, resulting in a considerable decrease in therapy-associated side effects with increasing antitumor and anticoagulant efficacy, as shown in Fig. (**4**). Although nanomedicine offers new and improved solutions for the treatment, there is a need for a better understanding of the nanodrugs interaction in the human body because this is a key factor in the success of potential nanotherapeutics. The key parameters in selecting NPs for drug delivery should be the optimization of the therapeutic index, the ratio of the therapeutic dose to toxic dose, kinetics, and nanoparticle distribution. Thus, systematic studies assessing the link between the biological effect of nanoparticles and their properties are essential.

LIST OF ABBREVIATIONS

6-MP	=	6-mercaptopurine
ALT	=	Altretamine
ATAs	=	Antithrombotic Agents
CLB	=	Chlorambucil
CNS	=	Central Nervous System
CVDs	=	Cardiovascular Diseases
DAUN	=	Daunorubicin
DOX	=	Doxorubicin
DVT	=	Deep Vein Thrombosis
GEM	=	Gemcitabine
GP IIb/IIIa	=	Glycoprotein IIb/IIIa

IFS	= Ifosfamide
IFS-LNC	= IFS-loaded-lipid-core Nanocapsules
MDA-MB-231	= Human Breast Cancer Cells
MLN	= Melphalan
NPs	= Nanoparticles
PEG	= Polyethylene Glycol
PTX	= Paclitaxel
RGD	= Peptide Arg-Gly-Asp
SLNs	= Solid-lipid-NPs
tPA	= Tissue Plasminogen Activators
U-87 MG cells	= Human Glioblastoma Cells
VTE	= Venous Thromboembolism

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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ORIGINAL RESEARCH

Effects of functionalized silver nanoparticles on aggregation of human blood platelets

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Purpose: We studied the effects of silver nanoparticles (AgNPs) on human blood platelet function. We hypothesized that AgNPs, a known antimicrobial agent, can be used as blood-compatible, "ideal material" in medical devices or as a drug delivery system. Therefore, the aim of the current study was to investigate if functionalized AgNPs affect platelet function and platelets as well as endothelial cell viability in vitro.

Methods: AgNPs, functionalized with reduced glutathione (GSH), polyethylene glycol (PEG) and lipoic acid (LA) were synthesized. Quartz crystal microbalance with dissipation was used to measure the effect of AgNPs on platelet aggregation. Platelet aggregation was measured by changes in frequency and dissipation, and the presence of platelets on the sensor surface was confirmed and imaged by phase contrast microscopy. Flow cytometry was used to detect surface abundance of platelet receptors. Lactate dehydrogenase test was used to assess the potential cytotoxicity of AgNPs on human blood platelets, endothelial cells, and fibroblasts. Commercially available ELISA tests were used to measure the levels of thromboxane B_2 and metalloproteinases (MMP-1, MMP-2) released by platelets as markers of platelet activation.

Results: 2 nm AgNPs-GSH, 3.7 nm AgNPs-PEG both at 50 and 100 µg/mL, and 2.5 nm AgNPs-LA at 100 µg/mL reduced platelet aggregation, inhibited collagen-mediated increase in total P-selectin and GPIIb/IIIa, TXB2 formation, MMP-1, and MMP-2 release. The tested AgNPs concentrations were not cytotoxic as they did not affect, platelet, endothelial cell, or fibroblast viability.

Conclusion: All tested functionalized AgNPs inhibited platelet aggregation at nontoxic concentrations. Therefore, functionalized AgNPs can be used as an antiplatelet agent or in design and manufacturing of blood-facing medical devices, such as vascular grafts, stents, heart valves, and catheters.

Keywords: functionalized silver nanoparticles, blood platelets, QCM-D, platelets receptors, TXB_2, MMP-1 and MMP-2

Introduction

Among metal nanoparticles, silver nanoparticles (AgNPs) are emerging as an attractive tool for many nanomedical applications.[1] They are endowed with anticancer, antibacterial, antifungal, and antiviral properties.[2] They can also be used to design an "ideal material" for bone prostheses, reconstructive orthopedic surgery, cardiac devices, extracorporeal circulation, catheters, and surgical appliances.[1,2] AgNPs have also potential to form a drug delivery platform.[3] For any blood-facing applications of AgNPs, blood and platelet biocompatibility are an essential characteristic.

Therefore, a number of research groups studied the effects of AgNPs on platelet reactivity. However, these studies yielded controversial results and both platelet activator and inhibitory properties of AgNPs have been claimed.[4–10]

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Others and we have indicated that the biocompatibility of nanoparticles with components of the circulation system depends on their functionalization and physicochemical properties.[4,10–12]

Conjugating nanoparticles and other nanomaterials with polyethylene glycol (PEG), known as PEGylation, is used widely as it offers a number of advantages. For example, PEGylated nanomaterials when used in protein delivery systems show increased protein solubility and stability, reduced immunogenicity, decreased clearance by the reticuloendothelial system, and increased plasma half-life leading to less frequent dosing.[13] It has been found by our team, that conjugation of gold nanoparticles with PEG protects platelets from activation.[4] Niidome et al, 2006demonstrated that PEG modification of gold nanorods also reduced their in vitro toxicity.[14] Moreover, we noticed that AgNPs coated with PEG, and lipoic acid (LA) decreased cytotoxicity against human cells compared to non-functionalized AgNPs and improved antibacterial, and antibiofilm activity.[8,13,15,16]

Based on our previous observations, we have synthesized AgNPs functionalized with PEG, LA, and reduced glutathione (GSH) and evaluate their effect on platelet aggregation under flow to mimic conditions encountered in microvasculature in vivo. We also evaluated the safety potential of thus synthesized AgNPs in regards to cytotoxicity. Finally, we focused on molecular mechanisms of platelet-AgNPs interactions including determination of surface abundance of platelet receptors, thromboxane B_2 formation, and release of MMP-2, MMP-9 from collagen-stimulated platelets.

Materials and methods

Synthesis and characterization of AgNPs

Glutathione stabilized silver nanoparticles (AgNPs-GSH)

Five hundred mg (2.7 mmol) of silver nitrate was dissolved in 320 mL of water and reaction mixture was placed in water/ice bath in dark. Then, 1.96 g (6.38 mmol) of reduced GSH was added slowly in small portions. After that, a white, flocculant appeared as precipitate. Reaction mixture was stirred further 1.5 hrs and next 9 mL of saturated solution of sodium bicarbonate was added dropwise, which increased pH to 4.5 and resulted in the disappearance of precipitate.

Next, 1.21 g (31.9 mmol) of sodium borohydride dissolved in 80 mL of water was added rapidly to vigorously stirring reaction mixture. After 15 mins, color of reaction mixture has changed to dark-brown. The resulting reaction mixture was stirred overnight at room temperature. After 12 hrs, the excess of unbounded GSH and aggregates formed during the synthesis of nanoparticles were precipitated by the addition 300 mL of isopropanol. The precipitate, thus formed, was centrifuged (10 mins, 5000 g, 0°C) and then nanoparticles were dissolved in 20 mL of water. Additionally, the above-described procedure of precipitation/centrifugation was repeated two times. Then, nanoparticles were put in SnakeSkin (3500-MW cut off) tube and dialyzed against distilled water for 3 days (water was changed every day).

Lipoic acid-stabilized silver nanoparticles (AgNPs-LA)

Briefly, 1.9 (9.2 mmol) g of LA and 700 mg (18.5 mmol) of sodium borohydride were dissolved in 1400 mL of water. Next, 70 mL of 25 mM silver nitrate and additional portion 1 g (26.43 mmol) of sodium borohydride in 200 mL of water in 200 mL of water were added and the resulting reaction mixture was stirred in dark for 5 hrs. Then, 300 mL of the mixture was placed in SnakeSkin (3500-MW cut off) tube and dialyzed against distilled water for 3 days (water was changed every day).

Polyethylene glycol-stabilized silver nanoparticles (AgNPs-PEG2000)

Polyethylene glycol (PEG, average molecular mass – 2000 Da) coated spherical silver nanoparticles were obtained as described by Chen and Wang and Vander Linden et al, with some modifications.[17,18] Briefly, dodecylamine (0.75 g, 4.04 mmol) was dissolved in cyclohexane (25 mL) and 6 mL of 37% aqueous formaldehyde solution was added. After 15 mins of vigorous stirring, a cyclohexane phase was separated by centrifugation and washed two times with water (2×10 mL). Next, an aqueous silver nitrate (0.2 g $AgNO_3$, 1.17 mmol, dissolved in 10 mL of water) was added under vigorous stirring and the organic phase was separated after 40 mins by centrifugation at 5000 g for 10 mins.

To the hexane solution of nanoparticles, 55 mg (0.0275 mmol) of PEG-2000 Da dissolved in 3 mL of DCM was added and the reaction mixture was stirred for 1 h. The precipitate of PEG 2000 Da coated nanoparticles was collected by centrifugation (10 mins, 5000 g), washed and then dissolved in 10 mL of deionized water. To remove the excess of unbonded thiol a dialysis (10,000-MW cut off) process in deionized water was applied for 3 days (water was changed every day).

The synthesized nanoparticles were covered with dodecyloamine molecules. To introduce PEG molecules onto nanoparticles surface ligand-exchange reaction was performed. The reaction relies on adding excess of thiol (which have stronger affinity to nanoparticles than amines), so PEG molecules replace all dodecyloamine molecules on NPs surface. Then, after purification of nanoparticles from excess of unbounded molecules by dialysis, the successful exchange reaction was confirmed by a series of HNMR, transmission electron microscopy (TEM), small-angle X-ray diffraction (SAXRD) measurements. To evidence that only PEG molecules are present on NPs surface, we performed additional thermogravimetry analysis (TGA) measurements.

Characterization of nanoparticles

To characterize particle size, surface coverage and purity of the obtained nanoparticles a series of TEM, SAXRD, TGA, and Fourier Transform Infrared Spectroscopy (FTIR) measurements were performed. FTIR and TGA were carried out to confirm the success of the ligand-exchange reactions and to calculate the number of molecules attached to the nanoparticle's surface measurements. Distribution of AgNPs size was determined and measured based on TEM images using ImageJ software (JEM-1200 EX II TEM ((JEOL, Tokyo, Japan)) which allow us to determine diameters of NPs in TEM image. According to those data, we prepared histograms of size distribution.

Blood collection and platelet isolation

Following informed consent, blood was withdrawn from healthy volunteers who had not been on any medication known to interfere with platelet function for at least 2 weeks prior to the study. Written informed consent was obtained from the volunteers in accordance with the Declaration of Helsinki. Whole blood was collected and carefully mixed with 3.15% sodium citrate (9:1). Platelet-rich plasma (PRP) and washed platelet (WP) suspensions were prepared from blood as described by Radomski and Moncada, using phosphate buffer solution at the final concentration of 250,000 platelets/μL. PRP was also separated from heparinized whole blood (250 g, 20 mins) for MMP-2 ELISA assay.[19]

Platelet-poor plasma (PPP), which served as a blank, was obtained by centrifugation of PRP at 1500 g for 10 mins. Approval for this study was obtained from the School of Pharmacy and Pharmaceutical Sciences Trinity College Dublin Research Ethics Committee (2016-03-01 [R8]).

Cell culture and exposure to AgNPs

Human umbilical vein endothelial cells (HUVEC) and human gingival fibroblast culture (HGF-1) cell line were obtained from the American Type Culture Collection (ATCC CRL-1730™ and ATCC CRL-2014™, respectively) and maintained as a monolayer culture in T-75 cm^2 tissue culture flasks. HUVEC were cultured in Ham's F12K medium adjusted to contain 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mg/mL heparin, 0.03 mg/mL endothelial cell growth supplement, and 10% fetal bovine serum. HGF-1 cells were grown in Dulbecco's Modified Eagle's Medium (Sigma Aldrich), a high glucose medium (4.5 g/L) containing sodium pyruvate (110 mg/L), and supplemented with 10% fetal bovine serum, 6 μg/mL penicillin-G, and 10 μg/mL streptomycin. Cells were cultured at 37°C in a humidified atmosphere of 95% O_2, 5% CO_2. When confluent, cells were detached enzymatically with trypsin-EDTA and sub-cultured into a new cell culture flask. The medium was replaced every 2nd day.

For experiments, the cells were seeded in 96-well plates at a density of 2×10^4 cells/mL and allowed to attach for 24 hrs, then treated with AgNPs suspended in appropriate serum-free cell culture medium for another 24 hrs. Before use, the suspensions of AgNPs were shaken for 1 min. Controls were supplied with an equivalent-volume of serum-free cell culture medium in the absence of AgNPs.

Platelet aggregation monitored by light aggregometry

To monitor platelet viability, the response of platelets to collagen (2 μg/mL) in PRP or WP was tested using light aggregometry. Briefly, PRP or WP samples (2.5×10^8 cells/mL) were placed in a PAP 8 aggregometer (Bio/Data Corporation, Horsham, PA, USA) and incubated for 20 mins at 37°C, with stirring at 900 r.p.m. in the presence or absence of collagen (2 μg/mL).

Platelet aggregation measured by quartz crystal microbalance with dissipation (QCM-D)

The effect of AgNPs on platelet aggregation was measured using the Q-Sense® E4 QCM-D system (Q-Sense AB, Vastra Frolunda, Sweden) under flow conditions using a peristaltic microflow system (ISM 935; Ismatec SA, Glattbrugg, Switzerland) as previously described by our group. The working principle of QCM-D relies on the changes in resonance frequency f and energy dissipation D when an

alternating current is applied through a quartz crystal and material is adsorbed on the sensor surface. The deposition of mass on the crystal induces a negative f shift from the fundamental resonant frequency of the crystal (Δf) and a positive energy dissipation shift (ΔD) that allows the measurement of the formation of platelet-microaggregates at real time on the quartz surface interface.[20,21]

For carrying out those experiments, polystyrene-coated quartz crystals with a frequency of 4.95 MHz were used as sensors following coating with fibrinogen. For fibrinogen coating, sensors were placed in fibrinogen dissolved in phosphate buffer solution (100 μg/mL) for 20 mins at room temperature. Fibrinogen-coated PC quartz crystals were then mounted in the flow chamber and perfused with PBS to ensure that fibrinogen loosely bound to the sensor was removed. Afterward, PRP suspensions were perfused through the device at 37°C at a flow rate of 100 μL/min in the presence of vehicle or AgNPs (50 and 100 μg/mL). AgNPs-platelet's interactions were monitored for 20 mins in real time by the acquisition of Q-Sense software (QSoft401) and platelet aggregation measured and analyzed as changes in frequency (Δf) and dissipation (ΔD).

To study whether or not the interaction of AgNPs with plasma proteins could be responsible for the shifts in frequency and dissipation during the aggregation studies, the interaction of NPs with PPP was also investigated. For this purpose, PPP was perfused at 100 μL/min in the presence and absence of NPs and Δf and ΔD were monitored also for 20 mins. Results from experiments using QCM-D are expressed as percentage of frequency and dissipation, where the maximal changes in frequency (negative shift) and dissipation (positive shift) at 20 mins of perfusion for the control (250,000 platelets/μL) are considered as 100%.

Phase contrast microscopy

PRP suspensions were perfused in the presence of vehicle or AgNPs on fibrinogen-coated polystyrene-coated quartz crystals for 20 mins through the device. Next, crystal surfaces were observed by phase contrast microscopy an Axiovert 200M optical microscope (Carl Zeiss) using a 5× objective and photomicrographs were taken using a digital camera and AxioVision software (v 4.7; Carl Zeiss).

Lactate dehydrogenase (LDH) release assay

The cytotoxic effect of AgNPs was assessed by detecting LDH release following exposure of the WP at 37°C for 20 mins and 24 hrs, human cell line (HUVEC, HGF-1) for 24 hrs to 50 and/or 100 μg/mL AgNPs using a Cytotoxicity Detection LDH kit (Promega, Poland), according to the manufacturers' instructions. WP or cells treated with lysis buffer (0.1% Triton X-100) was used as a positive control (total LDH release). Lysis buffer-treated cells were set to 100%. AgNPs were used as background control and their absorbance were subtracted from the reading of the samples. Results are given as % of the total LDH release from the cells.

Flow cytometry

The abundance of P-selectin and PAC-1 on the surface of platelets in the presence of AgNPs (50 and/or 100 μg/mL) was detected by flow cytometry. Collagen (2 μg/mL) induced platelets aggregation in PRP was used as a positive control whereas PRP without collagen (resting platelets) was used as a negative control. Platelets in PRP were pre-incubated with AgNPs-GSH, AgNPs-PEG (50 and 100 μg/mL), or AgNPs-LA (100 μg/mL) for 5 mins prior to the addition of collagen (2 μg/mL). When collagen-induced aggregation reached 50% maximal light transmission, samples were collected and incubated in the dark for 5 or 15 mins at room temperature in the presence of 10 μg/mL of P-selectin and GPIIb/IIIa (BD Biosciences, Oxford, UK), respectively. Subsequently, samples were diluted in FACSFlow™ and analyzed within 5 mins using a FACSArray™ bioanalyzer (BD Biosciences). Flow cytometry was performed on single-stained platelet samples as previously described.[19] Platelets were identified by forward and side scatter signals, and 10,000 platelet-specific events were analyzed by the flow cytometer for fluorescence. Data were analyzed using FACSArray software (v 1.0.3; BD Biosciences) and expressed as a percentage of control fluorescence. AgNPs were not detected by the bioanalyzer.

TXB_2 formation

PRP (2.5×10^8 platelets/mL) was pre-incubated with AgNPs (50 and/or 100 μg/mL) for 5 mins and 2 μg/mL collagen was added. Aggregation was halted when control reached maximum, by adding the equivalent-volume of 2 mM cold EDTA solution with 50 mM indomethacin. The samples were centrifuged at 12,000× g for 10 mins at 4°C and the supernatant was collected and stored at –80°C. TXB_2, the stable metabolite of TXA_2, was measured using a TXB_2 enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA), according to the instructions of the manufacturer. Absorbance values were also corrected with blank NPs.

MMP-1, MMP-2 release

Commercially available quantitative sandwiches ELISAs were used to measure concentrations of MMP-1 (total) (Abcam) and MMP-2 (both active and proactive) (R&D Systems, UK) released from platelets in PRP according to the manufacturer's protocol. Briefly, PRP (2.5×10^8 platelets/mL) was pre-incubated with AgNPs-GSH AgNPs-PEG, and AgNPs-LA (50 and/or 100 µg/mL) for 5 mins and 2 µg/mL collagen was added and aggregation was halted after 20 mins, the samples were collected, centrifuged at 12,000× g for 10 mins at 4°C and the supernatant was stored at –80°C until analysis. The lower limits of detection were 8 pg/mL for MMP-1 and 0.047 ng/mL for MMP-2.

Statistics

Data from at least three independent experiments were analyzed using GraphPad Prism (v 5.0; GraphPad Software, Inc, La Jolla, CA) and presented as means with standard deviation. Paired Student's *t*-tests, one-way analyses of variance, and Tukey–Kramer post hoc multiple comparison test were performed, where appropriate. Statistical significance was considered when $p<0.05$.

Results

Synthesis and characterization of functionalized AgNPs

We synthesized three types of functionalized AgNPs: AgNPs-GSH, AgNPs-LA, and AgNPs-PEG, as demonstrated in Figure 1.

TEM images show the prominence of spherical AgNPs-GSH with a diameter of 2.0±0.4 nm (Figure 2A).

It was also confirmed by SAXRD measurements that peaks location, as received by integration of obtained pattern, show the average distance between metallic cores about 2.2 nm which correspond well with TEM images (in case of SAXRD signal we have to also consider the length of molecules on the surface of nanoparticles so total distance between them is slightly larger). Both methods also

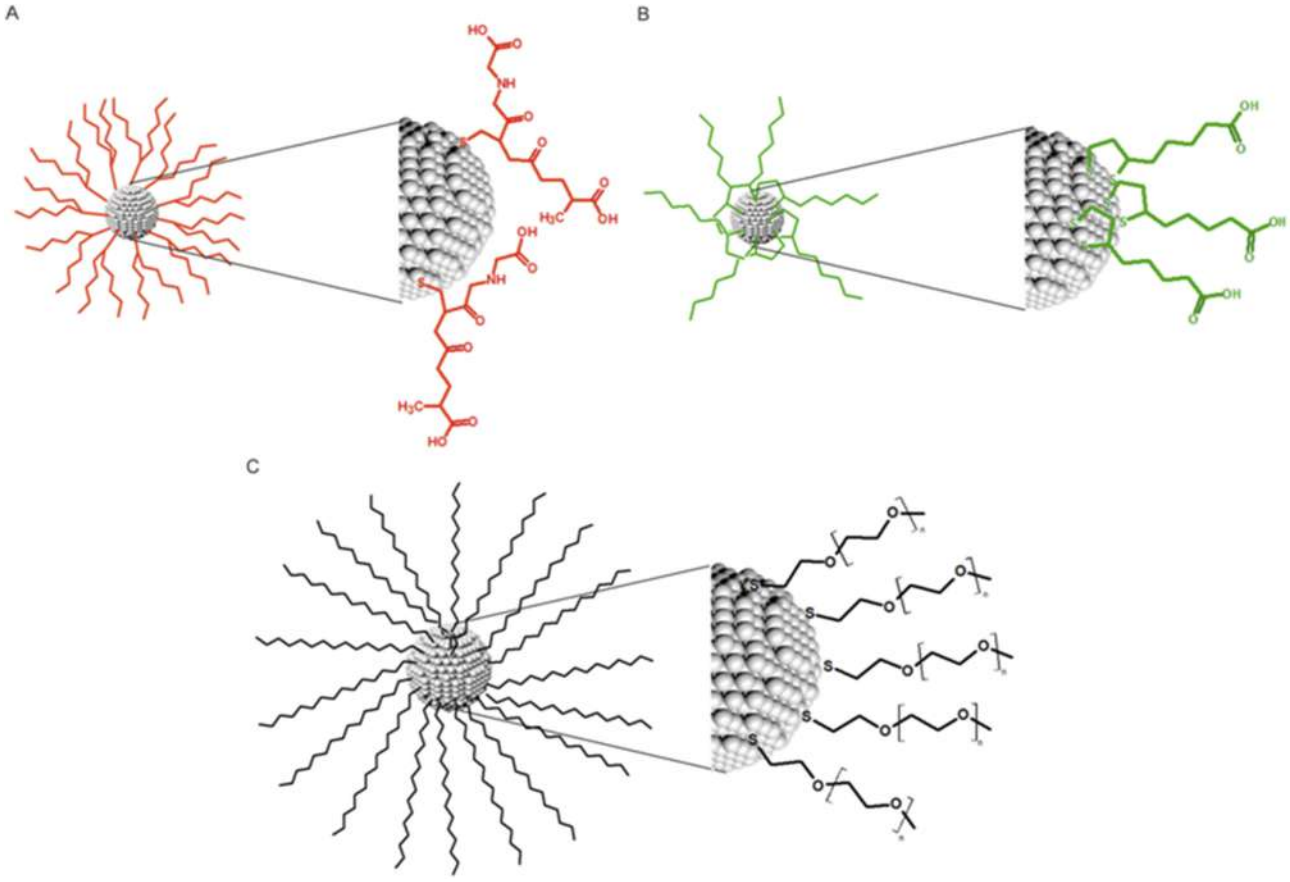

Figure 1 Schematic representation of spherical silver nanoparticles and their surface coverage: (**A**) 2 nm diameter AgNPs coated with GSH; (**B**) 2.5 nm diameter AgNPs coated with LA; (**C**) 3.7 nm diameter AgNPs coated with PEG-2000 Da.
Abbreviations: AgNPs, silver nanoparticles; GSH, glutathione; LA, lipoic acid.

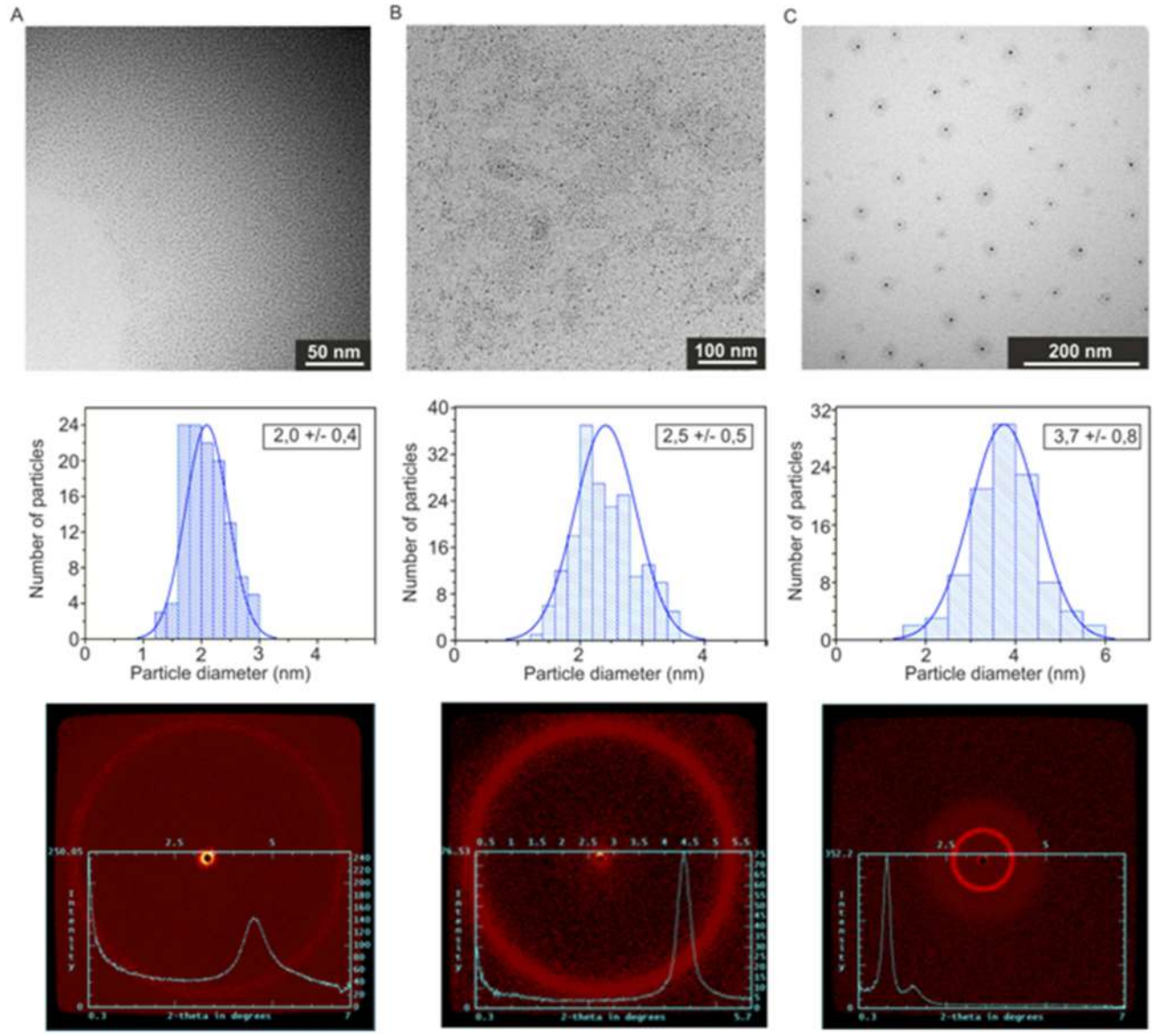

Figure 2 TEM images (top panel), histograms of NPs size distribution (middle panel), and SAXRD patterns (bottom panel): (**A**) AgNPs-GSH, (**B**) AgNPs-LA, (**C**) AgNPs-PEG 2000.
Abbreviations: TEM, transmission electron microscopy; NPs, nanopaticles; SAXRD, small-angle X-ray diffraction; AgNPs, silver nanoparticles; GSH, glutathione; LA, lipoic acid.

consistently confirm the formation of spherical AgNPs-LA and AgNPs-PEG with the average diameter of 2.5±0.5 nm (Figure 2B) and 3.7±0.8 nm (Figure 2C) as calculated by the ImageJ software, respectively. It worth to note that in the attached TEM image, PEG shells around each nanoparticle are clearly visible.

As can be observed in Figure 3, both the spectra of nanoparticles and ligands are characterized by analogous absorption bands, which indicates that the nanoparticles contain the same ligands as compared to controls. Some minor differences may be the result of entanglement of some groups in the molecule in the formation of covalent bonds on nanoparticles surface or non-covalent in the solid phase under the conditions of FTIR experiments.

To calculate the number of molecules attached to the nanoparticles surface a series of TGA measurements were also performed. Figure 4A–C shows mass loss during when heating the sample. This loss can be attributed to the removal of the organic shell and recalculated to the number of surface thiols. To recalculate the obtained data we first calculated the mass of a single nanoparticle, using the average diameter derived from TEM and the bulk density of the metal. The mass of the organic compounds removed from a single nanoparticle was calculated using

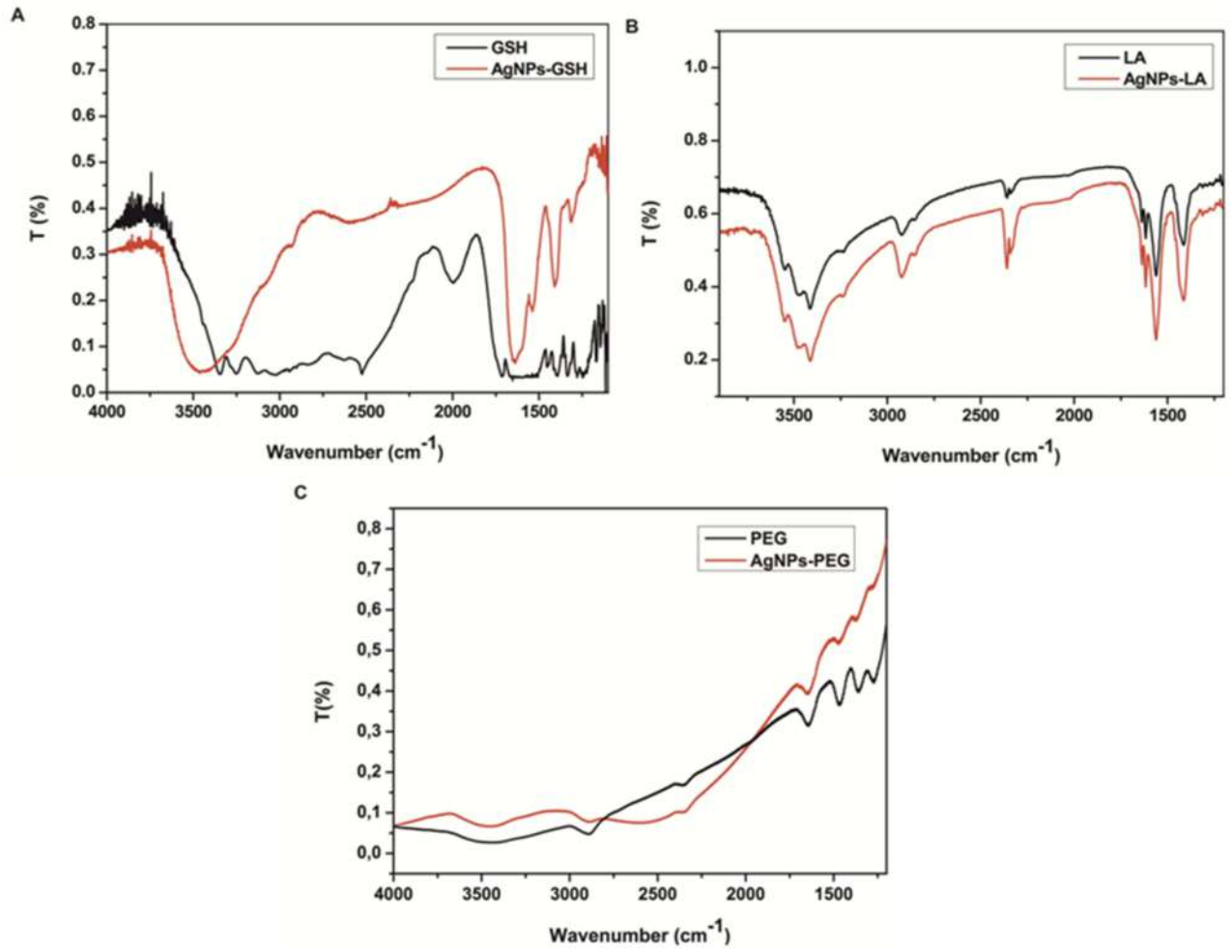

Figure 3 FTIR analysis of (**A**) AgNPs-GSH, (**B**) AgNPs-LA and (**C**) AgNPs-PEG.
Abbreviations: FTIR, Fourier Transform Infrared Spectroscopy; AgNPs, silver nanoparticles; GSH, glutathione; LA, lipoic acid; PEG, polyethylene glycol.

the % of mass left after the analysis. The calculated results are shown in Table 1

Functionalized AgNPs inhibit platelet aggregation as detected by QCM-D and microscopy

Before each QCM-D experiment platelet viability was confirmed using light aggregometry (Figure 5A). The perfusion of physiological concentrations of platelets (2.5×10^8 platelets/mL) on polystyrene-coated quartz crystals caused a decrease in frequency and an increase in dissipation indicating the deposition of platelet aggregates on the sensor surface (Figures 5B and 6). We found that incubation of platelets with AgNPs-GSH and AgNPs-PEG at concentrations 50 and 100 µg/mL or AgNPs-LA at 100 µg/mL significantly decreased dissipation and increased frequency as compared to controls (Figures 5B and 6A). The QCM-D data were confirmed by phase contrast microscopy (Figure 6B).

It is important to note that AgNPs did not cause deposition of plasma proteins on the crystal surface. Indeed, AgNPs did not induce changes in frequency and dissipation when plasma was perfused in the absence of platelets (Figures 5C and 7).

Moreover, we confirmed that the corresponding concentrations of GSH, LA, and PEG from 10 to 35 µM did not induce changes in dissipation and frequency (data not shown).

Functionalized AgNPs are not cytotoxic for blood platelets for human platelets, endothelial cells, and fibroblasts

The measurement of LDH enzyme release by inhibition of 3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) reduction is a commonly used assay to

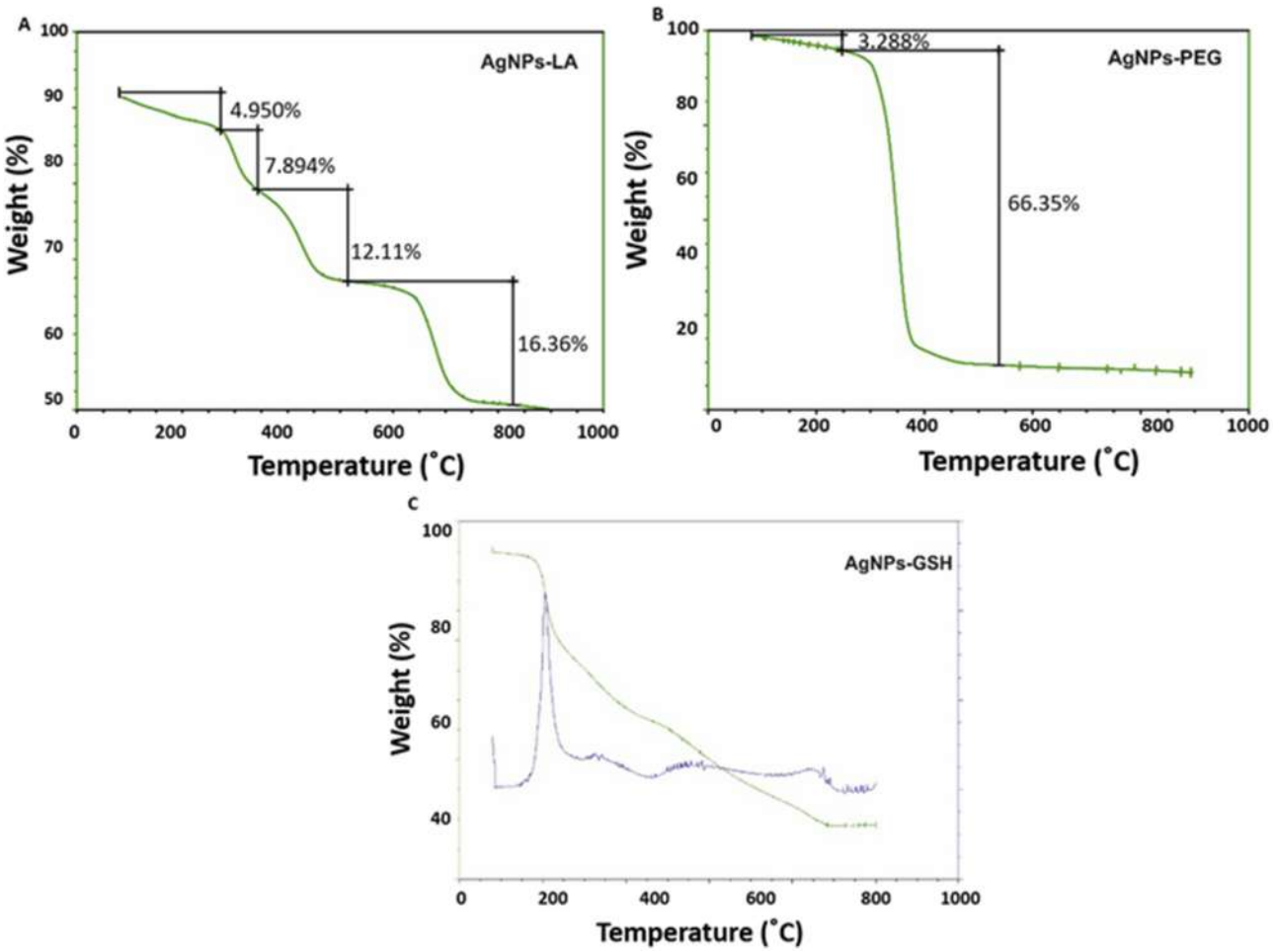

Figure 4 TGA analysis for (**A**) AgNPs-LA; (**B**) AgNPs-PEG; (**C**) AgNPs-GSH.
Abbreviations: TGA, thermogravimetry analysis; AgNPs, silver nanoparticles; LA, lipoic acid; PEG, polyethylene glycol; GSH, glutathione.

Table 1 %mass of organic and number of ligands attached to nanoparticles surface calculated from TGA analysis

Sample	Core size [nm] from TEM	Mas % of organic from TGA	Number of ligands
AgNPs-GSH	2±0.4	48.11	74
AgNPs-LA	2.5±0.5	41.31	176
AgNPs-PEG	3.7±0.8	69.64	192

Abbreviations: TGA, thermogravimetry analysis; TEM, transmission electron microscopy; AgNPs, silver nanoparticles; GSH, glutathione; LA, lipoic acid; PEG, polyethylene glycol.

evaluate potential cytotoxicity following exposure to investigated xenobiotics in vitro.[22] In our study, the LDH test was chosen because the MTT assay may not be suitable for platelets. Platelets share many of the same biological characteristics as other cells. However, unlike most cells, platelets lack a nucleus and are unable to adapt to changing biological settings by altered transcription.[23] To exclude possible cytotoxic effect of investigated AgNPs on human platelets, endothelial cells and fibroblasts we measured the release of cytosolic LDH from these cells. LDH is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. Necrotic, necroptotic, and late apoptotic cell death are evaluated by determining damage of the plasma membrane.[24] Incubation of 2 nm AgNPs-GSH, 2.5 nm AgNPs-PEG, and 3.7 nm AgNPs-LA at concentration of 50 or 100 µg/mL with WPs, for 20 mins and 24 hrs or umbilical vein endothelial cells (HUVEC) and gingival fibroblasts cells (HGF-1) for 24 hrs did not cause a significant release of total LDH (Figure 8). We incubated AgNPs with platelets for 20 mins, i.e., for the duration of our functional assay using QCM-D. The total release of LDH after 24 hrs incubation was higher than after 20

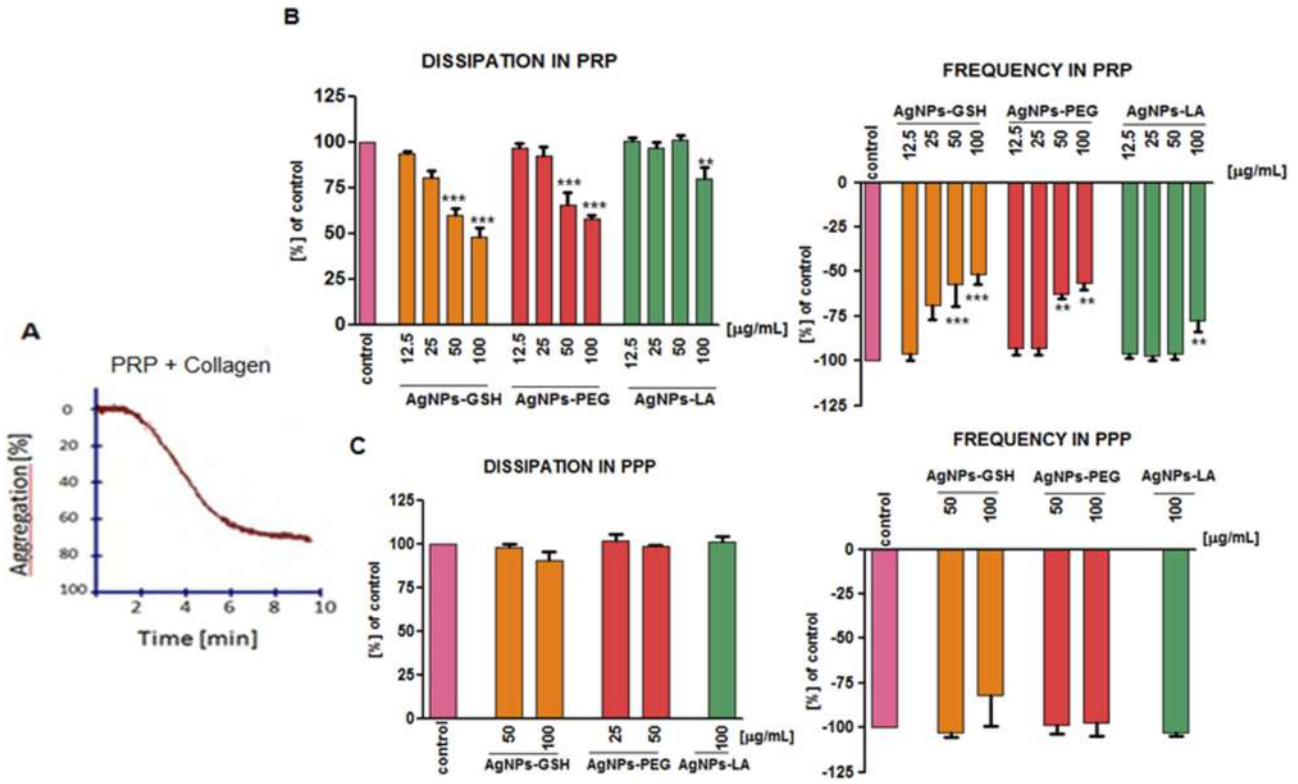

Figure 5 Measurement of the effects of AgNPs on platelet aggregation using light aggregometry and QCM-D.
Notes: (**A**) A representative light aggregometry tracing showing collagen (2 µg/mL)-induced platelet aggregation. (**B**) Perfusion of fibrinogen-coated polystyrene-coated quartz crystals with PRP in the presence of AgNPs-GSH, GSH-PEG or AgNPs-LA leads to the reduction of platelet aggregation. (**C**) Perfusion of fibrinogen-coated polystyrene-coated quartz crystals with PPP in the presence of AgNPs-GSH, GSH-PEG, and AgNPs-LA did not cause significant changes in frequency. Data are expressed as mean ± standard deviation; n=4 $^{**}P<0.01$; $^{***}P<0.001$ vs control.
Abbreviations: AgNPs, silver nanoparticles; QCM-D, quartz crystal microbalance with dissipation; PRP, platelet-rich plasma; GSH, glutathione; PEG, polyethylene glycol; LA, lipoic acid ; PPP, platelet-poor plasma.

mins, which could be due to the breakdown of platelets. However, the difference between the control and the platelets treated with AgNPs was not significant.

Effects of functionalized AgNPs on the expression of P-selectin and GPIIb/IIIa as measured by flow cytometry

Platelet activation leads to increased abundance of platelet surface receptors such as P-selectin and GPIIb/IIIa.[19] As expected, stimulation of platelets with collagen at 2 µg/mL induced a significant increase in the number of copies of P-selectin and activated GPIIb/IIIa, on the platelet surface. Incubation of PRP with AgNPs-GSH and AgNPs-PEG at 50 and 100 µg/mL or AgNPs-LA at 100 µg/mL significantly inhibited collagen-mediated increase in total P-selectin (Figure 9) and GPIIb/IIIa (Figure 10).

Functionalized AgNPs decrease TXB_2 formation in platelets

Incubation of platelets with AgNPs-GSH and AgNPs-PEG at 50 and 100 µg/mL or AgNPs-LA at 100 µg/mL significantly inhibited collagen-induced formation of TXB_2 (Figure 11).

Functionalized AgNPs decrease MMP-1 and MMP-2 release from platelets

Collagen at 2 µg/mL caused a significant enhancement of MMP-1 and MMP-2 release in PRP. Incubation of platelets with AgNPs-GSH and AgNPs-PEG significantly inhibited collagen-induced increase in MMP-1 in a concentration-dependent manner and MMP-2 at the highest concentration (100 µg/mL) as indicated in Figure 12.

Based on obtained results, we found that synthesized by our team AgNPs-GSH, AgNPs-PEG , and AgNPs-LA protected against blood platelets aggregation underflow condition and attenuated collagen-mediated increase in total P-selectin and GPIIb/IIIa, TXB_2 formation, MMP-1, MMP-2 release in PRP at concentration non-cytotoxicity to human platelets and human cells at non-cytotoxic concentration (Figure 13).

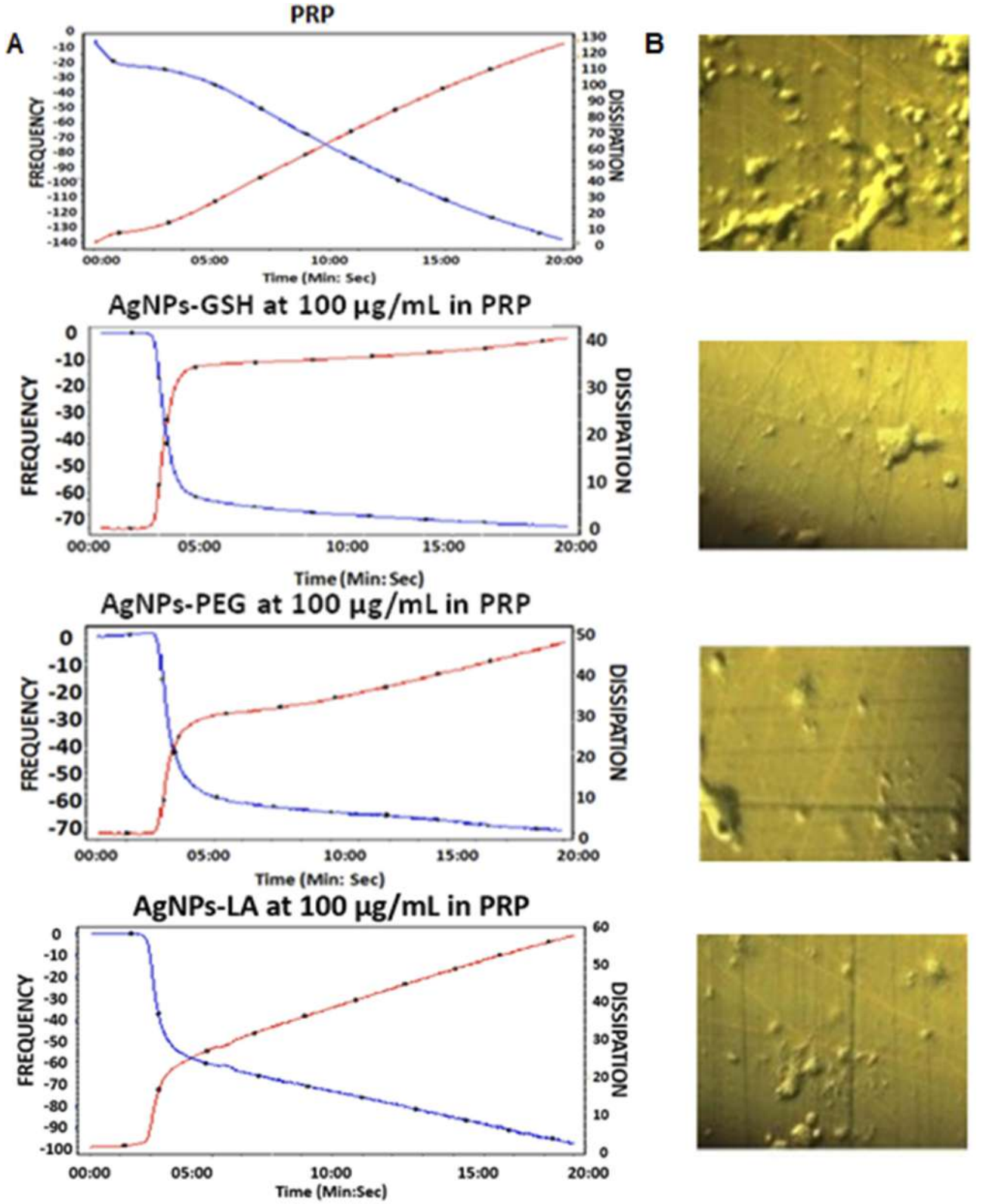

Figure 6 Effects of AgNPs on platelet aggregation as measured by QCM-D. Perfusion of sensor crystals with PRP in the presence of AgNPs-GSH, AgNPs-PEG, or AgNPs-LA (100 µg/mL) inhibited platelet aggregation.

Notes: (**A**) Representative tracings from the third overtone recorded by the device in the presence or absence of AgNPs (100 µg/mL) on frequency (blue line, left axis) and dissipation (red line, right axis). (**B**) Representative micrographs of the surface of sensors as viewed by phase contrast microscopy showing decreased accumulation of platelet aggregates in the presence of AgNPs.

Abbreviations: AgNPs, silver nanoparticles; QCM-D, quartz crystal microbalance with dissipation; PRP, platelet-rich plasma; GSH, glutathione; PEG, polyethylene glycol; LA, lipoic acid.

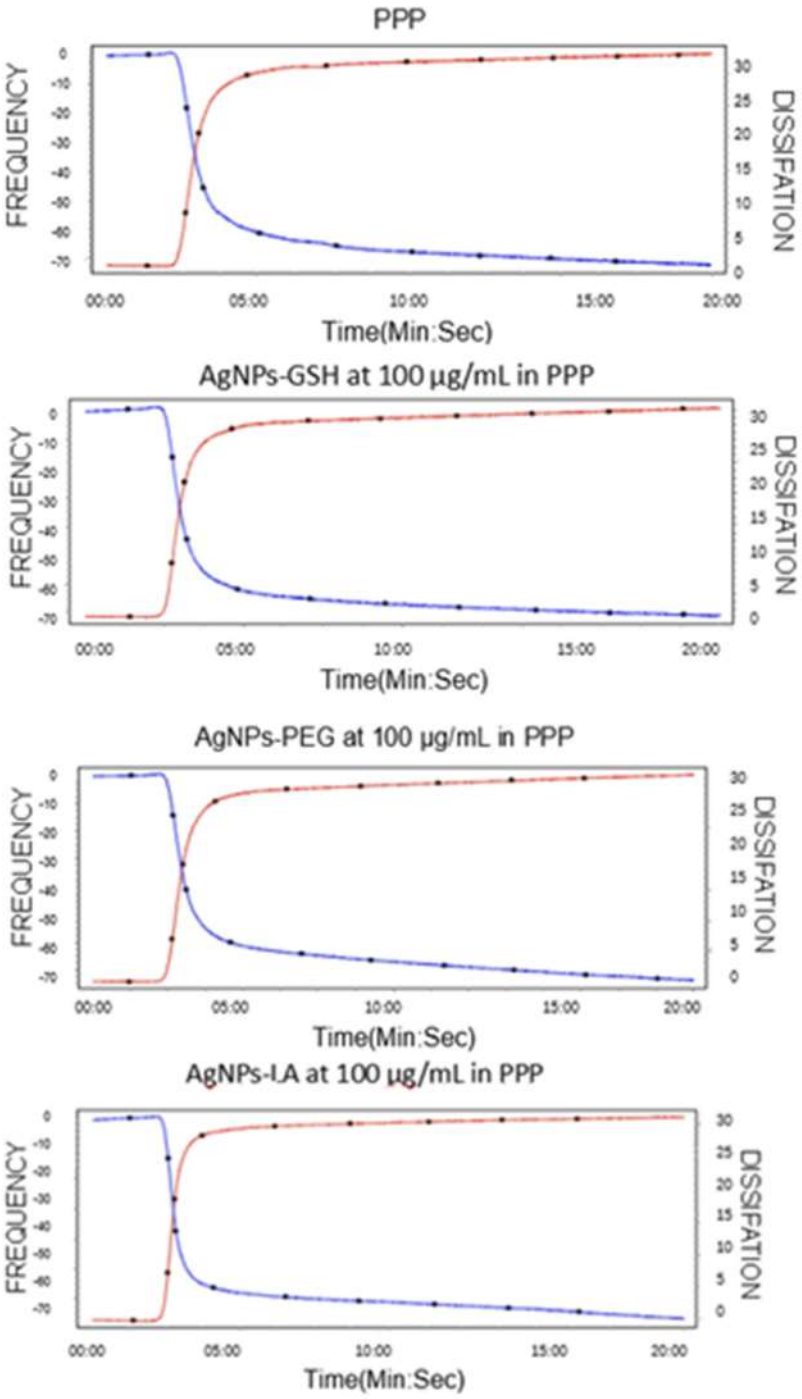

Figure 7 Effects of AgNPs on plasma protein accumulation as measured by QCM-D. In the absence of platelets (PPP) AgNPs did not cause deposition of proteins on the sensor surface. Representative traces from the third overtone recorded by the device showing effects of AgNPs (100 µg/mL) on frequency (blue line, left axis) and dissipation (red line, right axis).

Abbreviations: AgNPs, silver nanoparticles; QCM-D, quartz crystal microbalance with dissipation; PPP, platelet-poor plasma.

Discussion

Both pharmacological and toxicological actions of nanoparticles depend on their functionalization and physicochemical properties such as shape and size.[4,12,16,25,26] It has been shown that Ag nanowires (length of 1.5–25 µm; diameter of 100–160 nm) exerted a strong cytotoxic effect on human lung epithelial A549 cells, whereas spherical AgNPs (30 nm) had negligible effects on the cells.[27] The authors suggested that the direct contact of long wires with the cell surface may account for the toxicity. Indeed, we

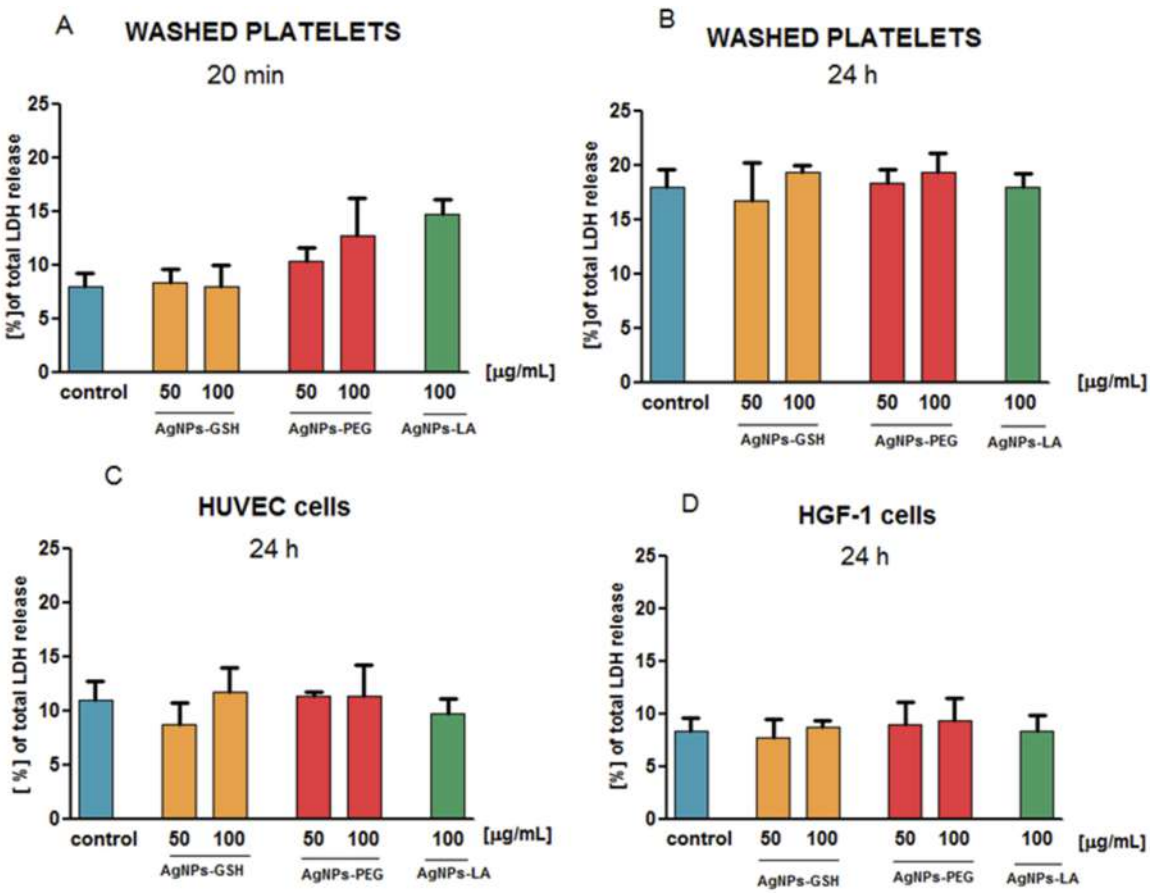

Figure 8 AgNPs do not cause LDH release from (**A**) washed platelets after 20 mins of incubation, (**B**) washed platelets, (**C**) HUVEC, and (**D**) HGF-1 cells after 24 hrs of incubation as compared with untreated WP or cells. Results are mean ± standard deviation; n=3. Lysis buffer-treated cells and WP were set to 100% (total LDH release).
Abbreviations: AgNPs, silver nanoparticles; LDH, lactate dehydrogenase; HUVEC, human umbilical vein endothelial cells; WP, washed platelet.

have demonstrated that spherical nanoparticles with the mean size of 2–3.7 nm are not cytotoxic against human cells at concentrations up to 100 µg/mL. On the other hand, Helmlinger et al, 2016 demonstrated that toxicity of spheres (20–60 nm), cubes (140–180 nm), and rods (diameter 80–120 nm, length >1000 nm) AgNPs on human mesenchymal cells was not shape dependent.[28] Several studies indicated that unmodified nanoparticles of Ag are cytotoxic to human cells.[26–29] Previously, we have found that treatment of human gingival fibroblast with 2 nm non-functionalized AgNPs causes cells death, apoptosis, inflammation, and oxidative stress.[29] We have also documented that exposure to non-functionalized AgNPs with the average size of 18 nm results in nanoparticle uptake by human osteoblast and changes in cell ultrastructure leading to apoptosis and necrosis. Moreover, the cell death was associated with increased level of iNOS mRNA, iNOS protein, and generation of increased amounts of NO.[26] In contrast, functionalized Ag nanoparticles may be more biocompatible.[4,5,8,10,13–16,30,31] A number of strategies are available to increase the biocompatibility of nanoparticles. Pegylation, i.e., coating the surface of nanoparticles with PEG is a commonly used approach for improving the efficiency of drug delivery to target cells and tissues. PEG coatings on nanoparticles shield the surface from aggregation and prolong systemic circulation time.[32] Glutathione is present in human tissues as the most abundant nonprotein thiol that defends against oxidative stress and it plays an important role in the detoxification processes.[33] In some studies, GSH was used as a stabilizer of nanoparticles.[33–36] Finally, LA, well known as an antioxidant, and agent improving biocompatibility of nanoparticles.[37] Also, our previous study demonstrated that 10 nm AgNPs functionalized with LA and PEG were less toxic against human gingival fibroblast in vitro than the unmodified ones.[16] Therefore, we synthesized GSH-PEG- and LA-functionalized AgNPs and studied the effects of these nanoparticles on platelet function. We

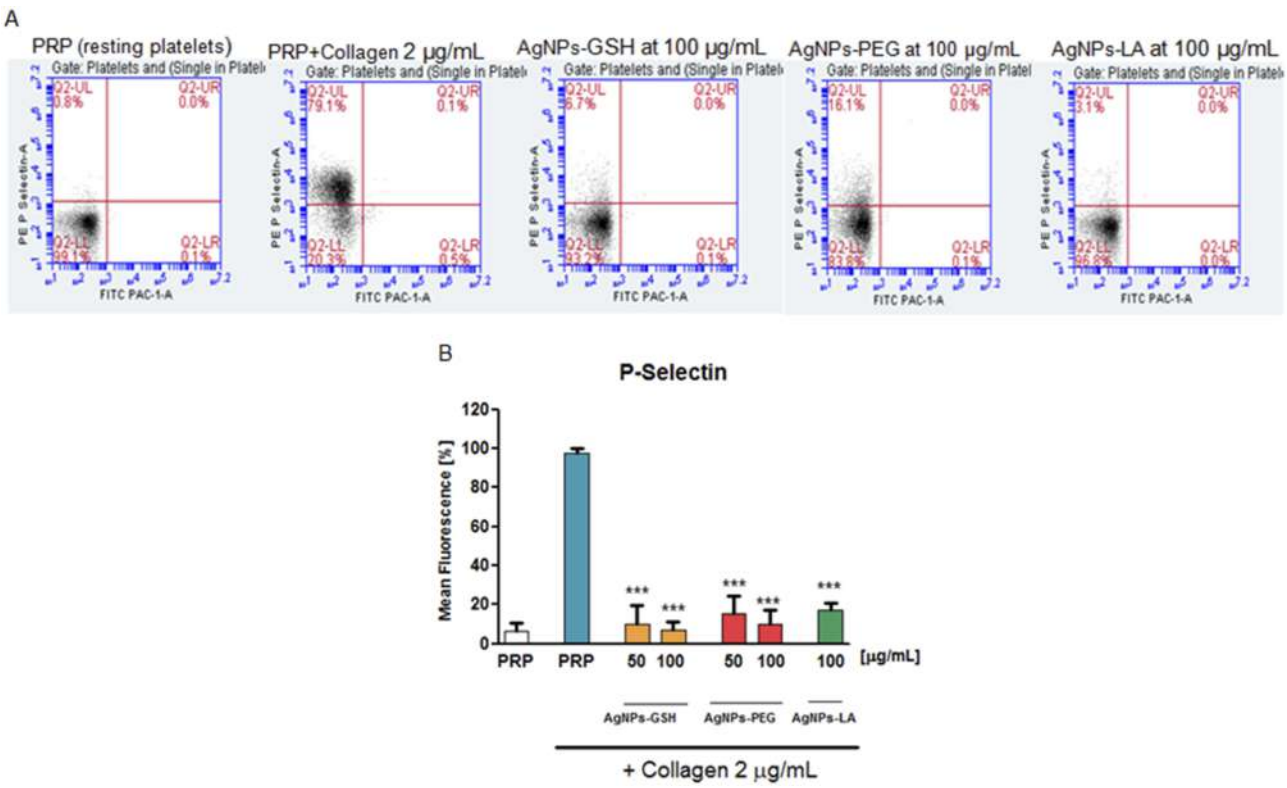

Figure 9 AgNPs-GSH. AgNPs-PEG, and AgNPs-LA attenuated collagen-stimulated increase in the abundance of P-selectin on the platelets.
Notes: (**A**) Representative flow cytometry recordings showing effects of AgNPs-GSH, AgNPs-PEG, and AgNPs-LA (100 µg/mL) on P-Selectin. The corresponding bar graph shows an analysis of the effects of AgNPs on (**B**) P-Selectin. Data are expressed as mean ± standard deviation; n=4; ***$P<0.001$ vs collagen-stimulated platelets or as indicated.
Abbreviations: AgNPs, silver nanoparticles; GSH, glutathione; PEG, polyethylene glycol; LA, lipoic acid.

found that all tested functionalized AgNPs exerted anti-platelet properties at non-cytotoxic concentrations as measured by QCM-D. The QCM-D method is able to measure nanoparticle-induced platelet microaggregation, underflow, mimicking conditions encountered in microvasculature, at concentrations undetectable by light aggregometry and flow cytometry.[20,21] Consistent with our results, Ragaseema et al, 2012 observed inhibitory effect of PEG-coated AgNPs on platelets aggregation.[8] In other research, 10 nm AgNPs showed dose-dependent antiplatelet activity and decreased ADP-induced aggregation as measured by light aggregometry.[30] Moreover, Shrivastava et al, 2009 presented that 10–15 nm AgNPs showed antiplatelet both in vitro and in vivo.[38] In contrast, 10–100 nm AgNPs-induced platelet aggregation both in isolated human platelets and in an animal model following intratracheal instillation.[6] Huang et al 2016 demonstrated that 20 nm AgNPs coated with polyvinyl pyrrolidone and citrate at the concentration of 500 µg/mL exerted no significant effect on human platelet aggregation.[5] Similarly, in 18 healthy human volunteers, after 2 weeks of daily exposure to orally ingested commercial 32 nm AgNPs, no effect on platelet aggregation was detected by light transmission aggregometry at peak serum silver concentrations <10 µg/L.[9] These contradictory results, i.e., inhibition, stimulation, or no significant effect of AgNPs on platelet function may be due to differences in the size of AgNPs, their stabilization, functionalization, as well as method of synthesis.

Mechanisms underlying modulation of platelet function by AgNPs might result from their cytotoxic effects. Some researchers found that AgNPs, can induce inflammation, oxidative stress, ultrastructural alteration, or apoptosis.[15,38] However, our findings do not support the notion that platelet-inhibitory effects of functionalized AgNPs are due to their cytotoxic effects as all three cell types: platelets, endothelial cells, and fibroblasts when exposed to these nanoparticles did not show cytotoxicity as measured by LDH release. This is consistent with work of Shrivastava et al, 2009 who confirmed that 10–15 nm AgNPs even at higher concentration (500 µg/mL), did not affect platelet membrane integrity.[39] Similarly, Krishnaraj and Berchmans, 2013 did not observe LDH release from platelets after exposure to 10 nm AgNPs at the

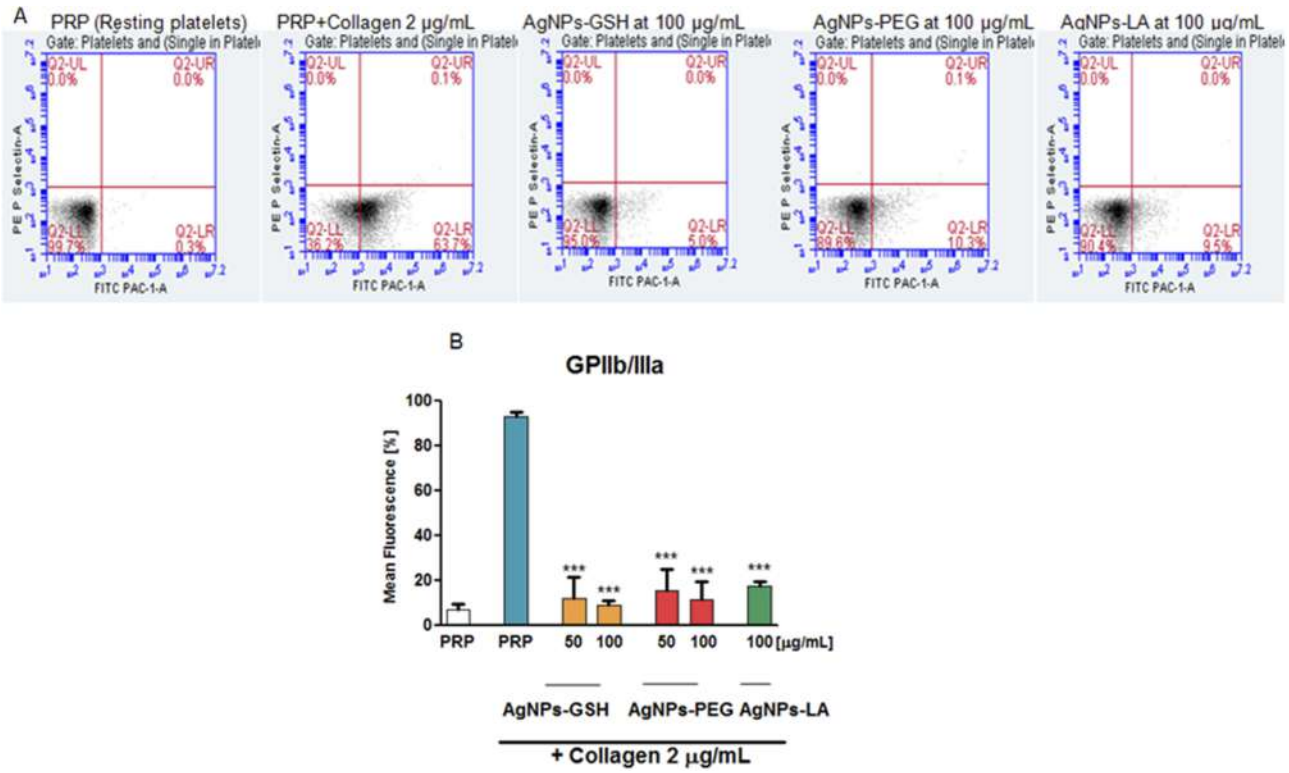

Figure 10 AgNPs-GSH. AgNPs-PEG, and AgNPs-LA attenuated collagen-stimulated increase in the abundance of GPIIb/IIIa on the platelet surface.
Notes: (**A**) Representative flow cytometry recordings showing effects of AgNPs-GSH, AgNPs-PEG, and AgNPs-LA (100 µg/mL) on GPIIb/IIIa. The corresponding bar graph shows analysis of the effects of AgNPs on (**B**) GPIIb/IIIa. Data expressed as mean ± standard deviation; n=4; ***P<0.001 vs collagen-stimulated platelets or as indicated.
Abbreviations: AgNPs, silver nanoparticles; GSH, glutathione; PEG, polyethylene glycol; LA, lipoic acid.

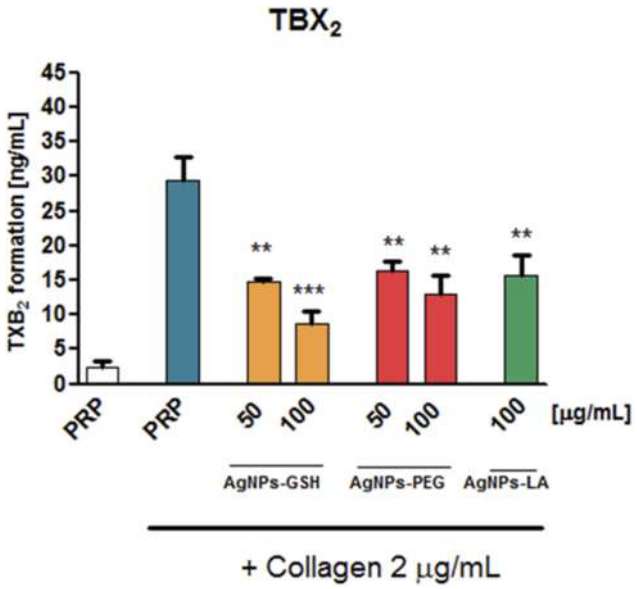

Figure 11 AgNPs-GSH, AgNPs-PEG, and AgNPs-LA decreased collagen-induced formation of TXB_2 by platelets.
Notes: Data expressed as mean ± standard deviation; n=4; **P<0.01; ***P<0.001 vs collagen-stimulated platelets.
Abbreviations: AgNPs, silver nanoparticles; GSH, glutathione; PEG, polyethylene glycol; LA, lipoic acid.

concentration range of 0.9–3.5 nM.[40] Furthermore, Kim et al, 2008 reported that nanosilver up to 300 mg/kg was nontoxic to rodents.[38]

It is rather more likely that platelet-inhibitory effects of functionalized AgNPs are due to interactions with platelet surface proteins. Platelet activation alters the composition of the platelet membrane, leading to surface expression of P-selectin and an increase in the number of integrin GPIIb/IIIa.[19,41] The activation of GPIIb/IIIa receptor is a common pathway leading to platelet activation and is also targeted by nanoparticles.[42] Indeed, in the current experiments, all tested functionalized AgNPs inhibited activation of GPIIb/IIIa on the platelet surface. Furthermore, it has been shown that the inhibition of platelets aggregation by AgNPs could be due to conformational modulation of platelet surface integrins GPIIb/IIIa.[41] Therefore, we propose that platelet-inhibitory activity of functionalized AgNPs is due to interactions with the function of this receptor complex. In addition to inhibition of activation of GPIIb/IIIa functionalized AgNPs may inhibit agglutination, i.e., passive mechanism of platelet

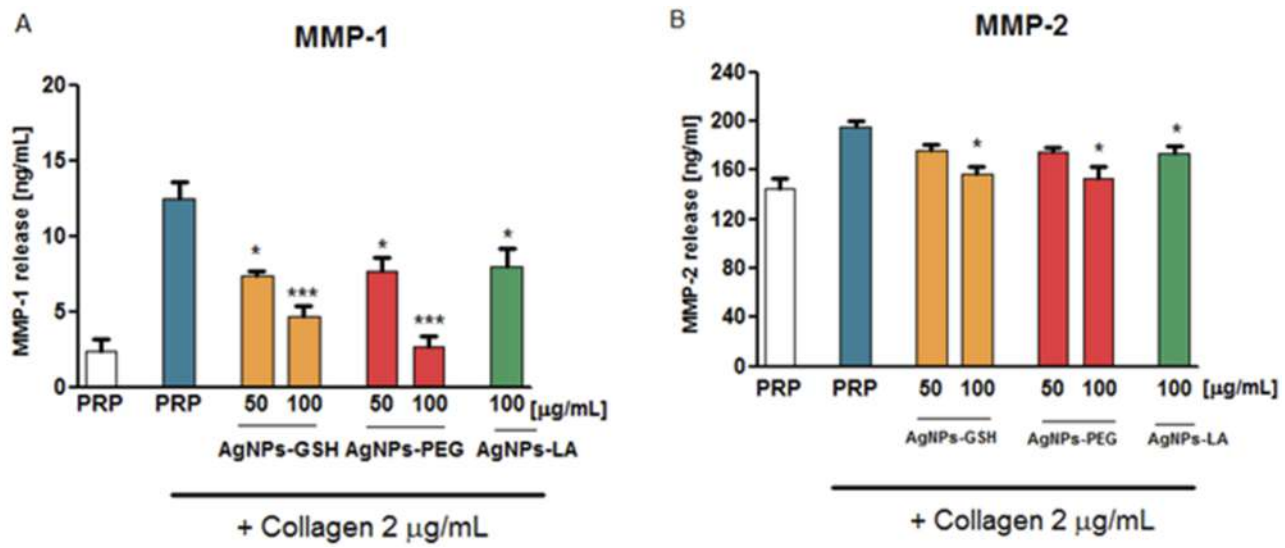

Figure 12 AgNPs-GSH, AgNPs-PEG, and AgNPs-LA attenuated collagen-induced increase of (**A**) MMP-1 and (**B**) MMP-2 levels from platelets.
Notes: Data expressed as mean ± standard deviation; n=4; *P<0.05; ***P<0.001 vs collagen-stimulated platelets or as indicated.
Abbreviations: AgNPs, silver nanoparticles; GSH, glutathione; PEG, polyethylene glycol; LA, lipoic acid.

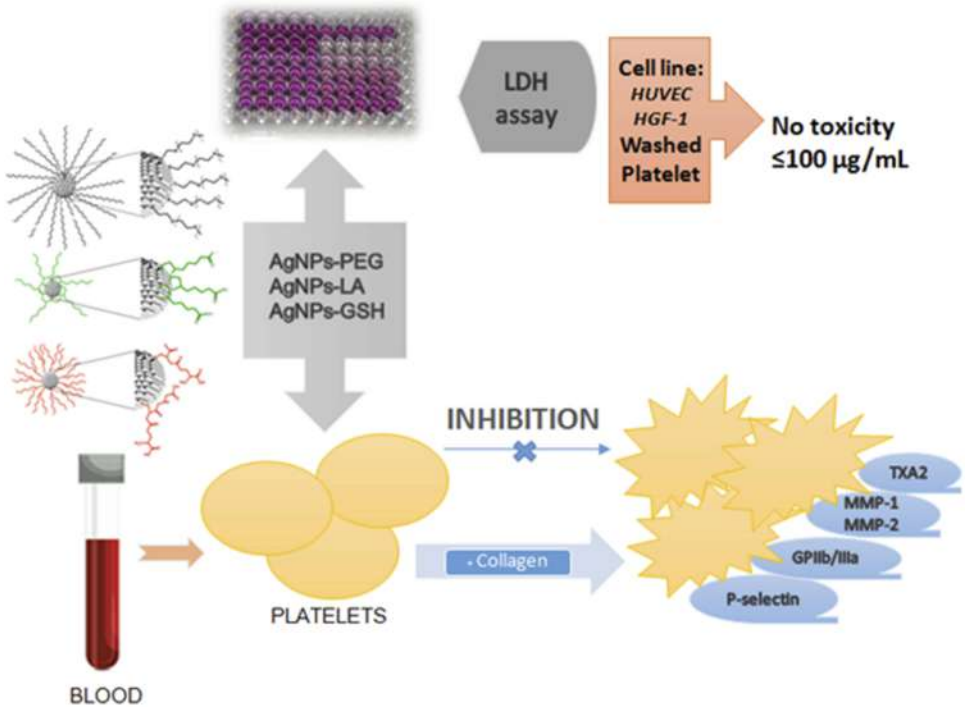

Figure 13 Effects of functionalized AgNPs on human platelet aggregation.
Abbreviation: AgNPs, silver nanoparticles.

aggregation.[43] Nanoparticle-induced platelet agglutination depends on the formation of nanoparticle–nanoparticle aggregates and these facilitate nanoparticle-made bridge formation between.[44] As functionalized AgNPs form less nanoparticle aggregates than uncoated NPs this property could also contribute to mechanisms that underlie the platelet-inhibitory activity of these NPs.

Finally, the net charge of functionalized AgNPs in interactions with platelets should be considered. Indeed, Dobovolskaia and colleagues showed that cationic but not

neutral or anionic PAMAM dendrimers have the ability to stimulate platelet aggregation. In our experiments, we used three types of organic molecules as surface ligands to modify nanoparticles: PEG-SH, α-LA, and L-Glutathione. Because thiol group of PEG-SH molecule creates a bond between the surface of the nanoparticle and the PEG moiety, it is not involved in any acid–base relationship. PEG stabilized nanoparticle should be considered as a neutral particle, which net charge cannot be influenced by acid or based environment. Moreover, PEG-ylated nanoparticles in contrast to other types of nanoparticles are subjected to well-known biological effects related to much weaker binding of macromolecules from physiological media. A weak interference of PEGylated nanoparticles with the immune system has been linked to the lower degree of protein binding to particles and prevents unwanted biological effects in many drug delivery systems based on this type of nanoparticles.[45] Coating of NPs with bulky molecules like PEG decreases protein binding to NPs as well as phagocytosis.[46] Therefore, the presence of PEG on the surface of PEG-AgNPs could limit interactions between platelet receptors such as GPIIb/IIIa and their ligands thus inhibiting platelet aggregation. The functionalization of AgNPs with GSH or LA may result in negative surface charge of GSH-AgNPs and LA-AgNPs. For α-LA, there is only one part of the molecule that can be protonated and deprotonated – it is carboxylic group which pKa =4.52, therefore in the biological experiment this molecule is deprotonated and gives negative net charge to the whole nanoparticle. The situation is more complicated for glutathione-stabilized nanoparticles because for L-GSH reduced pKa can be distinguished: pK1=2.12 (COOH), pK2=3.59 (COOH), pK3=8.75 (NH2), pK4=9.65 (SH). According to these data, GSH has a net negative charge at neutral pH and is highly water soluble. Therefore, it would be expected to reside in an aqueous environment and has less absorption on cell membranes. The influence of surface charge is difficult to interpret for GSH because effective surface charge may be modulated in physiological solution due to binding of proteins that have significant affinity to glutathione, very common molecule for cell environment. As an increase in platelet negative surface charge decreases platelet aggregation, platelet surface charge effects of GSH-AgNPs and LA-AgNPs could contribute to inhibition of platelet aggregation by these nanoparticles.[47] The function of platelet receptors is regulated by a number of downstream platelet mediators.

In the previous studies, it was indicated that the platelet release of MMP-1 and MMP-2 was caused by collagen-induced aggregation.[48,49] These MMPs rapidly relocate from granule structures inside the platelet to the plasma membrane of activated platelets and enhance P-selectin expression, platelet procoagulant activity, and thrombus formation.[48–51] Moreover, MMP-2 is involved in the modification of platelet glycoprotein $\alpha_{IIb}\beta_3$ resulting in enhanced platelet adhesion and aggregation.[48,49,52] MMP-1 was also indicated to associate with the $\alpha_{IIb}\beta_3$ integrin, as suggested by Galt et al, 2002.[52] It has been shown that MMP-2 contributed to platelets stimulation and aggregation by non-ADP and non-TXA_2 pathway.[51,53]

Thromboxane A_2 is yet another platelet mediator that acts as a potent vasoconstrictor and a stimulator of platelet aggregation. We found that all tested functionalized AgNPs, similar to aspirin, which represents commonly used antiplatelet agent, may exert a part of their effects through reduction of TXA_2 production.[51] Interestingly, Falcinelli et al, 2007 indicated that the lack of aspirin intake on MMP-2 release in vivo is probably implicated in the mechanism of aspirin resistance.[53] Thus, synthesized by our team functionalized AgNPs as inhibitors of MMP-2 and MMP-1 would be suggested as a novel, alternative strategy for developing therapeutics in thrombosis.[54–57] Furthermore, due to critical role in inducing platelet aggregate formation, GPIIb/IIIa has become a primary target for the development of antithrombotic agents. Currently, three integrin GPIIb/IIIa antagonists (abciximab, eptifibatide, and tirofiban) have been approved for clinical use.[58] Unfortunately, they exert severe side effects, especially bleeding complications and therefore studies on the development of more effective and safer antiplatelet agents are needed.

One needs to acknowledge that a significant limitation when developing functionalized AgNPs as antiplatelet agents may be due to the fact that non-functionalized may exert opposite effects and activate platelets. Indeed, Jun et al, 2011 observed that 10–100 nm AgNPs at a concentration of 100–250 μg/mL induced platelet activation and increase of P-selectin expression under in vitro condition. Moreover, they found that exposure of rats to AgNPs (0.05–0.1 mg/kg i.v. or 5–10 mg/kg intratracheal instillation) enhanced P-selectin expression.[6] Therefore, careful pharmacokinetics studies examing the fate of functionalized AgNPs would be crucial for pharmacological development of these agents.

According to the International Organization for Standardization (ISO 10993-4), five different categories (thrombosis, coagulation, platelets, hematology, and immunology (complement system and leukocytes)), are necessary for hemocompatibility evaluation.[59]

Our studies estimate influence for them, i.e., platelets and endothelial cells, which may suggest hemocompatibility. There is no doubt that to precisely define the safety profile of those AgNPs further studies for evaluating their effect on erythrocytes and white cells are warranted. Other authors have studied the effect of AgNPs to morphological blood elements that are also part of the immune system (NK cells, lymphocyte). These immune cells are belonging to the innate immune system and they can be rapidly activated upon recognition of a foreign invader such as a foreign material. So far, AgNPs affected the enzymatic and non-enzymatic antioxidant systems of red blood cells (RBCs), additionally were toxic at a high concentration, against low concentration (<0.4 μg/mL) of AgNPs was relatively safe.[60] Also exposure to AgNPs of NK cells, reduced the viability and the cytotoxic potential after polyriboinosinic-polyribocytidylic acid stimulation of NK cells and increased the expression of the inhibitory receptor CD159a. In the same, studies were confirmed that exposure to AgNPs changes NK cells' function and phenotype and may present a risk for modulating human immune responses.[61] For comparison, l-cysteine functionalized silver do not exhibit acute toxicity for plasma after intragastric and intraperitoneal administration.[62] Similarly, in Ferrer's research AgNPs functionalized with dextran did not affect morphology and cell viability of monocytes (THP-1).[63] But, another study shows that for RBCs, AgNP-PVP-20 at ~40 μg/mL could cause hemolysis to 19%. Both AgNP-PVP-20 and AgNP-CIT-20 were quite toxic to lymphocytes since they severely inhibited both lymphocyte proliferation and viability.[64]

Limitations and future perspectives

As it is an in vitro investigation, studies exploring, pharmaceutical, pharmacological, therapeutic, and clinical potential of functionalized AgNPs need to be performed. However, it is clear that the modification of AgNPs by functionalization holds a promise for further development of AgNPs as pharmaceutical or imaging agents. Having regard to successful clinical applications, it is necessary to precisely define safety profiles of AgNPs, therefore, effect of their exposure is a key issue that needs further in vivo assessment.[65]

Conclusion

We have shown that functionalized AgNPs (AgNPs-GSH, AgNPs-PEG, and AgNPs-LA) sized from 2 to 3.7 nm inhibit platelet aggregation underflow condition. Mechanisms of this action of functionalized AgNPs are likely to be dependent on down-regulation of P-selectin and GPIIb/IIIa receptor expression, TXA_2 formation, and MMP-1, MMP-2 release from platelets. Figure 13 summarizes the effects of functionalized AgNPs on platelets.

We believe that these findings are important for understanding interactions between AgNPs and human platelets and their pharmacological and toxicological significance. The lack of cytotoxicity against the important elements of the circulation system, i.e., platelets and endothelial cells in LDH assay, suggests potential hemocompatibility. Further studies are warranted for establishing the safety profile of these AgNPs prior to their potential clinical applications.

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Disclosure

The authors report no conflicts of interest in this work.

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Lipoic Acid-Coated Silver Nanoparticles: Biosafety Potential on the Vascular Microenvironment and Antibacterial Properties

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Purpose: To study and compare the antibacterial properties and the potential cytotoxic effects of commercially available uncoated silver nanoparticles (AgNPs) with lipoic acid coated silver nanoparticles (AgNPsLA) developed by our group. The antibacterial, cytotoxic, and hemolytic properties of those NPs were assessed with the main objective of investigating if AgNPsLA could maintain their antibacterial properties while improving their biosafety profile over uncoated AgNPs within the blood vessel's microenvironment.

Methods: Comercially available uncoated 2.6 nm AgNPs and 2.5 nm AgNPsLA synthesized and characterized as previously described by our group, were used in this study. Antimicrobial activity was assessed on a wide range of pathogens and expressed by minimal inhibitory concentrations (MIC). Assessment of cytotoxicity was carried out on human umbilical vein endothelial cells (HUVEC) using an MTT test. Detection of reactive oxygen species, cell apoptosis/necrosis in HUVEC, and measurement of mitochondrial destabilization in HUVEC and platelets were performed by flow cytometry. The potential harmful effect of nanoparticles on red blood cells (RBCs) was investigated measuring hemoglobin and LDH released after exposure to NPs. Transmission electron microscopy was also used to determine if AgNPs and AgNPsLA could induce any ultrastructural changes on HUVEC cells and *Staphylococcus aureus* bacteria.

Results: AgNPs and AgNPsLA had antimicrobial properties against pathogens associated with catheter-related bloodstream infections. AgNPs, in contrast to AgNPsLA, induced ROS production and apoptosis in HUVEC, ultrastructural changes in HUVEC and *S. aureus*, depolarization of mitochondrial membrane in HUVEC and platelets, and also hemolysis.

Conclusion: AgNPsLA synthesized by our group have antimicrobial activity and a better biosafety profile than uncoated AgNPs of similar size. Those observations are of critical

importance for the future *in vivo* investigations and the potential application of AgNPsLA in medical devices for human use.

Keywords: silver nanoparticles, biomaterial, lipoic acid coating, cytotoxicity, biocompatibility, endothelial cells, antimicrobial activity

INTRODUCTION

Nanomedicine is a field of science that applies the knowledge and tools of nanotechnology to the prevention and treatment of disease (Rani 2017). By the end of 2020 more than 200,000 scientific research articles were already published about nanomedicine (PubMed, 2020). Engineered nanoparticles (NPs) are endowed with exclusive characteristics, such as, among others, high surface area to volume ratio, high chemical reactivity, and even antimicrobial/fungicidal activity that makes them very attractive for their use in medicine (Medina et al., 2007). Modification of NPs surface may involve processes that can result in more desirable NPs properties, including reduction of cytotoxicity led by cellular events like oxidative stress and apoptosis (Abad et al., 2005). Turcu and co-workers (Turcu el al. 2017) developed LA-functionalized bioactive nanosystems using gold nanoparticles and demonstrated their biocompatibility at concentrations lower than 50 μg/ml using cell viability and cell cycle assays. Lipoic acid (LA) is a disulfide that it is reduced at intracellular levels into dihydrolipoic acid, a dithiol which has strong antioxidant properties, and it has been used for treating oxidative stress-related diseases like diabetic neuropathies (Salehi et al., 2019). Properties of LA including its metal chelating capacity, its ability to scavenge reactive oxygen species (ROS), its ability to regenerate endogenous antioxidants, and to repair oxidative damage have been demonstrated already. In fact, within the drug-related antioxidant pharmacology field, LA is a model compound that enhances understanding of the mode of action of antioxidants in drug therapy (Biewenga, Haenen, and Bast 1997).

It is estimated that up to 30% of all nosocomial bacteremias are associated with the use of intravascular devices such as central venous catheters with the involvement of strains such as *Enterococcus faecium, Staphylococcus aureus methicillin resistant, Klebsiella pneumoniae, Acinetobacter baumannii,* and *Pseudomonas aeruginosa* (Ruiz-Giardin et al., 2019). In addition, common complications associated with long-term use of central venous catheters are occlusions and catheter-related thrombosis. AgNPs are well known for having antimicrobial properties and unique characteristics that make them very attractive for biomedical applications (Hante et al., 2019). In one of our recent publications, we have demonstrated that AgNPsLA synthesized by our team are able to down-regulate P-selectin and GPIIb/IIIa receptor expression, inhibiting platelet aggregation under flow conditions. This effect is likely to be associated with thromboxane A2 formation and metalloproteinase release from platelets (Hajtuch et al., 2019). Based on these observations, it seems reasonable to think that, due to their anti-platelet and potential antibacterial properties, AgNPsLA could be useful to, for example, internally coat vascular catheters. However, it is necessary to accurately determine first if those NPs may also have some detrimental effects within the vascular microenvironment. The endothelium is particularly important for controlling macromolecules and fluid exchange between the blood and the interstitial space and it serves as a physiological physical barrier that controls the traffic of NPs from the vasculature into the surrounding tissue. In addition, the vascular endothelium is also involved in other physiological and pathological processes such as hemostasis and thrombosis, and inflammation and remodeling of the vascular wall (Engin et al., 2015). Changes in the mitochondrial membrane potential of platelets indicate mitochondria impairment. Mitochondria damage or dysfunction, as observed during several disease processes, results in attenuated platelet survival and increased risk for thrombovascular events (Melchinger et al., 2019). Red blood cells (RBCs) also play an important role within the circulating environment and the effect of NPs on them must be also investigated. In fact, although RBCs have been described as potential carriers of NPs for drug delivery, NPs may also have a deleterious effect inducing, for example, red cell lysis (Wadhwa et al., 2019).

In this work we aimed to investigate and compare the antibacterial properties and potential cytotoxic effects that commercially available bare silver nanoparticles (AgNPs) and lipoic acid coated AgNPsLA of similar size may exert within the blood vessel microenvironment. To this end, the effects of both types of nanoparticles on endothelial cells, platelets, and RBCs were examined. Moreover, their antimicrobial activity against a wide range of pathogens including those identified as etiological factors of potential catheter related infections was also investigated.

This study provides further support for the importance of the routine biosafety assessment of nanoparticles intended for future human applications and, in this particular case, for the potential use of those NPs as antiaggregating and antimicrobial agents for endoluminal catheter's coatings.

MATERIALS AND METHODS

Silver Nanoparticles

Uncoated silver nanoparticles (AgNPs) 2.6 nm size were purchased from United States Research Nanomaterials, Inc. (Houston, TX) and LA coated silver nanoparticles (AgNPsLA) 2.5 nm size synthesized by our group. Both types of NPs have been characterized and described in our previous research work (Zielinska et al., 2016; Zielinska et al., 2018; Hajtuch et al., 2019).

For measuring Ag release from the NPs, 1 ml sample of AgNPsLA and AgNPs (at concentrations of 10,000 and 2.000 μg/ml, respectively) were placed in a 3.5 kDa membrane

and dialyzed in an external HUVEC culture medium (7 ml). During dialysis, 0.5 ml samples were taken at intervals of 1, 12, and 24 h from the media and subjected to ICP-MS analysis.

It is well known that NPs can interfere with *in vitro* methods that are commonly used for toxicological studies (Kroll et al., 2009; Liang et al., 2015). For this reason, NPs on their own and with the substrates used when relevant, where tested as an internal control in the assays involving the measurement of absorbance and during flow cytometry studies.

Reference Strains of Microorganisms

Reference strains of bacteria that belong to ESKAPE pathogens (ESKAPE-bacterial pathogens commonly associated with antimicrobial resistance) were obtained from the American Type Culture Collection (ATCC); *Enterococcus faecium* (ATCC 700221), *Staphylococcus aureus methicillin resistant* (ATCC 33591), *Klebsiella pneumoniae* (ATCC 700603), *Acinetobacter baumannii* (ATCC 19606), *Pseudomonas aeruginosa* (ATCC 9027), and *Klebsiella aerogenes* (ATCC 13048) formerly *Enterobacter aerogenes* (ATCC 130480), and cultured following their recommendations. Briefly, all cultures were kept at −80°C using Roti®-Store Cryo-Vials (Carl Roth GmbH, Karlsruhe, Germany). Before the experiments, the cryo-protected bacteria were transferred into fresh Mueller-Hinton Broth media (Biocorp, Warsaw, Poland) and incubated for 24 h at 37°C on a rotating shaker (120 rpm). Cultures were then seeded on Mueller-Hinton Agarplates (Biocorp), incubated as mentioned above, and used for experiments. Cell densities for all assays were estimated and adjusted by optical density at 600 nm wavelength using a Multiskan™ 102 GO Microplate Spectrophotometer (Thermo Scientific).

Determination of Antimicrobial Activity

Antimicrobial activity and minimal inhibitory concentrations (MICs) of the NPs tested were determined using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. For this purpose, the initial inoculums of bacteria of 5×10^5 CFU/ml in Mueller–Hinton Broth were exposed to AgNPs and AgNPsLA (0.5–256 μg/ml) and incubated for 18 h at 37°C. The experiments were conducted on 96-well polystyrene plates with a final volume of 100 μl. The MIC was taken as the lowest concentration at which a noticeable growth of microorganisms was inhibited. All experiments were conducted in triplicate.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Sigma Aldrich (cat number: 200P-05N) and maintained as a monolayer culture in T-75 cm^2 tissue culture flasks. The tests were carried out in accordance with the manufacturer's specifications, which recommends culture up to eight passages. HUVEC were cultured with endothelial cell growth medium (Sigma Aldrich 211–500) in the presence of antibiotics (6 μg/ml of penicillin-G, and 10 μg/ml streptomycin) at 37°C in a humidified atmosphere of 5% CO_2. The medium was replaced every second day and when confluent, cells were detached with trypsin-EDTA and sub-cultured into new cell culture flasks.

Cellular Experiments with Nanoparticles

For all experiments involving HUVEC, cells were co-incubated with AgNPs and AgNPsLA for 24 h. Concentrations used in those experiments varied and were determined from results obtained from preliminary studies. Just before being added to the cells, NPs were diluted in serum-free media and vortex for 1 min to ensure equal dispersion of NPs in the solution. Control cells were incubated with serum-free media in the absence of NPs. The medium was not changed during the 24 h of the incubation process.

MTT Viability Assay

HUVEC were seeded in 96-well plates (15,000 cells per well) with complete media. After 24 h media was removed from the wells and cells were then co-incubated with NPs in a concentration range from 0.5 to 3.5 μg/ml for AgNPs or from 5 to 100 μg/ml for AgNPsLA. Following 24 h of incubation, the media was supplemented with water-soluble tetrazolium salt (at a final concentration of 0.5 μg/ml) and incubated for 2 h. Next, the media was removed, and the resultant crystals dissolved in DMSO. After 15 min, cell viability was assessed by measuring absorbance at 490 nm using a microplate reader (FLUOstar, OPTIMA). Viability was determined as a percentage of the control where the viability of the control cells was set as 100%. Absorbance values were corrected with blank NPs.

Detection of Reactive Oxygen Species

HUVEC were seeded into 6-well plates; the next day, the medium was replaced and cells co-incubated with AgNPs at 0.5–3.5 μg/ml or with AgNPsLA at 25–100 μg/ml for 24 h. After the incubation time, media from each well was discarded and replaced with a new solution supplemented with 10 μM 2, 7-dichlorofluorescein diacetate (DCF-DA). After 30 min of incubation, the fluorescence of oxidized DCF was measured by flow cytometry (excitation wavelength: 480 nm; an emission wavelength: 525 nm) using a BD FACS Calibur. The data obtained for every sample was expressed as a percentage of the control (cells in the absence of NPs) and analyzed using the CellQuest software.

Detection of Apoptosis/Necrosis

HUVEC were seeded into 6-well plates; the following day, the medium was replaced and cells were co-incubated with AgNPs at 1.5–3.5 μg/ml or with AgNPsLA at 25–100 μg/ml for 24 h. After the incubation time, cells were collected, washed twice with phosphate-buffered saline (PBS) (NaCl 0.138 M; KCl 0.0027 M; pH 7.4), and resuspended in binding buffer (50 mM HEPES: 4-(2-hydroxyethyl)-piperazineethanesulfonic acid, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4). Afterward, 5 μl of Annexin V and 5 μl propidium iodine were added to the cells, gently resuspended once again, and incubated at room temperature in the dark for 15 min. Cells were then further diluted in binding buffer before flow cytometry analysis. Flow cytometry was carried out using a BD FACSArray (BD Biosciences, San Jose, CA) and 20,000 cell-specific events were analyzed for each experiment using the CellQuest software.

Measurement of Mitochondrial Destabilization in Endothelial Cells and Platelets

HUVEC cells were seeded into 6-well plates; the next day, the medium was replaced, and cells treated with AgNPs at 1.5–3.5 μg/ml or with AgNPsLA at 25–100 μg/ml for 24 h. Afterward, the media for every well was discarded and replaced with a new solution supplemented with JC-1 (Cayman). Cells exposed to carbonyl cyanide 3-chlorophenylhydrazone (CCCP) were used as positive controls. In healthy cells with high mitochondrial membrane potential (ΔψM), JC-1 spontaneously forms complexes known as J-aggregates with intense red fluorescence. In case of apoptotic or unhealthy cells with low (ΔψM), JC-1 remains in the monomeric form, which shows only green fluorescence. The cells were detached, and 10,000 events counted by flow cytometry using a BD FACS Calibur. The fluorescence signals of JC-1 were detected in the fluorescence channels FL1. For microscope analysis, medium with JC-1 was replaced with fresh PBS and cells were observed under a fluorescence microscope (Olympus Life Science) and the data obtained analyzed using the CellQuest software.

To evaluate the potential mitochondrial destabilization in platelets, blood was withdrawn from healthy volunteers who had not been on any medication known to interfere with platelet function for at least 2 weeks prior to the study. The study was approved by the Bioethics Committee of the Medical University of Gdansk (NKBBN/552/2018-2019) and performed in accordance with the Code of Ethics of the World Medical Association, the ethical standards of the competent commission for Human Experiments (institutional and national) and the Helsinki Declaration of 1975 r., as amended in 2000. All volunteers gave informed consent before whole blood was collected and carefully mixed with 3.15% sodium citrate (9:1). Washed platelet (WP) suspensions were prepared from blood as described by Radomski and Moncada using Tyrode buffer at a final concentration of 250,000 platelets/μl (Radomski and Moncada 1983). Washed platelets were incubated with AgNPs or AgNPsLA for 1 h. After the incubation time, JC-1 was added, and 10,000 events measured by flow cytometry and the fluorescence signals of JC-1 were detected in the fluorescence channels FL1 (BD FACS Calibur) and data analyzed using the CellQuest software.

Analysis of Hemolytic Properties of AgNPs and AgNPsLA

To assess the effect of NPs on RBCs, peripheral blood was collected into a syringe containing sodium citrate (0.35% final conc.) and 10 ml centrifuged at 300 xg for 10 min at room temperature. Platelet rich plasma and buffy coat were removed by aspiration and the red blood cells (RBCs) were diluted in PBS (1:100), plated in 96-wells and co-incubated with AgNPs or AgNPsLA for 12 h. Hemolysis was determined by measuring hemoglobin and LDH released (Promega Cytotox 96). Absorbance was recorded at 540 nm for hemoglobin and at 492 nm for LDH, using a microplate reader (FLUOstar, OPTIMA). Data were expressed as % of LDH release from RBCs according to the manufacturer's instructions. For the data expressed as % of hemoglobin release, 0.1% Triton X -100 (Sigma-Aldrich, St. Louis, MO) was used as the positive control. One hundred percent lysis was corroborated by optical microscope.

Analysis of Ultrastructural Changes by Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to investigate if AgNPs or AgNPsLA could induced ultrastructural changes in HUVEC and *S. aureus*. HUVEC were plated into 6-well plates. After 24 h cells were co-incubated with AgNPs (3.5 μg/ml) or AgNPsLA (50 μg/ml). *S aureus* (ATCC 33591) were grown overnight. Afterward, bacteria were centrifuged (1.000 xg, 7 min) and resuspended in fresh Mueller-Hinton medium to obtain a cell suspension of 5×10^5 CFU/ml. Cells were then exposed to AgNPs or AgNPsLA at MIC concentrations and incubated for 18 h at 37°C. After treatment, bacteria were centrifuged (3.500 rpm, 10 min) and washed twice with PBS. HUVEC and *S. aureus* were fixed in 2.5% electron microscopy grade glutaraldehyde (Polysciences) in 0.1 M PBS (pH 7.4), post-fixed in 1% osmium tetroxide (Polysciences), dehydrated through a graded series of ethanol (30–100%), and embedded in Epon (Sigma). Ultrathin (65 nm) sections were cut using a Leica UC7 ultramicrotome, stained with Uranyless (Delta Microscopies) and Reynold's lead citrate (Delta Microscopies), and examined on a Tecnai G2 Spirit BioTWIN TEM.

Statistical Analysis

Data are expressed as mean ± SD of 3-4 independent experiments. The results are analyzed using one-way ANOVA and Tukey's post hoc test, and a p value <0.05 is considered statistically significant. IC50 and logIC50 were also calculated using the GraphPad Prism 5 program V.5 (GraphPad Software Inc., San Diego, CA) by non-linear regression analysis.

RESULTS

Silver Nanoparticles

AgNPs characterization data obtained previously confirmed stable and monodisperse negative charged NPs with sizes ranging from 1 to 5 nm with a mean diameter of 2.6 ± 0.8 nm by TEM EDS analysis in serum free media (Zielinska et al., 2016; Zielinska et al., 2018). In our previous study, we showed that our AgNPsLA are spherical with an average diameter of 2.5 ± 0.5 nm by TEM and SAXRD. The number of molecules attached to the NPs surfaces was determined by thermal gravimetric analysis (TGA) measurements. The % mass of organic was 41.31% and the number of ligands attached to the NPs was 176 (Hajtuch et al., 2019). ICP-MS analysis confirmed the stability of the AgNPsLA, as the silver content did not change significantly over time (0.015% after 24 h) when compared to the bare AgNPs that resulted in an increase in the content of silver ions over time in the HUVEC media (1.056% after 24 h) indicating a lower

TABLE 1 | Characterization of AgNPs and AgNPsLA

Nanoparticles	Size/method	Number of ligands attached to the AgNPs/method	Silver content in medium (after 1 h) ICP-MS method	Silver content in medium (after 12 h) ICP-MS method	Silver content in medium (after 24 h) ICP-MS method
AgNPs	2.6 ± 0.8 nm	–	8.32 μg/ml	15.7 μg/ml	21.12 μg/ml
	TEM		0.416%	0.785%	1.056%
AgNPsLA	2.5 ± 0.5 nm	176	1 μg/ml	1.27 μg/ml	1.51 μg/ml
	TEM, SAXRD	TGA	0.01%	0.0127%	0.015%

[a]*Based on results from our previous studies: Hajtuch et al., 2019; Zielinska et al., 2016; Zielinska et al., 2018).*

TABLE 2 | Minimal inhibitory concentrations of AgNPs and AgNPsLA against reference strains of microorganism

	MIC (μg/ml)					
	Enterococcus faecium	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Acinetobacter baumanni*	*Pseudomonas aeruginosa*	*Klebsiella aerogenes*
AgNPs	4	16	8	8	4	4
AgNPsLA	32	32	32	8	32	32

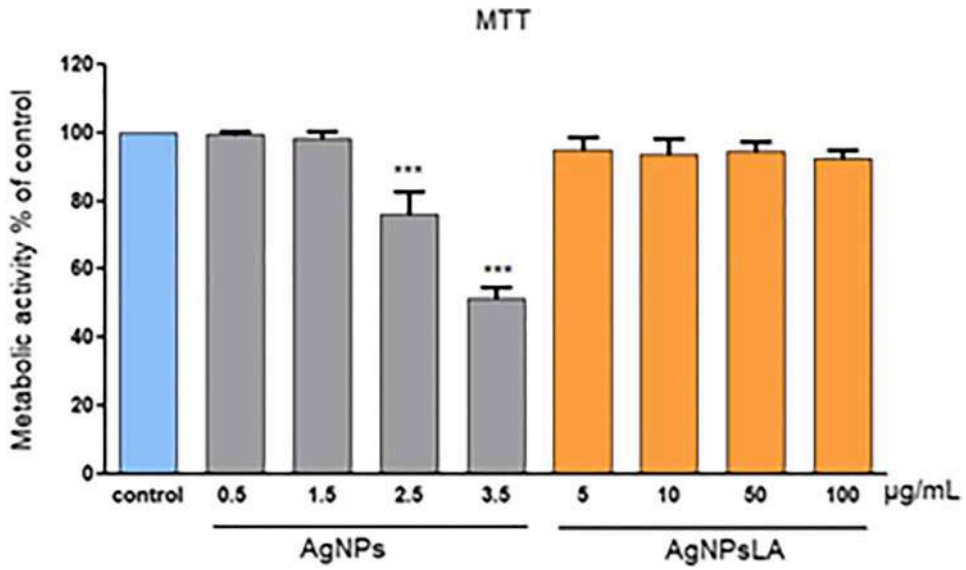

FIGURE 1 | Metabolic activity of HUVEC cells as measured by MTT assay. Co-incubation of HUVEC cells for 24 h with AgNPsLA did not modify the metabolic activity of HUVEC cells. Exposure to bare AgNPs resulted in a significant decrease of their metabolic activity when compared to cells in the absence of NPs. Data are presented as mean ± SD. ***$p < 0.001$ vs. control.

stability of these NPs under the conditions tested. Data are presented in **Table 1**.

Antimicrobial Activity of AgNPs and AgNPsLA and Their Impact on Metabolic Activity in HUVEC

Table 2 shows the MIC of AgNPs and Ag NPsLA for *E. faecium, S. aureus, K. pneumonia, A. baumanii, P. aeuruginosa,* and *K. aerogenes*. Although AgNPs had a higher efficacy than AgNPsLA against most of the microorganisms, AgNPsLA were also active with the lowest minimal inhibitory concentrations (8 μg/ml) obtained against *A. baumani.*

Figure 1 illustrates the changes induced in the metabolic activity of HUVEC as measured by MTT assay after their exposure to AgNPs and AgNPsLA. AgNPs decreased the metabolic activity in the cells in a concentration equal or higher than 2.5 μg/ml. At the highest tested concentration (3.5 μg/ml), they decreased the metabolic activity of cells to around 50%. In contrast, AgNPsLA did not affect the metabolic activity of the cells at any of the concentrations tested.

TABLE 3 | Selectivity index for AgNPs and AgNPsLA taking into account the average of MIC (X MIC) against *E. faecium, S.aureus, K. pneumonia, A. baumanii, P. aeuruginosa, K. aerogenes,* and the IC_{50} on HUVEC cells (SI = IC_{50}/MIC) by the MTT assay

NPs	IC_{50} µg/ml	X MIC µg/ml	Selectivity index
AgNPs	3.5	6.35	0.55
AgNPsLA	1,509	25.40	59.40

The selectivity index (IC_{50}/MIC) was also determined for AgNPs and AgNPsLA considering the average of MIC (X MIC) against *E. faecium, S. aureus, K. pneumonia, A. baumanii, P. aeuruginosa,* and *K. aerogenes,* and the IC_{50} on HUVEC (calculated based on the concentration of NPs that induced a 50% decrease of the metabolic activity in HUVEC as measured by the MTT assay). As shown in **Table 3** the selectivity index (SI) of AgNPs and AgNPsLA values were 0.55 and 59.40, respectively.

Impact of AgNPs and AgNPsLA on Reactive Oxygen Species Level

As shown in **Figure 2**, AgNPs induced reactive oxygen species (ROS) generation in HUVEC in a concentration-dependent manner, namely 1.4 times (for 2.5 µg/ml) and 1.7 times (for 3.5 µg/ml) compared with non-treated cells. In contrast, AgNPsLA did not increase the ROS level in HUVEC compared with control cells after 24 h incubation.

Impact of AgNPs and AgNPsLA on HUVEC Apoptosis

Next, we determined the type of cell death by dual staining of HUVEC with propidium iodide (PI) and Annexin V, which allows us to distinguish between the amount of early apoptotic (Annexin V^+ PI^-), late apoptotic/necroptotic (Annexin V^+ PI^+), and necrotic (Annexin V^- PI^+) cell populations (Lakshmanan 2016). After 24 h of incubation with AgNPs, a significant increase rate of late apoptotic population of HUVEC cells up to 28 and 45% was detected for 2.5 µg/ml and 3.5 µg/ml, respectively. In contrast, AgNPsLA caused neither apoptosis nor necrosis in HUVEC within the range of the investigated concentrations (**Figure 3**).

Impact of AgNPs and AgNPsLA on Mitochondrial Depolarization

Mitochondria play an essential role in activating caspase proteases through a pathway termed the mitochondrial or intrinsic pathway of apoptosis (Tait and Green 2013). To confirm further that AgNPs induce apoptosis, mitochondrial membrane potential was measured in HUVEC following incubation with AgNPs and AgNPsLA. JC-1 staining was used to determine NPs impact on mitochondria. **Figure 4** shows that AgNPs induced a significant damage to mitochondria after 24 h incubation, but no changes in membrane potential were detected in cells treated with AgNPsLA. The highest concentrations of AgNPs tested (2.5 and 3.5 µg/ml) significantly increased the polarization of mitochondrial membrane in HUVEC. Similarly, AgNPs also induced changes in membrane potential of human platelets, in contrast to AgNPsLA (**Figure 5**).

Evaluation of Hemolytic Activity of AgNPs and AgNPsLA

To evaluate the potential deleterious effect of AgNPs and AgNPsLA on RBCs, hemoglobin (Hb) and LDH release from RBCs in the presence of both NPs were measured. AgNPs led to concentration dependent hemolysis reflected by ~65% of Hb and ~80% of LDH release at the concentration of 3.5 µg/ml AgNPs

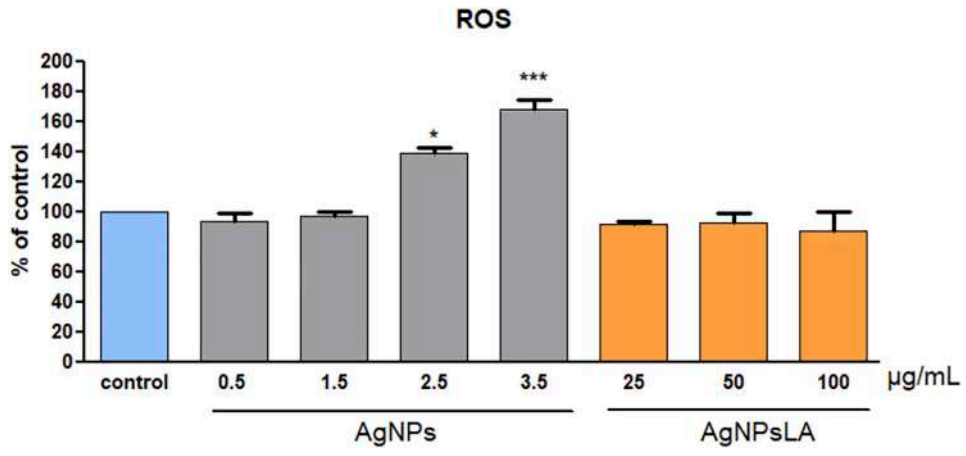

FIGURE 2 | Reactive oxygen species (ROS) generation in HUVEC cells. Co-incubation of HUVEC cells for 24 h with AgNPsLA did not modify ROS generation in HUVEC cells. AgNPs induced a significant increase in ROS generation when compared to cells in the absence of NPs. Data are presented as mean ± SD. *$p < 0.05$, ***$p < 0.001$ vs. control.

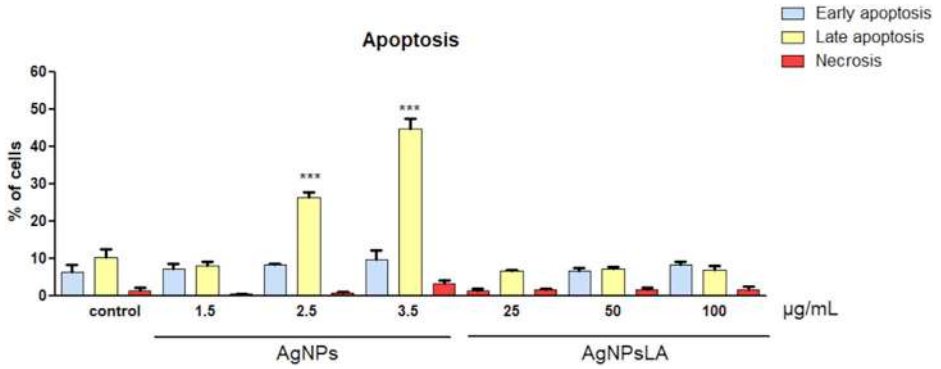

FIGURE 3 | Effects on apoptosis in HUVEC cells. Apoptotic rates of HUVEC cells incubated with different concentrations of AgNPs and AgNPsLA for 24 h determined by flow cytometry. AgNPsLA had no effect, AgNPs induced late apoptosis at the highest concentrations tested. Data are presented as mean ± SD of three independent experiments. ***$p < 0.001$ vs. untreated cells.

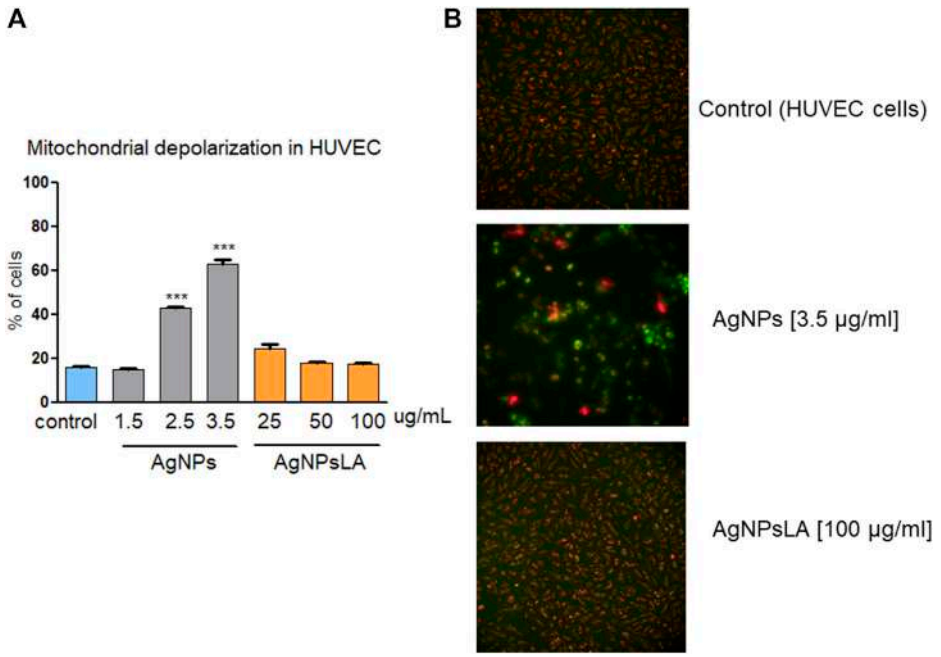

FIGURE 4 | Mitochondria depolarization in HUVEC. AgNPs induced mitochondria damage in HUVEC, no changes in membrane potential were detected in cells treated with AgNPsLA. **(A)** Percentage of cells associated to decreased mitochondrial membrane depolarization. Data are presented as mean ± SD of three independent experiments. ***$p < 0.001$ vs. control. **(B)** Representative images of JC-1 staining in HUVEC in the presence and absence of AgNPs and AgNPsLA. Red fluorescence is formed by JC-1 complexes in undamaged mitochondria, whereas green fluorescence is formed by JC-1 monomers in mitochondria with low membrane potential.

(**Figure 6A**). In contrast, AgNPsLA did not induce significant hemolysis leading to ~30% of Hb and ~20% of LDH release at the highest concentration tested (100 µg/ml) (**Figure 6B**).

Impact of AgNPs and AgNPsLA on Ultrastructural Changes

TEM analysis of HUVEC in the absence and presence of AgNPs and AgNPs is shown in **Figures 7A–C**. TEM images show the presence of both tested NPs located mainly in autolysosomes and vesicles. The morphology and structure of *S. aureus* were also examined by TEM after their exposure to AgNPs and AgNPsLA (**Figures 7D–F**). In contrast to unexposed bacteria, those incubated with both types of nanoparticles (AgNPs and AgNPsLA) showed structural changes mainly in the cell wall, which became thin, wrinkled, and sometimes broken. In addition, some bacteria showed signs of swelling or atrophy combined with deformation or rupture of the cell membrane and release of the cell contents.

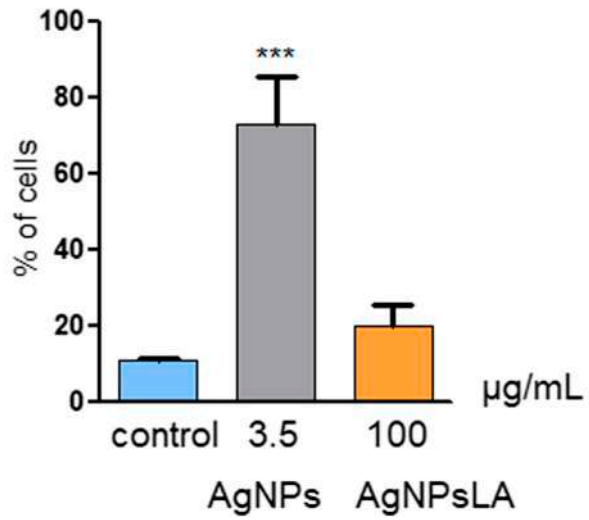

FIGURE 5 | Mitochondria depolarization in platelets. AgNPs caused significant changes in membrane potential of human platelets, in contrast to AgNPsLA. Percentage of cells with decreased mitochondrial membrane depolarization. Data are presented as mean ± SD of three independent experiments. ***p < 0.001 vs. control.

DISCUSSION

Nowadays, development of novel, efficient nanotechnological-based bactericidal agents against multidrug-resistant bacteria is among one of the priority areas in biomedical research (Dakal et al., 2016). *In vitro* studies available in the literature testing the effect of AgNPs have reported that they can be toxic to several human cell lines. Furthermore, and based on already published *in vivo* animal models, it is known that AgNPs tend to accumulate in the liver, spleen, and kidney and that they are able to cross the blood-brain barrier following intravenous, intraperitoneal, and intratracheal routes of administration (Liao et al., 2019). In this study, we assessed the biosafety of bare AgNPs and AgNPs coated with LA on the vascular microenvironment as they could be potentially used to coat medical devices such as vascular catheters.

The AgNPsLA synthesized by our team (2.5 nm size) demonstrated a fairly constant silver content over time in the media, while the commercially available AgNPs (2.6 nm size) that we used for this study showed an increase in the silver ion content over time, indicating a lower stability of these NPs. However, in our previous research, the soluble Ag present in the serum free medium after 24 and 48 h of exposure to 15 nm AgNPs was found to correspond to a release of less than 0.5% (Zielinska et al., 2016). Research data available in the literature indicate a correlation between the physicochemical behavior of AgNPs in cell culture medium or in a simulated biological medium with their toxicity toward cultured cells. In fact, the higher the *in vitro* silver release, the higher the toxicity of the AgNPs. In addition, coating of AgNPs can reduce the dissolution of silver particles in water for several days mitigating as well silver release (Veronesi et al., 2016; Cunningham et al., 2021; Zhang et al., 2021). We demonstrate that although the bare AgNPs used in this study induce cytotoxicity and hemolysis in a concentration-dependent manner, co-incubation with AgNPs coated with LA do not exert harmful effects on HUVEC, platelets, and RBCs. Moreover, those AgNPsLA have antimicrobial properties at concentrations that are safe for them.

AgNPs and AgNPsLA: Antimicrobial Properties and Metabolic Activity in HUVEC

With the emergence of pathogenic bacterial strains resistant to one or several antibiotics, there is an urgent need for new antibacterial agents. It has been demonstrated that AgNPs action is highly dependent on their surface reactivity (Le Ouay and Stellacci 2015). In our hands, bare AgNPs exhibited higher antimicrobial activity than AgNPsLA. We focused on six pathogens: *E. faecium, S. aureus, K. pneumonia, A. baumanii, P. aeuruginosa, and K. aerogenes*. Selected pathogens are well known as etiological factors of catheter related infections (Bouza et al., 2002; Shah et al., 2013). Many research studies have previously shown the antimicrobial activity of uncoated AgNPs (Kim et al., 2007; Qing et al., 2018; Prakash et al., 2019). However, when it comes to LA

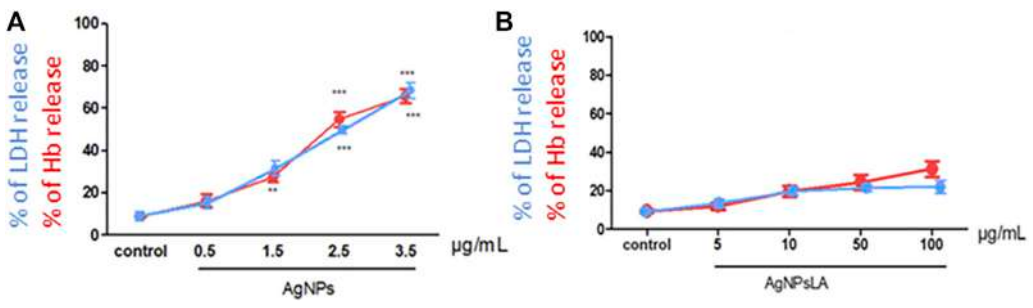

FIGURE 6 | Effect of AgNPs and AgNPsLA on red blood cells (RBCs). **(A)** % of LDH release (blue line) and % of hemoglobin (Hb) release (red line) from RBCs after incubation with AgNPs. **(B)** % of LDH release (blue line) and % Hb release (red line) from RBCs AgNPsLA treated. Data are presented as mean ± SD. **p < 0.01 ***p < 0.001 vs. control.

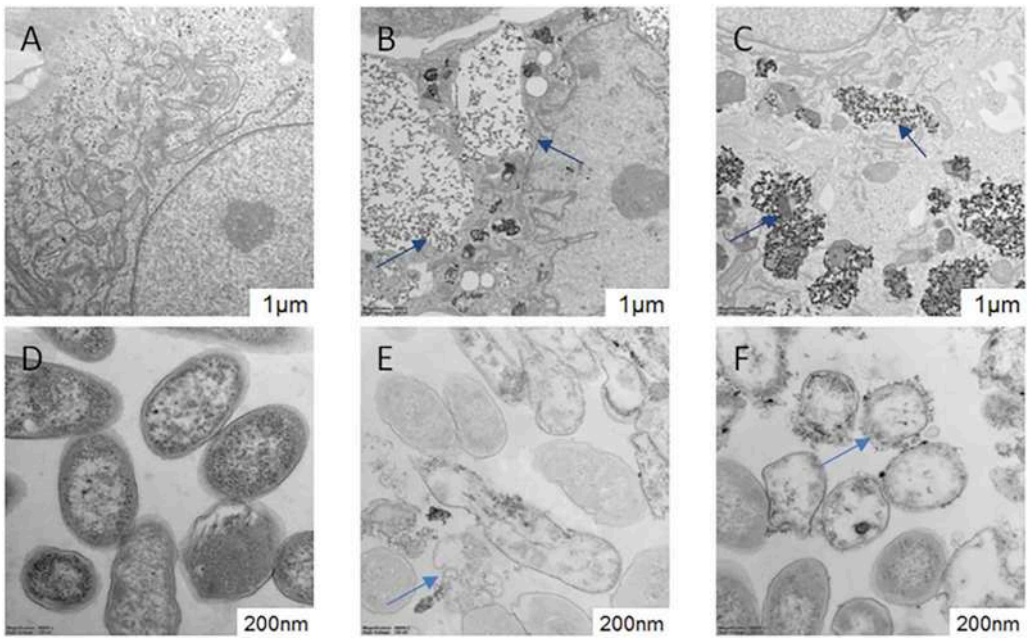

FIGURE 7 | TEM of HUVEC and *Staphylococcus aureus* morphology. **(A)** HUVEC control cells; **(B)** HUVEC treated with AgNPs (3.5 µg/ml). Blue arrows indicate AgNPs in autolysosomes and vesicles. **(C)** HUVEC treated with AgNPsLA (50 µg/ml). Blue arrows indicate AgNPsLA. **(D)** *S. aureus*. Control. **(E)** *S. aureus* treated with AgNPs (16 µg/ml). **(F)** *S. aureus* treated with AgNPsLA (32 µg/ml). The blue arrows point the nanoparticles.

coated NPs, only a few studies have examined their antibacterial properties (Cotton et al., 2019). Our team, has previously shown that AgNPsLA at concentrations ≤ 5–40 µg/ml inhibited growth of 70% of the tested bacteria (Niska et al., 2016). Our LA coated NPs exhibited a MIC range of 8–32 µg/ml for the reference strains.

Isolation of human umbilical vein endothelial cells (HUVEC) from umbilical cord was first described in 1973. To date, this model is still widely used due to the high success rate of HUVEC isolation and because this is an excellent model for studying a wide range of diseases, including cardiovascular and metabolic diseases. The vascular endothelium plays a crucial role in the regulation of blood pressure, blood flow, and coagulation (Leyte et al., 2020). The HUVEC model is in fact highly representative of the human vascular endothelium, allowing the study of the physiological and pathological effects of different factors (Maciag et al., 1981). We have previously demonstrated that AgNPsLA inhibit platelet aggregation (Hajtuch et al., 2019). So, based on our previous findings we hypothesized that those NPs could be used to internally coat an intravenous catheter for preventing thrombosis but to also fight against associated microbial infections. For this reason, we have focused on assessing further the potential effect that those NPs could exert not only on platelets but also on endothelial cells and red blood cells.

The MTT assay estimates cell viability by measuring mitochondrial metabolism. The enzymatic reduction of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan is catalyzed by mitochondrial succinate dehydrogenase. Hence, the MTT assay is dependent on mitochondrial respiration and indirectly serves to assess the cellular energy capacity of a cell (Chacon et al., 1997). Our results showed that although bare AgNPs exerted cytotoxic effects on HUVEC, AgNPsLA did not lead to toxicity even at the highest concentration tested (100 µg/ml). Those results are in agreement with our previous work where we demonstrated a lower cytotoxicity of LA coated AgNPs when compared to bare AgNPs using a gingival fibroblast model where AgNPsLA did not induce cell toxicity at concentrations up to 40 µg/ml (Niska et al., 2016). Selectivity index is the ratio that measures the window between cytotoxicity and antibacterial activity. The higher the SI index, the more effective and safer the material would be during *in vivo* treatment. Therefore, an ideal material should be cytotoxic only at very high concentrations but should show antibacterial activity at very low concentrations, which would result in a high SI value (Pritchett et al., 2014). In our research, SI of AgNPs and AgNPsLA values were 0.55 and 59.40, respectively, indicating the effectiveness and safety of AgNPsLA as a potential biomaterial.

Functionalization (Coating) With Lipoic Acid Prevents AgNPs-Induced ROS Production, Apoptosis and Mitochondrial Depolarization

ROS play a central role in cell signaling as well as in regulation of the main pathways of apoptosis mediated by mitochondria. Apoptosis is a tightly regulated and highly conserved process of cell death during which a cell undergoes self-destruction

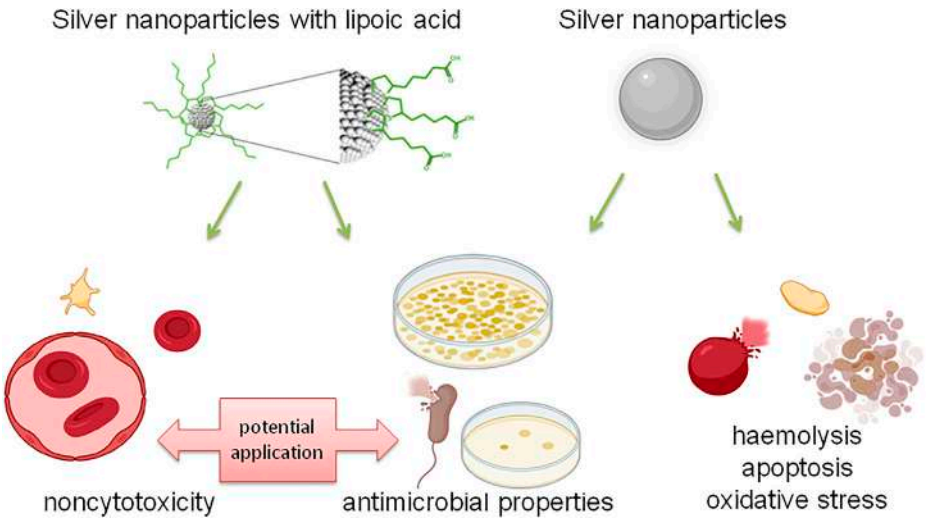

FIGURE 8 | Summary of the assessment of antibacterial properties and the potential of biosafety in the vascular microenvironment of new lipoic acid coated AgNPs and uncoated AgNPs.

(Redza-Dutordoir and Averill-Bates 2016). Mitochondrial dysfunction has been shown to participate in the induction of apoptosis and has even been suggested to be central to the apoptotic pathway. Indeed, opening of the mitochondrial permeability transition pore has been demonstrated to induce depolarization of the transmembrane potential, release of apoptogenic factors, and loss of oxidative phosphorylation (Gottlieb et al., 2003). Hence, we aimed to examine the impact of bare AgNPs and AgNPsLA on ROS, apoptosis, and mitochondrial membrane potential. BareAgNPs significantly increased ROS production, apoptosis, and mitochondrial depolarization, when compared to control and to AgNPsLA incubated cells. In this study, we have found that AgNPs induced mitochondrial membrane depolarization suggesting the formation of the mitochondrial permeability transition pore transmembrane protein in AgNPs incubated cells, which is consistent with ROS role in triggering cell damage processes including the formation of mitochondrial permeability transition pore (AshaRani et al., 2009). Many other studies have also reported the generation of ROS in different cell lines in the presence of AgNPs, which are in agreement with our results (Barcińska et al., 2018; El-Hussein and Hamblin 2018; Kang et al., 2012). However, we have reported for the first time that LA coating prevents the increase in ROS induced by bare AgNPs in HUVEC and that AgNPsLA do not affect the mitochondrial membrane potential of platelets, something that is of crucial importance. In fact, platelets do not have nuclei and therefore their function and span life largely depend on the functioning of their mitochondria (Melchinger et al., 2019) and the mitochondrial membrane potential of human platelets is considered a sensitive parameter of platelet quality (Verhoeven et al., 2005).

Impact of AgNPs and AgNPsLA on RBCs

Hemolysis refers to the breakdown of erythrocytes with subsequent release of their intracellular content. This can result in a dangerous situation that can ultimately lead to jaundice and severe anemia. Many natural and synthetic nanoparticles have elicited hemolytic action and hence, preclinical investigation of hemolytic activity is of massive importance for newly developed nanomedicines for human use that may come in contact with blood (Raval et al., 2018). Lactate dehydrogenase (LDH) can be physiologically present in serum due to cellular turnover but during hemolytic conditions LDH is often increased (Barcellini and Fattizzo 2015). Our results clearly show that bare AgNPs can induce hemolysis as demonstrated by an increase of hemoglobin and LDH release from erythrocytes co-incubated with those NPs. In fact, it has been previously found that AgNPs have hemolytic effects by changing membrane integrity and surface characteristics and that they might cause pore formation on the membrane of RBCs that ultimately results in osmotic lysis (Kwon et al., 2012; Krajewski et al., 2013). It has been described (Uchendu et al. (2014) that pretreatment with LA decreases erythrocyte fragility due to lipid peroxidation. Interestingly, the AgNPsLA tested in our work did not induce significant hemolysis when incubated with RBCs.

Impact of AgNPs and AgNPsLA on Ultrastructure of HUVEC and *S. aureus*

TEM analysis demonstrated that the exposure of HUVEC cells to AgNPs and AgNPsLA led to NPs accumulation in autolysosomes and vesicles, which is consistent with the earlier study published by Guo et al. (2016) that demonstrated AgNPs uptake by HUVEC cells. Moreover, both AgNPs and AgNPsLA significantly affect the cell membrane integrity when co-incubated with *S. aureus*. Those experiments confirmed the presence of cell wall damage caused by

those NPs, which is the basic mechanism of antibacterial action, as well as the accumulation of NPs in the bacterial membrane (Mirzajani et al., 2011; Bondarenko et al., 2018).

Research Limitations

Most experiments for characterizing the biological effects of nanomaterials are performed *in vitro* using cell lines. In fact, their potential toxicological effect, uptake mechanisms as well as other specific cellular responses, such as the measurements of ROS, can be carried out in different cell lines. However, one limitation of this approach is that the absorption and viability results recorded *in vitro* may differ from those observed in more organized systems, such as tissues or organs, or *in vivo* (Bakand and Hayes 2016). Under physiological conditions, endothelial cells are constantly exposed to shear stresses induced by blood flow and its viscosity, and the responses of these cells to stimuli may be determined by shear stress patterns. Indeed, endothelial cells are protected during laminar flow against harmful stimuli, while uneven shear stress can activate the endothelium (Matuszak et al., 2016). Recent studies indicate that endothelial uptake by non-targeted NPs depends on the presence and magnitude of shear stress (Fede et al., 2015; Klingberg et al., 2015). Therefore, and compared to traditional *in vitro* cytotoxicity assays performed under static conditions, NPs may exhibit higher and stress-dependent toxicity to endothelial cells under flow conditions (Shurbaji et al., 2020). It is worth to mention that advanced culture systems are expensive, technically demanding, and more difficult to standardize and that it is currently unclear to what extent advanced culture systems provide more predictive data on human toxicity than conventional systems. However, databases based on results obtained from conventional cell cultures, advanced models and animal experiments are crucial for defining the role of advanced culture systems in the toxicological assessment of NPs (Fröhlich 2018). There is no doubt that future research using models that mimic more closely physiological conditions, like in this particular case flow-through experiments, would provide a better inside on the effect of AgNPsLA on HUVEC cells under near-physiological conditions.

CONCLUSION

We compared the antimicrobial effect and potential deleterious effect that bare AgNPs and LA-coated AgNPs may had on endothelial cells, platelets, and RBCs. Co-incubation of RBC led to hemolysis with AgNPs but not with AgNPsLA. While considerable cytotoxicity was demonstrated in HUVEC cells for bare AgNPs at low concentrations (2.5 μg/ml), AgNPsLA did not affect cell viability of HUVEC when they were co-incubated up to 100 μg/ml for 24 h. Cell death associated to AgNPs incubations was found to be a ROS-dependent apoptosis mechanism accompanied by a depolarization of the mitochondrial membrane potential. In contrast, we showed, for the first time, that functionalization of AgNPs with LA maintains the antimicrobial activity associated and widely demonstrated for AgNPs but significantly reduces NPs cytotoxicity on endothelial, platelets, and red blood cells. Our work supports once again the importance for carrying out biocompatibility studies when investigating the potential application of nanotechnology in medicine.

Figure 8 summarizes assessment of the antibacterial properties and biosafety potential on the vascular microenvironment of novel lipoic acid-coated AgNPs and uncoated AgNPs.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethics Committe of the Medical University of Gdansk NKBBN/552/2018-2019. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

The authors confirm contribution to the paper as follows: study conception and design, draft manuscript preparation: I.I-S.; biocompatibility of AgNPs evaluation, data collection, analysis and interpretation of results, contribution in draft manuscript preparation: J.H.; draft manuscript preparation: M.S-M.; TEM analysis: M.N.; chemical synthesis and characterization: E.T. and M.W., microbiological analysis and interpretation of results: M.J. and W.K. All authors reviewed the results and approved the final version of the manuscript.

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ORIGINAL RESEARCH

The Pharmacological Effects of Silver Nanoparticles Functionalized with Eptifibatide on Platelets and Endothelial Cells

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Purpose: In the search for new drug delivery platforms for cardiovascular diseases and coating of medical devices, we synthesized eptifibatide-functionalized silver nanoparticles (AgNPs-EPI) and examined the pharmacological activity of AgNPs-EPI on platelets and endothelial cells in vitro and ex vivo.

Methods: Spherical AgNPs linked to eptifibatide were synthesized and characterized. Cytotoxicity was measured in microvascular endothelial cells (HMEC-1), platelets and red blood cells. Platelet mitochondrial respiration was measured using the Oxygraph-2k, a high-resolution modular respirometry system. The effect of AgNPs-EPI on the aggregation of washed platelets was measured by light aggregometry and the ex vivo occlusion time was determined using a reference laboratory method. The surface amount of platelet receptors such as P-selectin and GPIIb/IIIa was measured. The influence of AgNPS-EPI on blood coagulation science was assessed. Finally, the effect of AgNPs-EPI on endothelial cells was measured by the levels of 6-keto-PGF1alpha, tPa, cGMP and vWF.

Results: We describe the synthesis of AgNPs using eptifibatide as the stabilizing ligand. The molecules of this drug are directly bonded to the surface of the nanoparticles. The synthesized AgNPs-EPI did not affect the viability of platelets, endothelial cells and erythrocytes. Preincubation of platelets with AgNPs-EPI protected by mitochondrial oxidative phosphorylation capacity. AgNPs-EPI inhibited aggregation-induced P-selectin expression and GPIIb/IIIa conformational changes in platelets. AgNPs-EPI caused prolongation of the occlusion time in the presence of collagen/ADP and collagen/adrenaline. AgNPs-EPI regulated levels of 6-keto-PGF1alpha, tPa, vWf and cGMP produced in thrombin stimulated HMEC-1 cells.

Conclusion: AgNPs-EPI show anti-aggregatory activity at concentrations lower than those required by the free drug acting via regulation of platelet aggregation, blood coagulation, and endothelial cell activity. Our results provide proof-of-principle evidence that AgNPs may be used as an effective delivery platform for antiplatelet drugs.

Keywords: drug delivery, antiplatelet, RGD, aggregation, coagulation system, biocompatibility

Introduction

Hemostasis is a tightly regulated process that ensures vascular integrity. Following accidental vascular injury, the hemostatic system initiates vascular events, restores the hemostatic balance, and prevents a pathological extension of hemostasis, ie, thrombosis.[1] Hemostasis acts as an interplay between blood platelets, coagulation and fibrinolysis proteins, and endothelial cells. The endothelium was originally thought to be a simple passive barrier between the intravascular and extravascular compartment but is now viewed as an organ whose function is critical to maintaining hemostasis and promoting vascular health. The most important endothelial functions include regulation of vascular tone, cell adhesion, smooth muscle cell proliferation, and vascular wall inflammation. In addition, the endothelium serves as a hemocompatible lining that helps maintain blood flow and blood regulation coagulation system.[2] Endothelial cells secrete antiplatelet and anticoagulant agents that prevent platelet aggregation and fibrin formation. Endothelial dysfunction occurs due to a pathological process, which

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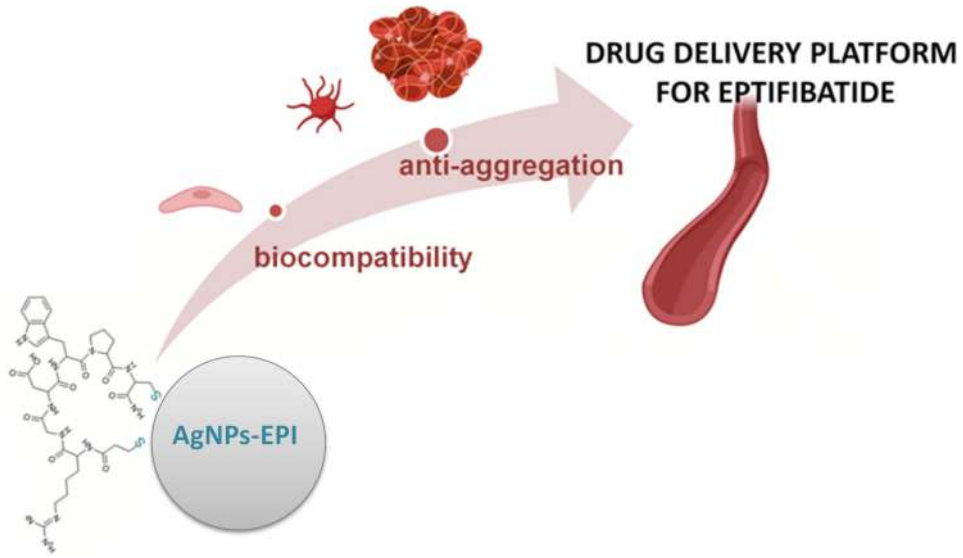
Graphical Abstract



may lead to increased platelet adhesion, aggregation, fibrin generation, and formation of occlusive thrombi.[3] Intact endothelium inhibits platelet adhesion by releasing nitric oxide and prostaglandin I_2, while activated endothelial cells secrete various molecules and receptors that increase platelet adhesion and aggregation to the injury site.[4] Thrombocytes play a significant role in the process of primary haemostasis, during which the platelet plug forms. It begins with the adhesion of platelets to the point where the continuity of a blood vessel has been broken. As a result of the injury, collagen fibers are exposed, with which the Ib/V/IX receptor complex present on the surface of blood platelets binds. The adhesion of platelets to the injured endothelium is also enhanced by the participation of von Willebrand factor, which forms a bond with glycoprotein Ib. Platelets adjacent to the site of damage are simultaneously activated, which leads to degranulation and the release of biologically active substances stored in granules.[5] Platelet aggregation is the major pathophysiological factor in the development of arterial ischaemic events, including coronary artery disease, cerebrovascular accidents, peripheral arterial disease.[6] Moreover, platelet activation and dysfunction have been implicated in diabetes, renal diseases, tumorgenesis, Alzheimer's, and cardiovascular diseases (CVD). CVD is the leading cause of death worldwide and encompasses diverse pathologies, including coronary heart disease, cerebrovascular disease, deep vein thrombosis, and pulmonary embolism.[7] The vascular therapy with antiplatelet agents is the cornerstone of clinical management of CVD. The way of antiplatelet therapy has changed over the past decade with the advent of several new drugs with different mechanisms of action.[6]

Eptifibatide is a synthetic cyclic heptapeptide deriving from the venom of southeastern pygmy rattlesnake that reversibly binds to platelet GPIIb/IIIa receptor resulting in a short-lasting receptor blockade and inhibition of platelet aggregation. In addition to tirofiban and abciximab, it belongs to the group of drugs that bind reversibly to the platelet GPIIb/IIIa receptor, causing short-term blockade of the receptor and inhibition of platelet aggregation. The compound also relaxes the coronary artery through endothelial-dependent NO-cGMP signaling. Blockade of platelet GPIIb/IIIa with eptifibatide has improved endothelial-dependent vasodilation.[8,9] The available studies suggest that eptifibatide is more safe than tirofiban in patients with acute coronary syndrome. The data also show that it increases survival during percutaneous coronary intervention compared to abciximab.[10,11] The major rate-limiting factor for the pharmacological use of EPI is its short half-life due to accumulation in non-targeted tissues and elimination by renal filtration. Advances in nanomedicine led to the pharmaceutical design of drug delivery platforms using nanoparticles allowing for controlled and targeted drug administration.[12,13] Silver nanoparticles (AgNPs) are an attractive tool for drug delivery platforms. Indeed, AgNPs can be functionalized in a specific way with molecular encapsulating agents such as proteins and ligands

connected via chemical bonds. In the last case, the synthesized nanoparticles exhibit high stability and tunable releasing of the bioactive ligands under the influence of the environment.[14] Especially beneficial is the connection via the Ag-S bond (thiolate bond) because it gives a possibility to obtain stable nanoparticles that can be separated in a solid-state and detailed characterized. Furthermore, such a form of potential pharmaceutics offers a broader range of drug preparation. This type of drug connection with the nanoparticle's surface is possible if the ligand contains mercaptyl or disulfide groups in its molecules. In the case of eptifibatide, disulfide groups in its molecules enable the connection directly to the silver surface without any linker. Noteworthy, the AgNPs show a strong antibacterial effect that improves infection treatment[15–17] Our recent studies have shown that the functionalized AgNPs synthesized by our team can inhibit platelet aggregation under flow conditions by reducing the expression of P-selectin and GPIIb/IIa receptors.[18] This paper reports the synthesis of narrow-dispersive, stable silver nanoparticles linked directly with eptifibatide molecules (AgNPs-EPI) via thiolate bonds. We also studied the impact of AgNPs-EPI on thrombin-induced endothelial dysfunction.[19,20] Our results indicate that AgNPs-EPI acts as a new inhibitor of platelet and endothelial cell activation and could be used as a platform for drug delivery in cardiovascular diseases and for coating medical devices.

Materials and Methods

Nanoparticle Synthesis and Characterization

30 mg of eptifibatide (EPI) was dissolved in 30 mL of H_2O and transferred to the flask immersing in an ice-water bath on a magnetic stirrer. After 15 minutes of stirring, 24 µL of 1 M $AgNO_3$ was added, and the mixture was stirred for the next 15 minutes; while stirring, the flask was covered with aluminum foil protecting against light. Next, 110 mg of $NaBH_4$ dissolved in 16 mL H_2O and cooled down in a fridge was added drop by drop. The mixture became first yellow, light brown, and finally dark brown during the addition of the reducing agent. The mixture was stirred for four h at room temperature. Protected against the light. Afterward, the solution was transferred to a dialysis bag, purified from inorganic compounds, and EPI molecules not connected with the NP's surface against ultrapure water for 48 h (the dialysate was changed three times). AgNPs were characterized by transmission electron microscopy (TEM), UV-vis spectrophotometry, thermogravimetric analyses (TGA), dynamic light scattering (DLS), transform infrared (FTIR) and zeta potential measurements. UV–vis absorption spectra were recorded using a Cary 50 UV/Vis spectrophotometer in ultra-pure water in the 200–800 nm range with a 1 cm quartz cell. Thermogravimetric analyses (TGA) were performed under N_2 using Q50-1261 TA Instruments (USA) with temperature compensated thermobalance (precision ±0.01%), a heating rate of 10 $K \cdot min^{-1}$. Weight loss during thermal decomposition was determined in the temperature range of 20–1000°C. Transmission electron microscopy (TEM) observations have been carried out using JEM 1400 JEOL Co. microscope at 120 kV acceleration voltage. The samples were obtained by casting the water solution of materials onto a carbon-coated copper microgrid (200 mesh) and air-dried overnight. Dynamic Light Scattering (DLS) measurements were performed with a Zetasizer Nano series apparatus (Malvern) with a backscattering detection at a constant 173 scattering angle equipped with a He-Ne laser (4 mW) at 632.8 nm using a thermostated cell holder. The hydrodynamic diameters of the particles were measured at 25°C, and size distribution by number was determined in ultra-pure water. The surfaces' potential zeta (ζ-potential) was determined for the same solution as hydrodynamic diameter measurements using Zetasizer folded capillary cells for electrophoresis. Fourier transform infrared (FTIR) spectroscopy measurements were performed on a Shimadzu FT-IR model 8400S spectrophotometer. FTIR spectra (absorption mode) were recorded with a resolution of 4 cm^{-1} from KBr pellets in a ratio of 1:1000 (w/w) in the case of AgNPs-EPI and 1:300 (w/w) for EPI. All compounds used in our studies show a purity >97%, determined by HPLC analyses. Eptifibatide acetate (≥98%, HPLC), silver nitrate (ACS reagent, ≥99%), sodium borohydride (≥98%), Milli-Q ultrapure water (resistivity 18.2 MΩ cm^{-1}, Millipore-Merk) was used in all the experiments. Dialysis cellulose membranes (Nadir, Bionovo) with molecular weight cut-off (MWCO) 10,000 Da were used to purify the synthesized nanoparticles.

Cell Culture

Immortalized human microvascular endothelial cell line (HMEC-1) were obtained from the ATCC (CRL-3243) and maintained as a monolayer culture in T-75 cm^2 tissue culture flasks. HMEC-1 cells were cultured in an MCDB131 medium (Life Technologies, cat# 10372019) in the presence of 6 µg/mL penicillin-G and 10 µg/mL streptomycin (Pan_Biotech, P06-07100).

Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. When confluent, cells were detached with trypsin-EDTA (Merck, T4049) and sub-cultured into a new cell culture flask. The medium was replaced every second day.

Incubations of Endothelial Cells with AgNPs-EPI, AgNPs, and Eptifibatide

For all cellular experiments, HMEC-1 cells were incubated in the presence of experimental agents for the time described in each method. Concentrations used in those experiments were selected from preliminary studies. AgNPs-EPI or AgNPs were diluted in FBS-free media and shaken well to ensure equal dispersion of NPs in the solution. In some experiments, eptifibatide acetate (Merck SML1042) was used at the concentration corresponding to the drug content in AgNPs-EPI to measure the effects of the free drug. Control cells were treated with NPs-free and FBS-free culture media.

Blood Collection and Platelet Isolation

The Bioethics Committee approved the study of the Medical University of Gdansk (NKBBN/552/2018–2021). It was also performed in accordance with the Code of Ethics of the World Medical Association, the ethical standards of the competent commission for Human Experiments (institutional and national), and the Helsinki Declaration of 1975 r., as amended in 2000. Blood was withdrawn from healthy volunteers who had not taken any drug known to affect platelet function for at least two weeks prior to the study. Each volunteer signed an informed consent form, had sufficient time to read the consent, and received comprehensive answers to the questions asked. For LDH assay, light aggregometry, and flow cytometry, whole blood was collected using 3.15% tri-sodium citrate (9:1, v:v). Washed platelet (WP) suspensions were isolated from blood as described by Radomski and Moncada and suspended using Tyrode's solution at the final concentration of 250,000 platelets/μL.[21] For mitochondria respiration experiments, platelets were prepared as previously described.[22] For coagulation studies, 3 mL of blood was collected using 3.8% buffered sodium citrate and prepared according to the reference recommendations for each method.

MTT Viability

HMEC-1 were seeded in 96-well plates (15,000 cells per well) with complete media. After 24 h, media was removed from the wells, and cells co-incubated with NPs at concentrations ranging from 0.5 to 150 μg/mL for AgNPsEPI. After 24 h, the media was supplemented with water-soluble tetrazolium salt (at a final concentration of 0.5 μg/mL) and incubated for two h. Next, the media was removed, and the resultant crystals dissolved in DMSO. After 15 min, cell viability was studied by measuring absorbance at 490 nm using a microplate reader (Synergy H1, BioTek). Viability was determined as a percentage of the control where the viability of control cells was set as 100%. Absorbance values were corrected with blank NPs.

Lactate Dehydrogenase (LDH) Release

The cytotoxic effect of AgNPs-EPI was measured by detecting LDH release following exposure of WP to AgNPs-EPI (10–150 μg/mL) at 37°C for 20 min by using a Cytotoxicity Detection LDH kit (Promega, Poland), according to the manufacturers' instructions. WP treated with lysis buffer (0.1% Triton X-100) was used as a positive control (total LDH release). Lysis buffer-treated cells were set to 100%. AgNPs-EPI was used as a background control, and their absorbance was subtracted from the reading of the samples. Results are given as % of the total LDH release from the cells. Absorbance was measured at 490 nm using a microplate reader (Synergy H1, BioTek).

Analysis of Hemolytic Properties of AgNPs-EPI

To assess the effect of AgNPs-EPI on RBCs, peripheral blood was collected into a syringe containing sodium citrate (0.35% final conc.) and 10 mL centrifuged at 300 xg for 10 min at room temperature. Platelet rich plasma and buffy coat were removed by aspiration and the red blood cells (RBCs) were diluted in PBS (1:100), plated in 96-wells and co-incubated with AgNP-EPI for 12 h. Hemolysis was determined by measuring hemoglobin. Absorbance was recorded at 540 nm for hemoglobin, using a microplate reader (Synergy H1, BioTek). Data expressed as % of hemoglobin release, 0.1% Triton X −100 (Sigma-Aldrich, St. Louis, MO) was used as the positive control. One hundred percent lysis was corroborated by optical microscope.

Platelet Function

To measure platelet aggregation under stirring, the response of platelets to collagen (2 μg/mL) in WP was tested using light aggregometry. Briefly, WP samples (2.5×10^8 cells/mL) were placed in a two-channel aggregometer (CHRONO-LOG-700) and incubated for 20 mins at 37°C, stirring at 900 r.p.m. in the presence or absence of AgNPs-EPI or EPI. Collagen (2 μg/mL) was added to the incubating, and the extent of aggregation was measured for 20 mins. To measure flow-induced platelet aggregation, in the presence or absence of AgNPs-EPI (50 μg/mL), the Innovate PFA-200 system (Siemens Healthcare Diagnostics Products, Munich, Germany) was used.

Flow Cytometry

The abundance of P-selectin and activated GPIIb/IIIa on the surface of platelets in the presence of AgNPs-EPI and EPI (25 and 50 μg/mL) was detected by flow cytometry. Collagen (2 μg/mL) induced platelet aggregation in WP was used as a positive control, whereas WP without collagen (resting platelets) was used as a negative control. Platelets in WP were pre-incubated with AgNPs-EPI and EPI (25 and 50 μg/mL) for 20 mins before adding collagen (2 μg/mL). Then, samples were collected and incubated in the dark for 15 mins at room temperature in the presence of 10 μg/mL of P-selectin (CD62P) and integrin GPIIb/IIIa (CD41/CD61) antibody (BioLegends, 304902 and 362803), respectively. Subsequently, samples were diluted in Tyrode's solution and analyzed using a BD FACS (Calibur). Platelets were identified by forward and side scatter signals, and the flow cytometer analyzed 10,000 platelet-specific events for fluorescence. The obtained data were analyzed using CellQuest software.

6-Keto-PGF1alpha, tPa, vWF and cGMP Levels

6-keto-PGF1alpha (a stable metabolite of prostacyclin), tissue plasminogen activator (tPa), von Willebrand factor (vWF), and cyclic guanosine monophosphate (CGMP) levels were measured in cell culture medium or cell lysate by commercially available enzyme linked immunosorbent assays (Abcam and BioFine). Cells were plated onto a 6-wells plate and incubated overnight. AgNPs-EPI and EPI were used at concentrations of 25 and 50 μg/mL. After 24h, Thrombin (Sigma Aldrich, T4393) was added to some of the wells (2U/mL for 1 hour) as a stimulator of endothelial dysfunction. Next, media were collected, aliquoted, and stored at −80°C. Cells were lysed in a lysis buffer containing protease inhibitors. The cell lysate was aliquoted and stored at −80°C. All tests were performed according to the manufacturer's protocols. The absorbance associated with samples, standard curves, and negative controls was measured using a Synergy H1 microplate reader (Biotek) at 450 nm with a wavelength correction of 540 nm.

High-Resolution Respirometry

Washed platelets (300 000 per each 2 mL chamber) were resuspended in MiR05 (mitochondrial respiration medium containing 0.5 mM EGTA), 3 mM MgCl2·6H2O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, and 1 g/L fatty acid BSA-free (pH 7.1). Mitochondrial respiration was measured in a high-resolution respirometer using an Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria), a modular system for high-resolution respirometry (HRR). Platelet samples were incubated at 37°C under constant stirring (750 rpm), ensuring a medium's homogenous oxygen distribution. Respiration of permeabilized platelets was determined using substrate-uncoupler inhibitor titration (SUIT) protocol as previously described.[23,24] After normal respiration was established, platelets were preincubated for 10 min in the presence or absence of AgNPs-EPI (50 μg/mL) or EPI (50 μg/mL). Following preincubation, collagen (2 μg/mL) was added to activate platelets. After another 5 min of incubation, titration was started with permeabilization of the cells with digitonin (10 μg/mL) and the addition of NADH-related substrates: pyruvate, glutamate, and malate (5 mM, 5 mM, and 0.5 mM, respectively) to induce leak respiration (state L). OXPHOS capacity of complex I (state CI) was evaluated by adding ADP (2.5 mM). Next, succinate (10 mM) was added to obtain maximal OXPHOS capacity with convergent input through complex I and II (state CI + CII). Oligomycin (1 μg/mL) was used to inhibit the ATP synthase. Titrations with the uncoupler FCCP (0.5 μM steps) were performed to determine electron transfer system (ETS) capacity (state E). Rotenone (0.5 μM, the inhibitor of complex I of ETS, state R) and Antimycin-A (2.5 μM, the inhibitor of to inhibit complex III of ETS) were added for determination of residual

oxygen consumption (ROX). Oxygen concentration (μM) and oxygen flux [pmol $O2 \cdot s^{-1} \cdot 10^{-6}$ cells] were recorded in real-time while obtained data were evaluated using DatLab software (Oroboros Instruments, Innsbruck, Austria). The OXPHOS coupling efficiency was calculated to measure mitochondrial quality and control. OXPHOS coupling efficiency, calculated with the formula (1 − (state L)/(state CI + CII)), reflects the coupling of respiration supported by electron transferring flavoprotein (ETF) with pyruvate, glutamate, malate, and succinate as substrates after addition of ADP (state CI + CII) and state L. A lower value of OCE denotes lesser coupling of the oxidation and phosphorylation after the addition of ADP.

Blood Coagulation

Several tests were used to measure the impact of AgNPs-EPI (50 μg/mL, 2 hours of incubation time) on the activity of factors involved in the coagulation cascade. Aqua pro-Injectione (Polpharma, Stargard Gdanski, Poland) was used in control experiments. Plasma fibrinogen was measured using the modified Clauss method. The assay measures the ability of fibrinogen to form fibrin clots after being exposed to a high concentration of purified thrombin. Under such conditions, the clotting time depends primarily on the presence of fibrinogen in the sample. APTT (Activated partial thromboplastin time) reflects the activity of coagulation factors of the intrinsic and common pathway (XII, XI, IX, X, VIII, II, and I). The test is performed by incubating plasma with an appropriate amount of phospholipids and a contact activator. The addition of calcium ions triggers the clotting process; the time until fibrin formation is measured. APTT ratio is the ratio of a patient's APTT to the regular laboratory reference APTT. The Prothrombin time (PT) measures the integrity of the extrinsic and final common pathways of the coagulation cascade. The coagulation cascade is activated by incubating the plasma with an optimal amount of calcium and thromboplastin, an activator of the extrinsic pathway; the clotting time is then measured. PT ratio is the ratio of a patient's PT to the standard laboratory reference PT. INR is derived from prothrombin time (PT). It is calculated as a ratio of the patient's prothrombin time to the control prothrombin time standardized for the potency of the thromboplastin reagent. Antithrombin III is converted by heparin to a direct inhibitor that inactivates the thrombin present in the sample. A kinetic test determines the residual thrombin content; the absorbance measured at 405nm is inversely proportional to the activity of antithrombin III in the sample. Thrombin time (TT) measures the last step in the clotting cascade. Standardized thrombin concentration is added to plasma, and time to fibrin clot formation is measured. D – Dimers - polystyrene molecules covalently bound to monoclonal antibodies (8D3) aggregate upon mixing with D-dimers samples. An aggregation reaction is then detected turbimetrically due to the increased turbidity of the sample. All tests were performed at the accredited hospital laboratory using a BCS-XP analyzer (Siemens Healthcare, Marburg, Germany).

Statistical Analysis

Each experiment was replicated independently three times. Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using Prism 5 software (GraphPad), the one-way analysis of variance (ANOVA), and Tukey's post hoc test.

Results

Nanoparticle Synthesis and Characterization

The conjugate AgNPs-EPI has been synthesized via the chemisorption of eptifibatide molecules onto a silver surface. In this process, silver nitrate was used as a source of silver ions, sodium borohydride as a reducing agent and eptifibatide as a stabilizing ligand. The reaction was carried out at a low temperature (around 4°C) to obtain nanoparticles with narrow size distribution.

Figure 1 shows the spectra recorded during the synthesis and purification steps of AgNPs-EPI. Namely, after adding the reducing agent, when the reaction was stopped (4h), after the dialysis process (20h), and the final product after 48 h of dialysis was dispersed in water. The position of the maximum Surface Plasmon Resonance (SPR) band in the UV-vis spectrum indicates the reaction's progress. The observed redshifting from 430 after adding reducing agent to 447 nm after 4 h of the reaction indicates increasing NPs size. The position of the maximum SPR band after a long time of

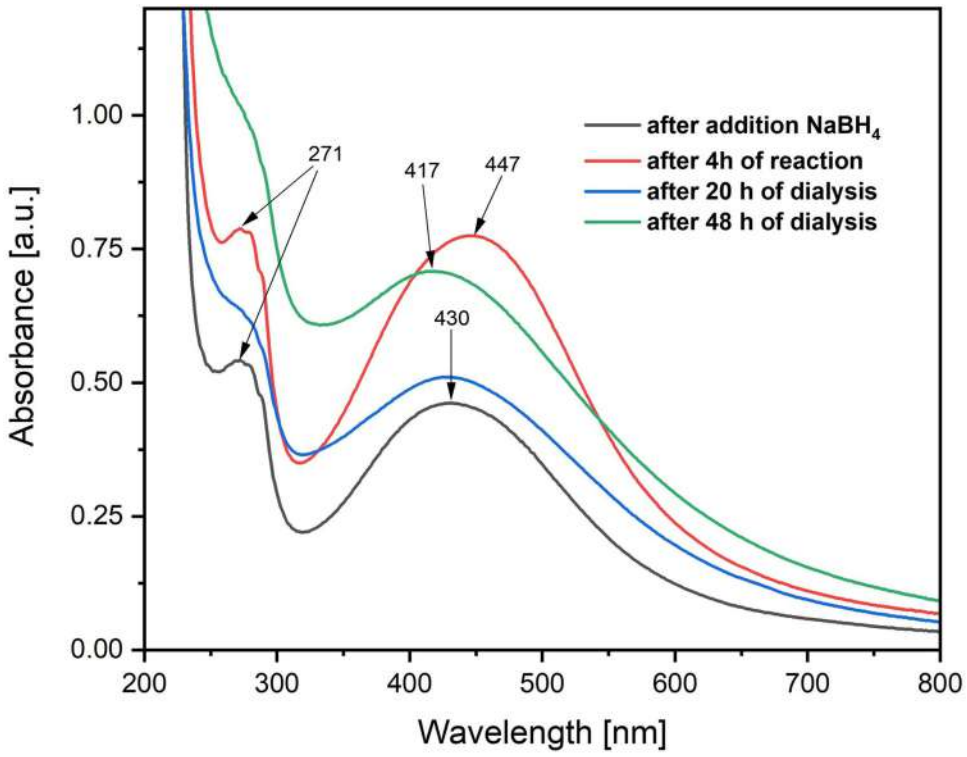


Figure 1 UV-vis spectra recorded during synthesis and purification of AgNPs-EPI. The bands with a maximum at 271 nm correspond to eptifibatide, and the bands with a maximum >400 nm correspond to surface plasmon resonance AgNPs.

dialysis (48 h) detected at 417 nm indicates that the NPs diameter is about 10 nm, which is consistent with results obtained from TEM analyses. The effectivity of dialysis in removing the excess of EPI's molecules not connected with nanoparticles surface is visible as decreasing the band's intensity with a maximum at 271 nm. The final product tested in all further experiments was obtained with the time of dialysis of 48 h to ensure that the synthesized material does not consist of eptifibatide molecules non-bounded with nanoparticles' surface.

Figure 2A shows a representative TEM image of the synthesized AgNPs-EPI and histogram 2 B with the size distribution of these nanoparticles. The synthesized NPs are spherical, narrow dispersive with an average diameter of metalcore 12.3 nm (more TEM images are presented in Supplementary Materials, Figure S1). Importantly, their propensity to self-assemble is detectable; they form groups of closely located objects that are not directly connected. Likely, molecules building organic layers on the surface of the NPs interact strongly via intermolecular interactions. This phenomenon may explain the results of DLS measurements. Compared with the average diameter of metal cores of the fabricated nanoparticles (from TEM measurements), their hydrodynamic diameters are surprisingly extensive, almost 200 nm (number size distribution). This difference may be explained based on the ability of the NPs to agglomerate via multidimensional interactions with amino acids building the peptide layer on their surface. Furthermore, the ability of amino acids to interact with water molecules via strong hydrogen interactions may also be responsible for the thick solvation shell forming around the NPs. The determined negative sign of zeta potential for the NPs shows that the functional groups in the protecting layer are ionized (mostly carboxylic groups); thus, the interactions with water molecules are more potent than in the case of neutral groups.

Figure 3 displays FTIR spectra recorded for the conjugate of silver nanoparticles with eptifibatide (AgNPs-EPI) and for this compound when it is non-bonded to the nanoparticles' surface (EPI). The characteristic bands corresponding to functional groups in the drug molecule appear in the AgNPs-EPI spectrum. The maxima of bands are located at the exact value of the wavenumber or shifted. Due to the presence of many functional groups (amine, carbonyl and amide) interacting mainly via hydrogen bonding groups, broadening the bands are observed in both spectra. The band

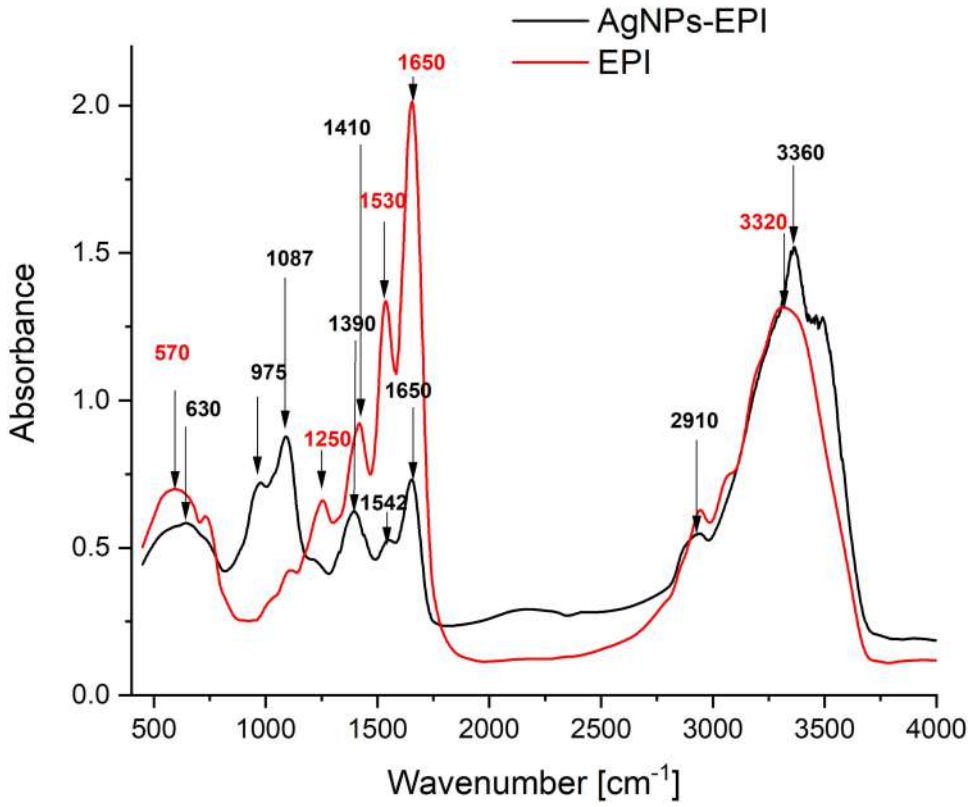


Figure 2 A representative TEM image of AgNPs-EPI (**A**) a histogram of size distribution obtained from the image: (**B**) and the results of DLS measurements: (**C**).

corresponding to the disulphide group appears at 570 cm^{-1} in the EPI molecule; meanwhile, in the conjugate spectrum, the band is significantly shifted to a higher wavelength with a maximum at 630 nm. The formation of the silver-sulphur bonds can explain it.[25] The most intensive bands in both spectra correspond to amine and amide groups. The vibration mode for amide carbonyl C=O bonds appears at 1650 cm^{-1} for both conjugate and free EPI molecules. It indicates a lack of interactions between these groups with the silver surface. However, the bands corresponding to C-N amide bonds (1530 and 1250 cm^{-1} in the EPI spectrum) are shifted in the AgNPs-EPI spectrum to lower wavenumbers. It suggests constraining these groups' motions in the conjugate, probably due to their interactions with silver (via nitrogen atoms). N-H stretch peaks characteristic of secondary amines at 3300 cm^{-1} are very strong in both spectra and located very close. Generally, the FTIR spectrum recorded for AgNPs-EPI confirmed the presence of the same functional groups as in EPI molecules, which means that they have not been transformed during the conjugate synthesis. Notably, the formation of Ag-S bonds between EPI molecule and nanoparticle's surface can also be observed in the FTIR spectrum.

Thermal gravimetric analyses (TGA) allowed for determining silver content in the synthesized NPs. Figure 4 shows TGA curves recorded for EPI and AgNPs-EPI. EPI decomposes in three steps (with maxima at 228°C, 320°C, and 711° C), and the process was completed at 800°C. In the case of AgNPs-EPI, decomposition of the organic fraction also occurs in three steps but at higher temperatures. Firstly, we observe that the drug connected with the metal core is more thermal stable than that not-connected, and it is a consequence of Ag-S bonds between the drug's molecules and nanoparticles. The final mass after decomposition of the organic fraction indicates that the content of silver is 29%, and the remaining EPI is 71%.

Assessment of Biocompatibility

The MTT and LDH assays were used to measure the viability of HMEC-1 cells and platelets following exposure to AgNPs-EPI (1–125 µg/mL). As shown in Figure 5, the viability of HMEC-1 was decreased only at 125 µg/mL.

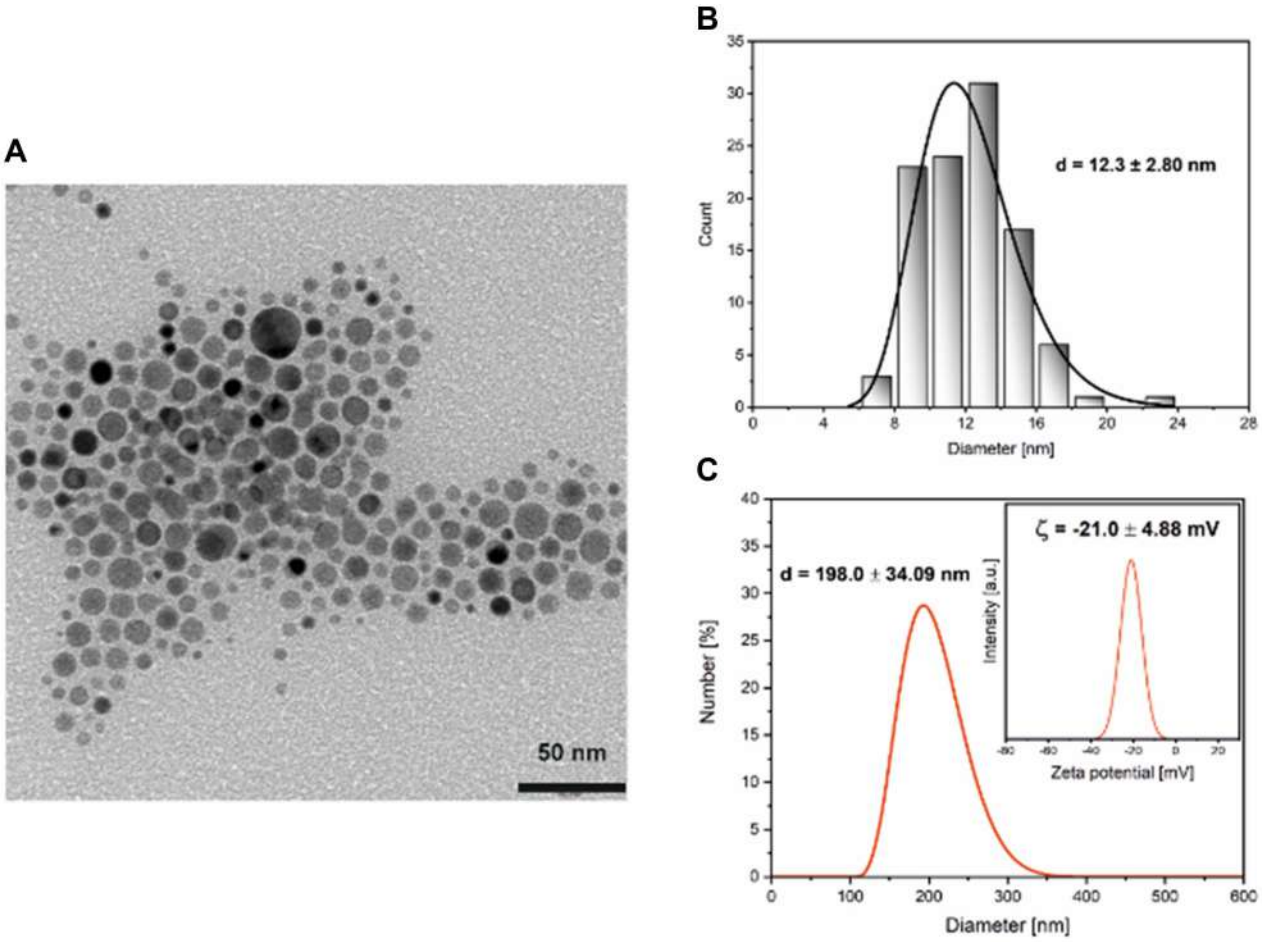


Figure 3 FTIR spectra recorded for synthesized conjugate and eptifibatide not bound with nanoparticles (samples prepared as pellets with KBr in a ratio of 1:200 (w/w)).

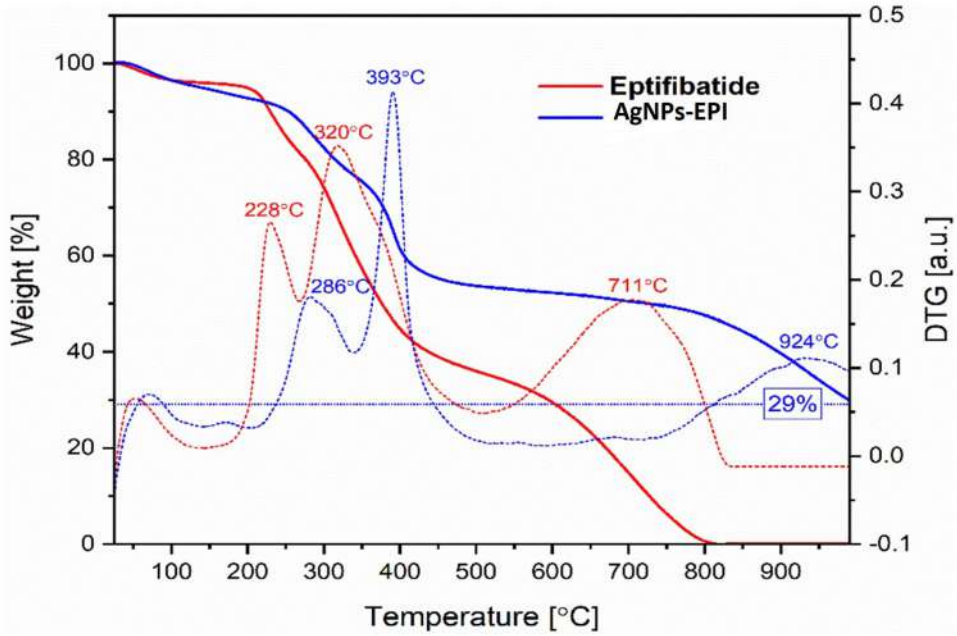


Figure 4 TGA curves recorded for AgNPs-EPI and eptifibatide (solid lines) and corresponding derivatives with temperature (dotted lines).

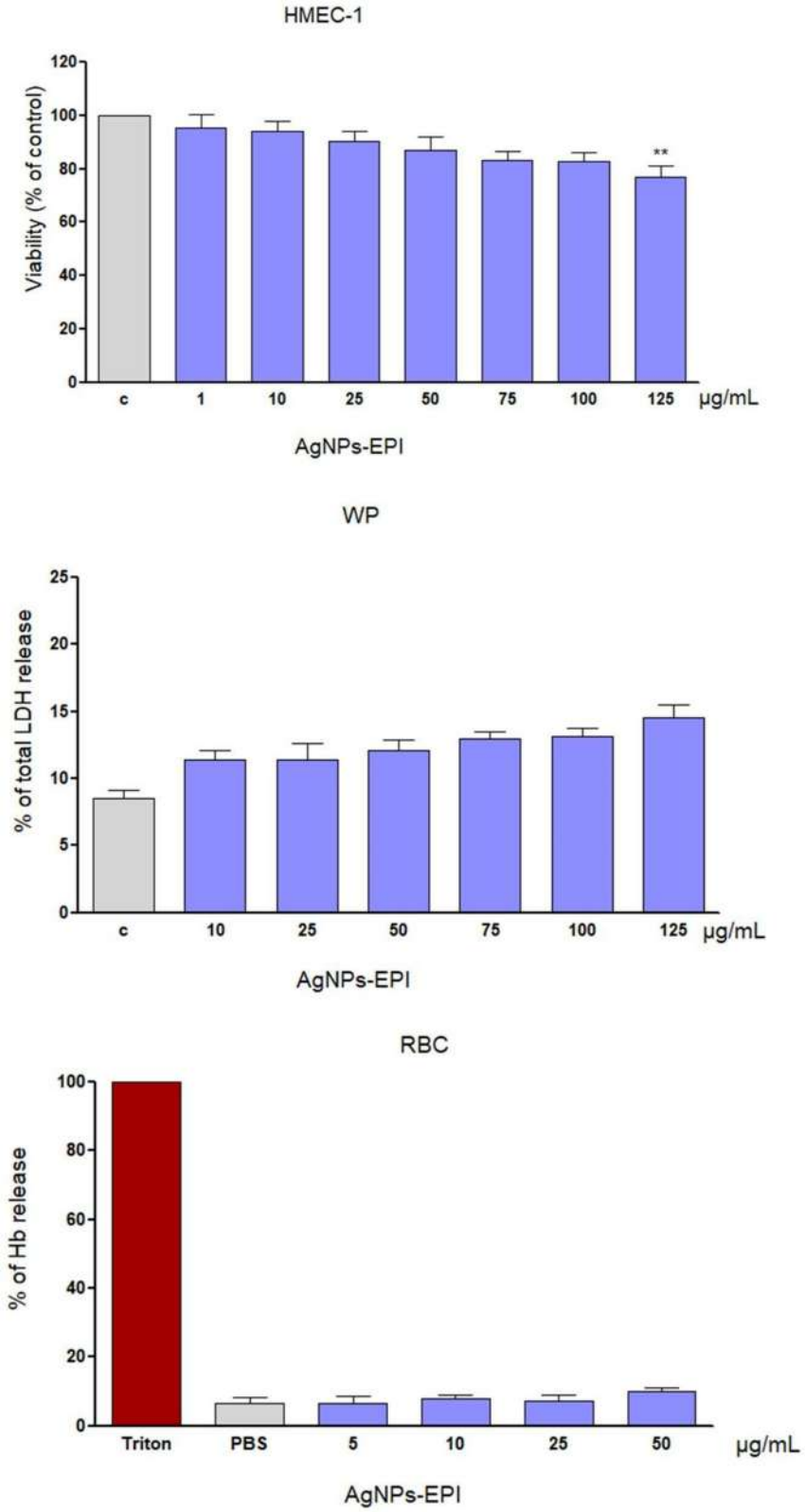


Figure 5 Effects of AgNPs-EPI on washed platelets (WP), endothelial cells (HMEC-1) viability following 24 h incubation and % Hb release from RBCs after 12 h incubationData are mean ±SD, n = 3. **p < 0.01.

Moreover, AgNPs-EPI did not induce significant hemolysis leading to ~10% of Hb at the highest concentration tested (50 µg/mL).

Inhibition of Collagen-Induced Washed Platelet (WP) Aggregation by AgNPs-EPI and EPI Under Stirring

Figure 6 shows the effects of AgNPs-EPI and EPI on collagen-induced platelet aggregation. Both compounds inhibited aggregation concentration-dependent; however, AgNPs-EPI were more potent than EPI, with IC_{50}= 14 and 23.4 for AgNPs-EPI and EPI, respectively.

Flow-Induced Inhibition of Platelet Aggregation with AgNPs-EPI and EPI

Figure 7 shows that AgNPs-EPI significantly inhibited flow-induced platelet aggregation, as shown by increased closure time in collagen/ADP and collagen/epinephrine cartridges.

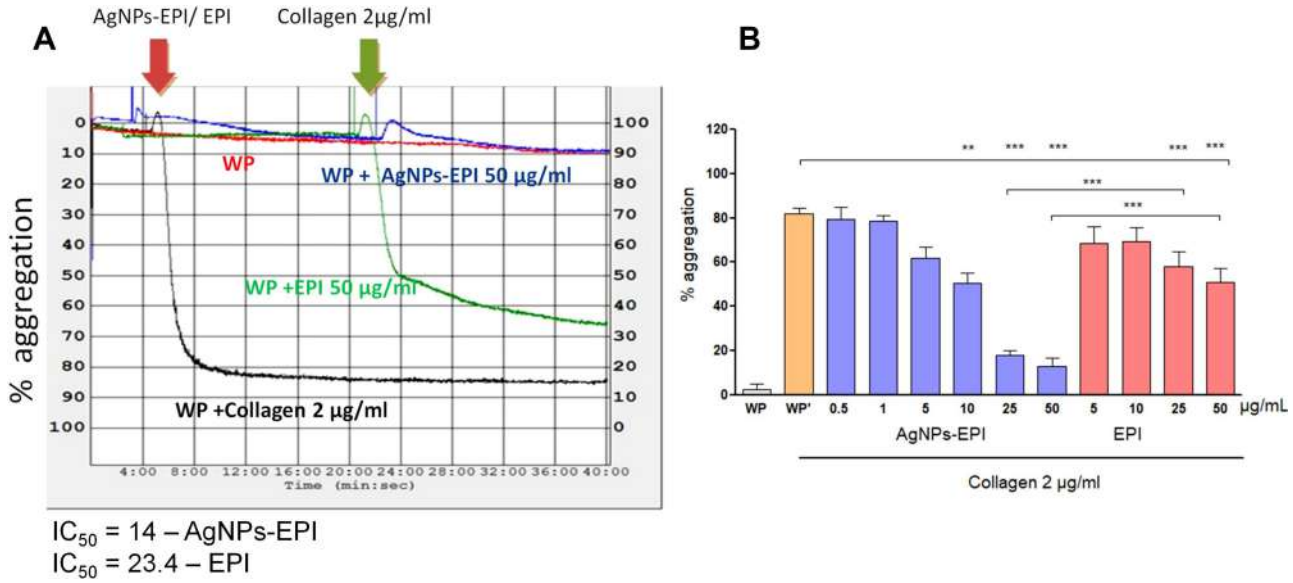


Figure 6 Inhibition of collagen-induced washed platelet (WP) aggregation by AgNPs-EPI and EPI, (**A**) – representative light aggregometry tracings showing the anti-aggregator effects of AgNPs-EPI and EPI both at 50 µg/mL. Collagen (black line) was used as a positive control; stirred platelets in the absence of NPs/drug were used as negative control (red line); platelet incubated with AgNPs-EPI with EPI (50µg/mL) and stimulated with collagen (blue line); platelet incubated with EPI (50µg/mL) and stimulated with collagen (green line). The brown arrow indicates the addition of NPs/drug, the green arrow indicates the addition of collagen. (**B**) – the statistical analysis of AgNPs-EPI and EPI effects on collagen-induced platelet aggregation. Data are mean ± standard deviation. **p < 0.001, ***p < 0.001 versus WP' (positive control).

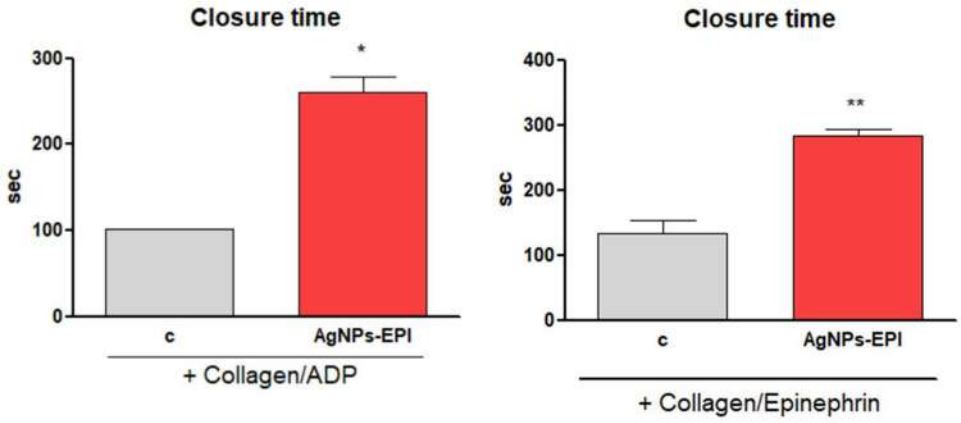


Figure 7 Inhibition flow-induced platelet aggregation with collagen/ADP and collagen/epinephrine cartridges by AgNPs-EPI, c – control closure time. Data are mean ±SD. *p < 0.05, **p < 0.005 versus control.

Effects of AgNPs-EPI and EPI on P-Selectin and GPIIb/IIIa Receptor Abundance

Figure 8 shows that both compounds concentration-dependently inhibited the abundance of P-selectin and GPIIb/IIIa on the platelet surface, and AgNPs-EP was more effective than EPI.

Effects of AgNPs-EPI on Platelet Mitochondrial Function

Figure 9A shows that activation of platelets by collagen resulted in a significant increase in LEAK respiration, an effect that was not significantly modified by AgNPs-EPI and EPI.

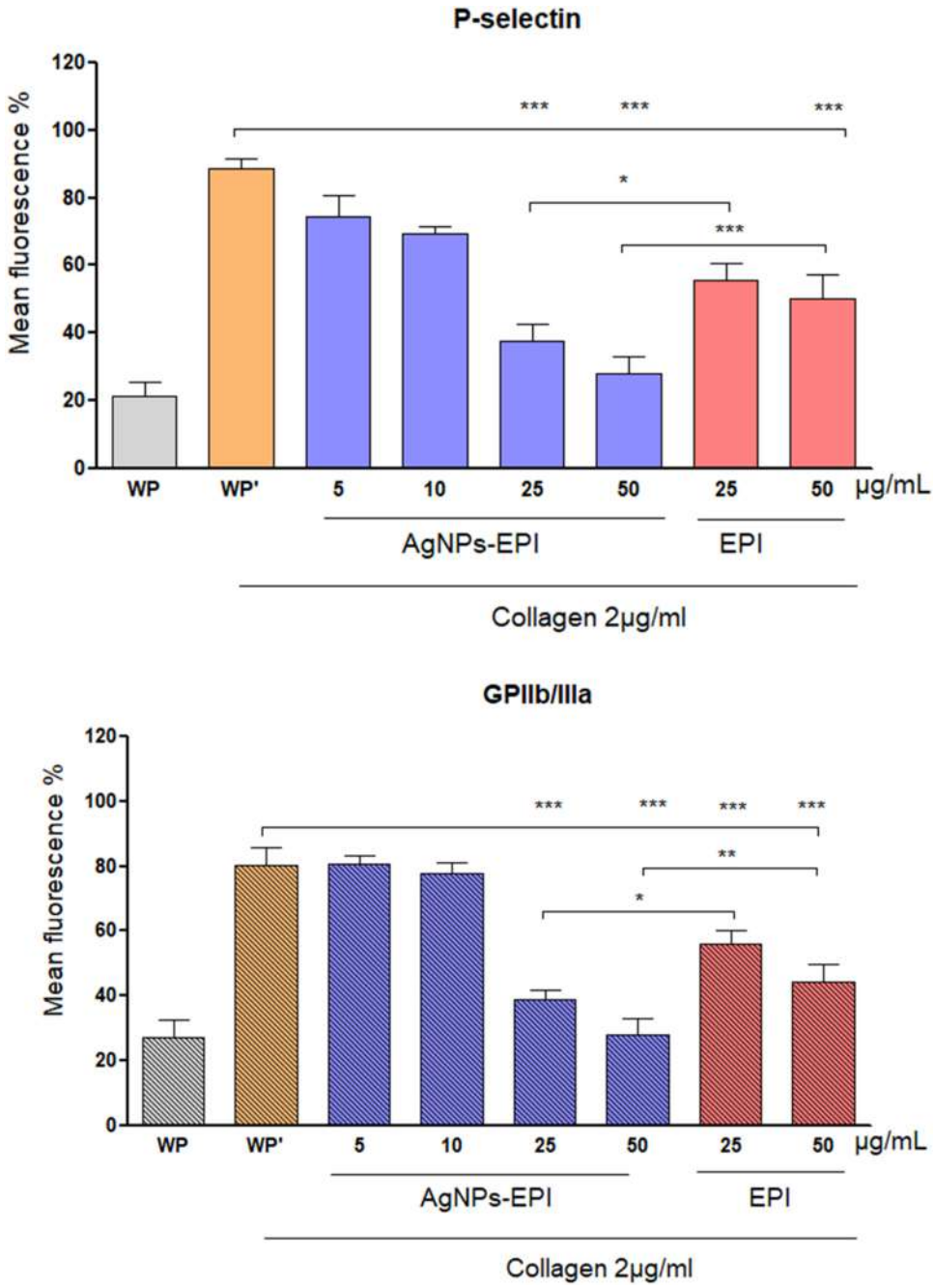


Figure 8 Inhibition of collagen-induced platelet receptor (GPIIb/IIIa and P-selectin) abundance by AgNPs-EPI and EPI. The statistical analysis of the effects of AgNPs-EPI and EPI. Data are expressed as mean ± standard deviation. *p < 0.05, **p < 0.001, ***p < 0.001 versus WP' (positive control).

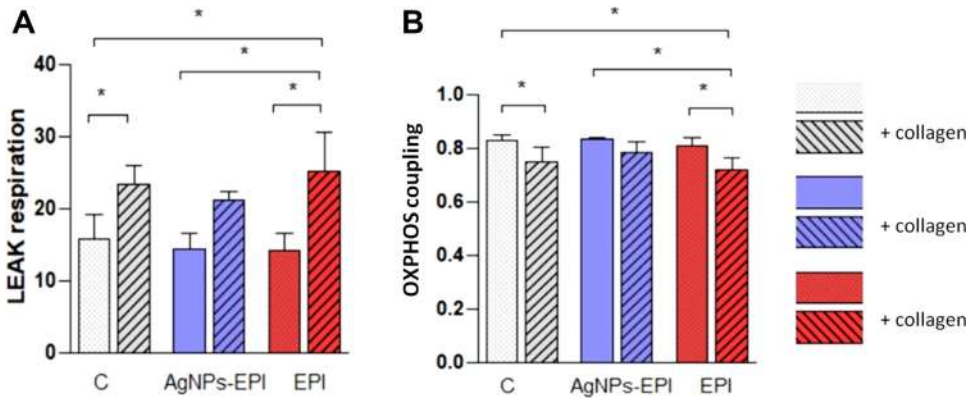


Figure 9 Effects of collagen-induced platelet aggregation on leak respiration (**A**) and OXPHOS coupling efficiency (**B**) in the presence or absence of AgNPs-EPI and EPI. Data are mean ±SD. *p < 0.05. Hatched bars show the effects of AgNPs-EPI and EPI in the presence of collagen (2 µg/mL).

In contrast, collagen resulted in a significant decrease in oxidative phosphorylation (OXPHOS) coupling efficiency. This effect was inhibited by AgNPs-EPI but not EPI (Figure 9B). Other respiration parameters (state CI, state CI + CII, state E, and state R) were not significantly changed (Table S1- Supplementary Materials).

Effects of AgNPs-EPI and EPI on the Secretion of Mediators by Endothelial Cells

The release of 6-keto-PGF1alpha, tPa, vWf, and cGMP from HMEC-1 cells was stimulated by thrombin. Figure 10 shows that AgNPs-EPI and EPI reduced the thrombin-induced release of these endothelial factors.

Coagulation System

The indices of the coagulation system (PT, APTT, APTT ratio, TT, INR, plasma fibrinogen, and D-dimers) were not significantly changed in the presence of AgNPs-EPI (Figure S2 -Supplementary Materials).

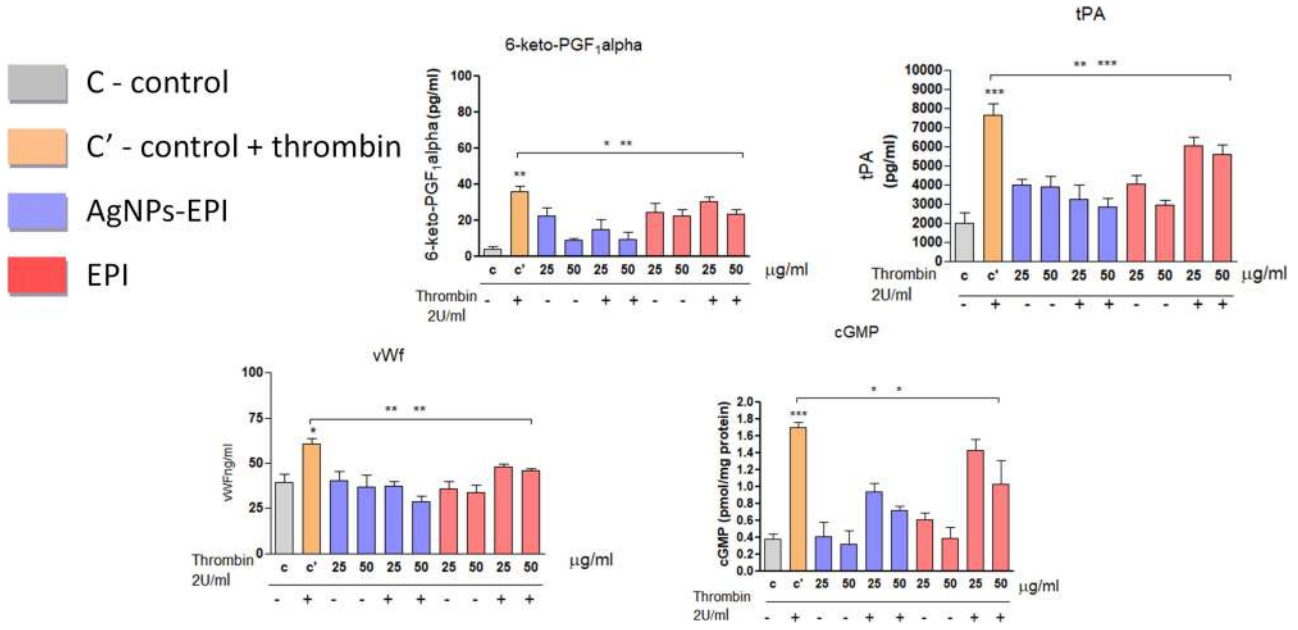


Figure 10 Inhibition of thrombin-induced release of 6-keto-PGF1alpha, tPA, vWF, and cGMP by AgNPs-EPI but not EPI. Preincubation of HMEC-1 cells with AgNPs-EPI inhibited the release of 6-keto-PGF1α, tPA, vWf, and cGMP. Data are mean ±SD. *p < 0.05, **p < 0.01, ***p < 0.001 to control or as indicated.

Discussion

This study aimed to synthesize and pharmacologically characterize AgNPs-EPI as a novel platelet aggregation inhibitor in vitro and ex vivo. We have found that: 1. AgNPs are an appropriate delivery platform for EPI, and 2. The synthesized AgNPs-EPI works as an effective inhibitor of platelet aggregation.

Pharmaceutical and Pharmacological Characterization of AgNPs-EPI

We have found that due to a disulfide bond in a molecule of EPI, it is possible to connect this drug to the nanosilver surface directly without any linker. According to this scheme, the chemisorption process of EPI on the AgNPs runs with the breaking of disulfide bonds. X-ray Photoelectron Spectroscopy and Electron Spin Resonance studies showed that the disulfide bonds could break onto the silver and gold surface during chemisorption and attached to the metal surface. [26–28] When designing a new pharmaceuticals based on silver nanoparticle platform, the potential cytotoxicity of silver nanomaterials should be taken into account.[29,30] The AgNPs-EPI synthesized for our experiments did not reduce the viability of human platelets and endothelial cells at concentrations<125 μg/mL, as shown by LDH and MTT tests. In addition, AgNPs-EPI tested in our work did not induce significant hemolysis when incubated with RBCs. Others and we have demonstrated that the cytotoxicity of AgNPs depends on the size, concentrations and functionalization of these materials.[31] As expected, the 12 nm AgNPs-EPI did not show significant cytotoxicity, similarly to the 2 nm AgNPs functionalized with glutathione, lipoic acid, or polyethylene glycol, as we have reported before.[32,33] Thus, AgNPs-EPI appear to be hemocompatible with endothelial cells, platelets and RBCs. We have then examined the pharmacological properties of AgNPs-EPI as a potential antiplatelet agent. There has been little research into the combination of eptifibatide and NPs. The encapsulation of the anticoagulant drug EPI in poly (DL-lactic-co-glycolic acid) (PLGA) nanoparticles was perfor med.[30] Experiments have shown effective drug delivery into the bloodstream and a low risk of platelet (membrane receptor) activation. Other researchers developed RGD-modified nanoliposomes (RGD-MNL) prepared as carriers for the targeted delivery of eptifibatide to activated platelets. The nanoliposomes had a size of about 10 nm, encapsulation efficiency of 37.5%, and good stability for 21 days, with little change in the size of the nanoliposomes. The results obtained in an ex vivo study showed that the antiplatelet activity of eptifibatide encapsulated nanoliposomes was higher compared to the free drug (81.63 vs 46.17% for RGD-MNL).[34] Other PC-based nanocapsules (2.8 × 1012) with encapsulated eptifibatide were incorporated into microfluidic blood perfusion tests and in vivo tail thrombosis and bleeding models. Shear-triggered delivery of eptifibatide in these nanocapsules selectively inhibited thrombus formation in vitro under conditions of stenosis and high shear flow above a shear rate of 1000 s^{-1}, leaving thrombus formation under the physiological conditions of shear rate.[35] Our earlier and other studies showed an inhibitory effect on platelet aggregation after stimulation.[18,36,37] Interestingly, new AgNPs-EPI synthesized by us inhibited aggregation more effectively than the free drug (10% vs 65% for AgNPs-EPI) under stirring and flow conditions (doubling closure time). This is not surprising as both AgNPs and EPI inhibit platelet aggregation as separate entities.[9,38] It should also be taken into account that silver nanoparticles have been shown to effectively inhibit integrin-dependent functional platelet responses such as aggregation, secretion, adhesion to immobilized fibrinogen or collagen, and the retraction of the fibrin clot in a dose-dependent manner irrespective of the nature of the agonists used. In vivo studies in mouse models also supported the antiplatelet effect properties of nanosilver, suggesting its additive effect on platelet inhibition.[39] For this reason and the available research data, it is believed that in the case of AgNPs-EPI, the drug EPI enhances the antiplatelet effect of conjugated AgNPs.Moreover, as functionalized AgNPs form less nanoparticle aggregates than uncoated NPs this property could also contribute to mechanisms that underlie the platelet-inhibitory activity of these possibilities.[18] The closure time, measured with the Platelet Function Analyzer (PFA-100/200®), is now used as the measurement of bleeding time. This is a system that simulates the vessel wall under physiological shear stress conditions.[40] Furthermore, closure time has some clinical utility in the exclusion/detection of primary hemostatic disorders and is sometimes referred to as an "in vitro" bleeding time.[41] These results confirm that AgNPs-EPI inhibits flow-induced platelet aggregation.

To study the mechanism(s) of AgNPs-EPI-induced inhibition of platelet aggregation, we first investigated the effects of this nanomaterial on platelet energy metabolism as mitochondria are often targeted by nanosystems.[42]

Despite the low mitochondria count in platelets (5–8 mitochondria per platelet), these organelles play an essential role during platelet activation. The mitochondrial pathway of platelet activation includes increased mitochondrial reactive oxygen species (ROS) formation, calcium release, or collapse of membrane potential.[43] Indeed, stimulation of platelets with collagen leads to mitochondrial ROS generation and increased intraplatelet calcium levels.[44] Our results confirmed this observation. We observed that collagen stimulates platelet mitochondria LEAK respiration, related to the leak of H^+, and may be considered a factor corresponding to ROS generation.[45] Additionally, the mitochondrial coupling efficiency is also reduced by collagen. Interestingly, preincubation of platelets with AgNPs-EPI alleviated these changes, contributing to the inhibition of platelet aggregation. The second, and likely the major, mechanism underlying the inhibitory activity of AgNPs-EPI on platelet aggregation is this nanoparticle-drug construct's ability to inhibit the activation and/or function of GPIIb/IIIa receptor by flow cytometry studies. We also investigated the impact of AgNPs-EPI on HMEC-1 endothelial cells derived from blood vessels.[46] We have shown that, as expected, HMEC-1 cells, when stimulated by thrombin, release a panel of antithrombotic factors such as 6-keto-PGF1alpha cyclic GMP and tPA as well as prothrombotic factors including vWf, 6-keto-PGF1alpha is the stable metabolite of PGI_2 (prostaglandin I_2), which is potent vasodilator and inhibitor of platelet aggregation and a significant product of arachidonic acid metabolism in endothelial cells that are derived from large blood vessels.[47]

Endothelium-dependent vasorelaxation mediated by nitric oxide (NO) release is stimulated by a number of blood-borne factors, including thrombin. It has been proven that thrombin is one of the mediators of the coagulation process, and its presence causes the release of these factors from the endothelial structures.[20,48] The nitric oxide-cGMP pathway is known to have vasoprotective, platelet-inhibitory, anti-inflammatory, and antioxidant effects on the vascular wall.[49] The drugs aimed at increasing the level of cGMP in the pulmonary vascular system are highly therapeutically effective.[50] Endothelial cells are thought to be the main source of plasma t-PA, a major fibrinolytic agent that limits the progression of occlusive thrombi. In cultured endothelial cells, t-PA synthesis is up-regulated in response to thrombin, which we have confirmed in our studies.[36] On the other hand, treatment of endothelial cells with thrombin causes an immediate increase in phospholipid methylation which ultimately leads to the release of von Willebrand factor (vWF).[51] Upon vascular damage leading to the exposure of subendothelial structures, vWF rapidly binds to GPIb alpha receptor on platelets facilitating platelet adhesion and thrombus formation.[52] The vWF derived from coronary endothelial cells is significantly increased in the event of damage to the coronary artery, which indicates the pathogenetic role of vWF in cardio-thrombotic diseases.[53] In addition, high vWF levels have been shown to be prognostic in patients with ischemic heart disease, peripheral vascular disease, and inflammatory vascular disease.[54] Importantly, we have shown that AgNPs-EPI, contrary to free drugs, effectively counteract the release of vWF.This would effectively decrease platelet-subendothelium interactions and work towards the preservation of vascular integrity. However, the impact of down-regulation of the release of endothelial mediators such as tPA nitric oxide and prostacyclin on these interactions needs to be determined. The results confirm that AgNPs-EPI acts as a novel inhibitor of platelet and endothelial cell activation and can be used as a platform for drug delivery in cardiovascular diseases and for coating medical devices. In addition, in a physiological environment, endothelial cells are exposed to shear stresses that are induced by blood flow and its viscosity. It is necessary to use models that mimic more physiological conditions, such as flow-through experiments, that would provide better results for the effects of AgNPs-EPI on cells under near physiological conditions. Nano-drugs with anticoagulant potential may have specific effects, but importantly, many of the cardiovascular coagulation factors will be affected and significant interactions may occur, potentially disrupting the haemostatic balance unintentionally, which may lead to thrombosis or bleeding complications.[55] ConclusionWe designed, synthesized and characterized new AgNPs conjugates with eptifibatide. In vitro pharmacological profile analysis of this conjugate shows that it is effective in inhibiting human platelet aggregation and thrombin-induced endothelial activation at concentrations that do not affect cell viability. Moreover, AgNPs-EPI effects thrombin-induced endothelial activation factors such as 6-keto-PGF1alpha, tPA, vWF, and cGMP, which are associated with platelet aggregation, fibrin production, and occlusive thrombus formation, which is essential to prevent vascular damage and maintaining blood flow. Despite significant preclinical data showing that nanoanticoagulants may have a specific effect, it should be noted that many clotting factors will be encountered in the circulatory system and may interact with them, potentially disrupting the haemostatic balance inadvertently, which potentially lead to thrombosis or bleeding complication. An in-depth understanding of the interface between NPs and the coagulation system is valuable for the engineering and successful translation of NPs into the clinical trials and they could be potentially used to coat medical devices such as vascular catheters. Thus, it is appropriate to move AgNPs-EPI to further stages of preclinical development.

Abbreviations

AgNPs-EPI, silver nanoparticles with eptifibatide; APTT, activated partial thromboplastin time; cGMP, guanosine monophosphate cyclic; CT, closure time; DLS, dynamic light scattering; EPI, eptifibatide; HMEC-1, immortalized human microvascular endothelial cell line; LDH, lactate dehydrogenase; NPs, nanoparticles; PGI_2, prostacyclin; PT, prothrombin time; TEM, transmission electron microscopy; TGA, thermogravimetric analyses; tPA, tissue plasminogen activator; TT, thrombin time; WP, washed platelet.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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