SZKOŁA DOKTORSKA
BioMedChem
Uniwersytetu Łódzkiego
i Instytutów Polskiej
Akademii Nauk w Łodzi

Marika Grodzicka

Praca doktorska:

Charakterystyka biofizyczna i ocena właściwości biologicznych polifenolowych dendrymerów modyfikowanych kwasem kawowym

Doctoral thesis:

Biophysical characterization and evaluation of biological properties of polyphenolic dendrimers modified with caffeic acid

- Promotor/Supervisor
  Prof. dr hab. Maksim Ionov
  Wydział Biologii i Ochrony Środowiska
  Uniwersytet Łódzki
- Promotor pomocniczy/Assistant Supervisor
  dr hab. Sylwia Michlewska
  Wydział Biologii i Ochrony Środowiska
  Uniwersytet Łódzki

Łódź, 2024

Składam serdeczne podziękowania Promotorowi niniejszej rozprawy
Prof. dr. hab. Maksimowi Ionovowi
za opiekę merytoryczną i wyrozumiałość w trakcie realizacji pracy.

Szczególne podziękowania składam Promotorowi pomocniczemu
Dr hab. Sylwii Michlewskiej
za jej entuzjazm, bycie nie tylko nauczycielką, ale także mentorką i przewodniczką.
Za nieocenione wsparcie, cenne wskazówki oraz poświęcony czas.

Dziękuję ***Pracownikom i Doktorantom*** *Katedry Biofizyki Ogólnej Uniwersytetu Łódzkiego*
za życzliwość, stworzenie przyjaznej atmosfery oraz wszelką pomoc
w trakcie wykonywania badań.

Osobne podziękowania pragnę złożyć
Dr Małgorzacie Grygiel
za wprowadzenie do laboratorium i pomoc w stawianiu pierwszych kroków.

Na koniec, przede wszystkim, chciałabym podziękować mojej
Rodzinie i Przyjaciołom
za wiarę we mnie, wsparcie i pomoc w trudnych chwilach

oraz

Rodzicom
za możliwość rozwijania pasji, ich wsparcie na całej mojej drodze edukacji
od początków w szkole podstawowej aż do dzisiaj,
kiedy to składam moją pracę doktorską.

Źródła finansowania

Badania zostały wykonane w ramach projektów:

- Międzynarodowy projekt badawczy współfinansowany przez NCN w ramach konkursu „BEETHOVEN LIFE 1" UMO-2018/31/F/NZ5/03454. „NP-HALE", pt.: „Nowe polimerowe nanocząstki do przenoszenia kwasów nukleinowych w leczeniu chorób płuc: synteza, analiza toksykologiczna i ocena biologiczno-terapeutyczna" 2020-2024.
- Międzynarodowy projekt badawczy M-ERA.NET współfinansowany przez NCN, UMO-2018/30/Z/NZ1/00911. "NanoTENDO", pt.: "Transfer nanocząstek przez barierę śródbłonkową" 2019-2024.
- Projekt wymiany bilateralnej PL-SK współfinansowany przez NAWA, PPN/BIL/2018/1/00150/U/00001, pt.: „Nowe nanosystemy dostarczania leków cytostatycznych z wykorzystaniem technologii łączącej właściwości dendrymerów i liposomów" 2019–2021.
- Projekt „EUROPARTNER" finansowany przez NAWA, PPI/APM/2018/1/00007/U/001, pt.: „Działania wzmacniające i upowszechniające międzynarodowe partnerstwo Wydziału Biologii i Ochrony Środowiska na rzecz interdyscyplinarnych badań i innowacji Uniwersytetu Łódzkiego" 2019-2022.
- Projekt "MINIATURA7" finansowany przez NCN Nr.: 2023/07/X/ NZ7/00026, pt.: „Polifenolowe dendrymery nowej generacji, jako nośniki paklitakselu w terapii lekoopornego drobnocząsteczkowego raka płuc" 2023-2024.

Współpraca z krajowymi i zagranicznymi instytucjami naukowymi:

Katedra Chemii Organicznej i Chemii Nieorganicznej, Uniwersytet w Alcalá de Henares

Katedra Fizyki Jądrowej i Biofizyki, Uniwersytet Komeńskiego w Bratysławie,

Katedra Chemii Fizycznej, Zakład Chemii Biofizycznej, Wydział Chemii, Uniwersytet Łódzki

Laboratorium Biofizyki Molekularnej, Zakład Mikrobiologii i Biotechnologii, Wydział Biologii, Uniwersytet w Białymstoku

Collegium Medicum, Wydział Lekarski, Akademia Mazowiecka w Płocku

Spis treści

Załączniki:

1. Publikacje wchodzące w skład rozprawy doktorskiej

2. Oświadczenia współautorów publikacji wchodzących w skład rozprawy doktorskiej

Wykaz skrótów

AAPH – 2,2'-Azobis(2-amidinopropane) dihydrochloride

BJ – *ang. Human fibroblasts* – fibroblasty ludzkie

CD – *ang. Circular Dichroism* – dichroizm kołowy

DLS – *ang. Dynamic light scattering* – Dynamiczne rozpraszanie światła

DMPC – 1,2-dimyristoyl-sn-glycero-3-phosphocholine

DPH – 1,6-diphenyl-1,3,5-hexatriene

DPPG – 1,2-dihexadecanoyl-sn-glycero-3-phospho-(1′-rac-glycerol)

DPPH – 2,2 diphenyl 1 picrylhydrazyl

FBS – *ang. Fetal Bovine Serum* – płodowa surowica bydlęca

H_2DCF-DA – 2′,7′-dichlorodihydrofluorescein diacetate

HSA – *ang. Human Serum Albumin* – albumina ludzka

LDE – *ang. Laser Doppler electrophoresis* – Laserowa Elektroforezę Dopplera

MTT – dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide

NSCLC – *ang. Non-Small Cell Lung Cancer* – niedrobnokomórkowy rak płuc

PAMAM – *ang. Poly(amidoamine)* – dendrymery poliamidoaminowe

PD – *ang. Polyphenolic Dendrimers* – dendrymery polifenolowe

PEG – *ang. polyethylene glycol* – glikol polietylenowy

PPI – *ang. Poly(propylene imine*) – nanoczastki polipropylenoiminowe

RFT – Reaktywne formy tlenu

SCLC – *ang. Small Cell Lung Cancer* –drobnokomórkowy rak płuc

siRNA – *ang. Small Interfering RNA* – małe interferujące RNA

TEM – *ang. Transmission Electron Microscopy* – Transmisyjna mikroskopia elektronowa

TMA-DPH – 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluene sulfonate

Wprowadzenie

Na początku lat 80 zespół prof. D. Tomalii po raz pierwszy zsyntetyzował rozgałęzione, kuliste, monodyspersyjne nanocząstki nazywane dendrymerami [1,2]. Dzięki unikalnej strukturze dendrymery uważane są za doskonałe transportery leków i kwasów nukleinowych. Zastosowanie tych nanocząstek może przyczyniać się do zwiększenia skuteczności terapii różnych chorób. Dendrymery są dobrze zdefiniowane i stosunkowo łatwe do syntezy [3–8]. Najlepiej poznane są właściwości fizykochemiczne i biologiczne takich dendrymerów jak PAMAM, PPI czy dendrymery fosforowe lub karbokrzemowe. Obecnie wiadomo, że dendrymery skoniugowane z lekami poprawiają ich rozpuszczalność i biodostępność. Ponadto powolne uwalnianie leków z kompleksów dendrymer/lek wydłuża ich czas krążenia w krwiobiegu. Dendrymery posiadające ładunek kationowy mogą tworzyć stabilne kompleksy z kwasami nukleinowymi, dlatego też są sugerowane jako odpowiednie nośniki DNA lub RNA. Otwiera to możliwość ich wykorzystania w terapiach genowych.

Kationowe dendrymery mogą oddziaływać z ujemnie naładowanymi błonami lipidowymi [5,8–13]. Jednak, dodatnio naładowane dendrymery z amidowymi grupami powierzchniowymi mogą być wysoce toksyczne [8,12,14,15]. Jednym ze sposobów zmniejszenia toksyczności dendrymerów kationowych jest ich koniugacja z glikolem polietylenowym (PEG) [16,17].

PEGylacja dendrymerów stabilizuje ich interakcję z kwasami nukleinowymi i ogranicza odziaływanie z białkami surowicy [16,17]. Ponadto PEG jest rozpuszczalny w wodzie i jest zatwierdzony do stosowania przez Agencję Food and Drug Administration (FDA) [16]. PEG nie jest immunogenny i toksyczny, a jego obecność w strukturze dendrymeru zwiększa biokompatybilność nanocząstek [17,18].

Pomimo licznych zalet dendrymerów naukowcy wciąż starają się ulepszyć ich właściwości biomedyczne. Przykładowo zmiany struktury dendrymeru poza dołączeniem PEG, obejmują zakotwiczenie w szkielecie nanocząsteczek różnych biomolekuł, np.: polifenoli, takich jak kwas kawowy, ferulowy lub galusowy [4].

Spożywanie polifenoli, takich jak kwas kawowy w codziennej diecie, niewątpliwie ma korzystny wpływ na zdrowie człowieka i ochrania komórki przed stresem oksydacyjnym [4,19]. Stres oksydacyjny odgrywa kluczową rolę w procesie starzenia się komórek i towarzyszy rozwojowi wielu poważnych chorób, takich jak choroba Alzheimera i Parkinsona, cukrzyca, reumatoidalne zapalenie stawów, choroby sercowo-naczyniowe, a nawet nowotwory [20,21]. Sugeruje się, że kawa ma właściwości przeciwnowotworowe,

które są ściśle powiązane z jej właściwościami antyoksydacyjnymi. Korzystny wpływ kawy opisano w leczeniu raka wątroby [22], raka jelita grubego [23,24], raka jamy ustnej [25] i czerniaka skóry [26,27]. Te zalety kawy mogą być związane z obecnością w niej kwasu kawowego, polifenolu będącego jednym ze znanych przeciwutleniaczy [28]. Ponadto, kwas kawowy ma działanie przeciwzakrzepowe, przeciwnadciśnieniowe, i przeciwwirusowe [29,30]. W związku z tym, że niektóre z polifenoli, nie są łatwo biodostępne, ich koniugacja z dendrymerami stanowi korzystne rozwiązanie, pozwalające na stworzenie nanocząstek o właściwościach przeciwutleniających do zastosowań biomedycznych [31,32].

W obecnej pracy przeprowadzono biofizyczną charakterystykę nowo zsyntetyzowanych polifenolowych dendrymerów zawierających kwas kawowy i PEG. Oceniono ich aktywność jako przeciwutleniaczy, a także przeanalizowano charakter interakcji z albuminą ludzką i błonami biologicznymi. Końcowym etapem badań była analiza potencjalnego zastosowania dendrymerów do przenoszenia proapoptotycznego siRNA do komórek A549 niedrobnokomórkowego raka płuc [19,33–35].

Cele pracy

Głównym celem niniejszej pracy była biofizyczna charakterystyka polifenolowych dendrymerów karbokrzemowych pierwszej generacji zawierających w swojej strukturze kwas kawowy (CA) i glikol polietylenowy (PEG). Dodatkowym celem była ocena właściwości biologicznych dendrymerów takich jak aktywność przeciwutleniająca, cytotoksyczność, charakter odziaływania z błonami biologicznymi i białkiem osocza oraz analiza możliwości zastosowania tych dendrymerów jako nośników terapeutycznego siRNA.

Do zrealizowania celów pracy zaplanowano następujące zadania badawcze:

1. Charakterystyka biofizyczna polifenolowych dendrymerów karbokrzemowych oraz analiza ich cytotoksyczności i właściwości przeciwutleniających.
2. Analiza oddziaływań dendrymerów z albuminą ludzką oraz błonami biologicznymi.
3. Ocena zdolności dendrymerów do tworzenia kompleksów z terapeutycznymi siRNA i przenoszenia ich do komórek.

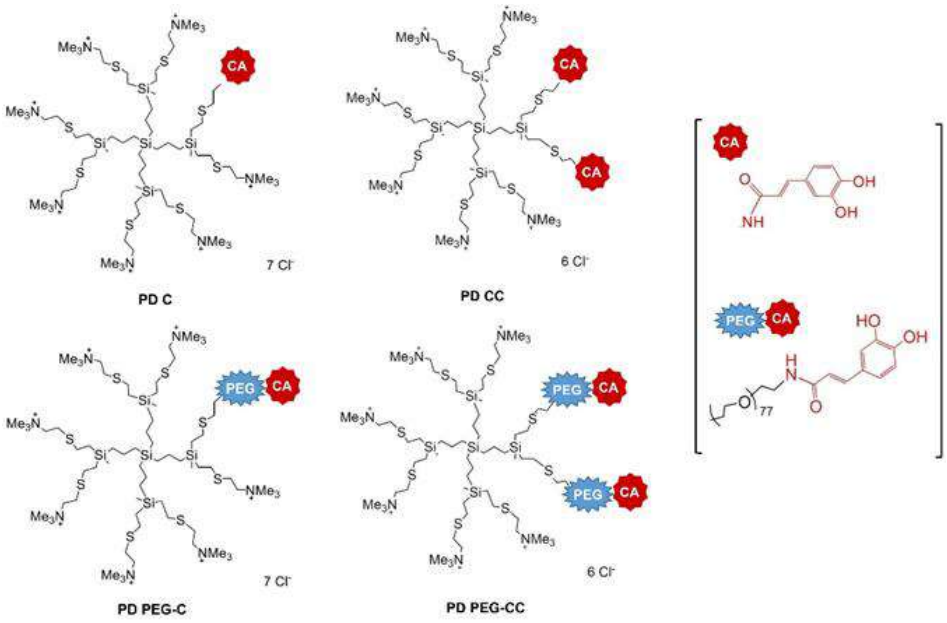
4. Ocena skuteczności działania powstałych kompleksów dendrymer/siRNA w warunkach *in vitro.*

Hipoteza badawcza

Polifenolowe dendrymery karbokrzemowe pierwszej generacji wykazują potencjał przeciwutleniający, chronią komórki przed negatywnymi skutkami stresu oksydacyjnego, wchodzą w interakcję z albuminą ludzką i błonami biologicznymi, a także tworzą kompleksy z terapeutycznymi siRNA i przenoszą je do komórek.

Materiały i metody

Przedmiotem badań były polifenolowe dendrymery karbokrzemowe pierwszej generacji zawierające w swojej strukturze kwas kawowy dołączony do dendrymerów bezpośrednio lub za pośrednictwem PEG (Rys. 1, Tab. 1). Dendrymery zostały zsyntezowane w zespole partnerskim profesora Javiera de la Maty w Uniwersytecie Alcalá de Henares w Hiszpanii. W pracy wykorzystano proapoptotyczne siRNA: siMcl-1, siBcl-2 (ABO Sp. z o.o, Polska) (Tab. 2).



Rys. 1. Struktura karbokrzemowych dendrymerów polifenolowych modyfikowanych kwasem kawowym i PEG: PD C, PD CC, PD PEG-C, PD PEG-CC.

Tabela 1. Masa molowa karbokrzemowych dendrymerów polifenolowych

	PD C	PD CC	PD PEG-C	PD PEG-CC
Masa molowa [g/mol]	1914,33	1997,94	5306,42	8782,10

Tabela 2. Sekwencja siRNA (siMcl-1, Bcl-2).

siRNA	siMcl-1	siBcl-2
Sense	5'-GGACUUUUAUACCUGUUAUtt 3'	5'-GCUGCACCUGACGCCCUUCtt 3'
Antisense	5'-AUAACAGGUAUAAAAGUCCtg 3'	5'-GAAGGGCGUCAGGUGCAGCtt 3'

W pracy dodatkowo zostały wykorzystane:

- fibroblasty ludzkie (BJ)
- komórki niedrobnokomórkowego raka płuc (A549)
- erytrocyty i błony erytrocytarne izolowane z kożucha leukocytarno-płytkowego zdrowych dawców pozyskane z Regionalnego Centrum Krwiodawstwa i Krwiolecznictwa w Łodzi
- liposomy (DMPC/DPPG)
- albumina ludzka (HSA)

Podczas wykonywania zadań badawczych zastosowano następujące techniki: Laserowa Elektroforeza Dopplera (LDE) oraz technika dynamicznego rozpraszania światła (DLS), transmisyjna mikroskopia elektronowa (TEM), spektroskopia fluorescencyjna, dichroizm kołowy (CD), elektroforeza w żelu agarozowym, mikroskopia konfokalna, cytometria przepływowa, a także testy *in vitro*.

Omówienie prac wchodzących w skład rozprawy doktorskiej

W pierwszej pracy wchodzącej w skład rozprawy doktorskiej **M. Grodzicka, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov. Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity. *Sust, Mat. Technol*. 33, 2022, doi: 10.1016/j.susmat.2022.e00497** jest opisana synteza karbokrzemowych dendrymerów polifenolowych pierwszej generacji: 1 - $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$, 2 - $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$, 3- $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$, 4 - $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$. Ta część pracy została przygotowana przez zespół hiszpański, kierowany przez profesora Javiera de la Mata, w Katedrze Chemii Organicznej i Nieorganicznej Uniwersytetu Alcala de Henares. W trakcie syntezy, dendrymery karbokrzemowe zostały wzbogacone o jedną lub dwie cząsteczki kwasu kawowego dołączonego do dendrymeru bezpośrednio lub za pośrednictwem glikolu polietylenowego (PEG). Druga cześć publikacji została poświęcona biofizycznej charakterystyce dendrymerów oraz ocenie ich cytotoksyczności. Wyznaczono średnicę hydrodynamiczną, indeks polidyspersyjności i potencjał zeta dendrymerów oraz określono ich morfologię z wykorzystaniem transmisyjnej mikroskopii elektronowej. Otrzymane wyniki wykazały, że badane dendrymery mają dodatni ładunek. Dendrymery nie zawierające PEG charakteryzowały się wyższym potencjałem zeta niż dendrymery z PEG. Obrazy otrzymane za pomocą transmisyjnej mikroskopii elektronowej pokazały, że dendrymery z PEG maja większy rozmiar niż dendrymery niezawierające PEG. Wszystkie badane dendrymery wykazywały stosunkowo niską cytotoksyczność zarówno wobec fibroblastów (BJ), jak i komórek raka płuc (A549). W kolejnych badaniach zaobserwowano, że dendrymery polifenolowe wykazywały wyraźną aktywność przeciwutleniającą. Skuteczność dendrymerów w zmiataniu wolnych rodników została sprawdzona w odniesieniu do znanych przeciwutleniaczy: kwasu askorbinowego, kwasu kawowego i melatoniny. Najbardziej efektywne były dendrymery zawierające PEG, zwłaszcza dendrymer z dwiema resztami kwasu kawowego. Sprawdzano również czy dendrymery polifenolowe mogą wykazywać efekt ochronny przy indukowanej peroksydacji lipidów. Pokazano, że wszystkie badane nanocząstki zmniejszały poziom produktów peroksydacji lipidów, a także obniżały poziom hemolizy erytrocytów indukowanej AAPH, chroniąc erytrocyty przed uszkodzeniami wywołanymi stresem oksydacyjnym. Ponieważ wszystkie badane dendrymery wykazywały istotny potencjał przeciwutleniający, sprawdzono również ich aktywność w tym zakresie na fibroblastach ludzkich. Wszystkie badane związki

znacząco zmniejszały poziom reaktywnych form tlenu (RFT) w komórkach. Co więcej, dendrymery wykazywały większy efekt ochronny przed uszkodzeniami spowodowanymi stresem oksydacyjnym niż klasyczne przeciwutleniacze. Podsumowując, badane dendrymery charakteryzowały się niską cytotoksycznością, a także wysokim potencjałem przeciwutleniającym, porównywalnym z klasycznymi przeciwutleniaczami.

Zastosowanie dendrymerów jako narzędzi dostarczania leków i kwasów nukleinowych do komórek docelowych wymaga scharakteryzowania ich interakcji z różnymi systemami biologicznymi, a przede wszystkim z białkami surowicy. W związku z tym, w drugiej pracy **M. Grodzicka, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion, *Sci. Rep,* 14:5946, 2024, https://doi.org/10.1038/s41598-024-56509-0** została zbadana zdolność dendrymerów polifenolowych do interakcji z albuminą ludzką. Albumina ludzka jest białkiem osocza, zbudowanym z 585 aminokwasów, z trzema homologicznymi domenami [36]. Każda domena ma dwie subdomeny, do których mogą być przyłączone różne cząsteczki. Subdomena IIA, zawiera resztę tryptofanu i jest głównym hydrofobowym miejscem wiązania ligandów [36]. Dendrymery poprzez interakcje hydrofobowe oddziałują głównie z subdomeną IIA [37,38]. Efekt ten ma kluczowe znaczenie dla medycznego zastosowania nanocząstek, ponieważ absorpcja na powierzchni albuminy może zmienić jej właściwości i biodostępność. Wyniki przedstawione w wymienionej publikacji pokazały, że obecność dendrymerów w roztworze albuminy spowodowała zwiększenie rozmiaru obserwowanych cząsteczek. Najwyższe wartości średnicy hydrodynamicznej kompleksów albumina/dendrymer zaobserwowano z dendrymerami zawierającymi PEG. Wskaźnik polidyspersyjności kompleksów utworzonych przez dendrymery bez PEG wynosił około 0,4, podczas gdy z PEG, około 0,5. Dodanie dendrymerów z wyjątkiem dendrymeru zawierającego dwie reszty kwasu kawowego i PEG zmniejszało wartość potencjału zeta zaobserwowanego dla HSA. Efekt ten był najbardziej wyraźny w przypadku dendrymeru bez PEG i z jedną resztą kwasu kawowego. Na obrazach TEM albumina była widoczna jako struktura fibrylarna. Obecność dendrymerów sprawiła, że cząsteczki albuminy stały się bardziej elektronowo gęste.

Zbadano wygaszanie fluorescencji reszt tryptofanu obecnego w HSA wywołane obecnością dendrymerów. Odziaływanie dendrymerów z HSA spowodowało przesunięcie widma fluorescencji w kierunku dłuższych fal. Najbardziej wyraźny efekt zaobserwowano dla dendrymeru zawierającego dwie reszty kwasu kawowego i PEG. Analiza widm

dichroizmu kołowego (CD) wskazała, że struktura HSA została zmieniona nieznacznie w obecności dendrymerów. Wyniki otrzymane za pomocą techniki izotermicznej kalorymetrii miareczkowej (ITC) pozwoliły na oszacowanie przybliżonego stosunku molowego kompleksów albumina/dendrymer wynoszącego 6-10 cząsteczek dendrymerów na jedną cząsteczkę albuminy. Podsumowując, dendrymery polifenolowe oddziałują z albuminą surowicy ludzkiej, nieznacznie wpływając na strukturę drugorzędową białka, a interakcja ta zależy od obecności reszt kwasu kawowego i PEG w strukturze nanocząstek.

Zastosowanie dendrymerów jako nośników leków wymaga również zrozumienia sposobu ich odziaływania z błonami biologicznymi. W związku z tym kolejna praca **M. Grodzicka, S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, *Chem. Phys. Lipids*. 265, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444** została poświęcona określeniu mechanizmów interakcji badanych dendrymerów z błonami lipidowymi, przygotowanymi z lipidów syntetycznych lub izolowanymi z erytrocytów krwi obwodowej.

Do analizy interakcji pomiędzy dendrymerami, a sztucznymi błonami lipidowymi (liposomami) wykorzystano techniki TEM, LDE oraz DLS. Liposomy o średnicy 100 nm uzyskano z mieszaniny fosfolipidów DMPC/DPPG w stosunku molowym 97:3. Po dodaniu dendrymerów zawierających jedną resztę kwasu kawowego do zawiesiny liposomów ich średnica hydrodynamiczna zmieniła się nieznacznie. Z kolei obecność dendrymerów z dwiema resztami kwasu kawowego spowodowało istotny wzrost wielkości nanokompleksów. Wyniki te zostały potwierdzone poprzez analizę obrazów uzyskanych z wykorzystaniem techniki transmisyjnej mikroskopii elektronowej, na których pokazano, że dendrymery oddziałują z powierzchnią liposomów powodując widoczne zmiany morfologiczne. Liposomy skompleksowane z dendrymerami miały zmieniony kształt i strukturę. Dodatkowo, obecność dendrymerów powodowała zmianę ładunku powierzchniowego liposomów z ujemnego na dodatni, przy czym dendrymery nie zawierające PEG powodowały większy wzrost wartości potencjału zeta niż dendrymery z PEG. Uzyskane wyniki mogą wskazywać na elektrostatyczny charakter interakcji między badanymi składnikami. Oddziaływanie dendrymerów polifenolowych z błonami lipidowymi przygotowanymi z mieszaniny lipidów DMPC/DPPG zostało dodatkowo zbadane za pomocą techniki DSC. Obecność dendrymerów powodowała wyostrzenie piku topnienia lipidów, wskazując na wyższą kooperatywność topnienia, w porównaniu z błonami

lipidowymi niezawierającymi dendrymerów. Zaobserwowane zmniejszenie entalpii wskazywało na relaksację molekuł fosfolipidów w regionie niepolarnym, co sugeruje, że dendrymery oddziałują również z hydrofobowym obszarem dwuwarstwy lipidowej.

Do analizy zmian płynności błon spowodowanych obecnością dendrymerów zastosowano technikę fluorymetryczną z wykorzystaniem znaczników fluorescencyjnych DPH i TMA-DPH. Sonda DPH lokalizuje się w hydrofobowym regionie dwuwarstwy lipidowej, natomiast TMA-DPH w obszarze hydrofilowym. Dodanie dendrymerów do zawiesiny błon zawierających sondy DPH i TMA DPH spowodowało wzrost rejestrowanej anizotropii fluorescencji, co wskazuje na zmniejszenie płynności błony zarówno w regionie hydrofilowym, jak i hydrofobowym. Dendrymery zawierające PEG usztywniały błonę lipidową w mniejszym stopniu niż nanocząstki bez PEG. Najsilniejszy efekt usztywnienia błony stwierdzono dla dendrymeru bez PEG, zawierającego jedną resztę kwasu kawowego. Otrzymane wyniki pokazały, że badane dendrymery wykazywały stosunkowo słabe działanie hemotoksyczne, a efekt ten został całkowicie zniesiony w obecności FBS. Zjawisko to można tłumaczyć oddziaływaniem dendrymerów z białkami surowicy.

Końcowy etap pracy doktorskiej obejmował ocenę zdolności dendrymerów polifenolowych do tworzenia kompleksów z proapoptotyczymi siRNA (siMcl-1 i siBcl-2). Dodatkowo zbadano efektywność przeciwnowotworowego działania kompleksów dendrymer/siRNA w stosunku do komórek A549 niedrobnokomórkowego raka płuc. Wyniki tych badań zostały opublikowane w pracy **M. Grodzicka, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Polyphenolic dendrimers as carriers of anticancer siRNA, *Int. J. Pharm*. 658, 2024, https://doi.org/10.1016/j.ijpharm.2024. 124199**. Pokazano, że wszystkie testowane dendrymery nie tylko tworzyły kompleksy z siRNA, ale również chroniły go przed degradacją w obecności nukleaz. Analiza danych dotyczących stabilności utworzonych kompleksów dendrymer/siRNA wykazała, że kompleksy zawierające dendrymery z PEG były bardziej stabilne niż dendrymery bez PEG. Wyniki oceny biofizycznej powstałych kompleksów pozwoliły na oszacowanie stosunków molowych pomiędzy składnikami kompleksu dendrymer/siRNA, 1:16 dla dendrymerów nie zawierających PEG, 1:40 dla dendrymerów z PEG i jedna reszta kwasu kawowego oraz 1:30 dla dendrymerów z PEG i dwiema resztami kwasu kawowego. Efekt cytotoksyczny kompleksów określano w stosunku do fibroblastów ludzkich (BJ) oraz komórek A549. Podczas, gdy kompleksy nie wykazywały cytotoksyczności w stosunku do fibroblastów, w komórkach nowotworowych

zaobserwowano spadek żywotności. Dodatkowo, za pomocą cytometrii przepływowej oraz przy użyciu mikroskopii konfokalnej sprawdzono zdolność dendrymerów do wprowadzenia siRNA do komórek nowotworowych. Wszystkie badane dendrymery skutecznie wprowadzały siRNA zarówno w hodowli 2D, jak i 3D, a efektywność internalizacji była większa po 3 niż po 24 godzinach. Sprawdzono wpływ utworzonych kompleksów na zdolność komórek nowotworowych do adhezji oraz migracji. W przypadku kompleksów z siBcl-2 jedynie kompleks utworzony z dendrymerem bez PEG i jedna resztą kwasu kawowego powodował znaczący spadek liczby zadherowanych komórek. Z kolei zastosowanie wszystkich testowanych kompleksów utworzonych z siMcl-1 prowadziło do zmniejszenia adhezji komórek nowotworowych, przy czym najbardziej wyraźny efekt wykazał kompleks zawierający siMcl-1 i dendrymer bez PEG, z dwiema resztami kwasu kawowego. Zaobserwowano również, że wszystkie badane kompleksy hamowały proces migracji komórek, a efekt ten był najbardziej widoczny po zastosowaniu kompleksu z siMcl-1 i dendrymerem bez PEG, i jedną resztą kwasu kawowego. W celu sprawdzenia, czy kompleksy dendrymerów z proapoptotycznym siRNA indukują apoptozę zastosowano test podwójnego barwienia (aneksyną V/ jodkiem propidyny) i analizowano poszczególne frakcje komórek techniką cytometrii przepływowej. 72-godzinna inkubacja komórek A549 z utworzonymi kompleksami doprowadziła do zmniejszenia frakcji żywych komórek nowotworowych. Obecność nanokompleksów spowodowała zwiększenie liczby komórek wczesno-apoptotycznych, podczas gdy frakcja komórek nekrotycznych była nadal nieznaczna. Największą ilość komórek apoptotycznych, wynoszącą około 50%, zaobserwowano po zastosowaniu kompleksów utworzonych z dendrymerem z PEG i dwiema resztami kwasu kawowego. Uzyskany efekt został potwierdzony przez obserwację komórek za pomocą mikroskopii konfokalnej. Na podstawie otrzymanych wyników można stwierdzić, że dendrymery polifenolowe skutecznie dostarczają siRNA do komórek A549 i kierują je na drogę programowanej śmierci jaką jest apoptoza. Spośród czterech badanych związków najskuteczniejszy okazał się być dendrymer $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$, zawierający dwie reszty kwasu kawowego, pozbawiony łańcuchów glikolu polietylenowego.

Wnioski

- Karbokrzemowe dendrymery polifenolowe pierwszej generacji wykazują niską cytotoksyczność, a modyfikacja kwasem kawowym czyni je skutecznymi przeciwutleniaczami;
- Dendrymery polifenolowe oddziałują z albuminą surowicy ludzkiej oraz błonami lipidowymi;
- Dendrymery polifenolowe tworzą stabilne kompleksy z proapoptotycznymi siRNA (siMcl-1 i siBcl-2) oraz chronią je przed degradacją w obecności nukleaz;
- Proapoptotyczne siRNA wprowadzone do komórek nowotworowych linii A549 z wykorzystaniem dendrymerów polifenolowych indukuje apoptozę.

Streszczenie w języku polskim

Dendrymery to rozgałęzione struktury polimerowe, które są szeroko badane jako nośniki leków i kwasów nukleinowych. Obecnie naukowcy poszukują nowych terapeutyków opartych na dendrymerach, które będą posiadały cechy niezbędne do zastosowań biomedycznych, m.in. odpowiednia biodostępność, niska toksyczność i wysoki profil transfekcji. Unikalne właściwości karbokrzemowych dendrymerów w dostarczaniu leków zostały wielokrotnie udowodnione, a ich skuteczność została dodatkowo ulepszona przez koniugacje z polifenolami — wtórnymi metabolitami roślinnymi o szerokim spektrum aktywności biologicznej, w tymo działaniu przeciwutleniającym korzystnym dla zdrowia człowieka. Badania zaprezentowane w obecnej pracy koncentrują się na charakterystyce dwóch nowych typów karbokrzemowych układów dendrytycznych. Pierwsza grupa zawiera jedną lub dwie jednostki kwasu kawowego oraz grupy amonowe na powierzchni, co sprawia, że są one rozpuszczalne w wodzie. Druga grupa zawiera dodatkowo jedną lub dwie cząsteczki PEG w swojej strukturze, co zwiększa biokompatybilność układu. Oba typy dendrymerów polifenolowych wykazały niską cytotoksyczność oraz chroniły erytrocyty przed hemolizą oksydacyjną. Dodatkowo, badane dendrymery ograniczały produkcję RFT, indukowaną przez AAPH w ludzkich fibroblastach. Uzyskane wyniki wykazały, że dendrymer z dwiema cząsteczkami kwasu kawowego i PEG charakteryzował się najlepszą aktywnością antyoksydacyjną.

Zastosowanie dendrymerów jako nośników leków i kwasów nukleinowych wymaga zrozumienia ich interakcji z różnymi systemami biologicznymi, szczególnie z białkami surowicy i błonami biologicznymi. Dlatego zbadano interakcje dendrymerów polifenolowych z albuminą ludzką oraz błonami biologicznymi. Uzyskane wyniki wykazały, że dendrymery oddziałują z albuminą ludzką, nieznacznie zmieniając jej strukturę drugorzędową, a obecność kwasu kawowego oraz PEG w strukturze dendrymeru wpływała na właściwości termodynamiczne błon lipidowych.

W końcowym etapie badań oceniono potencjał dendrymerów polifenolowych w dostarczaniu terapeutycznych siRNA do komórek nowotworowych A549. Dendrymery tworzyły stabilne kompleksy z siRNA i chroniły kwasy nukleinowe przed degradacją w obecności nukleaz. Wszystkie badane dendrypleksy hamowały migrację i adhezję komórek nowotworowych oraz zwiększały populację komórek wczesno-apoptotycznych. Spośród czterech testowanych związków, dendrymer zawierający dwie reszty kwasu kawowego miał najlepszy profil transfekcji. Podsumowując, wydaje się być on obiecującym kandydatem do dostarczania siRNA do komórek nowotworowych.

Streszczenie w języku angielskim

Dendrimers are branched polymer structures that have been extensively studied as carriers for drugs and nucleic acids. Currently, scientists are trying to develop new dendrimer-based therapeutics that possess required characteristics for biomedical applications, such as improved bioavailability, low toxicity, and high transfection efficiency. The unique properties of carbosilane dendrimers as drug carriers have already been demonstrated, and their effectiveness has been further enhanced by conjugation with polyphenols — secondary plant metabolites with a broad range of biological activities, including antioxidant activity beneficial to human health. The research presented in this doctoral thesis focuses on characterization of two types of carbosilane dendritic systems. The first group contains one or two caffeic acid residues and ammonium groups on the surface, making them water-soluble. The second group additionally contains one or two PEG chains, which increases the system's biocompatibility. Both types of polyphenolic dendrimers showed low cytotoxicity and protected erythrocytes from oxidative hemolysis. Furthermore, the dendrimers reduced AAPH-induced ROS production in human fibroblasts.

The results indicated that the dendrimer with two caffeic acid units and PEG exhibited best antioxidant properties.

The application of dendrimers as carriers for drugs and nucleic acids requires an understanding of their interactions with various biological systems such as serum proteins and biological membranes. Therefore, the interactions of a new class of dendrimers functionalized with caffeic acid residues with human albumin and biological membranes were studied. The results showed that polyphenolic dendrimers interact with human albumin, altered its secondary structure insignificantly. The presence of caffeic acid and PEG in the dendrimer structure influenced the thermodynamic properties of the lipid membrane.

In the final stage of the study, the potential of polyphenolic dendrimers to deliver pro-apoptotic siRNAs to A549 cancer cells was evaluated. The dendrimers formed stable complexes with siRNA and protected the nucleic acids from degradation by nucleases. All the studied dendrimer/siRNA complexes inhibited A549 cell migration and adhesion, while also increased the population of early apoptotic cells. Among the four tested compounds, the dendrimer containing two caffeic acid residues complexed with siRNA had the best transfection profile. In conclusion, it appears this dendrimer can be a promising candidate for delivering siRNA to cancer cells.

Bibliografia

[1] D.A. Tomalia, J.M.J. Fréchet, Discovery of dendrimers and dendritic polymers: A brief historical perspective, J Polym Sci A Polym Chem 40 (2002) 2719–2728. https://doi.org/10.1002/pola.10301.

[2] D.A. Tomalia, H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder, P. Smith, A New Class of Polymers : Starburst-Dendritic, Polym J 17 (1985) 117–132. http://www.japsonline.com/abstract.php?article_id=1465.

[3] P. Kesharwani, S. Banerjee, U. Gupta, M.C.I. Mohd Amin, S. Padhye, F.H. Sarkar, A.K. Iyer, PAMAM dendrimers as promising nanocarriers for RNAi therapeutics, Materials Today 18 (2015) 565–572. https://doi.org/10.1016/j.mattod.2015.06.003.

[4] N.S. Del Olmo, C.E.P. González, J.D. Rojas, R. Gómez, P. Ortega, A. Escarpa, F.J. de la Mata, Antioxidant and antibacterial properties of carbosilane dendrimers functionalized with polyphenolic moieties, Pharmaceutics 12 (2020) 1–16. https://doi.org/10.3390/pharmaceutics12080698.

[5] N.S. Del Olmo, M. Holota, S. Michlewska, R. Gómez, P. Ortega, M. Ionov, F.J. de la Mata, M. Bryszewska, Copper (II) metallodendrimers combined with pro-apoptotic

sirnas as a promising strategy against breast cancer cells, Pharmaceutics 12 (2020) 1–14. https://doi.org/10.3390/pharmaceutics12080727.

[6] P. Pandi, A. Jain, N. Kommineni, M. Ionov, M. Bryszewska, W. Khan, Dendrimer as a new potential carrier for topical delivery of siRNA: A comparative study of dendriplex vs. lipoplex for delivery of TNF-α siRNA, Int J Pharm 550 (2018) 240–250. https://doi.org/10.1016/j.ijpharm.2018.08.024.

[7] S. Michlewska, M. Ionov, D. Shcharbin, M. Maroto-Díaz, R. Gomez Ramirez, F. Javier de la Mata, M. Bryszewska, Ruthenium metallodendrimers with anticancer potential in an acute promyelocytic leukemia cell line (HL60), Eur Polym J 87 (2017) 39–47. https://doi.org/10.1016/j.eurpolymj.2016.12.011.

[8] S. Michlewska, M. Ionov, M. Maroto-Díaz, A. Szwed, A. Ihnatsyeu-Kachan, S. Loznikova, D. Shcharbin, M. Maly, R.G. Ramirez, F.J. de la Mata, M. Bryszewska, Ruthenium dendrimers as carriers for anticancer siRNA, J Inorg Biochem 181 (2018) 18–27. https://doi.org/10.1016/j.jinorgbio.2018.01.001.

[9] O.A. Krasheninina, E.K. Apartsin, E. Fuentes, A. Szulc, M. Ionov, A.G. Venyaminova, D. Shcharbin, F.J. de la Mata, M. Bryszewska, R. Gómez, Complexes of pro-apoptotic sirnas and carbosilane dendrimers: Formation and effect on cancer cells, Pharmaceutics 11 (2019). https://doi.org/10.3390/pharmaceutics11010025.

[10] A. Jain, S. Mahira, J.P. Majoral, M. Bryszewska, W. Khan, M. Ionov, Dendrimer mediated targeting of siRNA against polo-like kinase for the treatment of triple negative breast cancer, J Biomed Mater Res A 107 (2019) 1933–1944. https://doi.org/10.1002/jbm.a.36701.

[11] K. Białkowska, K. Miłowska, S. Michlewska, P. Sokołowska, P. Komorowski, T. Lozano-Cruz, R. Gomez-Ramirez, F.J. de la Mata, M. Bryszewska, Interaction of cationic carbosilane dendrimers and their siRNA complexes with MCF-7 cells, Int J Mol Sci 22 (2021). https://doi.org/10.3390/ijms22137097.

[12] M. Ionov, D. Wróbel, K. Gardikis, S. Hatziantoniou, C. Demetzos, J.P. Majoral, B. Klajnert, M. Bryszewska, Effect of phosphorus dendrimers on DMPC lipid membranes, Chem Phys Lipids 165 (2012) 408–413. https://doi.org/10.1016/j.chemphyslip.2011.11.014.

[13] D. Wrobel, A. Kłys, M. Ionov, P. Vitovic, I. Waczulikowa, T. Hianik, R. Gomez-Ramirez, J. De La Mata, B. Klajnert, M. Bryszewska, Cationic carbosilane dendrimers-lipid membrane interactions, Chem Phys Lipids 165 (2012) 401–407. https://doi.org/10.1016/j.chemphyslip.2012.01.008.

[14] P. Kesharwani, K. Jain, N.K. Jain, Dendrimer as nanocarrier for drug delivery, Prog Polym Sci 39 (2014) 268–307. https://doi.org/10.1016/j.progpolymsci.2013.07.005.

[15] J. Lazniewska, K. Milowska, M. Zablocka, S. Mignani, A.M. Caminade, J.P. Majoral, M. Bryszewska, T. Gabryelak, Mechanism of cationic phosphorus dendrimer toxicity against murine neural cell lines, Mol Pharm 10 (2013) 3484–3496. https://doi.org/10.1021/mp4003255.

[16] S. Thakur, P. Kesharwani, R.K. Tekade, N.K. Jain, Impact of pegylation on biopharmaceutical properties of dendrimers, Polymer (Guildf) 59 (2015) 67–92. https://doi.org/10.1016/j.polymer.2014.12.051.

[17] S. Somani, P. Laskar, N. Altwaijry, P. Kewcharoenvong, C. Irving, G. Robb, B.S. Pickard, C. Dufès, PEGylation of polypropylenimine dendrimers: Effects on cytotoxicity, DNA condensation, gene delivery and expression in cancer cells, Sci Rep 8 (2018) 1–13. https://doi.org/10.1038/s41598-018-27400-6.

[18] W. Wang, W. Xiong, J. Wan, X. Sun, H. Xu, X. Yang, The decrease of PAMAM dendrimer-induced cytotoxicity by PEGylation via attenuation of oxidative stress, Nanotechnology 20 (2009). https://doi.org/10.1088/0957-4484/20/10/105103.

[19] M. Grodzicka, C.E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F.J. de la Mata, M. Ionov, Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity, Sustainable Materials and Technologies 33 (2022) e00497. https://doi.org/10.1016/j.susmat.2022.e00497.

[20] M.A. Antunes, M. Lopes-Pacheco, P.R.M. Rocco, Oxidative Stress-Derived Mitochondrial Dysfunction in Chronic Obstructive Pulmonary Disease: A Concise Review, Oxid Med Cell Longev 2021 (2021). https://doi.org/10.1155/2021/6644002.

[21] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, International Journal of Biochemistry and Cell Biology 39 (2007) 44–84. https://doi.org/10.1016/j.biocel.2006.07.001.

[22] P. Muriel, J. Arauz, Coffee and liver diseases, Fitoterapia 81 (2010) 297–305. https://doi.org/10.1016/j.fitote.2009.10.003.

[23] S.L. Schmit, O. Nwogu, M. Matejcic, A. DeRenzis, L. Lipworth, W.J. Blot, L. Raskin, Coffee consumption and cancer risk in African Americans from the Southern Community Cohort Study, Sci Rep 10 (2020) 1–8. https://doi.org/10.1038/s41598-020-72993-6.

[24] P.J. Boekema, M. Samsom, G.P. Van Berge Henegouwen, A.J.P.M. Smout, Coffee and gastrointestinal function: Facts and fiction: A review, Scand J Gastroenterol Suppl 33 (1999) 35–39. https://doi.org/10.1080/003655299750025525.

[25] Y.M. Li, J. Peng, L.Z. Li, Coffee consumption associated with reduced risk of oral cancer: A meta-analysis, Oral Surg Oral Med Oral Pathol Oral Radiol 121 (2016) 381-389.e1. https://doi.org/10.1016/j.oooo.2015.12.006.

[26] E. Loftfield, N.D. Freedman, B.I. Graubard, A.R. Hollenbeck, F.M. Shebl, S.T. Mayne, R. Sinha, Coffee drinking and cutaneous melanoma risk in the NIH-AARP diet and health study, J Natl Cancer Inst 107 (2015) 1–9. https://doi.org/10.1093/jnci/dju421.

[27] M. Lukic, M. Jareid, E. Weiderpass, T. Braaten, Coffee consumption and the risk of malignant melanoma in the Norwegian Women and Cancer (NOWAC) Study, BMC Cancer 16 (2016). https://doi.org/10.1186/s12885-016-2586-5.

[28] C.L. Ky, J. Louarn, S. Dussert, B. Guyot, S. Hamon, M. Noirot, Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild Coffea arabica L. and C. canephora P. accessions, Food Chem 75 (2001) 223–230. https://doi.org/10.1016/S0308-8146(01)00204-7.

[29] D. Guo, D. Dou, L. Ge, Z. Huang, L. Wang, N. Gu, A caffeic acid mediated facile synthesis of silver nanoparticles with powerful anti-cancer activity, Colloids Surf B Biointerfaces 134 (2015) 229–234. https://doi.org/10.1016/j.colsurfb.2015.06.070.

[30] K.M. Monteiro Espíndola, R.G. Ferreira, L.E. Mosquera Narvaez, A.C. Rocha Silva Rosario, A.H. Machado Da Silva, A.G. Bispo Silva, A.P. Oliveira Vieira, M. Chagas Monteiro, Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma, Front Oncol 9 (2019) 3–5. https://doi.org/10.3389/fonc.2019.00541.

[31] M. Yousefi, A. Narmani, S.M. Jafari, Dendrimers as efficient nanocarriers for the protection and delivery of bioactive phytochemicals, Adv Colloid Interface Sci 278 (2020). https://doi.org/10.1016/j.cis.2020.102125.

[32] G. Mencia, N.S. Del Olmo, L. Muñoz-Moreno, M. Maroto-Diaz, R. Gomez, P. Ortega, M. José Carmena, F. Javier de la Mata, Polyphenolic carbosilane dendrimers as anticancer agents against prostate cancer, New Journal of Chemistry 40 (2016) 10488–10497. https://doi.org/10.1039/c6nj02545e.

[33] M. Grodzicka, S. Michlewska, A. Buczkowski, S. Sekowski, C.E. Pena-Gonzalez, P. Ortega, F.J. de la Mata, J. Blasiak, M. Bryszewska, M. Ionov, A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion, Sci Rep 14 (2024) 1–9. https://doi.org/10.1038/s41598-024-56509-0.

[34] M. Grodzicka, S. Michlewska, A. Buczkowski, P. Ortega, F.J. de la Mata, M. Bryszewska, M. Ionov, Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chem Phys Lipids 265 (2024) 105444. https://doi.org/10.1016/j.chemphyslip.2024.105444.

[35] M. Grodzicka, S. Michlewska, J. Blasiak, P. Ortega, F.J. de la Mata, M. Bryszewska, M. Ionov, Polyphenolic dendrimers as carriers of anticancer siRNA, Int J Pharm 658 (2024) 124199. https://doi.org/10.1016/j.ijpharm.2024.124199.

[36] M. Kubczak, M. Grodzicka, S. Michlewska, M. Karimov, A. Ewe, A. Aigner, M. Bryszewska, M. Ionov, The effect of novel tyrosine-modified polyethyleneimines on human albumin structure – Thermodynamic and spectroscopic study, Colloids Surf B Biointerfaces 227 (2023). https://doi.org/10.1016/j.colsurfb.2023.113359.

[37] K. Tokarczyk, B. Jachimska, Characterization of G4 PAMAM dendrimer complexes with 5-fluorouracil and their interactions with bovine serum albumin, Colloids Surf A Physicochem Eng Asp 561 (2019) 357–363. https://doi.org/10.1016/j.colsurfa.2018.10.080.

[38] B. Klajnert, D. Appelhans, H. Komber, N. Morgner, S. Schwarz, S. Richter, B. Brutschy, M. Ionov, A.K. Tonkikh, M. Bryszewska, B. Voit, The influence of densely organized maltose shells on the biological properties of poly(propylene imine) dendrimers: New

effects dependent on hydrogen bonding, Chemistry - A European Journal 14 (2008) 7030–7041. https://doi.org/10.1002/chem.200800342.

Dorobek naukowy

Spis publikacji wchodzących w skład rozprawy doktorskiej:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity,* Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497. **IF = 8,6; Punkty MEiN = 200.**

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena‑Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure‑dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0. **IF = 3,8; Punkty MEiN = 140;**

3. **M. Grodzicka**, S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Effect of polyphenolic dendrimers on biological and artificial lipid membranes,* Chemistry and Physics of Lipids, Elsevier, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444, **IF = 3,4; Punkty MEiN = 100;**

4. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA,* International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199. **IF = 5,3; Punkty MEiN = 100;**

Publikacje wchodzące w skład rozprawy doktorskiej

IF:21,1; Punkty MEiN: 540

Spis pozostałych publikacji:

1. M. Kubczak, **M. Grodzicka**, S. Michlewska, M. Karimov, A. Ewe, A. Aigner, M. Bryszewska, M. Ionov; *The effect of novel tyrosine-modified polyethyleneimines*

on human albumin structure – thermodynamic and spectroscopic study, Colloids and Surfaces B: Biointerfaces, Elsevier, 2023, https://doi.org/10.1016/j.colsurfb.2023.113359. **IF = 5,4; Punkty MEiN = 100;**

2. S. Michlewska, Z. Garaiova, V. Šubjakova, M. Hołota, M. Kubczak, **M. Grodzicka**, E. Okła, N. Naziris, Ł. Balcerzak, P. Ortega, F. J. de la Mata, T. Hianik, I. Waczulikova , M. Bryszewska, M. Ionov; *Lipid-coated ruthenium dendrimer conjugated with doxorubicin in anti-cancer drug delivery: Introducing protocols,* Colloids and Surfaces B: Biointerfaces, Elsevier, 2023, https://doi.org/10.1016/j.colsurfb.2023.113371. **IF = 5,4; Punkty MEiN = 100;**

3. **M. Grodzicka** S. Michlewska, P. Ortega, C.E. Pena-Gonzalez, F. Javier de la Mata, M. Bryszewska, M. Ionov, *Polyphenolic dendrimers as possible nucleicacid carriers in small cell lung cancer therapy,* FEBS Open Bio , 2023, https://doi.org/10.1002/2211-5463.13646. **IF = 2,792; Punkty MEiN = 70;**

4. M. Strachowska, K. Gronkowska, M. Sobczak, **M. Grodzicka**, S. Michlewska, K. Kołacz, T. Sarkar, J. Korszun, M. Ionov, A. Robaszkiewicz, *I-CBP112 declines overexpression of ATP-binding cassette transporters and sensitized drug-resistant MDA-MB-231 and A549 cell lines to chemotherapy drugs,* Biomedicine & Pharmacotherapy, Elsevier, 2023, https://doi.org/10.1016/j.biopha.2023.115798. **IF = 6,9; Punkty = MEiN 140;**

5. **M. Grodzicka**, M. Kubczak, S. Michlewska, M. Ionov, M. Bryszewska; *Oddziaływanie dendrymerów zawierających atomy miedzi z aminotransferazą asparaginianową (AST)*, Wydawnictwo TYGIEL sp. z o.o.(2021) Tom 2; s.188-196. **Punkty MEiN = 80.**

   IF: 20,492; Punkty MEiN: 490

   Wszystkie publikacje

   Łącznie **IF: 41,592; Punkty MEiN**:**1030**

   H-index (na podstawie bazy Web of Science): 2

   Liczba cytowań: 18

Działalność naukowa

Komunikaty zjazdowe

- 48th FEBS Congress 2024; **M. Grodzicka,** S. Michlewska, Paula Ortega, C.E. Pena-Gonzalez, F. J.de la Mata, M. Bryszewska, M. Ionov, poster naukowy pt.: „Carbosilane dendrimers conjugated with caffeic acid and PEG interact with human serum albumin" (29.06 — 03.07.2024 r.);

- 47th FEBS Congress 2023; **M. Grodzicka,** S. Michlewska, Paula Ortega, C.E. Pena-Gonzalez, F. J.de la Mata, M. Bryszewska, M. Ionov, poster naukowy pt.: „Polyphenolic dendrimers as possible nucleic acid carriers in Non-Small Cell Lung Cancer therapy" (08.07 — 12.07.2023 r.);

- International Conference „InterNanoPoland2022"; **M. Grodzicka,** S. Michlewska, C. E. Pena-Gonzalez, R. Lozano, P. Ortega, M. Bryszewska, F. J. de la Mata, M. Ionov, poster naukowy pt.: „Interaction of polyphenol dendrimers with human serum albumin" (11.10 — 12.10.2022 r.);

- XIII Interdyscyplinarna Konferencja naukowa TYGIEL 2021 „Interdyscyplinarność kluczem do rozwoju"; **M. Grodzicka,** M. Kubczak, S. Michlewska, poster naukowy pt.: „Oddziaływanie dendrymerów zawierających atomy miedzi z aminotransferazą asparaginianową (AST)" (25.03 — 28.03.2021 r.).

Udział w projektach

- Wykonawca w projekcie MINIATURA – 7 „Polifenolowe dendrymery nowej generacji, jako nośniki paklitakselu w terapii lekoopornego drobnocząsteczkowego raka płuc"; Nr.: 2023/07/X/ NZ7/00026 (2023 — 2024);

- Wykonawca w projekcie IDUB „Metalodendrymery zawierające atomy miedzi, jako potencjalne nanonośniki terapeutycznego siRNA w leczeniu nowotworu wątroby". Finansowanym z subwencji otrzymanej w ramach programu "Inicjatywa Doskonałości - Uczelnia Badawcza"; Nr.: 44/2021 (2022 — 2024);

- Wykonawca w projekcie Projekt Lider „Przełamywanie oporności wielolekowej nowotworów na poziomie genomu: opracowanie metody zapobiegającej nadekspresji"; Nr.: LIDER/22/0122/L-10/18/NCBR/2019 (2021 — 2022);

- Bilateralny PL-SK projekt programu wymiany naukowej miedzy Rzecząpospolitą Polską a Republiką Słowacką „Nowe nanosystemy dostarczania leków cytostatycznych z wykorzystaniem technologii łączącej właściwości dendrymerów i liposomów"; Nr.: PPN/BIL/2018/1/00150/ U/00001;

- EUROPARTNER „Działania wzmacniające i upowszechniające międzynarodowe partnerstwo Wydziału Biologii i Ochrony Środowiska na rzecz interdyscyplinarnych badań i innowacji Uniwersytet Łódzkiego"; Nr.: PPI/APM/2018/1/00007/U/00;

- Wykonawca w projekcie Beethoven Live 1 „Nowe polimerowe nanocząstki do przenoszenia kwasów nukleinowych w leczeniu chorób płuc – synteza, analiza toksykologiczna i ocena biologiczno-terapeutyczna"; Nr.: 2018/31/F/N25 /03454.

Działalność organizacyjna i promocyjna

- Przeprowadzenie warsztatów pt.: „Poznaj tajemnice życia z Panem Kleksem!", w ramach ogólnopolskiej akcji „Noc Biologów" promującej wiedzę biologiczną w formie popularnonaukowej (2024);

- Wygłoszenie wykładu na Wydziale Biologii i Ochrony Środowiska pt.: „Woda jako główny składnik organizmu człowieka" w ramach ogólnopolskiej akcji „Noc Biologów" promującej wiedzę biologiczną w formie popularnonaukowej (2023);

- Współudział w prowadzeniu zajęć (ćwiczeń): „Fizyka z elementami biofizyki" dla kierunku biologia, studiów stacjonarnych; Wydział Biologii i Ochrony Środowiska, Uniwersytet Łódzki (w latach 2021 — 2023);

- Wygłoszenie wykładu pt.: „Czy wirus zawsze jest naszym wrogiem? – fagoterapia" w ramach Festiwalu Nauki, Techniki i Sztuki, którego zadaniem jest popularyzacja nauki i sztuki oraz promocja osiągnięć łódzkich środowisk naukowych i artystycznych (2021);

- Opiekowanie się gośćmi zagranicznymi z instytucji partnerskiej podczas ich pobytu w Katedrze Biofizyki Ogólnej BiOŚ UŁ (mgr Judith Recio Ruiz i mgr Silvia Muñoz Sánchez) z Uniwersytetu w Alcalá de Henares, Hiszpania (2021).

Pełnione funkcje

1. Członek Polskiego Towarzystwa Biofizycznego (2023 -obecnie).

2. Członek Polskiego Towarzystwa Biochemiczne (2022 -obecnie).

Staże naukowe

- Staż naukowy w Łotewskim Centrum Badań i Studiów Biomedycznych, Ryga, Łotwa. W ramach Programu PROM 2023 – NAWA „Międzynarodowa wymiana stypendialna doktorantów i kadry akademickiej" (17.06. — 30.06.2023 r.);

- Staż naukowy w Katedrze Fizyki Jądrowej i Biofizyki, Wydział Matematyki i Fizyki Uniwersytet Komeńskiego w Bratysławie, Słowacja. W ramach międzynarodowego projektu NAWA o akronimie: „EUROPARTNER", pt.: „Działania wzmacniające i upowszechniające międzynarodowe partnerstwo Wydziału Biologii i Ochrony Środowiska na rzecz interdyscyplinarnych badań i innowacji Uniwersytetu Łódzkiego". Nr: PPI/APM/2018/1/00007/U/00 (05.05. — 18.05.2022 r.);

- Staż naukowy w Katedrze Fizyki Jądrowej i Biofizyki, Wydział Matematyki i Fizyki Uniwersytet Komeńskiego w Bratysławie, Słowacja. W ramach programu wymiany bilateralnej miedzy Rzecząpospolitą Polską a Republiką Słowacką, „Nowe nanosystemy dostarczania leków cytostatycznych z wykorzystaniem technologii łączącej właściwości dendrymerów i liposomów", Nr.: PPN/BIL/2018/1/00150/ U/00001 (06.12. — 15.12.2021 r.);

- Staż naukowy w Zakładzie Chemii Organicznej i Nieorganicznej, Wydział Farmacji Uniwersytetu w Alcalá de Henares, Hiszpania. W ramach projektu NAWA AMP o akronimie: „EUROPARTNER" „Działania wzmacniające i upowszechniające międzynarodowe partnerstwo Wydziału Biologii i Ochrony Środowiska na rzecz interdyscyplinarnych badań i innowacji Uniwersytetu Łódzkiego" Nr: PPI/APM/2018/1/00007/U/00 (03.06. — 03.07.2021 r.).

Nagrody i wyróżnienia

- Stypendium wyjazdowe Federation of European Biochemical Societies „48 th FEBS Congress – Bursaries" (2024). Stypendium na sfinansowanie udziału w konferencji 48th FEBS Congress, 29.06 — 03.07.2024 r., Mediolan, Włochy;

- I Nagroda Naukowa Rektora Uniwersytetu Łódzkiego dla doktorantek i doktorantów szkół doktorskich UŁ za osiągnięcia w zakresie pracy naukowej i badawczej, publikacji i umiędzynarodowienia kształcenia (2024);

- Stypendium wyjazdowe PROM 2023 – NAWA „Międzynarodowa wymiana stypendialna doktorantów i kadry akademickiej" (2023). Stypendium na odbycie stażu naukowego w terminie: 17.06. — 30.06.2023 r. w Łotewskim Centrum Badań i Studiów Biomedycznych, Ryga, Łotwa;

- Stypendium wyjazdowe Federation of European Biochemical Societies „47 th FEBS Congress – Bursaries" (2023). Stypendium na sfinansowanie udziału w konferencji 47th FEBS Congress, w terminie: 08.07 — 12.07.2023 r., Tours, Francja.

Sustainable Materials and Technologies 33 (2022) e00497

Contents lists available at ScienceDirect

Sustainable Materials and Technologies

journal homepage: www.elsevier.com/locate/susmat

Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity

Marika Grodzicka [a,b,1], Cornelia E. Pena-Gonzalez [c,1], Paula Ortega [c,d], Sylwia Michlewska [e,*], Rebeca Lozano [c], Maria Bryszewska [a], Francisco Javier de la Mata [c,d], Maksim Ionov [a]

[a] Department of General Biophysics, Faculty of Biology & Environmental Protection, University of Lodz, Pomorska 141/143, Lodz 90–236, Poland
[b] BioMedChem Doctoral School of the UL and Lodz Institutes of the Polish Academy of Science, Banacha 12/16, 0-237 Lodz, Poland
[c] Universidad de Alcalá, Department of Organic and Inorganic Chemistry, and Research Institute in Chemistry "Andrés M. del Río" (IQAR), Spain and Instituto Ramon y Cajal de Investigacion Sanitaria, IRYCIS, Colmenar Viejo Road, Km 9, 100, 28034 Madrid, Spain
[d] Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain
[e] Laboratory of Microscopic Imaging & Specialized Biological Techniques, Faculty of Biology & Environmental Protection, University of Lodz, Banacha12/16, Lodz 90–237, Poland

ARTICLE INFO

Keywords:
Polyphenolic dendrimers
Synthesis
Caffeic acid
Antioxidant activity
Toxicity

ABSTRACT

Dendrimers, branched polymer structures, have been widely studied as efficient drug carriers. Scientists are trying to find new dendrimer-based formulations with the properties needed for biomedical applications such as improved bioavailability, low toxicity and high transfection profiles. The unique drug delivery properties of carbosilane dendrimers have already been demonstrated. Their efficacy has been further improved by conjugation with polyphenols, plant secondary metabolites with a wide range of biological activities, including antioxidant effects that are beneficial for human health. The present study focuses on synthesis and characterization of two new types of carbosilane dendritic systems, one family presents one or two caffeic acid units and ammonium groups on the surface to make them water soluble. The other family has, in addition to the two mentioned functionalities, one or two polyethylene glycol (PEG) chains in the structure to increase the biocompatibility of the system. Carbosilane dendrimers with caffeic acid have low toxicity and protect erythrocytes against oxidative hemolysis. These dendrimers also decrease AAPH-induced ROS production in human fibroblasts.

Various techniques demonstrating such antioxidant activities have been applied in the current research. The best antioxidant properties were shown for the dendrimer with two PEG-caffeic acid moieties. Further aspects of the biochemical characterization of the dendrimers are also considered and discussed.

1. Introduction

During the early 1980s, super-branched monodispersive nanoparticles named dendrimers were first synthesized by Tomalia's team [1,2]. Dendrimers are spherical, with a densely packed surface and free internal spaces. Owing to their unique architecture they are considered excellent transporters of drugs and genes [3]. They could be used to make cancer or neurodegenerative disease therapies more efficient. They are well defined and relatively easy to synthesize [4–10]. Dendrimers such as PAMAM, PPI, (see Table 1 "Abbreviations"), and carbosilane have been repeatedly described and their physicochemical and biological properties have been reported. Conjugation of drugs with dendrimers improves their solubility and bioavailability. Slow release of a drug from a drug/dendrimer complex sustains its circulation in the bloodstream and reduces the side effects of chemotherapeutics. Cationic dendrimers can form stable complexes with nucleic acids and have been suggested as promising carriers for genes for use in gene therapy. Moreover, nucleic acids complexed with dendrimers can interact with negatively-charged cell membranes [8,11–14]. On the other hand, positively charged dendrimers can be cytotoxic [8,15,16]. One way to decrease such toxicity is to conjugate them with polyethylene glycol (PEG) [17–20]. PEGylation can stabilize their interactions with nucleic acids and reduce interactions with serum proteins [6,20]. PEG also increases the solubility of dendrimers in water and limits their uptake by

* Corresponding author.
E-mail address: sylwia.michlewska@biol.uni.lodz.pl (S. Michlewska).
[1] These authors contributed equally to the work

https://doi.org/10.1016/j.susmat.2022.e00497
Received 20 April 2022; Received in revised form 26 August 2022; Accepted 16 September 2022
Available online 21 September 2022

Table 1
Abbreviations.

A549	Cancer human alveolar basal epithelial cells
AAPH	2,2′-azobis-2-methyl-propanimidamide, dihydrochloride, Cayman, USA
BJ	Normal human fibroblasts
BODIPY581/591	(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-un-decanoic acid; invitrogen, Thermo Fisher Scientific, USA
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
DCF	2′,7′-dichlorofluorescein
DLS	Dynamic light-scattering
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylfuran
DMPA	2,2-dimethoxy-2-phenylaceto-phenone
DMSO	Dimethyl Sulfoxide, Avantor, Gliwice, Poland
DPPH	2,2′-diphenyl-1-picrylhydrazyl; Sigma Aldrich,
EDCI-HCl	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
FBS	Fetal Bovine Serum
FRAP	Ferric Reducing Antioxidant Power
H_2DCF-DA	2′,7′-dichlorofluorescindiacetate; Thermo Fisher, Waltham, MA, USA
HOBt	1-Hydroxybenzotriazole hydrate
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Methylthiazolyldiphenyl-tetrazolium bromide; Sigma Aldrich, USA
NMR	Nuclear magnetic resonance
PAMAM	Poly(amidoamine)
PB	Na-phosphate buffer
PBS	Phosphate Buffered Saline
PDI	Polydispersity index
PEG	Polyethylene glycol
PPI	Poly(propylene imine)
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute
TEAC	Trolox Equivalent Antioxidant Capacity
TEM	Transmission electron microscopy
TPTZ	2,4,6-tripyridyl-striazine

reticuloendothelial systems, prolonging their circulation time and increasing haemocompatibility [20,21].

Scientists continue to improve the properties of dendrimers, for example, by attaching anticancer metals such as gold, ruthenium, or copper to their surfaces [7,22–26]. Such metallodendrimers have been intensively studied for anticancer therapy. Other strategies include anchoring active biomolecules e.g. polyphenols such as caffeic, ferulic, and gallic acids in the nanoparticle structure. These modifications can increase the bioavailability of the polyphenols and confer antioxidative, antibacterial and other biological activities on the dendrimers [27,28]. This can be crucial in some cases since oxidative stress contributes to the development of such conditions as Alzheimer's and Parkinson's diseases, diabetes, rheumatoid arthritis, cardiovascular diseases, and cancers [29,30]. Antioxidants are undoubtedly beneficial for human health and protect cells against injuries caused by oxidative stress [31].

The polyphenol caffeic acid, present at high levels in coffee, has good antioxidant properties [32]. Numerous studies have indicated that coffee benefits people with diabetes [33], obesity [34] and cognitive deficits [35], suggesting that it contains physiologically active substances and could have anticancer effects. Coffee is reportedly beneficial in treating gastric [36], colorectal [37] and oral [38] cancers, and melanoma [39,40], possibly because it contains polyphenols and especially caffeic acid [41]. Apart from its antioxidant and anticancer properties, caffeic acid also has anticoagulant, antihypertensive, antifibrotic, and antiviral activities [42,43].

In order to combine these medical properties of caffeic acid with the drug delivery properties of dendrimers, a new class of polyphenolic dendrimers has been synthesized [44]. The present study investigates these cationic dendrimers, which comprise a carbosilane core functionalized with caffeic acid moieties. The manuscript focuses mainly on analyzing their antioxidant and antiradical properties. Their biophysical characterization, haemotoxicity and cytotoxicity are also considered. The scheme of the research is outlined in Fig. 1.

2. Material and methods

2.1. Synthesis and chemical characterization

All reactions took place under an inert atmosphere and the solvents used were bought in dry conditions. NMR spectra were recorded in a Varian 500 Hz spectrometer using $CDCl_3$ and CD_3OD as solvents. Chemical shifts (δ) are given in ppm. A LECO CHNS-932 instrument was used for all elemental analyses. The reagents HS-PEG-NH_2·HCl, 2,2-dimethoxy-2-phenylaceto-phenone (DMPA), HS-$(CH_2)_2NH_3Cl$, HS-$(CH_2)_2N(CH_3)_2$HCl, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI·HCl), 1-Hydroxybenzotriazole hydrate (HOBt), $NaBH_4$, Amberlite IRA-Cl and caffeic acid were purchased from sigma addrich.

The supporting material details the synthesis and characterization of dendrimers **II**, **III**, and **1**–**10**. We describe the polyphenolic derivatives here.

2.1.1. Synthesis of G_2-[(S-$(CH_2)_2$NH(CO)CH=CHCH$_2$Ph(OH)$_2$)(S-N$(CH_3)_2)_7$] (11)

First, caffeic acid (96.8 mg, 0.537 mM) was activated with EDCI-HCl (102.73 mg, 0.537 mM) and HOBt (75.3 mg, 0.537 mM) using dry Dimethylformamide (DMF) as solvent. The mixture was stirred for 1 h at room temperature, then a DMF solution of the dendrimer G_2-[(S-NH_2)(S-N$(CH_3)_2)_7$] (**7**) (375.8 mg, 0.268 mM) was added dropwise at 0 °C with stirring. The mixture was maintained in this condition for 5 min, incubated at 60 °C overnight, and then treated with Na_2CO_3 (243.6 mg, 2.25 mM) for 3 h, filtered, and purified by size exclusion chromatography in DMF. The solvent was eliminated under vacuum, leaving compound **11** as an orange oil (267.81 mg, 59.3%).

^{1}H NMR (CD_3OD): δ (ppm) 0.06 (s, 12H, SiC*H*$_3$), 0.64 (m, 8H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.70 (m, 8H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.93 (m, 16H, SiC*H*$_2$CH$_2$S), 1.40 (m, 8H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.27 (s, 42H, NC*H*$_3$), 2.55 (m, 14H, SCH$_2$C*H*$_2$NCH$_3$), 2.57–2.67 (m, overlapping of signals, 28H, SiCH$_2$C*H*$_2$S and SC*H*$_2$CH$_2$NCH$_3$), 2.71 (m, 2H, SC*H*$_2$CH$_2$NH), 3.47 (m, 2H, SCH$_2$C*H*$_2$NH), 6.36 (d, 1H, $^3J_{(H-H)}$ = 15.7 Hz, PhC*H*=CH(*CO*)NH), 6.76 (d, 1H, $^3J_{(H-H)}$ = 8.1 Hz, 1H$_{Ar}$, *meta-CH=CH*), 6.90 (d, 1H, $^3J_{(H-H)}$ = 8.2 Hz, 1H$_{Ar}$, *orto-CH=CH*), 7.01 (s, 1H, 1H$_{Ar}$, *orto-CH=CH, orto-OH*), 7.41 (d, 1H, $^3J_{(H-H)}$ = 15.7 Hz, PhCH=C*H*(*CO*)NH). **^{13}C {^{1}H}-NMR (CD_3OD):** δ (ppm) -6.2 SiCH$_3$), 14.3 (SiCH$_2$CH$_2$S), 17.2 (SiCH$_2$CH$_2$CH$_2$Si), 18.5 (SiCH$_2$CH$_2$CH$_2$Si), 27.2 (SiCH$_2$CH$_2$S), 28.4 (SCH$_2$CH$_2$NCH$_3$), 30.7 (SCH$_2$CH$_2$NH), 39.2 (SCH$_2$CH$_2$NH), 44.0 (NCH$_3$), 58.9 (SiCH$_2$CH$_2$NCH$_3$), 113.7 (CAr, *orto-OH, orto-CH=CH*), 115.1 (CAr, *meta-CH=CH*), 116.9 (PhCH=CH(*CO*)NH), 120.8, (CAr, *orto-CH=CH*), 141.0 (PhCH=CH(*CO*)NH), not observed (Cipso), not observed (NHC=O). **Elemental Analysis (%):** Calc for $C_{71}H_{150}N_8O_3S_8Si_5$ (1560.94 g/mol). C, 54.63; H, 9.69; N, 7.18. Exp.: C, 54.51; H, 8.727; N, 7.328;

2.1.2. Synthesis of G_2-[(S-$(CH_2)_2$NH(CO)CH=CHCH$_2$Ph(OH)$_2)_2$(S-N$(CH_3)_2)_6$] (12)

Dendrimer **12** was prepared by the same method as **11** using the following reagents: caffeic acid (104.8 mg, 0.546 mM), EDCI·HCl (105.5 mg, 0.545 mM), HOBt (72.6 mg, 0.545 mM), G_2-[(S-NH_2)(S-N$(CH_3)_2)_7$] (**8**) (187.0 mg, 0.136 mM) and Na_2CO_3 (86.74 mg, 0.818 mM). Compound **12** was obtained as an orange oil (138.7 mg, 68%).

^{1}H NMR (CD_3OD): δ (ppm) 0.06 (s, 12H, SiC*H*$_3$), 0.66 (m, 8H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.72 (m, 8H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.95 (m, 16H, SiC*H*$_2$CH$_2$S), 1.42 (m, 8H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.28 (s, 36H, NC*H*$_3$), 2.55 (m, 12H, SC*H*$_2$CH$_2$NCH$_3$), 2.58–2.69 (m, overlapping of signals, 32H, SiCH$_2$C*H*$_2$S, SCH$_2$C*H*$_2$NCH$_3$ and SC*H*$_2$CH$_2$NH), 3.47 (m, 4H, OCH$_2$C*H*$_2$NH), 6.41 (d, 2H, $^3J_{(H-H)}$ = 15.7 Hz, PhC*H*=CH(*CO*)NH), 6.76 (d, 2H, $^3J_{(H-H)}$ = 8.1 Hz, H$_{Ar}$, *meta-CH=CH*), 6.90 (d, 2H, $^3J_{(H-H)}$ = 8.2

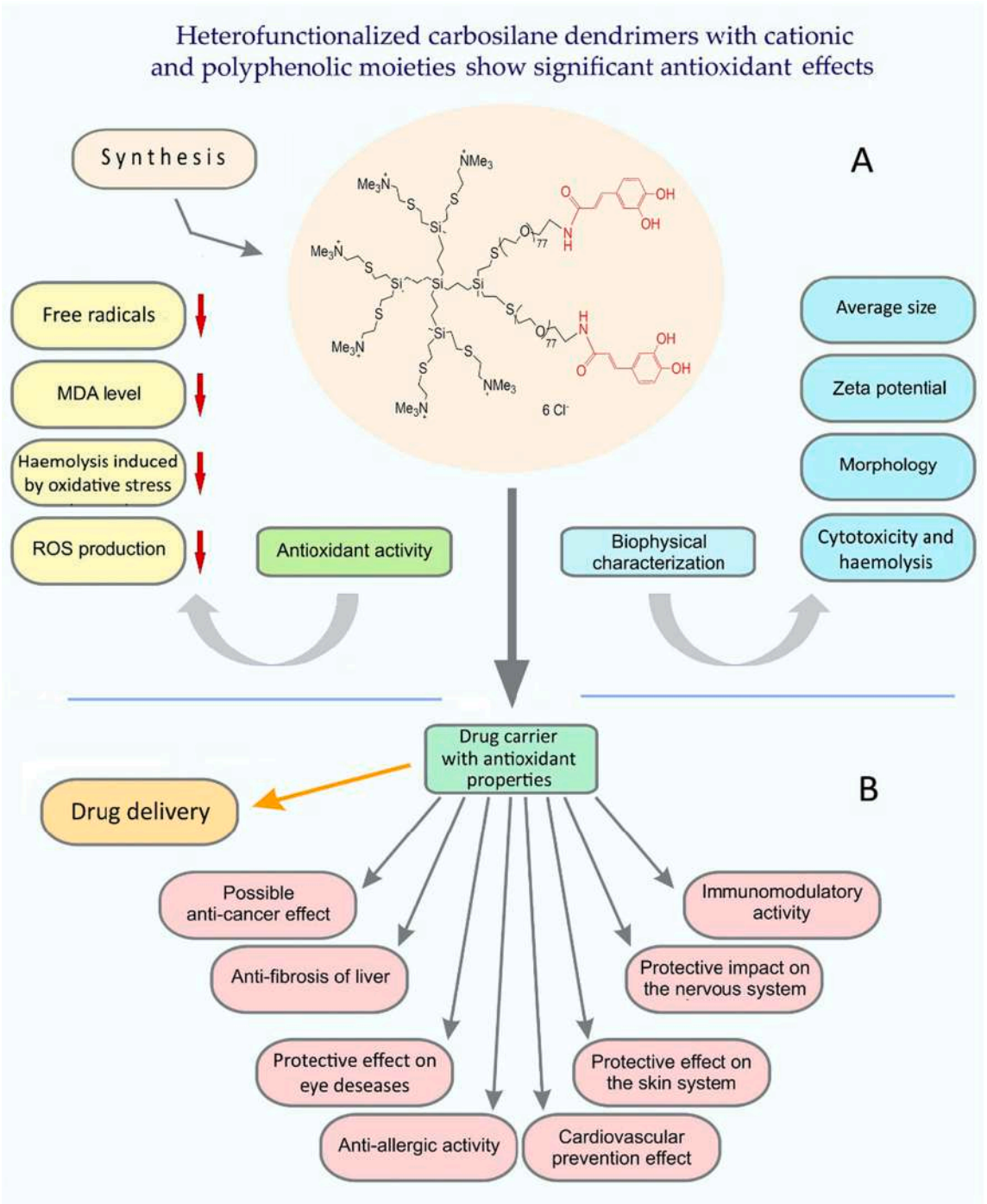


Fig. 1. (A) Schematic drawing of the research. Antioxidant effects (left) and biophysical properties (right) of polyphenolic dendrimers considered in this study. (B) Possible (expected) protective effects against various injuries to body systems resulting from antioxidant activity.

Hz, H_{Ar}, *orto-CH=CH*), 7.01 (s, 2H, H_{Ar}, *orto-CH=CH, orto-OH*), 7.41 (d, 2H, $^{3}J_{(H-H)}$ = 15.7 Hz, PhCH=C*H*(*CO*)NH). **^{13}C {^{1}H}-NMR (CD_3OD):** δ (ppm) -6.1 Si*C*H_3), 14.3 (Si*C*$H_2CH_2CH_2Si$), 17.1 (Si*C*$H_2CH_2CH_2Si$), 18.0 ($SiCH_2CH_2CH_2Si$), 18.3 ($SiCH_2CH_2CH_2Si$), 27.2 ($SiCH_2CH_2S$), 28.4 ($SCH_2CH_2NCH_3$), 30.7 (SCH_2CH_2NH), 39.2 (SCH_2CH_2NH), 44.0 (NCH_3), 58.9 ($SCH_2CH_2NCH_3$), 113.7 (CAr, *orto-OH, orto-CH=CH*), 115.1 (CAr, *meta-CH=CH*), 116.9 (PhCH=CH(*CO*)NH), 120.8, (CAr, *orto-CH=CH*), 126.8 (Cipso, *meta-OH, para-OH*), 141.0 (PhCH=CH(*CO*)NH), not observed (NHC=O). **Elemental Analysis (%):** Calc for $C_{78}H_{152}N_8O_6S_8Si_5$ (1695.03 g/mol). C, 55.27; H, 9.04; N, 6.61. Exp.: C, 54.58; H, 8.85; N, 6.542.

2.1.3. Synthesis of G_2-[(S-PEG-NH(CO)CH=CHCH$_2$Ph(OH)$_2$)(S-N(CH$_3$)$_2$)$_7$] (13)

Dendrimer **13** was prepared by the same method as **11** using the following reagents: caffeic acid (19.09 mg, 0.106 mM), EDCI·HCl (20.3 mg, 0.106 mM), HOBt (14.11 mg, 0.106 mM), G_2-[(S-PEG-NH_2)(S-N$(CH_3)_2)_7$] (**9**) (253.6 mg, 0.053 mM), Na_2CO_3 (47.19 mg, 0.445 mM). Compound **13** was obtained an orange oil (123.2 mg, 45%).

^{1}H NMR (CD_3OD):δ (ppm) 0.08 (s, 12H, SiCH_3), 0.65 (m, 8H, $SiCH_2CH_2CH_2Si$), 0.71 (m, 8H, $SiCH_2CH_2CH_2Si$), 0.96 (m, 16H, $SiCH_2CH_2S$), 1.42 (m, 8H, $SiCH_2CH_2CH_2Si$), 2.29 (s, 42H, NCH_3), 2.55 (m, 14H, $SCH_2CH_2NCH_3$), 2.59–2.75 (m, overlapping of signals, 32H, $SiCH_2CH_2S$, $SCH_2CH_2NCH_3$ and S-CH_2CH_2O), 3.48 (m, 2H,

OCH_2CH_2NH), 3.58–3.69 (m, 308H, OCH_2), 6.42 (d, 1H, $^3J_{(H—H)} = 15.7$ Hz, PhC*H*=CH(*CO*)NH), 6.77 (d, 1H, $^3J_{(H—H)} = 8.2$ Hz, $1H_{Ar}$, *meta-CH=CH*), 6.92 (dd, 1H, $^3J_{(H—H)} = 8.2$ Hz, $^5J_{(H—H)} = 2.1$ Hz, $1H_{Ar}$, *ortho-CH=CH*), 7.02 (d, 1H, $^5J_{(H—H)} = 2.1$ Hz, $2H_{Ar}$, *ortho-CH=CH, ortho-OH*), 7.40 (d, 1H, $^3J_{(H—H)} = 15.7$ Hz, PhCH=C*H*(*CO*)NH). **^{13}C {^{1}H}-NMR (CD_3OD):** *δ* (ppm) -6.4 $SiCH_3$), 14.3 ($SiCH_2CH_2S$), 16.9 ($SiCH_2CH_2CH_2Si$), 18.0 ($SiCH_2CH_2CH_2Si$), 27.1 ($SiCH_2CH_2S$), 28.4 ($SCH_2CH_2NCH_3$ and SCH_2CH_2O), 30.7 (SCH_2CH_2NH), 39.2 (OCH_2CH_2NH), 44.0 (NCH_3), 58.9 ($SiCH_2CH_2NCH_3$), 70.4 (**O**CH_2), 113.6 (CAr, *orto-OH, orto-CH=CH*), 115.1 (CAr, *meta-CH=CH*), 117.1 (PhCH=CH(*CO*)NH), 120.8, (CAr, *orto-CH=CH*), 140.8 (PhCH=CH(*CO*)NH), not observed (Cipso), not observed (NH*C*=O). **Elemental Analysis (%):** Calc for $C_{225}H_{458}N_8O_{80}S_8Si_5$ (4953.02 g/mol). C, 54.56; H, 9.32; N, 2.26; S, 5.18. Exp.: C, 54.51; H, 8.727; N, 2.223; S, 5.631.

2.1.4. Synthesis of G_2-[(S-PEG-NH(CO)CH=CHCH$_2$Ph(OH)$_2$)$_2$(S-N(CH$_3$)$_2$)$_6$] (14)

Dendrimer **14** was again prepared by the same method as **11** using the following reagents: caffeic acid (33.73 mg, 0.187 mM), EDCI·HCl (35.81 mg, 0.187 mM), HOBt (24.92 mg, 0.187 mM), G_2-[(S-PEG-$NH_2)_2$(S-N$(CH_3)_2)_6$] (**10**) (381.13 mg, 0.046 mM) and Na_2CO_3 (35.72 mg, 0.337 mM). Compound **13** was obtained as a brown solid (178.3 mg, 48%).

^{1}H NMR (CD_3OD):*δ* (ppm) 0.08 (s, 12H, $SiCH_3$), 0.66 (m, 8H, $SiCH_2CH_2CH_2Si$), 0.72 (m, 8H, $SiCH_2CH_2CH_2Si$), 0.95 (m, 16H, $SiCH_2CH_2S$), 1.42 (m, 8H, $SiCH_2CH_2CH_2Si$), 2.28 (s, 36H, NCH_3), 2.55 (m, 12H, $SCH_2CH_2NCH_3$), 2.60–2.75 (m, overlapping of signals, 32H, $SiCH_2CH_2S$, $SCH_2CH_2NCH_3$ and SCH_2CH_2O), 3.49 (m, 4H, O-CH_2CH_2NH), 3.55–3.71 (m, 616H, OCH_2), 6.41 (d, 2H, $^3J_{(H—H)} = 15.7$ Hz, PhCH=CH(*CO*)NH), 6.76 (d, 2H, $^3J_{(H—H)} = 8.2$ Hz, $1H_{Ar}$, *meta-CH=CH*), 6.90 (dd, 2H, $^3J_{(H—H)} = 8.2$ Hz, $^5J_{(H—H)} = 2.1$ Hz, $2H_{Ar}$, *ortho-CH=CH*), 7.01 (d, 2H, $^5J_{(H—H)} = 2.1$ Hz, $2H_{Ar}$, *ortho-CH=CH, ortho-OH*), 7.40 (d, 2H, $^3J_{(H—H)} = 15.7$ Hz, PhCH=C*H*(*CO*)NH). **^{13}C {^{1}H}-NMR (CD_3OD):** *δ* (ppm) -6.1 $SiCH_3$), 14.3 ($SiCH_2CH_2S$), 17.1 ($SiCH_2CH_2CH_2Si$), 17.9 ($SiCH_2CH_2CH_2Si$), 18.2 ($SiCH_2CH_2CH_2Si$), 27.0 ($SiCH_2CH_2S$), 28.4 ($SCH_2CH_2NCH_3$ and SCH_2CH_2O), 30.7 (SCH_2CH_2NH), 38.8 (OCH_2CH_2NH), 43.8 (NCH_3), 58.9 ($SiCH_2CH_2NCH_3$), 70.0 (**O**CH_2), 113.3 (CAr, *orto-OH, orto-CH=CH*), 115.9 (CAr, *meta-CH=CH*), 116.8 (PhCH=CH(*CO*)NH), 120.5, (CAr, *orto-CH=CH*), 139.7 (PhCH=CH(*CO*)NH), not observed (Cipso), not observed (NH*C*=O). **Elemental Analysis (%):** Calc for $C_{386}H_{768}N_8O_{160}S_8Si_5$ (8479.19 g/mol). C, 54.68; H, 9.13; N, 1.32; S, 3.02. Exp.: C, 54.58; H, 8.672; N, 1.54; S, 2.99.

2.1.5. Synthesis of G_2-[(S-(CH$_2$)$_2$NH(CO)CH=CHCH$_2$Ph(OH)$_2$)(S-N(CH$_3$)$_3$Cl)$_7$] (15)

Methyl iodide (MeI) (0.15 mL, 2.443 mM) was added over a tetrahydrofuran (THF) solution of dendrimer G_2-[(S-$(CH_2)_2$NH(*CO*)CH=CHCH$_2$Ph$(OH)_2)$(S-N$(CH_3)_2)_7$] (**11**) (453 mg, 0.290 mM) and stirred for 24 h. The volatiles were removed under vacuum, the remaining solid was dissolved in water, and the iodide ion was exchanged for chloride through amberlite IRA—Cl. After the solvents were evaporated the solid obtained was washed several times with Et_2O and dried under vacuum, leaving compound **15** as a brown solid in a moderate yield (376.2 mg; 67.6%).

^{1}H NMR (CD_3OD):*δ* (ppm) 0.12 (s, 12H, $SiCH_3$), 0.67 (m, overlapping signals, 16H, $SiCH_2CH_2CH_2Si$), 0.98 (m, 16H, $SiCH_2CH_2S$), 1.42 (m, 8H, $SiCH_2CH_2CH_2Si$), 2.74 (m, 8H, $SiCH_2CH_2S$), 3.00 (m, 16H, $SCH_2CH_2N^+CH_3$ and SCH_2CH_2NH), 3.22 (broad s, 63H, N^+CH_3), 3.53–3.83 (m, overlapping signals, 16H, $SCH_2CH_2N^+CH_3$ and SCH_2CH_2NH), 7.36 (broad m, 1H, PhC*H*=CH(*CO*)NH), 7.47–7.68 (C_6H_4)), 7.98 (broad m, 1H, PhCH=C*H*(*CO*)NH). **Elemental Analysis (%):** Calc for $C_{78}H_{171}Cl_7N_8O_3S_8Si_5$ (1914.33 g/mol). C, 48.94; H, 9.00; N, 5.85; Exp.: C, 44.64; H, 8.430; N, 5.191.

2.1.6. Synthesis of G_2-[(S-(CH$_2$)$_2$NH(CO)CH=CHCH$_2$Ph(OH)$_2$)$_2$(S-N(CH$_3$)Cl)$_6$] (16)

Dendrimer **16** was prepared by the same method as 15 using the following reagents: MeI (0.026 mL, 0.420 mM); G_2-[(S-$(CH_2)_2$NH(*CO*)CH=CHCH$_2$Ph$(OH)_2)_2$(S-N$(CH_3)_2)_6$] (**12**) (85.5 mg; 0.05 mM). Compound **16** was obtained as a yellow solid (62.83 mg, 62.9%).

^{1}H NMR (CD_3OD):*δ* (ppm) 0.12 (s, 12H, $SiCH_3$), 0.67 (m, overlapping signals, 16H, $SiCH_2CH_2CH_2Si$), 0.98 (m, 16H, $SiCH_2CH_2S$), 1.42 (m, 8H, $SiCH_2CH_2CH_2Si$), 2.74 (m, 8H, $SiCH_2CH_2S$), 3.00 (m, 16H, $SCH_2CH_2N^+CH_3$ and SCH_2CH_2NH), 3.22 (broad s, 54H, N^+CH_3), 3.53–3.83 (m, overlapping signals, 16H, $SCH_2CH_2N^+CH_3$ and SCH_2CH_2NH), 7.36 (broad m, 2H, PhC*H*=CH(*CO*)NH), 7.47–7.68 (C_6H_4)), 7.98 (broad m, 2H, PhCH=C*H*(*CO*)NH). **Elemental Analysis (%):** Calc for $C_{80}H_{166}Cl_6N_8O_8S_8Si_5$ (1977.86 g/mol). C, 48.58; H, 8.46; N, 5.67; Exp.: C, 43.82; H, 7.859; N, 6.562.

2.1.7. Synthesis of G_2-[(S-PEG-NH(CO)CH=CHCH$_2$Ph(OH)$_2$)(S-N(CH$_3$)$_3$Cl)$_7$] (17)

Dendrimer **17** was again prepared by the same method as **15** using the following reagents: MeI (0.027 mL, 0.444 mM); G_2-[(S-PEG-NH(*CO*)CH=CHCH$_2$Ph$(OH)_2$)(S-N$(CH_3)_2)_7$] (**13**) (262.5 mg; 0.053 mM). Compound **17** was obtained as a yellow solid (110 mg, 42.3%).

^{1}H NMR (CD_3OD):*δ* (ppm) 0.10 (s, 12H, $SiCH_3$), 0.65 (m, overlapping signals, 16H, $SiCH_2CH_2CH_2Si$), 0.96 (m, 16H, $SiCH_2CH_2S$), 1.42 (m, 8H, $SiCH_2CH_2CH_2Si$), 2.74 (m, 8H, $SiCH_2CH_2S$), 3.00 (m, 32H, $SCH_2CH_2N^+CH_3$, SCH_2CH_2NH and S-CH_2CH_2O), 3.22 (broad s, 63H, N^+CH_3), 3.53–3.83 (m, overlapping signals, 387H, N^+CH_3, $SCH_2CH_2N^+CH_3$, SCH_2CH_2NH, OCH_2), The signal corresponding to aromatic rings not appreciated **Elemental Analysis (%):** Calc for $C_{232}H_{479}Cl_7N_8O_{80}S_8Si_5$ (5306.42 g/mol). C, 52.51; H, 9.10; N, 2.11. Exp.: C,52.99; H,9.60; N, 2.45.

2.1.8. Synthesis of G_2-[(S-PEG-NH(CO)CH=CHCH$_2$Ph(OH)$_2$)$_2$(S-N(CH$_3$)$_3$Cl)$_6$] (18)

Dendrimer **18** was prepared by the same method using the following reagents: MeI (55.8 mg, 0.024 mL); G_2-[(S-PEG-NH(*CO*)CH=CHCH$_2$Ph$(OH)_2)_2$(S-N$(CH_3)_2)_6$] (14) (371.3 mg; 0.046 mM). Compound **18** was obtained as a pallid yellow solid (211.5 mg, 51.4%).

^{1}H NMR (CD_3OD):*δ* (ppm) 0.99 (s, 12H, $SiCH_3$), 0.66 (m, overlapping signals, 16H, $SiCH_2CH_2CH_2Si$), 0.96 (m, 16H, $SiCH_2CH_2S$), 1.42 (m, 8H, $SiCH_2CH_2CH_2Si$), 2.74 (m, 8H, $SiCH_2CH_2S$), 3.00 (m, 32H, $SCH_2CH_2N^+CH_3$, SCH_2CH_2NH and S-CH_2CH_2O), 3.22 (broad s, 54H, N^+CH_3), 3.53–3.83 (m, overlapping signals, 677H, N^+CH_3, $SCH_2CH_2N^+CH_3$, SCH_2CH_2NH, OCH_2), The signal corresponding to aromatic rings not appreciated). The signal corresponding to aromatic rings not appreciated. **Elemental Analysis (%):** Calc for $C_{392}H_{786}Cl_6N_8O_{160}S_8Si_5$ (8782.10 g/mol). C, 53.61; H, 9.02; N, 1.28. Exp.: C, 54.01; H,9.33; N,2.92.

2.2. Hydrodynamic diameter, zeta potential and transmission Electron microscopy

The hydrodynamic diameters of the polyphenolic dendrimers were measured by dynamic light-scattering (DLS) using a photon correlation spectrometer (Zetasizer Nano-ZS, Malvern Instruments, UK) in DTS0012 plastic cells (Malvern), in 10 mM Na-phosphate buffer (disodium hydrogen phosphate & monosodium phosphate, 4:1), pH 7.4 at 25 °C. Dendrimer concentration was 10 μM. To analyze the surface charge parameters, the zeta potential was measured in 10 mM Na-phosphate buffer (disodium hydrogen phosphate & monosodium phosphate, 4:1), pH 7.4 at 25 °C using the same Malvern spectrometer. The final dendrimer concentration in the samples was 10 μM. The zeta potential was calculated directly from the Helmholtz-Smoluchowski Eq. [45]. Malvern software was used for data analysis. Three separate experiments were conducted, each in seven replicates.

Dendrimer morphology and size were examined by transmission

electron microscopy (TEM). Dendrimer samples (10 μL), 1 mM in Na-phosphate buffer (PB), were placed on 200 mesh copper grids with a carbon surface, stained with 2% uranyl acetate for 20 min, washed with deionized water and dried at room temperature. Images were obtained using a JEOL1010 transmission electron microscope (JEOL, Tokyo, Japan).

2.3. Cell viability assay

To evaluate the cytotoxic effects of dendrimers, BJ (normal human fibroblasts) and A549 (cancer human alveolar basal epithelial cells) were used in RPMI 1640 and DMEM (Gibco), respectively, supplemented with 10% bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin, 1:1) at 37 °C in an atmosphere of 5% CO_2 and 95% humidity. The Colorimetric cell viability assay (MTT) assay was used to determine percentage cell viability. Cells were seeded in a 96-well plate (10,000 cells/well) and incubated (24 h, 37 °C, 5% CO_2), and dendrimers (12.5–100 μM) were added to the wells and incubated for 24 h. MTT (0.5 mg/mL per well) was added for 2 h, and then 100 μL DMSO was added to each well to dissolve the formazan crystals. Sample absorbance was measured at $\lambda = 580$ nm (background correction at 720 nm) using a multiwell plate reader (BioTek PowerWave HT, BioTek Instruments, Inc. Winooski, VT, USA). The percentage cell viability was calculated by the formula:

$$\text{Viability } [\%] = \frac{A_{\text{sample}} \times 100}{A_{\text{control}}}$$

2.4. Oxidative stress: Antioxidant and antiradical activity

2.4.1. Free radical scavenging activity

The free radical scavenging activity of the dendrimers was measured using the free radical DPPH (2,2′-diphenyl-1-picrylhydrazyl); antioxidants change the colour of a DPPH solution from purple to yellow. Aliquots of 0.25 mM DPPH in ethanol (180 μL) were mixed with (20 μL) dendrimer solutions, to the final concentrations of dendrimers 12.5–100 μM and incubated in the dark at room temperature for 30 min. The absorbance was measured at $\lambda = 517$ nm using a Jasco V-650 spectrophotometer. The scavenging activity was determined from the percentage decrease in DPPH absorbance according to the formula:

$$\text{DPPH}_{\text{inhibition}} [\%] = 100(A_0 - A_P)/A_0.$$

where A_0 is the absorbance of the DPPH solution and A_P is the absorbance of DPPH samples after incubation with dendrimers.

2.4.2. Ferric Reducing Antioxidant Power (FRAP) assay presented as Trolox Equivalent Antioxidant Capacity (TEAC)

Antioxidant activity was also measured by the reduction of Fe^{3+} to Fe^{2+} in the presence of TPTZ (2,4,6-tripyridyl-striazine), forming an intense blue Fe^{+2}–TPTZ complex with maximum absorption at 593 nm. Aliquots of FRAP solution (180 μL) were placed in 96 well plates and 20 μL of 10–100 μM dendrimer in methanol was added. After incubation in the dark at room temperature for 30 min, the absorbance at $\lambda = 517$ nm was measured using a microplate reader (EpochTM, BioTek Instruments, Winooski, VT, USA). The antioxidant activity of FRAP was expressed as TEAC (Trolox Equivalent Antioxidant Capacity) using the standard curve (Figure Si.22) prepared with a methanol solution of Trolox at concentrations ranging from 10 to 100 μM. The results are presented as μM trolox/μM compound. All tests were performed in triplicate and methanol was used as control.

2.4.3. Lipid peroxidation

The level of lipid peroxidation in erythrocyte membranes in the presence of polyphenolic dendrimers was determined using the fluorescent probe 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-un-decanoic acid (BODIPY581/591). Lipid peroxidation was induced by adding α,α'-Azodiisobutyramidine dihydrochloride (AAPH) to a suspension of human erythrocyte membranes. Red blood cells were haemolysed in PB and centrifuged at 15,000 ×*g* for 15 min at 4 °C. The membranes were washed several times with dilute Na-phosphate buffer and incubated for 60 min with 12.5–100 μM dendrimers and 50 mM AAPH, with gentle vortexing. BODIPY581/591 (2.5 μM) was added to the samples. Fluorescence was measured at excitation $\lambda = 485$ nm and emission $\lambda = 530$ nm wavelengths using a multiwell plate reader (BioTek PowerWave HT, BioTek Instruments, Inc. Winooski, VT, USA). The results were calculated as:

$$\text{Lipid peroxidation } [\%] = \frac{A_s \times 100}{A_{0.}}$$

Where: A_0 is fluorescence of the sample incubated with AAPH; A_s fluorescence of samples with AAPH and dendrimers.

2.4.4. AAPH-induced haemolysis

Erythrocytes isolated as described above (hematocrit 7%) were incubated with PBS (control) and preincubated with dendrimers at 12.5–100 μM and 50 mM AAPH in PBS. The mixtures were gently shaken and incubated for 3 h at 37 °C. PBS was added and the samples were centrifuged at 3000 rpm for 10 min at room temperature. The absorbance of the supernatant was determined at $\lambda = 535$ nm using a Jasco V-650 spectrophotometer. Reference values were measured using the same volume of erythrocytes with AAPH but without dendrimers. The percentage of AAPH-induced haemolysis was calculated from the formula:

$$\text{H } [\%] = \frac{A_{\text{sample}} \times 100}{A_{\text{control positive}}}$$

2.4.5. Inhibition of cellular reactive oxygen species (ROS) production

The ability to decrease the level of cellular reactive oxygen species (ROS) was tested using BJ cells seeded on black plates and treated with 12.5–100 μM dendrimers. Oxidative stress and ROS production were induced by adding 50 mM AAPH. To measure the ROS level, non-fluorescent 2′,7′-dichlorofluorescindiacetate (H_2DCF-DA) (10 μM) was added. Under oxidative stress, H_2DCF-DA is converted to highly fluorescent 2′,7′-dichlorofluorescein (DCF). After 30 min. Incubation, DCF fluorescence was measured at $\lambda_{exc} = 485$ nm and $\lambda_{em} = 530$ nm in a multiwell plate reader (BioTek PowerWave HT, BioTek Instruments, Inc. Winooski, VT, USA).

Confocal microscopy was used to visualize the ability of dendrimers to decrease the level of ROS in BJ cells. Cells were seeded on labtec plates and treated with dendrimers at 50 μM. Oxidative stress was induced by adding 50 mM AAPH, followed by the probe H_2DCF-DA (10 μM). Microphotographs were taken at $\lambda_{em} = 490$ nm, $\lambda_{em} = 527$ nm using a confocal microscope (Leica TCS LSI, Leica Microsystems, Frankfurt, Germany) with a 63×/1.40 (HC PL APO CS2, Leica Microsystems) objective. Leica Application Suite X software (LAS X, Leica Microsystems, Frankfurt, Germany) was used to obtain and analyze the images.

2.5. Statistical analysis

A *t*-test was used to compare two groups of samples. For more than two groups the Kruskal-Wallis test was used.

3. Results

3.1. Synthesis and characterization

To prepare dendritic systems with a caffeic acid and $-NMe_3^+$ moieties on the surface, we used a random approach, starting from spherical carbosilane dendrimers with vinyl groups at the periphery non-soluble in water. The vinyl groups allowed primary amino groups to be introduced by thiol-ene click chemistry. These groups are necessary for

attaching caffeic acid to the dendritic surface by straightforward amidation (Fig. 2) [46].

In this work, heterofunctionalized dendrimers were obtained using two types of thiol derivative, which allowed the primary amino groups necessary for the subsequent amidation to be introduced. Following the methods in the literature [47], the reaction of vinyl dendrimer G2 (vinil) 8 (**I**) with one or two equivalents of (i) cysteamine hydrochloride HS $(CH_2)_2NH_3Cl$ or (ii) a commercial thiol HS-PEG-NH_2·HCl (MW = 3.5 kDa) generated the heterofunctionalized derivatives G_2[(vinyl)n (NH_2 . HCl)m] (n = 7, m = 1 (**II**); n = 6, m = 2 (**III**)) and G_2[(Vinyl)n(PEG-NH_2.HCl)m] (n = 7, m = 1 (**1**); n = 6, m = 2 (**2**)). ^{1}H NMR spectra confirmed the introduction of the new chain in each case. Compounds **I** and **II** showed the methylene group resonances of the new chain Si $(CH_2)_2$S at δ ca. 2.57 for the single bond to the sulphur atom, and 0.99 ppm for the methylene group next to the silicon atom, respectively. They also showed the resonances of polyethylene glycol methylene groups at 3.62 ppm (Fig. 3). Likewise, ^{1}H NMR was used to determine the number of amino groups in the dendritic system. For this, the integral relationship between the signals corresponding to the methylene group bonded to the sulphur atom (Si$(CH_2)_2$S) of the newly-introduced chain and the resonances of the vinyl groups was used [47]. The remaining vinyl groups were functionalized with an excess of the thiol HS $(CH_2)_2NMe_2$·HCl, producing the systems G_2[(NMe_2•HCl)n(NH_2•HCl)m] (n = 7, m = 1 (**3**); n = 6, m = 2 (**4**)) and G2[(NMe_2HCl)n(PEG-NH_2.HCl)m] (n = 7, m = 1 (**5**); n = 6, m = 2 (**6**)). Again, ^{1}H NMR spectra confirmed the total functionalization of the remaining vinyl groups by the disappearance of their corresponding resonances and the appearance of a new signal at δ ca. 2.91 belonging to the methyl groups bound to the nitrogen of the new chain. The neutralization of cationic compounds **3**–**6** with Na_2CO_3 allowed the corresponding dendritic derivatives with amino group G_2[(NMe_2)n(NH_2)m] ((n = 7, m = 1 (**7**); n = 6, m = 2 (**8**)) and G2[(NMe_2)n(PEG-NH_2)m] (n = 7, m = 1 (**9**); n = 6, m = 2 (**10**)) to be obtained. Neutralization of the amino groups was corroborated by ^{1}H NMR: the signal corresponding to methyl groups bound to the nitrogen atom was displaced from 2.91 to 2.21 ppm. Once obtained compounds **7**–**10** with two different terminal amine groups, the formation of an amide bond by condensation of a carboxylic acid present in caffeic acid and an primary amine located in the dendritic branched using N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDCI·HCl) and 1-hydroxybenzotriazole (HOBt) as coupling reagents, which prevent the acid-base reaction and makes the carboxylic acid susceptible to a nucleophilic attack, allowing the obtention of the heterofunctionalized dendrimers G_2[(NMe_2)n(NH-CA)m] (n = 7, m = 1 (**11**); n = 6, m = 2 (**12**)) and G_2[(NMe_2)n(PEG-NH-CA)m] (n = 7, m = 1 (**13**); n = 6, m = 2 (**14**)). Amide formation was confirmed by ^{1}H and ^{13}C NMR; the spectra showed the displacement of the methylene group bound to the amide fragment from 2.99 (-C$H_2$$NH_2$) to 3.40 ppm (-C(*O*)NHCH_2) in ^{1}H NMR and from 28.2 (-*C*$H_2$$NH_2$) to 39 ppm (-C(*O*)NH*C*H_2-) in ^{13}C NMR. Furthermore, signals assigned to the alkene fragment at 6.45 and 7.42 ppm in ^{1}H NMR and 118.5 and 142.2 ppm in ^{13}C NMR were observed, along with a set of signals belonging to aromatic protons at 6.80, 6.95, and 7.05 ppm in ^{1}H NMR and between 115.0 and 148.9 ppm in ^{13}C NMR (Figs. 4 and 5). Finally, the amino groups present in the dendritic structure of compounds **11**–**14** were quaternized with methyl iodide in order to obtain water soluble macromolecules. Afterwards, iodide ions were exchanged for chloride through amberlite IRA-Cl and the reaction mixtures were purified by size exclusion chromatography allowing polyphenolic carbosilane dendrimers G_2[(NMe_3Cl)n(NH-CA)m] (n = 7, m = 1 (**15**); n = 6, m = 2 (**16**)) and G_2[(NMe_3Cl)n(PEG-NH-CA)m] (n = 7, m = 1 (**17**); n = 6, m = 2 (**18**)) to be obtained as brown solids in moderate yields and soluble in water. The ^{1}H and ^{13}C NMR spectra of ionic heterofunzionalized dendrimers **15**–**18** showed

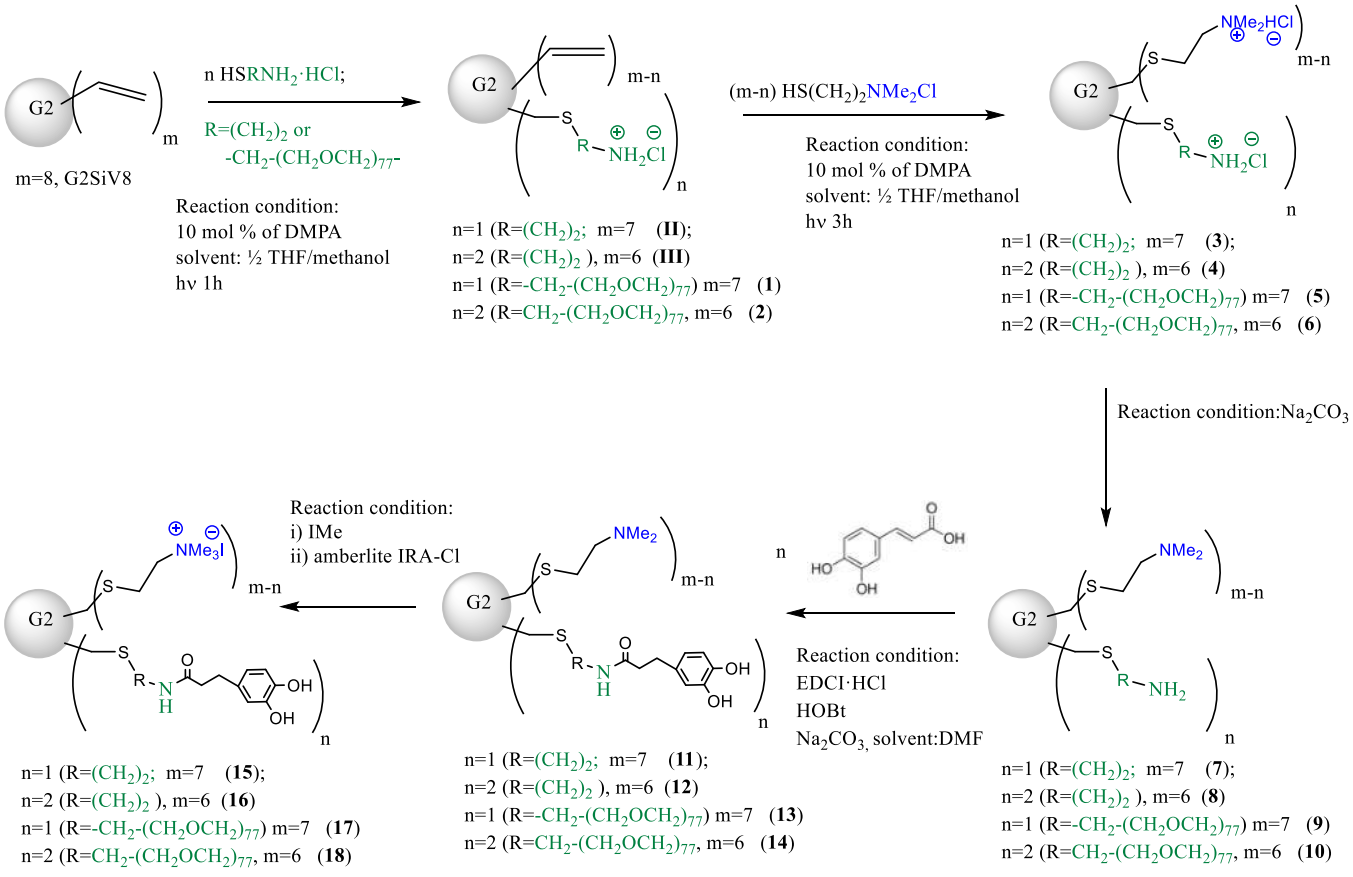


Fig. 2. Schematic representation of the synthetic steps of polyphenolic dendrimers.

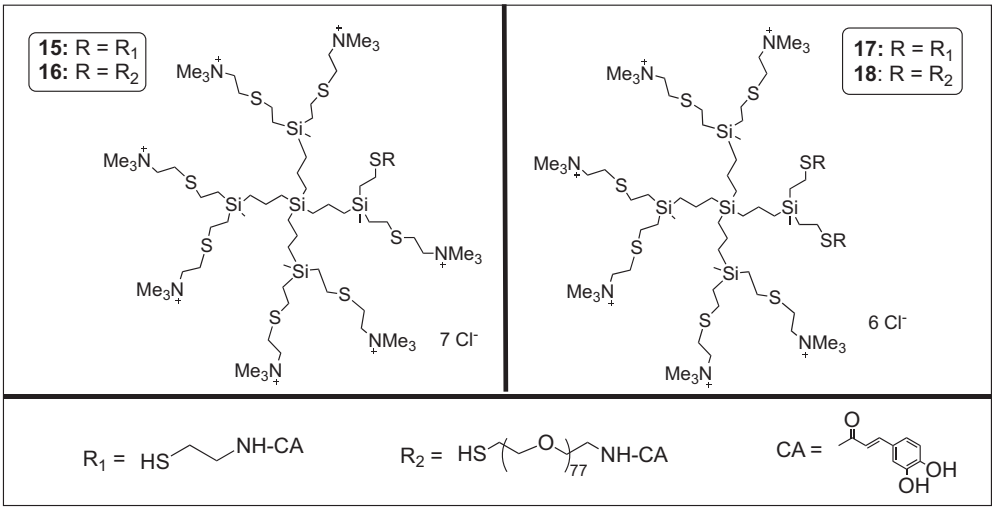


Fig. 3. Proposed structure of heterofunctionalized polyphenolic carbosilane dendrimers.

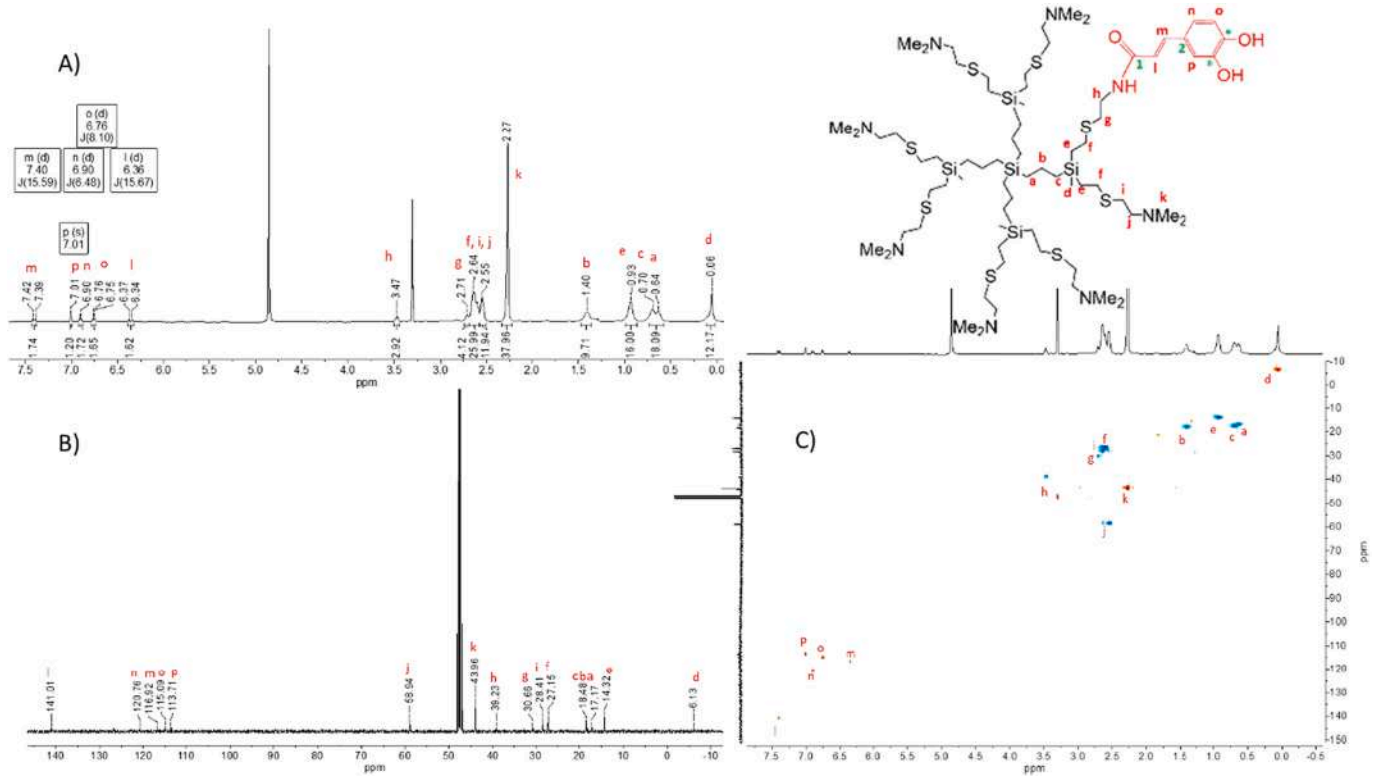


Fig. 4. NMR spectra in CD_3OD of compound $G_2[(NMe_2)_7(NH\text{-}CA)]$ (**11**): (A) ^1H NMR, (B) ^{13}C NMR, and (C) {^1H–^{13}C}-HSQC-2D-NMR (500 MHz, CD_3OD) (d, doublet; dd, doublet of doublets).

resonance patterns identical to those of their neutral precursors **11**–**14** for the carbosilane framework; quaternization of the amine groups revealed a deshelling of about $\Delta d = 1$ ppm with respect to the neutral derivatives in the chemical shifts of the signal attributed to the outer chain $S(CH_2)_2N$ and the methyl group NMe_3 that appears at 3.00 ppm.

Please see Supplementary Material Information for more synthesis details, including figs. S1-S23.

3.2. Biophysical characterization

3.2.1. Average size, surface charge and polydispersity index

To characterize the new compounds, their mean diameter, zeta potential and polydispersity index were measured. The average dendrimer sizes are shown in Fig. 6A. The histograms show the percentage distribution of nanoparticle numbers, indicating their hydrodynamic diameter. The dendrimer **15** formulations ranged in diameter from 50 to 200 nm and other dendrimers from 100 to 800 nm. The average dendrimer sizes were: $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$ (**15**), 215.6 ± 8.6 nm; $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$ (**16**), 440.2 ± 20.0 nm; $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$ (**17**), 460.8 ± 23.3 nm; $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$ (**18**), 476.4 ± 12.9 nm. The polydispersity index (PDI), a parameter indicating nanoparticle heterogeneity, is shown in Fig. 6D. The PDIs of the polyphenolic dendrimers were: **15**: 0.45 ± 0.03, **16**: 0.6 ± 0.02, **17**: 0.46 ± 0.01, **18**:0.44 ± 0.01.

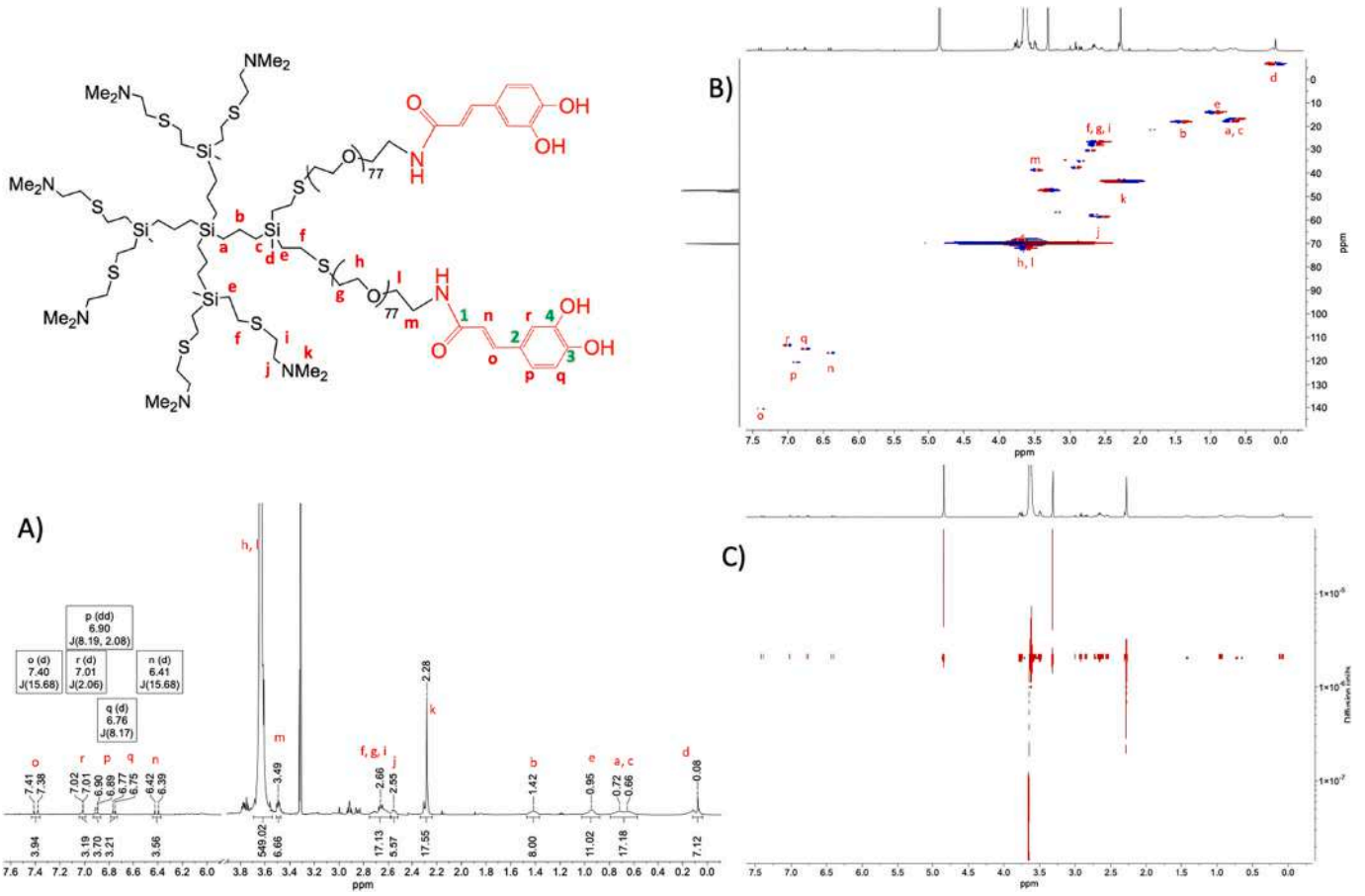


Fig. 5. NMR spectra in CD_3OD of compound $G_2[(NMe_2)_6(PEG\text{-}NH\text{-}CA)_2]$ (**14**): (A) ^1H NMR, (B) {^1H–^{13}C}-HSQC-2D-NMR, and (C) ^1H-DOSY-2D-NMR (d, doublet; dd, doublet of doublets).

The zeta potential values are presented in Fig. 6C. All nanoparticles were positively charged. The PEG-free dendrimers had the highest zeta potentials: **15**, 18.6 ± 0.3 mV and **16**, 21.1 ± 0.5 mV. Dendrimers with PEG anchored in their scaffolds had lower zeta potentials: **17**, 2.8 ± 0.1 mV and **18**, 2.9 ± 0.05 mV.

3.2.2. *Transmission Electron Microscopy (TEM)*

To characterize dendrimer morphology and size, TEM microimages were examined (Fig. 6B). Nanoparticles were visible as single dots and they were smaller than 5 nm. Images show that dendrimers with PEG (**17**, **18**) were bigger than PEG-free dendrimers (**15**, **16**). Substances with low electron density were observed between the nanoparticles. This could explain the big differences in DLS and TEM results. Perhaps dendrimers can form structures containing several molecules attached to each other, which are bigger than a single dendrimer molecule and were probably detected with the Zetasizer spectrometer.

3.3. *Dendrimer cytotoxicity*

The cytotoxic effect of polyphenol dendrimers towards BJ (normal) and A549 (cancer) cells was evaluated after 24 h incubation (Fig. 7). All dendrimers had relatively low cytotoxicity against both cell lines at 12.5–25 μM; the cytotoxic effect on BJ cells was more pronounced. For A549 cells, the most cytotoxic dendrimer was $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$ (**17**), which at 100 μM decreased cell viability up to 77% vs control. For BJ cells, dendrimer **17** had similar values at all concentrations tested, the most cytotoxic being PEG-free dendrimer **15** at 50–100 μM. Dendrimer $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$ (**18**) had similar tendency and decreased the viability of BJ cells up to 50–55% of control.

3.4. *Antioxidant activity*

3.4.1. *DPPH scavenging activity*

The ability of dendrimers to scavenge free radicals was studied using the DPPH free radical assay. Incubation of dendrimers with ethanolic solutions of DPPH significantly reduced its light absorbance in a concentration-dependent manner (Fig. 8A).

The effectiveness of dendrimers for free radical scavenging was compared with the antioxidants melatonin, ascorbic acid and caffeic acid. Melatonin had the lowest DPPH radical scavenging activity; caffeic acid was most effective. At the highest concentration, its efficiency reached 87.5 ± 1.1%. The effect of dendrimers was slightly lower than that of caffeic acid, but comparable. The dendrimers at 100 μM showed the highest antiradical activities: PD PEG-CC: 71.7 ± 5.1%, PD PEG—C: 73.9 ± 4.3%, PD-CC: 76.8 ± 5.5%, and PD-C: 78.0 ± 5.7%. The scavenging parameters of ascorbic acid and melatonin at the same concentration were 70.4 ± 16.9% and 14.5 ± 5.1%, respectively.

3.4.2. *FRAP assay*

This involves the use of a metal complex and can reflect the antioxidant potential of ligands through the reduction of ferric iron, Fe^{3+} to ferrous iron, Fe^{2+}. The Trolox equivalent antioxidant capacity (TEAC) of compounds was established for this assay. The TEAC assay compared the highest mean antioxidant capacity of compounds **15–18** with the standard antioxidant Trolox (Fig. 8B). Dendrimer **18,** with two PEG-caffeic acid moieties, showed the highest antioxidant effect, even higher than free caffeic acid. Other dendrimers were generally less effective, but comparable with caffeic acid. The FRAP assay results presented as Trolox equivalent antioxidant capacity were similar to the DPPH results;

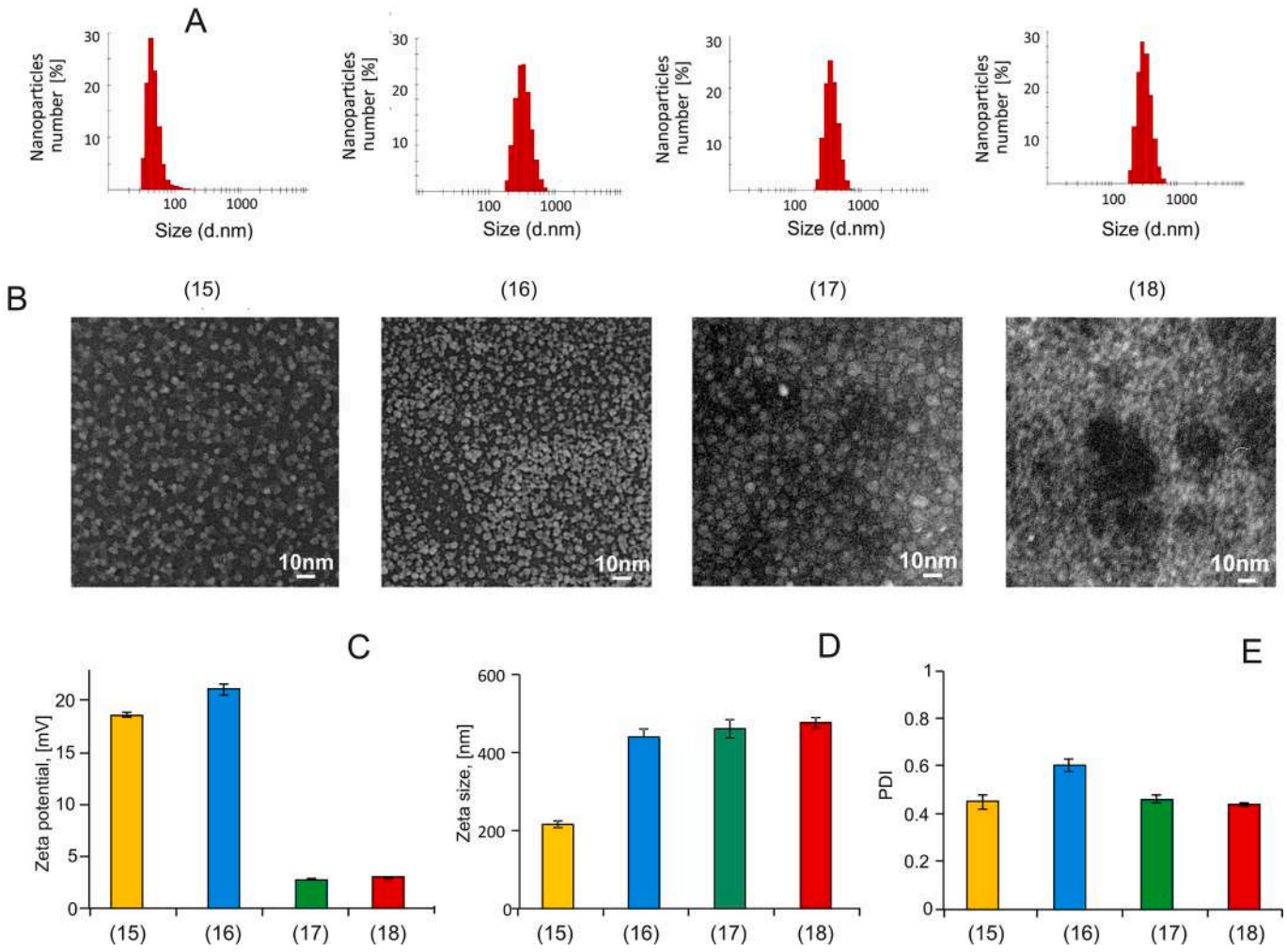


Fig. 6. (A) - Average hydrodynamic diameter of dendrimers: $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$ (**15**); $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$ (**16**); $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$ (**17**) and $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$ (**18**) shown as percentage of particle numbers; (B) - TEM images showing morphological characteristics of dendrimers. Samples at the concentration of 1 mM for TEM were dissolved in 10 mM Na-phosphate buffer, placed on copper girds with carbon surfaces and dried. Magnification x100,000; Bars = 10 nm. To obtain greater contrast the colours have been inverted. (C) - Zeta potential (D) – Zeta size and (E) -PDI index of 10 μM dendrimers in Na-phosphate buffer, pH 7.4. Bars represent mean ± SE of three separate experiments and each experiment was done in seven replicates.
TEM: transmission electron microscopy; **PDI**: polydispersity index; **SE**: standard errors.

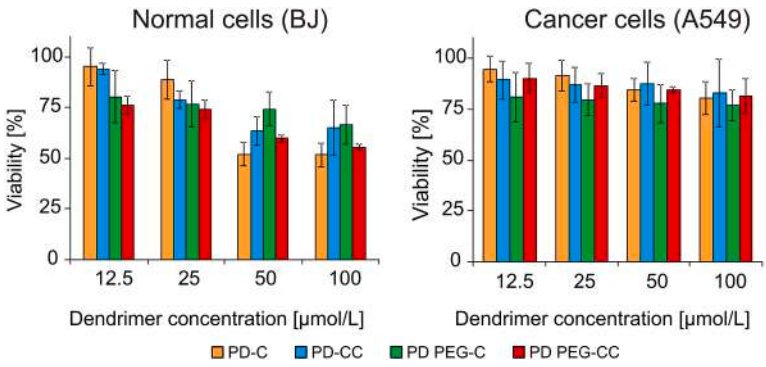


Fig. 7. Viability of BJ (left) and A549 (right) cells in the presence of polyphenolic dendrimers. PBS 10 mM, 10,000 cells/well, MTT 0.5 mg/mL per well, V = 0.2 mL, pH 7.4, incubation time 24 h, $T = 37$ °C, 5% CO_2. Dendrimer concentrations 12.5–100 μM. Values are expressed as mean ± SD ($n = 3$).
BJ: normal human fibroblast cell line; **A549**: human alveolar basal epithelial cancer cell line; **MTT**: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; **PBS**: phosphate buffered saline; **SD** standard deviation.

all the dendrimers studied showed significant antioxidant potential.

3.4.3. *Lipid peroxidation*

The effect of the dendrimers on the lipid peroxidation level was analyzed by the BODIPY581/591 fluorescence assay. BODIPY581/591 is widely used for measuring lipid peroxidation in various biological membranes. A higher BODIPY581/591 fluorescence intensity indicates more peroxidation. All dendrimers studied reduced the level of AAPH-induced lipid peroxidation; the effect was concentration-dependent (Fig. 8C). The most effective concentration was 100 μM. At 12.5 μM, dendrimers $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$ (**15**) and $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$ (**18**) conferred the best protection (reduction up to ~30%). In

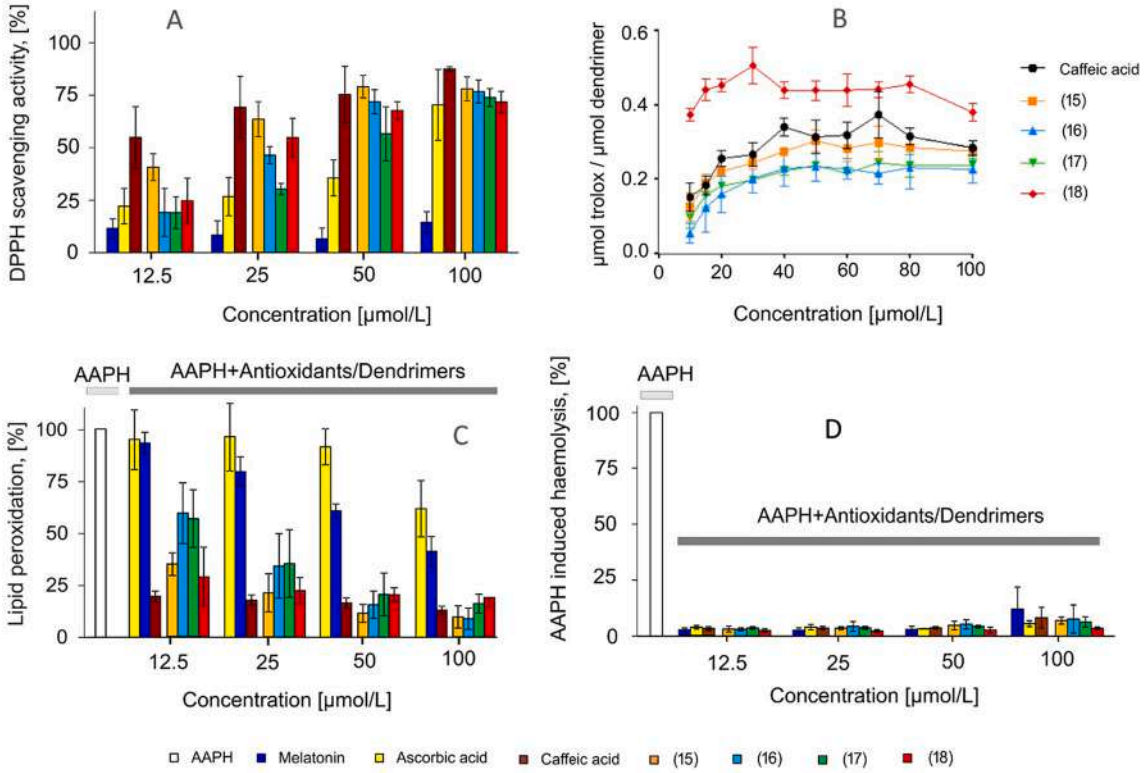


Fig. 8. (A) Percentage of DPPH free radical scavenging by the polyphenolic dendrimers over the concentration range of 12.5–100 μM. Incubation time 30 min. DPPH 0.25 mM, Values are expressed as mean ± SD ($n = 3$). (B) - Antioxidant behavior of dendrimers estimated by FRAP (Ferric Reducing Antioxidant Power**)** assay presented as Trolox Equivalent Antioxidant Capacity (TEAC). Values are expressed as mean ± SE (n = 3). (C) - Dendrimers suppress AAPH-induced lipid peroxidation in erythrocyte membranes. PBS 10 mM, pH 7.4, AAPH 50 mM, BODIPY 100 μM, incubation time 60 min, $T = 37$ °C. Values are expressed as mean ± SD (n = 3). (D) - Antihaemolytic effects of dendrimers on AAPH-induced oxidative haemolysis of human erythrocytes. Hematocrit 7%, PBS buffer 10 mM, pH 7.4, AAPH 50 mM, incubation time 3 h, T = 37 °C. Values are expressed as mean ± SD (n = 3). Dendrimer concentrations ranged from 12.5 to 100 μM. The effects of dendrimers were compared with those of caffeic acid and other antioxidants (except FRAP assay) such as melatonin or ascorbic acid at the same concentrations.
DPPH: 2,2-Diphenyl-1-picrylhydrazyl; **BODIPY®581/1591:** (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a, 4a-diaza-s-indacene-3-un-decanoic acid, **FRAP**: Ferric Reducing Antioxidant Power; **TEAC**: Trolox Equivalent Antioxidant Capacity; **AAPH**: 2,2′-azobis(2-amidinopropane) dihydrochloride; PBS: phosphate buffered saline; **SD:** standard deviation.

contrast, at higher concentrations (50–100 μM), dendrimers **15** and **18** provided the most effective protection (up to ~9–8%). Among antioxidants, the most effective was caffeic acid (100 μM), which decreased lipid peroxidation up to 13.1 + 4.96% vs control (AAPH). Melatonin or ascorbic acid, used as controls at 100 μM, showed weaker protective effects than the dendrimers or caffeic acid: 41.3 + 12.8% (melatonin) and 61.7 + 24.4% (ascorbic acid) vs. control.

3.4.4. AAPH-induced haemolysis

The ability of polyphenol dendrimers to protect against AAPH-induced haemolysis was studied. Incubation of human erythrocytes with 50 mM AAPH (oxidant) significantly increased haemolysis. Oxidative AAPH haemolysis reached about 70.7 ± 1.2% vs control after 3 h incubation.

Preincubation of the samples with dendrimers at 12.5–100 μM significantly decreased the level of AAPH-induced hemolysis. After 3 h preincubation at 12.5–50 μM, the haemolysis level decreased up to 5% vs the AAPH control. The slight increase in haemolysis (up to 14%) at 100 μM concentration can be explained by dendrimer haemotoxicity. The reduction of haemolysis by the compounds implies their ability to quench free radicals and increase the antioxidant capacity of erythrocytes, alleviating destructive oxidative haemolysis. The protective effect of dendrimers was comparable with those of ascorbic acid, caffeic acid and melatonin (Fig. 8D).

3.4.5. AAPH-induced ROS production

Since dendrimers demonstrated significant antioxidant and antiradical activities, we were interested in their ability to protect living cells against increased production of ROS under oxidative stress conditions. All the compounds tested at 50 μM and above inhibited ROS production by the water-soluble free radical generator AAPH in human fibroblasts. The dendrimers were more protective than classical antioxidants such as ascorbic acid or melatonin at the same concentrations (Fig. 9A). This can be explained by the low antioxidant concentrations; their action was not strong enough. On the other hand, caffeic acid caused the most pronounced ROS reduction, decreasing the ROS level up to 30% vs control at 12.5 μM and up to 3% at 25 μM.

Confocal microscopy was also used to visualize the ability of dendrimers to decrease ROS levels. Microimages of BJ cells (Fig. 9B) show decreased fluorescence intensity when dendrimers are present in the cell suspension. The effects of the dendrimers were compared with those of antioxidants. Melatonin (50 μM) did not change the fluorescence intensity of DCF much, indicating weak influence on the cellular ROS level; ascorbic acid had a more pronounced effect (Fig. 9B). Caffeic acid or dendrimers (50 μM) reduced the fluorescence intensity significantly, demonstrating decreased cellular ROS production under oxidative stress conditions.

4. Discussion

The use of dendritic systems in biomedicine is developing

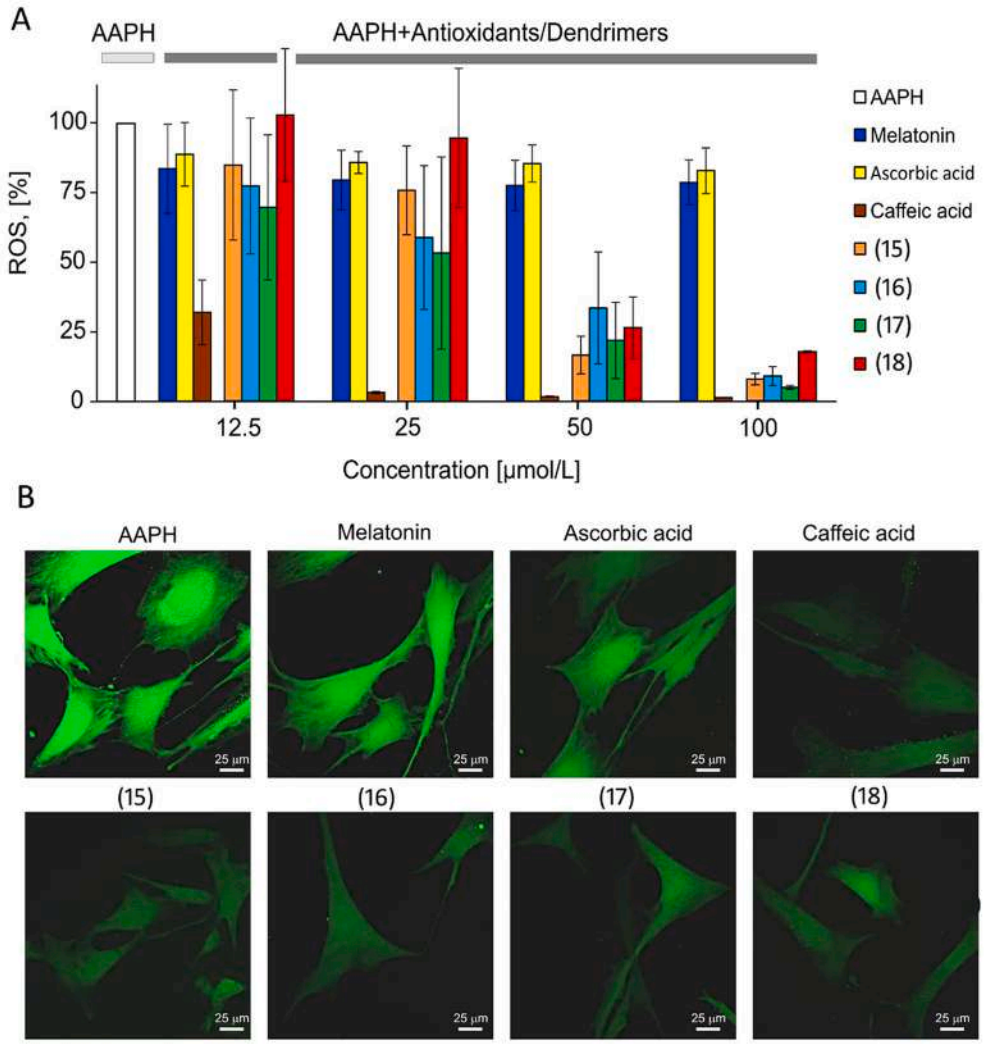


Fig. 9. Effect of polyphenolic dendrimers on AAPH-induced ROS production in BJ cells detected by fluorescence intensity (A) or confocal microscopy (B). PBS 10 mM, pH 7.4, AAPH 50 mM, H_2DCF-DA 10 μM, incubation time 30 min, $T = 37$ °C. Dendrimer concentrations 12.5–100 μM (fluorescence), 50 μM (confocal microscopy). The effects of dendrimers were compared with those of caffeic acid and other antioxidants such as melatonin or ascorbic acid at the same concentrations. Values are expressed as mean ± SD ($n = 3$).

continuously [48–53]. Functionalizing these systems, exploiting the multivalency of dendrimer skeletons using molecules with various therapeutic activities, has made it possible to reduce toxicity, increase bioavailability and improve activity. The properties of these nanoparticles can be improved for biomedical purposes. This improvement can take several directions, the first being to modify dendrimers with other active molecules such as metals [23,24,26,55,56] or polyethylene glycol [57–59] to improve their anticancer properties [23,24,56,60], reduce toxicity and increase bioavailability [24,59,61,62]. In another direction, the medical properties of dendrimers can be enhanced by conjugating them with natural biomolecules such as polyphenols [31,44,63].

Polyphenols are good natural antioxidants. Their physicochemical properties enable them to participate in various cellular redox-type metabolic reactions and prevent cells from being injured by ROS [44,64,65]. However, they are generally poorly absorbed by the intestines, rapidly metabolized, and quickly excreted. These factors limit their bioavailability [44,66].

To address these shortcomings, we have synthesized carbosilane dendritic systems containing one or two caffeic acid units, and ammonium groups on the surface to make them water soluble. The polyphenol is anchored to the dendritic skeleton by amidation, either on a dendritic branch functionalized with $HS(CH_2)_2NH_2$ (compounds **7–8**) or HS-PEG-NH_2 (MW = 3.5 kDa, compounds **9–10**). Cationic systems **15**–**18** were obtained by quaternization of the dimethylammonium groups on the remaining dendritic branches. Their structural characterization by one- and two-dimensional NMR and elemental analysis is consistent with their proposed structures. Considering their potential use as antioxidants, we have studied their antioxidant activity including their ability to protect living cells under oxidative stress conditions. We have also characterized their biophysical properties.

Their hydrodynamic diameter ranged from 200 to 500 nm depending on the dendrimer, but the sizes ranged widely from 10 to 1000 nm. This could indicate the tendency of dendrimers to form assembled structures. The size of other carbosilane dendrimers has been reported as 150 nm to 500 nm [7],. The PDI of the dendrimers, which indicates the uniformity and homogeneity of a nanoparticle distribution, was about 0.4, except for dendrimer **18,** which contains two caffeic acid moieties and PEG.

To confirm the results from DLS we examined the morphology of dendrimers **15**–**18** by TEM. They were visible as single dots less than 5–10 nm in diameter. Dendrimers are usually shown as aggregate structures on TEM images [68,69], probably because interaction between nanoparticles leading to larger structures. The discrepancy between DLS and TEM results has been described in more detail previously [4].

Another aim was to investigate the surface charge of the dendrimers studied. On the basis of zeta potential values, we inferred that all heterofunctionalized dendrimers were positively charged. However, dendrimers containing PEG had lower zeta potentials than PEG-free dendrimers, 3 mV (**17**–**18**) vs 20 mV (**15**–**16**). These results indicate that

PEG in a dendrimer scaffold decreases the net charge, with beneficial effects such as prolonged circulation time, lower cytotoxicity, reduced reactions with serum proteins, and stabilization of interactions with nucleic acids [6,20].

Toxicity profiles of potential therapeutic agents should be defined in vitro before their application in vivo. Therefore, the next step in this study was to evaluate dendrimer cytotoxicity. The cytotoxicity towards both cell lines tested (BJ and A549) was low. Similarly, other polyphenolic dendrimers showed no negative effect on PBM [70], HFF-1 [44] or CHO-K1 cells [71]. Dendrimers containing vanillin were cytotoxic against PC3 and HeLa cells [63]. It should be noted that the types of terminal end group in carbosilane dendrimers determined their toxicity towards different kinds of cells [60].

One of the most important findings of this study was that polyphenolic dendrimers have antioxidant activity. They were effective in scavenging free radicals, inhibiting AAPH-induced haemolysis and lipid peroxidation, and decreasing the ROS level. For the first time we investigated the ability of dendrimers to scavenge free radicals using the DPPH and FRAP assay. The addition of any of the dendrimers to an ethanol solution of the free radical was effective in free radical scavenging. Among the concentrations used, 100 μM had the most pronounced effect, having a scavenging effect of 7% or more. Similar results for other polyphenolic dendrimers had been demonstrated previously. For example, carbosilane dendrimers containing vanillin exhibited high antiradical potential [63]. The same effect was shown for dendritic polyphenol molecules [71] and polyphenol-based dendrimers [71]. Analysis of the antiradical activity in reaction with DPPH showed that PEG-free dendrimers showed weak change in antiradical activity. However, there was a visible difference for dendrimers containing PEG. A similar trend was observed in the FRAP assay. It has been shown that heterofunctionalized systems have lower activity than homofunctionalized ones [44]. Nevertheless, their activity is comparable to free caffeic acid, with the advantage of increasing the bioavailability of caffeic acid owing to the presence of ammonium groups on the surface, which confer high solubility in water.

The antioxidant properties of polyphenols have been presented many times [72,73]. Free radicals are produced during metabolic activity in cells, but if they reach high levels then cell structures can be damaged irreversibly [71].

We have also demonstrated that polyphenolic dendrimers with ammonium moieties on the surface inhibit AAPH-induced lipid peroxidation in erythrocyte membranes. The new dendritic systems could protect erythrocyte membranes against oxidative damage. AAPH increased the level of lipid peroxidation, but preincubation with dendrimers significantly inhibited this effect in a concentration-dependent manner; 50–100 μM concentrations showed most pronounced effect, almost equal to that of caffeic acid.

Since the newly synthesized systems had proved highly effective in free radical scavenging and inhibition of lipid peroxidation, we hypothesized that the dendrimers could protect human erythrocytes from induced oxidative stress. The anti-haemolytic effect of dendrimers on AAPH-induced oxidative haemolysis was investigated by preincubating erythrocytes with them. AAPH increases haemolysis by increasing intracellular free radical production [74,75]. The free radicals attack the erythrocyte membrane, altering the constituent lipids and proteins and inducing haemolysis.

Treating the erythrocytes with dendrimers before AAPH exposure drastically decreased the level of oxidative haemolysis. Other natural compounds such as catechins and polyphenols efficiently protect human erythrocytes against haemolysis induced by oxidative stress [76–78].

Evidence shows that increased haemolysis induced by oxidative stress is associated with activation of cellular ROS production [74]. Our results confirm this, showing an increased ROS level in human fibroblasts after treatment with AAPH. Treatment of BJ cells with polyphenolic dendrimers (50–100 μM) significantly decreased the intracellular level of ROS, protecting the cells against damage induced by oxidative stress. We previously reported a similar effect when BJ cells were treated with antioxidant plant extracts isolated from *Hippophae rhamnoides* L. and *Rosa canina* L. [79,80]. Both extracts were rich in natural antioxidants including polyphenols [79,80]. It has been documented that polyphenols decrease ROS levels in different kinds of cells such as dermal fibroblasts [81], PC12 [82], and kidney mesangial cells [83].

Summarizing, the dendrimers studied had low cytotoxicity and showed high antioxidant potential comparable with known antioxidants. Therefore, caffeic acid in their structure can quench free radicals, protecting the cells against oxidative stress.

5. Conclusions

This work has demonstrated that conjugation of polyphenols with cationic carbosilane dendrimers could be a promising way of harnessing the potential of these powerful antioxidants, significantly increasing their bioavailability. Therefore, a new water-soluble family of carbosilane dendrimers functionalized with caffeic acid and surface ammonium groups has been synthesized. The most important outcome is that these dendritic systems have low toxiciy and are highly effective against oxidative stress in vitro. Their antioxidant property could help to diminish oxidative haemolysis, lipid peroxidation, and ROS levels via their ability to scavenge free radicals. These findings are confirmed by various biological tests including in vitro studies on human fibroblasts, erythrocytes and A549 cells.

CRediT authorship contribution statement

Marika Grodzicka: Investigation, Data curation, Writing – original draft. **Cornelia E. Pena-Gonzalez:** Investigation, Data curation, Writing – original draft. **Paula Ortega:** Methodology, Validation, Supervision, Data curation, Writing – review & editing. **Sylwia Michlewska:** Visualization, Validation, Investigation, Writing – review & editing. **Rebeca Lozano:** Investigation, Software, Validation. **Maria Bryszewska:** Writing – review & editing. **Francisco Javier de la Mata:** Supervision, Conceptualization, Methodology, Writing – review & editing. **Maksim Ionov:** Conceptualization, Methodology, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This work was co-financed by the Project “EUROPARTNER” of Polish National Agency for Academic Exchange (NAWA) project No: PPI/APM/2018/1/00007/U/001 and partly granted by the Project “Np-Hale” under Beethoven Life 1 program of National Science Centre of Poland (NCN) project No: 2018/31/F/NZ5/03454. Grants from PID2020-112924RBI00 (MINECO), and project SBPLY/17/180501/000358 Junta de Comunidades de Castilla-La Mancha (JCCM), PID2019-104070RB-C22 (MICINN) consortiums IMMUNOTHERCAN-CM B2017/BMD-3733 and NANODENDMED II-CM ref. B2017/BMD-3703. CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008-2011, Iniciativa Ingenio 2010, the Consolider Program, and CIBER Actions and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.susmat.2022.e00497.

References

[1] Z. Lyu, L. Ding, A.Y.T. Huang, C.L. Kao, L. Peng, Poly(amidoamine)dendrimers: covalent and supramolecular synthesis, Mater. Today Chem. 13 (2019) 34–48, https://doi.org/10.1016/j.mtchem.2019.04.004.

[2] D.A. Tomalia, H. Baker, J. Dewald, M. Hall, G. Kallos, S. Martin, J. Roeck, J. Ryder, P. Smith, A new class of polymers: starburst-dendritic macromolecules, Polym. J. 17 (1985) 117–132, https://doi.org/10.1295/polymj.17.117.

[3] P. Pandi, A. Jain, N. Kommineni, M. Ionov, M. Bryszewska, W. Khan, Dendrimer as a new potential carrier for topical delivery of siRNA: A comparative study of dendriplex vs. lipoplex for delivery of TNF-α siRNA, Int. J. Pharm. 550 (2018) 240–250, https://doi.org/10.1016/j.ijpharm.2018.08.024.

[4] M. Ionov, K. Ciepluch, B. Klajnert, S. Glińska, R. Gomez-Ramirez, F.J. de la Mata, M.A. Munoz-Fernandez, M. Bryszewska, Complexation of HIV derived peptides with carbosilane dendrimers, Colloids Surf. B: Biointerfaces 101 (2013) 236–242, https://doi.org/10.1016/j.colsurfb.2012.07.011.

[5] P. Kesharwani, L. Xie, G. Mao, S. Padhye, A.K. Iyer, Hyaluronic acid-conjugated polyamidoamine dendrimers for targeted delivery of 3,4-difluorobenzylidene curcumin to CD44 overexpressing pancreatic cancer cells, Colloids Surf. B: Biointerfaces 136 (2015) 413–423, https://doi.org/10.1016/j.colsurfb.2015.09.043.

[6] S. Thakur, P. Kesharwani, R.K. Tekade, N.K. Jain, Impact of pegylation on biopharmaceutical properties of dendrimers, Polymer (Guildf). 59 (2015) 67–92, https://doi.org/10.1016/j.polymer.2014.12.051.

[7] S. Michlewska, M. Ionov, D. Shcharbin, M. Maroto-Díaz, R. Gomez Ramirez, F. Javier de la Mata, M. Bryszewska, Ruthenium metallodendrimers with anticancer potential in an acute promyelocytic leukemia cell line (HL60), Eur. Polym. J. 87 (2017) 39–47, https://doi.org/10.1016/j.eurpolymj.2016.12.011.

[8] S. Michlewska, M. Ionov, M. Maroto-Díaz, A. Szwed, A. Ihnatsyeu-Kachan, S. Loznikova, D. Shcharbin, M. Maly, R.G. Ramirez, F.J. de la Mata, M. Bryszewska, Ruthenium dendrimers as carriers for anticancer siRNA, J. Inorg. Biochem. 181 (2018), https://doi.org/10.1016/j.jinorgbio.2018.01.001.

[9] N.S. Del Olmo, M. Holota, S. Michlewska, R. Gómez, P. Ortega, M. Ionov, F.J. de la Mata, M. Bryszewska, Copper (II) metallodendrimers combined with pro-apoptotic sirnas as a promising strategy against breast cancer cells, Pharmaceutics. 12 (2020) 1–14, https://doi.org/10.3390/pharmaceutics12080727.

[10] E. Pedziwiatr-Werbicka, K. Milowska, V. Dzmitruk, M. Ionov, D. Shcharbin, M. Bryszewska, Dendrimers and hyperbranched structures for biomedical applications, Eur. Polym. J. 119 (2019) 61–73, https://doi.org/10.1016/j.eurpolymj.2019.07.013.

[11] O. Sytar, I. Hemmerich, M. Zivcak, C. Rauh, M. Brestic, Comparative analysis of bioactive phenolic compounds composition from 26 medicinal plants, Saudi, J. Biol. Sci. 25 (2018) 631–641, https://doi.org/10.1016/j.sjbs.2016.01.036.

[12] O.A. Krasheninina, E.K. Apartsin, E. Fuentes, A. Szulc, M. Ionov, A. G. Venyaminova, D. Shcharbin, F.J. de la Mata, M. Bryszewska, R. Gómez, R. Gómez, Complexes of pro-apoptotic sirnas and carbosilane dendrimers: formation and effect on cancer cells, Pharmaceutics. 11 (2019) 25, https://doi.org/10.3390/pharmaceutics11010025.

[13] A. Jain, S. Mahira, J.-P. Majoral, M. Bryszewska, W. Khan, M. Ionov, Dendrimer mediated targeting of siRNA against polo-like kinase for the treatment of triple negative breast cancer, J. Biomed. Mater. Res. Part A. 107 (2019) 1933–1944, https://doi.org/10.1002/jbm.a.36701.

[14] K. Białkowska, K. Miłowska, S. Michlewska, P. Sokołowska, P. Komorowski, T. Lozano-Cruz, R. Gomez-Ramirez, F.J. de la Mata, M. Bryszewska, Interaction of cationic carbosilane dendrimers and their siRNA complexes with MCF-7 cells, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/ijms22137097.

[15] M. Ionov, Z. Garaiova, I. Waczulikova, D. Wróbel, E. Pędziwiatr-Werbicka, R. Gomez-Ramirez, F.J. De La Mata, B. Klajnert, T. Hianik, M. Bryszewska, siRNA carriers based on carbosilane dendrimers affect zeta potential and size of phospholipid vesicles, Biochim. Biophys. Acta Biomembr. 2012 (1818) 2209–2216, https://doi.org/10.1016/j.bbamem.2012.04.019.

[16] J. Lazniewska, K. Milowska, N. Katir, A. El Kadib, M. Bryszewska, J.P. Majoral, T. Gabryelak, Viologen-phosphorus dendrimers exhibit minor toxicity against a murine neuroblastoma cell line, Cell. Mol. Biol. Lett. 18 (2013) 459–478, https://doi.org/10.2478/s11658-013-0100-5.

[17] K. Jain, P. Kesharwani, U. Gupta, N.K. Jain, Dendrimer toxicity: Let's meet the challenge, Int. J. Pharm. 394 (2010) 122–142, https://doi.org/10.1016/j.ijpharm.2010.04.027.

[18] S. Svenson, Dendrimers as versatile platform in drug delivery applications, Eur. J. Pharm. Biopharm. 71 (2009) 445–462, https://doi.org/10.1016/j.ejpb.2008.09.023.

[19] V. Patel, C. Rajani, D. Paul, P. Borisa, K. Rajpoot, S.R. Youngren-Ortiz, R.K. Tekade, Dendrimers as Novel Drug-Delivery System and its Applications, Elsevier Inc., 2019, https://doi.org/10.1016/B978-0-12-814487-9.00008-9.

[20] S. Somani, P. Laskar, N. Altwaijry, P. Kewcharoenvong, C. Irving, G. Robb, B. S. Pickard, C. Dufès, PEGylation of polypropylenimine dendrimers: effects on cytotoxicity, DNA condensation, gene delivery and expression in cancer cells, Sci. Rep. 8 (2018) 1–13, https://doi.org/10.1038/s41598-018-27400-6.

[21] Y. Jin, X. Ren, W. Wang, L. Ke, E. Ning, L. Du, J. Bradshaw, A 5-fluorouracil-loaded pH-responsive dendrimer nanocarrier for tumor targeting, Int. J. Pharm. 420 (2011) 378–384, https://doi.org/10.1016/j.ijpharm.2011.08.053.

[22] S. Spreckelmeyer, C. Orvig, A. Casini, Cellular transport mechanisms of cytotoxic metallodrugs: an overview beyond cisplatin, Molecules. 19 (2014) 15584–15610, https://doi.org/10.3390/molecules191015584.

[23] S. Michlewska, M. Ionov, A. Szwed, A. Rogalska, N.S. Del Olmo, P. Ortega, M. Denel, D. Jacenik, D. Shcharbin, F.J. de la Mata, M. Bryszewska, Ruthenium dendrimers against human lymphoblastic leukemia 1301 cells, Int. J. Mol. Sci. 21 (2020) 1–13, https://doi.org/10.3390/ijms21114119.

[24] S. Michlewska, M. Maroto, M. Hołota, M. Kubczak, N. Sanz del Olmo, P. Ortega, D. Shcharbin, F.J. de la Mata, M. Bryszewska, M. Ionov, Combined therapy of ruthenium dendrimers and anti-cancer drugs against human leukemic cells, Dalton Trans. (2021) 9500–9511, https://doi.org/10.1039/d1dt01388b.

[25] M. Hołota, J. Magiera, S. Michlewska, M. Kubczak, N.S.D.N.S. Del Olmo, S. García-Gallego, P. Ortega, F.J.J. De La Mata, M. Ionov, M. Bryszewska, In vitro anticancer properties of copper metallodendrimers, Biomolecules. 9 (2019) 1–15, https://doi.org/10.3390/biom9040155.

[26] N. Sanz del Olmo, M. Maroto-Díaz, R. Gómez, P. Ortega, M. Cangiotti, M. F. Ottaviani, F.J. de la Mata, Carbosilane metallodendrimers based on copper (II) complexes: synthesis, EPR characterization and anticancer activity, J. Inorg. Biochem. 177 (2017) 211–218, https://doi.org/10.1016/j.jinorgbio.2017.09.023.

[27] S. Alfei, B. Marengo, C. Domenicotti, Polyester-based dendrimer nanoparticles combined with etoposide have an improved cytotoxic and pro-oxidant effect on human neuroblastoma cells, Antioxidants. 9 (2020) 1–23, https://doi.org/10.3390/antiox9010050.

[28] S. Ku, F. Yan, Y. Wang, Y. Sun, N. Yang, L. Ye, The blood-brain barrier penetration and distribution of PEGylated fluorescein-doped magnetic silica nanoparticles in rat brain, Biochem. Biophys. Res. Commun. 394 (2010) 871–876, https://doi.org/10.1016/j.bbrc.2010.03.006.

[29] E. Lopez-Lopez, W.E. Evans, New insights into methotrexate accumulation in leukemia cells in vivo, Mol. Cell. Oncol. 8 (2021) 1865086, https://doi.org/10.1080/23723556.2020.1865086.

[30] M. Valko, D. Leibfritz, J. Moncol, M.T.D. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease, Int. J. Biochem. Cell Biol. 39 (2007) 44–84, https://doi.org/10.1016/j.biocel.2006.07.001.

[31] S. Alfei, B. Marengo, G. Zuccari, F. Turrini, C. Domenicotti, Dendrimer nanodevices and gallic acid as novel strategies to fight chemoresistance in neuroblastoma cells, Nanomaterials. 10 (2020) 1–30, https://doi.org/10.3390/nano10061243.

[32] C.-L. Ky, J. Louarn, S. Dussert, B. Guyot, S. Hamon, M. Noirot, Caffeine, trigonelline, chlorogenic acids and sucrose diversity in wild *Coffea arabica* L. and *C. canephora* P. accessions, Food Chem. 75 (2001) 223–230, https://doi.org/10.1016/S0308-8146(01)00204-7.

[33] J.A. Greenberg, C.N. Boozer, A. Geliebter, Coffee, diabetes, and weight control, Am. J. Clin. Nutr. 84 (2006) 682–693, https://doi.org/10.1093/ajcn/84.4.682.

[34] E. Choi, K.-H. Choi, S.M. Park, D. Shin, H.-K. Joh, E. Cho, The benefit of bone health by drinking coffee among Korean postmenopausal women: A cross-sectional analysis of the fourth & fifth Korea National Health and nutrition examination surveys, PLoS One 11 (2016), e0147762, https://doi.org/10.1371/journal.pone.0147762.

[35] B. Shukitt-Hale, M.G. Miller, Y.F. Chu, B.J. Lyle, J.A. Joseph, Coffee, but not caffeine, has positive effects on cognition and psychomotor behavior in aging, Age (Omaha). 35 (2013) 2183–2192, https://doi.org/10.1007/s11357-012-9509-4.

[36] S. Ni, Y. Xie, Y. Tang, Y. Liu, J. Chen, S. Zhu, N-2-hydroxypropyltimehyl ammonium chloride chitosan nanoparticles for siRNA pulmonary delivery: preparation, characterization and in vitro evaluation, J. Drug Target. 25 (2017) 451–462, https://doi.org/10.1080/1061186X.2016.1278219.

[37] Pevzner, 乳鼠心肌提取 HHS public access, Physiol. Behav. 176 (2017) 139–148, https://doi.org/10.1158/1055-9965.EPI-15-0924.Coffee.

[38] Y.-M. Li, J. Peng, L.-Z. Li, Coffee consumption associated with reduced risk of Oral cancer: a meta-analysis, Oral Surg, Oral Med. Oral Pathol. Oral Radiol. 121 (2016) 381–389.e1, https://doi.org/10.1016/j.oooo.2015.12.006.

[39] E. Loftfield, N.D. Freedman, B.I. Graubard, A.R. Hollenbeck, F.M. Shebl, S. T. Mayne, R. Sinha, Coffee drinking and cutaneous melanoma risk in the NIH-AARP diet and health study, J. Natl. Cancer Inst. 107 (2015) 1–9, https://doi.org/10.1093/jnci/dju421.

[40] M. Lukic, M. Jareid, E. Weiderpass, T. Braaten, Coffee consumption and the risk of malignant melanoma in the Norwegian women and Cancer (NOWAC) study, BMC Cancer 16 (2016), https://doi.org/10.1186/s12885-016-2586-5.

[41] M. Koga, S. Nakagawa, A. Kato, I. Kusumi, Caffeic acid reduces oxidative stress and microglial activation in the mouse hippocampus, Tissue Cell 60 (2019) 14–20, https://doi.org/10.1016/j.tice.2019.07.006.

[42] K.M. Monteiro Espíndola, R.G. Ferreira, L.E. Mosquera Narvaez, A.C. Rocha Silva Rosario, A.H. Machado Da Silva, A.G. Bispo Silva, A.P. Oliveira Vieira, M. Chagas Monteiro, Chemical and pharmacological aspects of caffeic acid and its activity in hepatocarcinoma, Front. Oncol. 9 (2019) 3–5, https://doi.org/10.3389/fonc.2019.00541.

[43] D. Guo, D. Dou, L. Ge, Z. Huang, L. Wang, N. Gu, A caffeic acid mediated facile synthesis of silver nanoparticles with powerful anti-cancer activity, Colloids Surf. B: Biointerfaces 134 (2015) 229–234, https://doi.org/10.1016/j.colsurfb.2015.06.070.

[44] N.S. Del Olmo, C.E.P. González, J.D. Rojas, R. Gómez, P. Ortega, A. Escarpa, F.J. de la Mata, Antioxidant and antibacterial properties of carbosilane dendrimers functionalized with polyphenolic moieties, Pharmaceutics. 12 (2020) 1–16, https://doi.org/10.3390/pharmaceutics12080698.

[45] E.M. Egorova, The validity of the Smoluchowski equation in electrophoretic studies of lipid membranes, Electrophoresis. 15 (1994) 1125–1131, https://doi.org/10.1002/elps.11501501170.

[46] M. Galán, E. Fuentes-Paniagua, F.J. de la Mata, R. Gómez, Heterofunctionalized Carbosilane dendritic systems: Bifunctionalized Dendrons as building blocks versus statistically decorated dendrimers, Organometallics. 33 (2014) 3977–3989, https://doi.org/10.1021/om500464k.

[47] E. Fuentes-Paniagua, M.J. Serramía, J. Sánchez-Nieves, S. Álvarez, M.Á. Muñoz-Fernández, R. Gómez, F.J. De La Mata, Fluorescein labelled cationic carbosilane dendritic systems for biological studies, Eur. Polym. J. 71 (2015) 61–72, https://doi.org/10.1016/j.eurpolymj.2015.07.043.
[48] X. Yan, Y. Yang, Y. Sun, Dendrimer applications for Cancer therapies, J. Phys. Conf. Ser. 1948 (2021), https://doi.org/10.1088/1742-6596/1948/1/012205.
[49] B. Noriega-Luna, L.A. Godínez, F.J. Rodríguez, A. Rodríguez, G. Zaldívar-Lelo De Larrea, C.F. Sosa-Ferreyra, R.F. Mercado-Curiel, J. Manríquez, E. Bustos, Applications of dendrimers in drug delivery agents, diagnosis, therapy, and detection, J. Nanomater. 2014 (2014), https://doi.org/10.1155/2014/507273.
[50] A.P. Sherje, M. Jadhav, B.R. Dravyakar, D. Kadam, Dendrimers: A versatile nanocarrier for drug delivery and targeting, Int. J. Pharm. 548 (2018) 707–720, https://doi.org/10.1016/j.ijpharm.2018.07.030.
[51] S. Svenson, D.A. Tomalia, Dendrimers in biomedical applications - reflections on the field, Adv. Drug Deliv. Rev. 57 (2005) 2106–2129, https://doi.org/10.1016/j.addr.2005.09.018.
[52] P. Kesharwani, S. Banerjee, U. Gupta, M.C.I. Mohd Amin, S. Padhye, F.H. Sarkar, A. K. Iyer, PAMAM dendrimers as promising nanocarriers for RNAi therapeutics, Mater. Today 18 (2015) 565–572, https://doi.org/10.1016/j.mattod.2015.06.003.
[53] N. Martinho, H. Florindo, L. Silva, S. Brocchini, M. Zloh, T. Barata, Molecular modeling to study dendrimers for biomedical applications, Molecules. 19 (2014) 20424–20467, https://doi.org/10.3390/molecules191220424.
[55] M. Maroto-Díaz, B.T. Elie, P. Gómez-Sal, J. Pérez-Serrano, R. Gómez, M. Contel, F. Javier De La Mata, Synthesis and anticancer activity of carbosilane metallodendrimers based on arene ruthenium(ii) complexes, Dalton Trans. 45 (2016) 7049–7066, https://doi.org/10.1039/c6dt00465b.
[56] M. Maroto-Diaz, N. Sanz del Olmo, L. Muñoz-Moreno, A.M. Bajo, M.J. Carmena, R. Gómez, S. García-Gallego, F.J. de la Mata, In vitro and in vivo evaluation of first-generation carbosilane arene Ru(II)-metallodendrimers in advanced prostate cancer, Eur. Polym. J. 113 (2019) 229–235, https://doi.org/10.1016/j.eurpolymj.2019.01.047.
[57] A.M. Master, M.E. Rodriguez, M.E. Kenney, N.L. Oleinick, A. Sen Gupta, Delivery of the photosensitizer Pc 4 in PEG–PCL micelles for in vitro PDT studies, J. Pharm. Sci. 99 (2010) 2386–2398, https://doi.org/10.1002/jps.
[58] T. Sadat, J. Kashi, S. Eskandarion, M. Esfandyari-Manesh, S. Mahmoud, A. Marashi, N. Samadi, S.M. Fatemi, F. Atyabi, S. Eshraghi, R. Dinarvand, Improved drug loading and antibacterial activity of minocycline-loaded PLGA nanoparticles prepared by solid/oil/water ion pairing method, Int. J. Nanomedicine 2 (2012) 221–234, https://doi.org/10.2147/ijn.s27709.
[59] A. Janaszewska, J. Lazniewska, P. Trzepiński, M. Marcinkowska, B. Klajnert-Maculewicz, Cytotoxicity of dendrimers, Biomolecules. 9 (2019) 1–23, https://doi.org/10.3390/biom9080330.
[60] S. Michlewska, M. Ionov, M. Maroto-Díaz, A. Szwed, A. Ihnatsyeu-Kachan, V. Abashkin, V. Dzmitruk, A. Rogalska, M. Denel, M. Gapinska, D. Shcharbin, R. Gomez Ramirez, F.J. De La Mata, M. Bryszewska, Ruthenium dendrimers against acute promyelocytic leukemia: in vitro studies on HL-60 cells, Future Med. Chem. 11 (2019), https://doi.org/10.4155/fmc-2018-0274.
[61] Y. Chang, N. Liu, L. Chen, X. Meng, Y. Liu, Y. Li, J. Wang, Synthesis and characterization of DOX-conjugated dendrimer-modified magnetic iron oxide conjugates for magnetic resonance imaging, targeting, and drug delivery, J. Mater. Chem. 22 (2012) 9594–9601, https://doi.org/10.1039/c2jm16792a.
[62] D. Luong, P. Kesharwani, R. Deshmukh, M.C.I. Mohd Amin, U. Gupta, K. Greish, A. K. Iyer, PEGylated PAMAM dendrimers: enhancing efficacy and mitigating toxicity for effective anticancer drug and gene delivery, Acta Biomater. 43 (2016) 14–29, https://doi.org/10.1016/j.actbio.2016.07.015.
[63] G. Mencia, N.S. Del Olmo, L. Muñoz-Moreno, M. Maroto-Diaz, R. Gomez, P. Ortega, M. José Carmena, F. Javier de la Mata, Polyphenolic carbosilane dendrimers as anticancer agents against prostate cancer, New J. Chem. 40 (2016) 10488–10497, https://doi.org/10.1039/c6nj02545e.
[64] F. Visioli, G. Bellomo, C. Galli, Free radical-scavenging properties of olive oil polyphenols, Biochem. Biophys. Res. Commun. 247 (1998) 60–64, https://doi.org/10.1006/bbrc.1998.8735.
[65] Y. Hanasaki, S. Ogawa, S. Fukui, The correlation between active oxygens scavenging and antioxidative effects of flavonoids, Free Radic. Biol. Med. 16 (1994) 845–850, https://doi.org/10.1016/0891-5849(94)90202-X.
[66] D.D. Milinčić, D.A. Popović, S.M. Lević, A. Kostić, Ž.L. Tešić, V.A. Nedović, M. B. Pešić, Application of polyphenol-loaded nanoparticles in food industry, Nanomaterials. 9 (2019), https://doi.org/10.3390/nano9111629.
[68] N. Katir, N. Marcotte, S. Michlewska, M. Ionov, N. El Brahmi, M. Bousmina, J. P. Majoral, M. Bryszewska, A. El Kadib, Dendrimer for templating the growth of porous catechol-coordinated titanium dioxide frameworks: toward Hemocompatible nanomaterials, ACS Appl. Nano Mater. 2 (2019), https://doi.org/10.1021/acsanm.9b00382.
[69] S. Michlewska, M. Kubczak, M. Maroto-Díaz, N.S. Del Olmo, P. Ortega, D. Shcharbin, R.G. Ramirez, F.J. De La Mata, M. Ionov, M. Bryszewska, Synthesis and characterization of FITC labelled ruthenium dendrimer as a prospective anticancer drug, Biomolecules. 9 (2019), https://doi.org/10.3390/biom9090411.
[70] E. Pedziwiatr-Werbicka, E. Fuentes, V. Dzmitruk, J. Sánchez-Nieves, M. Sudas, E. Drozd, A. Shakhbazau, D. Shcharbin, F.J. de la Mata, R. Gomez-Ramirez, M. A. Munoz-Fernandez, M. Bryszewska, Novel "SiC" carbosilane dendrimers as carriers for anti-HIV nucleic acids: studies on complexation and interaction with blood cells, Colloids Surf. B: Biointerfaces 109 (2013) 183–189, https://doi.org/10.1016/j.colsurfb.2013.03.045.
[71] W.H. Lee, C.Y. Loo, D. Traini, P.M. Young, Inhalation of nanoparticle-based drug for lung cancer treatment: advantages and challenges, Asian, J. Pharm. Sci. 10 (2015) 481–489, https://doi.org/10.1016/j.ajps.2015.08.009.
[72] M.E.E.-D. Ibrahim, R.M. Alqurashi, Anti-fungal and antioxidant properties of propolis (bee glue) extracts, Int. J. Food Microbiol. 361 (2022), 109463, https://doi.org/10.1016/j.ijfoodmicro.2021.109463.
[73] S.E. Owumi, C.E. Irozuru, U.O. Arunsi, A.K. Oyelere, Caffeic acid protects against DNA damage, oxidative and inflammatory mediated toxicities, and upregulated caspases activation in the hepatorenal system of rats treated with aflatoxin B1, Toxicon. 207 (2022) 1–12, https://doi.org/10.1016/j.toxicon.2021.12.021.
[74] H.-L. Yang, M. Korivi, M.-K. Lin, H.C.-W. Chang, C.-R. Wu, M.-S. Lee, W.T.-L. Chen, Y.-C. Hseu, Antihemolytic and antioxidant properties of pearl powder against 2,2′-azobis(2-amidinopropane) dihydrochloride-induced hemolysis and oxidative damage to erythrocyte membrane lipids and proteins, J. Food Drug Anal. 25 (2017) 898–907, https://doi.org/10.1016/j.jfda.2016.10.007.
[75] V.M. Barodka, E. Nagababu, J.G. Mohanty, D. Nyhan, D.E. Berkowitz, J.M. Rifkind, J.J. Strouse, New insights provided by a comparison of impaired deformability with erythrocyte oxidative stress for sickle cell disease, blood cells, Mol. Dis. 52 (2014) 230–235, https://doi.org/10.1016/j.bcmd.2013.10.004.
[76] T. Baccarin, M. Mitjans, E. Lemos-Senna, M.P. Vinardell, Protection against oxidative damage in human erythrocytes and preliminary photosafety assessment of Punica granatum seed oil nanoemulsions entrapping polyphenol-rich ethyl acetate fraction, Toxicol. in Vitro 30 (2015) 421–428, https://doi.org/10.1016/j.tiv.2015.09.020.
[77] C. Widén, A. Ekholm, M.D. Coleman, S. Renvert, K. Rumpunen, Erythrocyte antioxidant protection of rose hips (*Rosa* spp.), Oxidative Med. Cell. Longev. 2012 (2012), 621579, https://doi.org/10.1155/2012/621579.
[78] K. Naparlo, G. Bartosz, I. Stefaniuk, B. Cieniek, M. Soszynski, I. Sadowska-Bartosz, Interaction of catechins with human erythrocytes, Molecules. 25 (2020) 1–16, https://doi.org/10.3390/molecules25061456.
[79] M. Kubczak, A.B. Khassenova, B. Skalski, S. Michlewska, *Hippophae rhamnoides* L. leaf and twig extracts as rich sources of nutrients and bioactive compounds with antioxidant activity, Sci. Rep. (2022) 1–14, https://doi.org/10.1038/s41598-022-05104-2.
[80] M. Kubczak, A.B. Khassenova, B. Skalski, S. Michlewska, M. Wielanek, A. N. Aralbayeva, M.K. Murzakhmetova, M. Zamaraeva, M. Skłodowska, M. Bryszewska, M. Ionov, Bioactive compounds and antiradical activity of the Rosa canina L. leaf and twig extracts, Agronomy 10 (2020) 1–10.
[81] A. Darawsha, A. Trachtenberg, J. Levy, Y. Sharoni, The protective effect of carotenoids, polyphenols, and estradiol on dermal fibroblasts under oxidative stress, Antioxidants 10 (2021), https://doi.org/10.3390/antiox10122023.
[82] J.A.G. Crispo, D.R. Ansell, M. Piche, J.K. Eibl, N. Khaper, G.M. Ross, T.C. Tai, Protective effects of polyphenolic compounds on oxidative stress-induced cytotoxicity in PC12 cells, Can. J. Physiol. Pharmacol. 88 (2010) 429–438, https://doi.org/10.1139/Y09-137.
[83] H. Wang, D. Li, Z. Hu, S. Zhao, Z. Zheng, W. Li, Protective effects of green tea polyphenol against renal injury through ROS-mediated JNK-MAPK pathway in Lead exposed rats, Mol. Cell 39 (2016) 508–513, https://doi.org/10.14348/molcells.2016.2170.

Supplementary Material

Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity

Marika Grodzicka [⊥a,b], Cornelia E. Pena-Gonzalez [⊥c], Paula Ortega [c,d], Sylwia Michlewska [e*], Rebeca Lozano [c], Maria Bryszewska [a], Francisco Javier de la Mata [c,d], Maksim Ionov [a]

[a] *Department of General Biophysics. Faculty of Biology & Environmental Protection. University of Lodz. Pomorska 141/143. Lodz 90–236. Poland*
[b] *BioMedChem Doctoral School of the UL and Lodz Institutes of the Polish Academy of Science, Banacha 12/16, 0-237 Lodz, Poland*
[c] *Universidad de Alcalá. Department of Organic and Inorganic Chemistry, and Research Institute in Chemistry "Andrés M. del Río" (IQAR), Spain and Instituto Ramon y Cajal de Investigacion Sanitaria, IRYCIS, Colmenar Viejo Road, Km 9, 100, 28034 Madrid, Spain*
[d] *Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain.*
[e] *Laboratory of Microscopic Imaging & Specialized Biological Techniques. Faculty of Biology & Environmental Protection. University of Lodz. Banacha12/16. Lodz 90–237. Poland*

[⊥] *These authors contributed equally to the work.*

Corresponding author:
Sylwia Michlewska
e-mail: sylwia.michlewska@biol.uni.lodz.pl

1) Synthesis of G_2-[(S-NH_3Cl)V_7] (II).

Dendrimer **II** was prepared according to the published synthetic protocol [1].

European Polymer Journal, 2015, Volume 71, Pages 61-72.
https://doi.org/10.1016/j.eurpolymj.2015.07.043**.**

G_2SiV_8 (62.5 mg, 0.106 mmol), cysteamine hydrochloride (12.13 mg, 0.106 mmol) and 10 mol % of DMPA (2.74 mg, 0.010 mmol) were combined in 4 mL of ½ THF/methanol solution. The reaction mixture was deoxygenated and irradiated for 0.5 h. The reaction mixture was directly used to obtain **3**.

^{1}H-NMR (CD_3OD): δ (ppm) 0.10 (s, 12 H, SiCH_3), 0.41-0.75 (m, overlapping of signals, 16 H, SiCH_2CH_2CH_2Si), 0.90 (m, 2 H, SiCH_2CH_2S), 1.30 (m, 8 H, SiCH_2CH_2CH_2Si), 2.57 (m, 2 H,

$SiCH_2CH_2S$), 2.95 (m, 2 H, SCH_2CH_2N), 3.19 (m, 2 H, $SCH_2CH_2NH_3Cl$), 5.60-5.74 (m, 7 H, $SiCHCH_2$), 5.88-6.19 (m, 14 H, $SiCHCH_2$), 8.27 (s, 3 H, NH_3).

2) Synthesis of G_2-[(S-$NH_3Cl)_2V_6$] (III)

Dendrimer **III** was prepared according to the published synthetic protocol [1].
European Polymer Journal, 2015, Volume 71, Pages 61-72.
https://doi.org/10.1016/j.eurpolymj.2015.07.043**.**

G_2SiV_8 (80.2 mg, 0.137 mmol), cysteamine hydrochloride (31.14 mg, 0.274 mmol) and 10 mol % of DMPA (7.0 mg, 0.027 mmol) were combined in 4 mL of ½ THF/methanol solution. The reaction mixture was deoxygenated and irradiated for 0.5 h. The reaction mixture was directly used to obtain **4**.

^{1}H-NMR ($CDCl_3$): δ (ppm) 0.15 (s, 12 H, $SiCH_3$), 0.54-0.82 (m, overlapping of signals ,16 H, $SiCH_2CH_2CH_2Si$), 0.98 (m, 4 H, $SiCH_2CH_2S$), 1.41 (m, 8 H, $SiCH_2CH_2CH_2Si$), 2.67 (m, 4 H, $SiCH_2CH_2S$), 2.86 (m, 4 H, SCH_2CH_2N), 3.14 (m, 4 H, $SCH_2CH_2NH_3Cl$), 5.66-5.82 (m, 6 H, $SiCHCH_2$), 5.88-6.19 (m, 12 H, $SiCHCH_2$), not observed (s, 3 H, NH_3).

3) Synthesis of G_2-[(S-PEG-$NH_3Cl)V_7$] (1)

G_2SiV_8 (31.0 mg, 0.053 mmol), commercial thiol HS-PEG-NH_2·HCl (MW = 3.5 KD) (186.4 mg, 0.053 mmol) and 10 mol % of DMPA (1.35 mg, 0.005 mmol) were combined in 4 mL of ½ THF/methanol solution. The reaction mixture was deoxygenated and irradiated for 0.5 h. The reaction mixture was directly used to obtain **5**.

^{1}H-NMR (CD_3OD): δ (ppm) 0.06 (s, 12 H, $SiCH_3$), 0.64 (m, 8 H, $SiCH_2CH_2CH_2Si$), 0.70 (m, 8 H, $SiCH_2CH_2CH_2Si$), 0.93 (m, 2 H, $SiCH_2CH_2S$), 1.40 (m, 8 H, $SiCH_2CH_2CH_2Si$), 3.70 (overlapping of signals m, 308 H, SCH_2CH_2O, m, 2 H, $OCH_2CH_2NH_3$); 5.66-5.82 (m, 7 H, $SiCHCH_2$), 5.88-6.19 (m, 14 H, $SiCHCH_2$), not observed (s, 3 H, NH_3).

4) Synthesis of G_2-[(S-PEG-$NH_3Cl)_2V_6$] (2)

G_2SiV_8 (24.0 mg, 0.0468 mmol), commercial thiol HS-PEG-NH_2·HCl (MW = 3.5 KD) (324.6 mg, 0.093 mmol) and 10 mol % of DMPA (2.38 mg, 0.009 mmol) were combined in 4 mL of ½ THF/methanol solution. The reaction mixture was deoxygenated and irradiated for 0.5 h. The reaction mixture was directly used to obtain **6**.

^{1}H-NMR (CD_3OD): δ (ppm) 0.06 (s, 12 H, $SiCH_3$), 0.64 (m, 8 H, $SiCH_2CH_2CH_2Si$), 0.70 (m, 8 H, $SiCH_2CH_2CH_2Si$), 0.93 (m, 4 H, $SiCH_2CH_2S$), 1.40 (m, 8 H, $SiCH_2CH_2CH_2Si$), 3.70 (overlapping of signals m, 616 H, SCH_2CH_2O, m, 2 H, OCH_2CH_2NH), 5.66-5.82 (m, 6 H, $SiCHCH_2$), 5.88-6.19 (m, 12 H, $SiCHCH_2$), not observed (s, 3 H, NH_3).

5) Synthesis of G_2-[(S-NH_3Cl)(S-$N(CH_3)_2$·HCl)$_7$] (3)

1, 2-(dimethylamino)ethanothiol hydrochloride (108.2 mg, 0.762 mmol) and DMPA (19.5 mg, 0.07 mmol) were added to the reaction mixture of compound **II**. Afterward, the reaction mixture was irradiated for 3 h and monitored by ^{1}H NMR until the disappearance of vinyl groups resonances which confirm that reaction is completed. The final reaction mixture was concentrated by rotatory evaporation and was directly used to obtain **7**.

^{1}H-NMR (CD_3OD): δ (ppm) 0.09 (s, 12 H, SiC*H*$_3$), 0.61 (m, 8 H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.71 (m, 8 H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.97 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.38 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.70 (m, 16 H, SiCH$_2$C*H*$_2$S), 2.91 (s, 42 H, N$^+$C*H*$_3$), 2.95 (m, 16 H, SC*H*$_2$CH$_2$N), 3.16 (m, 2 H, SCH$_2$C*H*$_2$NH$_3$Cl), 3.35 (m, 14 H, SCH$_2$C*H*$_2$N$^+$CH$_3$).

6) Synthesis of G$_2$-[(S-NH$_3$Cl)$_2$(S-N(CH$_3$)$_2$·HCl)$_6$] (4)

Dendrimer **4** has been prepared through the same method as described in **3** by using the following reagents: 1, 2-(dimethylamino)ethanothiol hydrochloride (118.8 mg, 0.838 mmol) and DMPA (21.3 mg, 0.08 mmol). Compound **4** was directly used to obtain **8**.

^{1}H-NMR (CD_3OD): δ (ppm) 0.09 (s, 12 H, SiC*H*$_3$), 0.63 (m, 8 H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.71 (m, 8 H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.97 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.35 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.72 (m, 16 H, SiCH$_2$C*H*$_2$S), 2.93 (s, 36 H, N$^+$C*H*$_3$), 2.95 (m, 16 H, SC*H*$_2$CH$_2$N), 3.18 (m, 4 H, SCH$_2$C*H*$_2$N$^+$H$_3$Cl), 3.36 (m, 12 H, SCH$_2$C*H*$_2$NCH$_3$).

7) Synthesis of G$_2$-[(S-PEG-NH$_3$Cl)(S-N(CH$_3$)$_2$·HCl)$_7$] (5)

Dendrimer **5** has been prepared through the same method as described in **3** by using the following reagents: 1, 2-(dimethylamino)ethanothiol hydrochloride (52.5 mg , 0.371 mmol) and DMPA (9.5 mg, 0.03 mmol). Compound 5 was directly used to obtain **9**.

^{1}H-NMR (CD_3OD): δ (ppm) 0.08 (s, 12 H, SiC*H*$_3$), 0.65 (m, 8 H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.71 (m, 8 H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.96 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.42 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.29 (s, 42 H, NC*H*$_3$), 2.55 (m, 14 H, SCH$_2$C*H*$_2$NCH$_3$), 2.59-2.75 (m, overlapping of signals, 34 H, SiCH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NCH$_3$ and S-C*H*$_2$CH$_2$O;) 3.09 (m, 2 H, SCH$_2$C*H*$_2$N$^+$H$_3$Cl) 3.55-3.71 (m, 618 H, OC*H*$_2$)., 3.58-3.69 (m, 308 H and OCH$_2$).

8) Synthesis of G$_2$-[(S-PEG-NH$_3$Cl)$_2$(S-N(CH$_3$)$_2$·HCl)$_6$] (6)

Dendrimer **6** has been prepared through the same method as described in **3** by using the following reagents: 1, 2-(dimethylamino)ethanothiol hydrochloride (39.77 mg , 0.280 mmol) and DMPA (7.2 mg, 0.03 mmol). Compound **6** is isolated as a white solid (296.5 mg, 75 %).

^{1}H-NMR (CD_3OD): δ (ppm) 0.09 (s, 12 H, SiC*H*$_3$), 0.72 (m, 8 H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.72 (m, 8 H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.95 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.45 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.28 (s, 36 H, NC*H*$_3$), 2.55 (m, 12 H, SCH$_2$C*H*$_2$NCH$_3$), 2.60-2.75 (m, overlapping of signals, 36 H, SiCH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NCH$_3$ and SC*H*$_2$CH$_2$O) 3.18 (m, 4 H, SCH$_2$C*H*$_2$N$^+$H$_3$Cl) 3.55-3.71 (m, 616 H, OC*H*$_2$).

9) Synthesis of G_2-[(S-NH_2)(S-$N(CH_3)_2$)$_7$] (7)

To a water solution of compound **3**, Na_2CO_3 (53.93 mg, 0.508mmol) was added and the mixture keeps on magnetic stirring during 1 h. Afterward, extraction with CH_2Cl_2 was performed and the organic phase was dried with magnesium sulphate. The solution was filtered, and volatiles remove under vacuum obtaining dendrimer **7** as a pallid orange oil (96.37 mg; 65 %).

^{1}H-NMR (CD_3OD): δ (ppm) 0.11 (s, 12 H, SiC*H*$_3$), 0.72 (m, 16 H, SiC*H*$_2$CH$_2$C*H*$_2$Si), 0.98 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.45 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.31 (s, 42 H, NC*H*$_3$), 2.50-2.58 (m, overlapping of signals, 46 H, SiCH$_2$C*H*$_2$S and SC*H*$_2$C*H*$_2$N), 2.88 (m, 2 H, SCH$_2$C*H*$_2$NH$_2$). **^{13}C {^{1}H}-NMR (CD_3OD):** δ (ppm) -6.1 Si*C*H$_3$), 14.4 (Si*C*H$_2$CH$_2$S), 17.2 (Si*C*H$_2$CH$_2$CH$_2$Si), 18.3 (SiCH$_2$*C*H$_2$CH$_2$Si), 18.5 (SiCH$_2$CH$_2$*C*H$_2$Si), 27.1, 27.2, 28.4, 28.5 (Si*C*H$_2$*C*H$_2$S, S*C*H$_2$CH$_2$NCH$_3$ and SCH$_2$*C*H$_2$NCH$_3$), 44.1 (N*C*H$_3$), 59.0 (SCH$_2$*C*H$_2$NCH$_3$). **Elemental Analysis (%):** Calc for $C_{62}H_{144}N_8S_8Si_5$ (1398.80 g/mol). C, 53.24; H, 10.38; N, 8.01. Exp.: C, 53.56; H, 9.98; N, 7.96.

10) Synthesis of G_2-[(S-NH_2)$_2$(S-$N(CH_3)_2$)$_6$] (8)

Dendrimer **8** has been prepared through the same method as described in **7** from a solution of compound **4**. Compound **8** was obtained as a pallid orange oil (133.06 mg, 71 %).

^{1}H-NMR (CD_3OD): δ (ppm) 0.11 (s, 12 H, SiC*H*$_3$), 0.74 (m, 16 H, SiC*H*$_2$CH$_2$C*H*$_2$Si), 0.99 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.47 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.35 (s, 36 H, NC*H*$_3$), 2.50-2.58 (m, overlapping of signals, 46 H, SiCH$_2$C*H*$_2$S and SC*H*$_2$C*H*$_2$N), 2.89 (m, 4 H, SCH$_2$C*H*$_2$NH$_2$). **^{13}C {^{1}H}-NMR (CD_3OD):** δ (ppm) -6.1 Si*C*H$_3$), 14.4 (Si*C*H$_2$CH$_2$S), 17.2 (Si*C*H$_2$CH$_2$CH$_2$Si), 18.3 (SiCH$_2$*C*H$_2$CH$_2$Si), 18.5 (SiCH$_2$CH$_2$*C*H$_2$Si), 27.1, 27.2, 28.4, 28.5 (Si*C*H$_2$*C*H$_2$S, S*C*H$_2$CH$_2$NCH$_3$ and SCH$_2$*C*H$_2$NCH$_3$), 44.1 (N*C*H$_3$), 59.0 (SCH$_2$*C*H$_2$NCH$_3$). **Elemental Analysis (%):** Calc for $C_{62}H_{144}N_8S_8Si_5$ (1368.80 g/mol). C, 52.57; H, 10.30; N, 8.17 . Exp.: C, 52.98; H, 10.45; N, 8.36.

11) Synthesis of G_2-[(S-PEG-NH_2)(S-$N(CH_3)_2$)$_7$] (9)

Dendrimer **9** has been prepared through the same method as described in **7** from a solution of compound **5**. Compound **9** was obtained as a pallid orange oil (167.58 mg, 66 %).

^{1}H-NMR (CD_3OD): δ (ppm) 0.08 (s, 12 H, SiC*H*$_3$), 0.65 (m, 8 H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.71 (m, 8 H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.96 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.42 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.29 (s, 42 H, NC*H*$_3$), 2.55 (m, 14 H, SCH$_2$C*H*$_2$NCH$_3$), 2.59-2.75 (m, overlapping of signals, 34 H, SiCH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NCH$_3$ and S-C*H*$_2$CH$_2$O; OCH$_2$C*H*$_2$NH), 3.58-3.69 (m, , 308 H, OC*H*$_2$). **^{13}C {^{1}H}-NMR (CD_3OD):** δ (ppm) -6.4 Si*C*H$_3$), 14.3 (Si*C*H$_2$CH$_2$S), 16.9 (Si*C*H$_2$CH$_2$CH$_2$Si), 18.0 (SiCH$_2$CH$_2$CH$_2$Si), 27.1 (SiCH$_2$CH$_2$S), 28.4 (SCH$_2$CH$_2$NCH$_3$ and SCH$_2$CH$_2$O), 30.7 (SCH$_2$CH$_2$NH), 39.2 (OCH$_2$CH$_2$NH), 44.0 (N*C*H$_3$), 58.9 (SiCH$_2$CH$_2$NCH$_3$), 70.4 **(**OCH$_2$**). Elemental Analysis (%):** Calc for $C_{216}H_{452}N_8O_{77}S_8Si_5$ (4790.88 g/mol). C, 54.15; H, 9.51; N, 2.34; S, 5.35. Exp.: C, 53.73; H, 8.98; N, 5.15.

12) Synthesis of G_2-[(S-PEG-NH_2)$_2$(S-$N(CH_3)_2$)$_6$] (10)

Dendrimer **10** has been prepared through the same method as described in **7** from a solution of compound **6**. Compound **10** was obtained as a pallid orange oil (187.56 mg, 50 %).

^{1}H-NMR (CD_3OD): δ (ppm) 0.08 (s, 12 H, SiC*H*$_3$), 0.66 (m, 8 H, SiC*H*$_2$CH$_2$CH$_2$Si), 0.72 (m, 8 H, SiCH$_2$CH$_2$C*H*$_2$Si), 0.95 (m, 16 H, SiC*H*$_2$CH$_2$S), 1.42 (m, 8 H, SiCH$_2$C*H*$_2$CH$_2$Si), 2.28 (s, 36 H, NC*H*$_3$), 2.55 (m, 12 H, SCH$_2$C*H*$_2$NCH$_3$), 2.60-2.75 (m, overlapping of signals, 36 H, SiCH$_2$C*H*$_2$S, SC*H*$_2$CH$_2$NCH$_3$, SC*H*$_2$CH$_2$O and O-CH$_2$C*H*$_2$NH), 3.55-3.71 (m, , 616 H, OC*H*$_2$. **^{13}C {^{1}H}-NMR (CD_3OD):** δ (ppm) -6.1 SiCH$_3$), 14.3 (SiCH$_2$CH$_2$S), 17.1 (SiCH$_2$CH$_2$CH$_2$Si), 17.9 (SiCH$_2$CH$_2$CH$_2$Si), 18.2 (SiCH$_2$CH$_2$CH$_2$Si), 27.0 (SiCH$_2$CH$_2$S), 28.4 (SCH$_2$CH$_2$NCH$_3$ and SCH$_2$CH$_2$O), 30.7 (SCH$_2$CH$_2$NH), 38.8 (OCH$_2$CH$_2$NH), 43.8 (NCH$_3$), 58.9 (SiCH$_2$CH$_2$NCH$_3$), 70.0 (OCH$_2$).**Elemental Analysis (%):** Calc for $C_{368}H_{756}N_8O_{154}S_8Si_5$ (8154.90 g/mol). C, 54.20; H, 9.34; N, 1.37; S, 3.15. Exp.: C, 55.10; H, 9.80; N, 1.03.

Table of contents

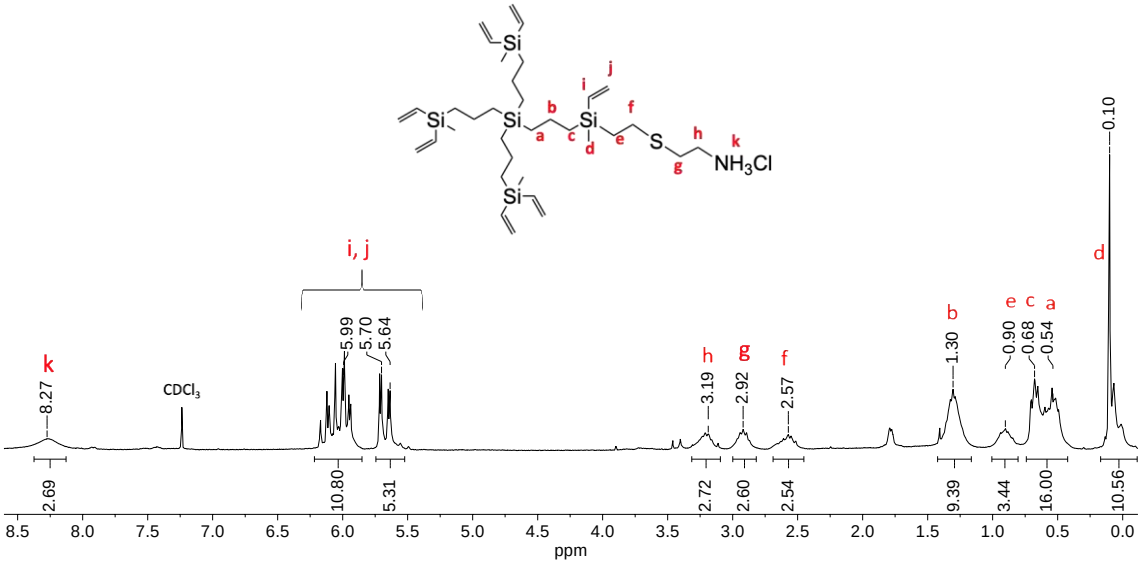


Figure S1. ^{1}H-NMR (500 MHz, $CDCl_3$) of compound **(II)**

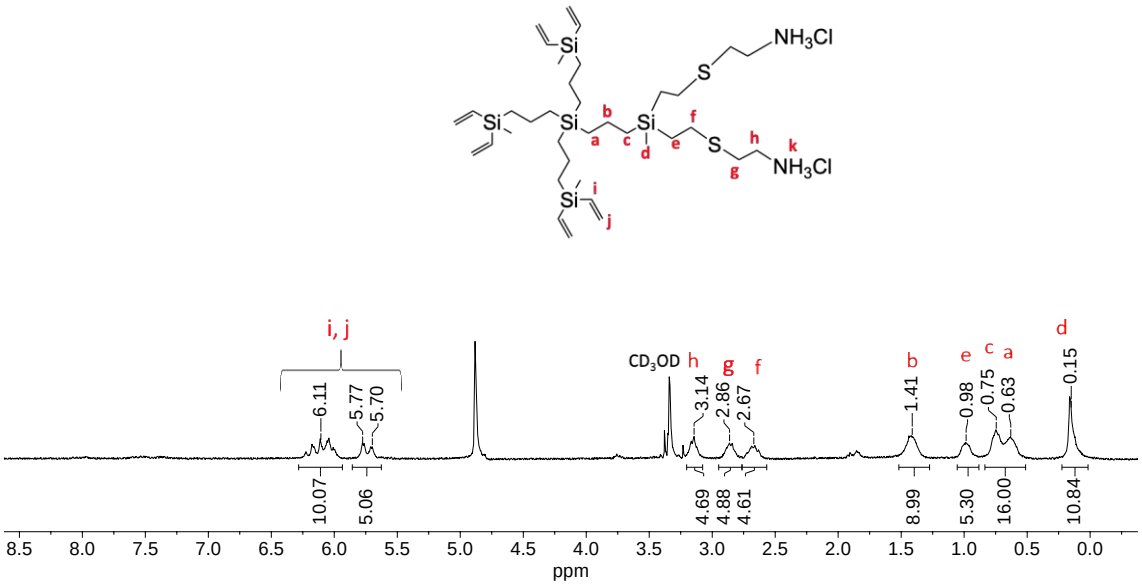


Figure S2. ^{1}H-NMR (500 MHz, CD_3OD) of compound **(III)**

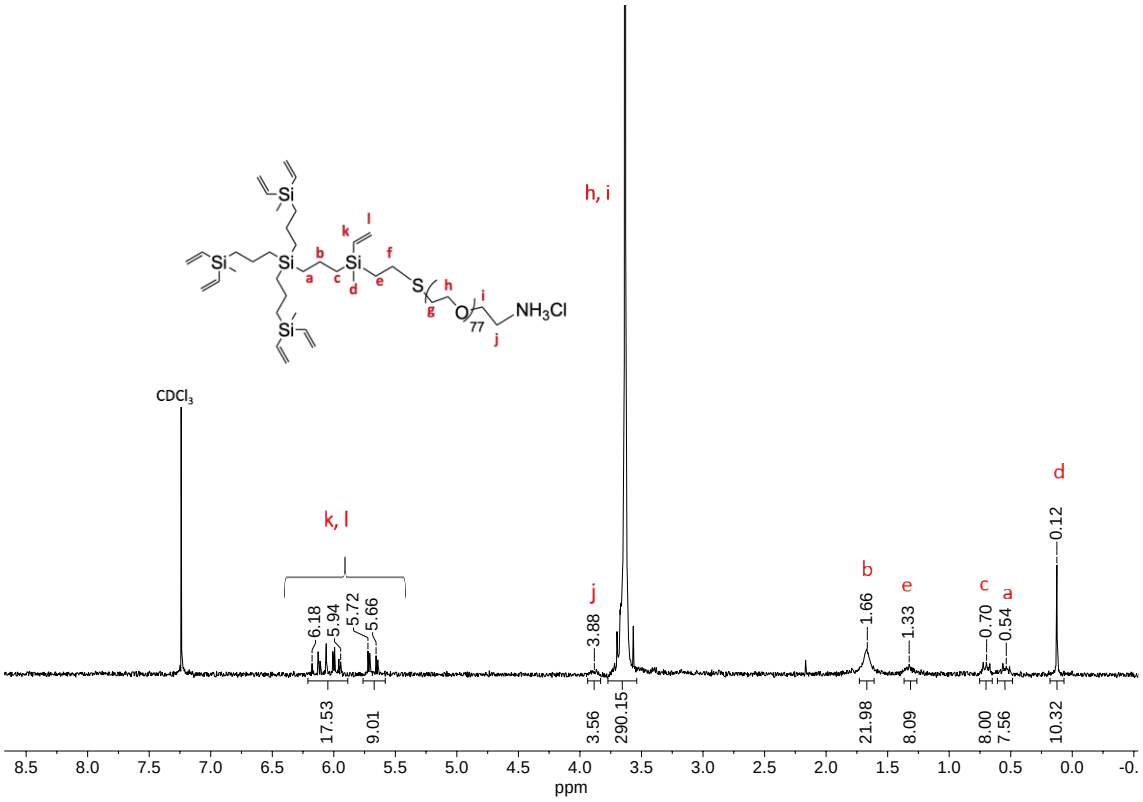


Figure S3. ^{1}H-NMR (500 MHz, $CDCl_3$) of compound **(1)**

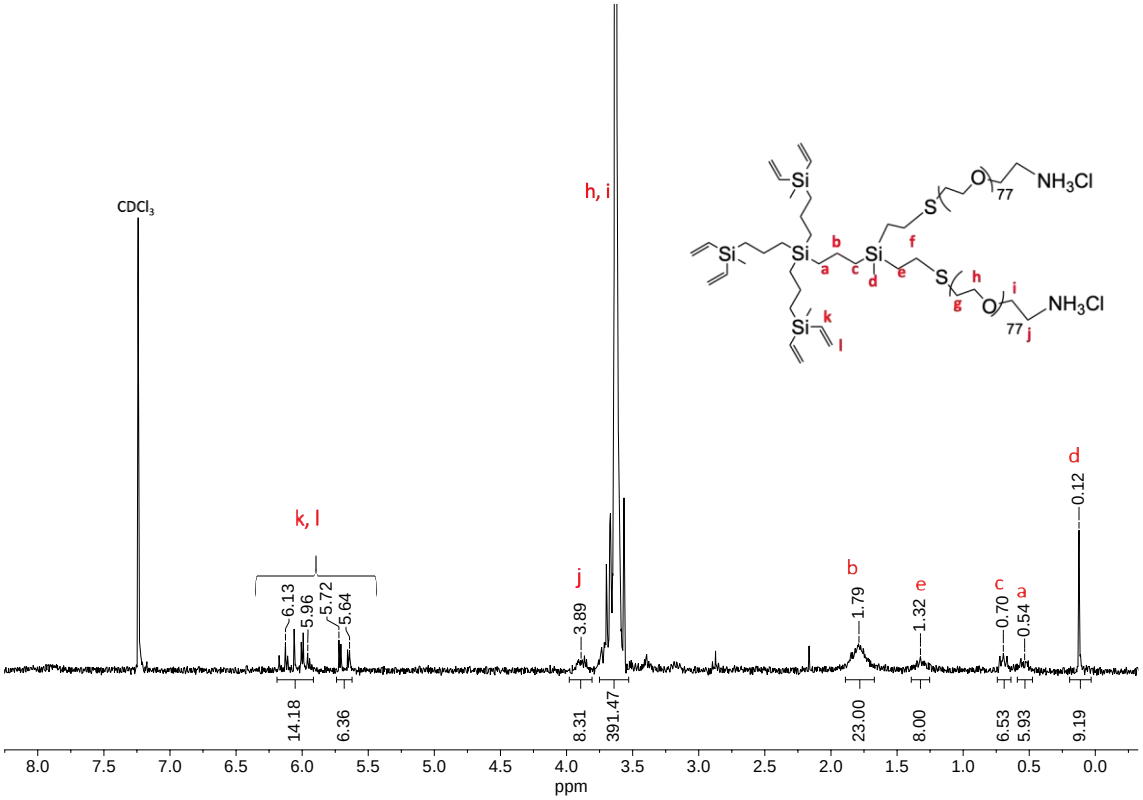


Figure S4. ^{1}H-NMR (500 MHz, $CDCl_3$) of compound **(2)**

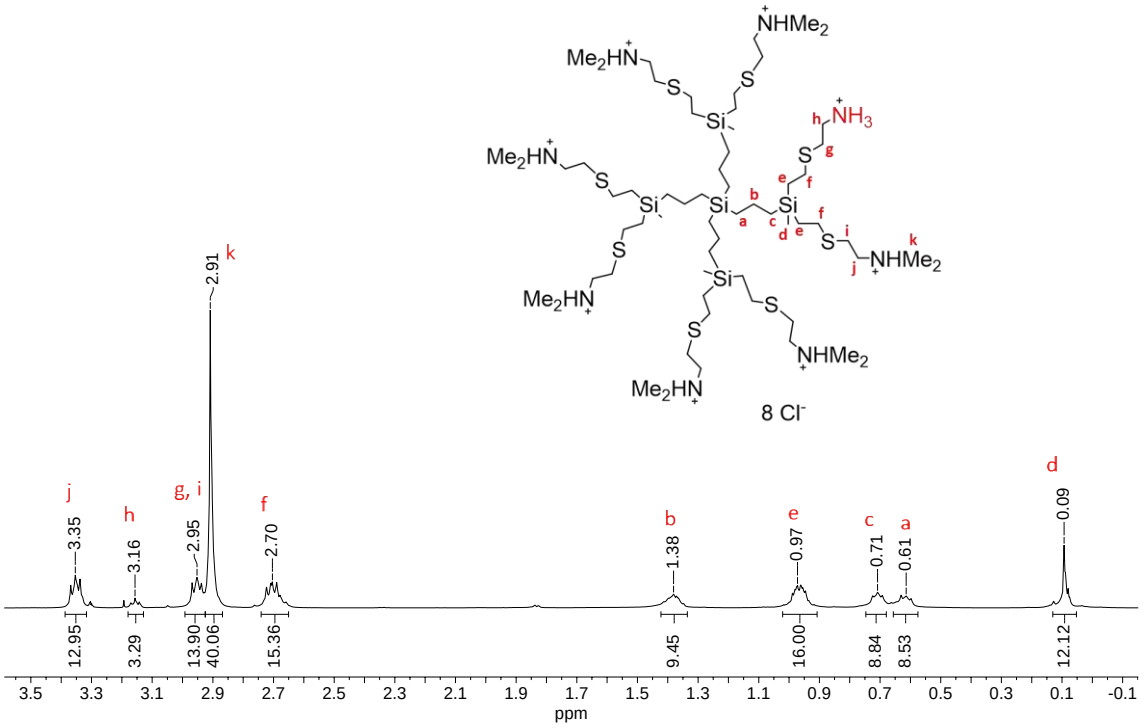


Figure S5. ^{1}H-NMR (500 MHz, CD_3OD) of compound **(3)**

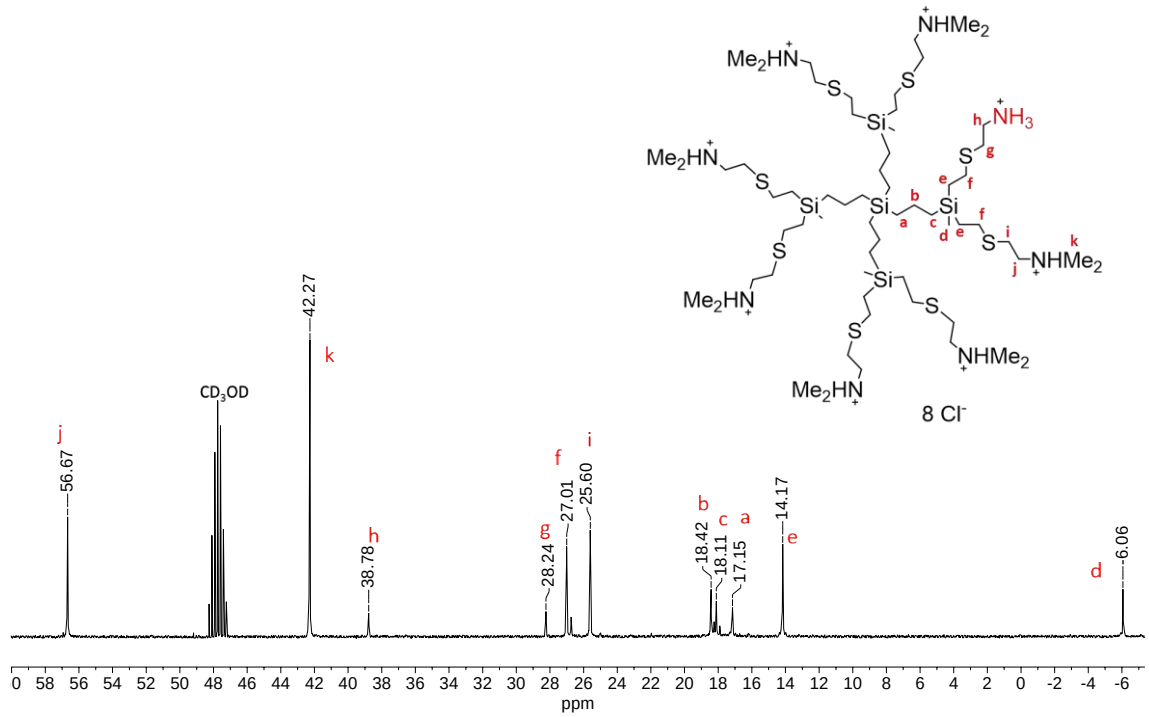


Figure S6. ^{13}C-NMR (500 MHz, CD_3OD) of compound **(3)**

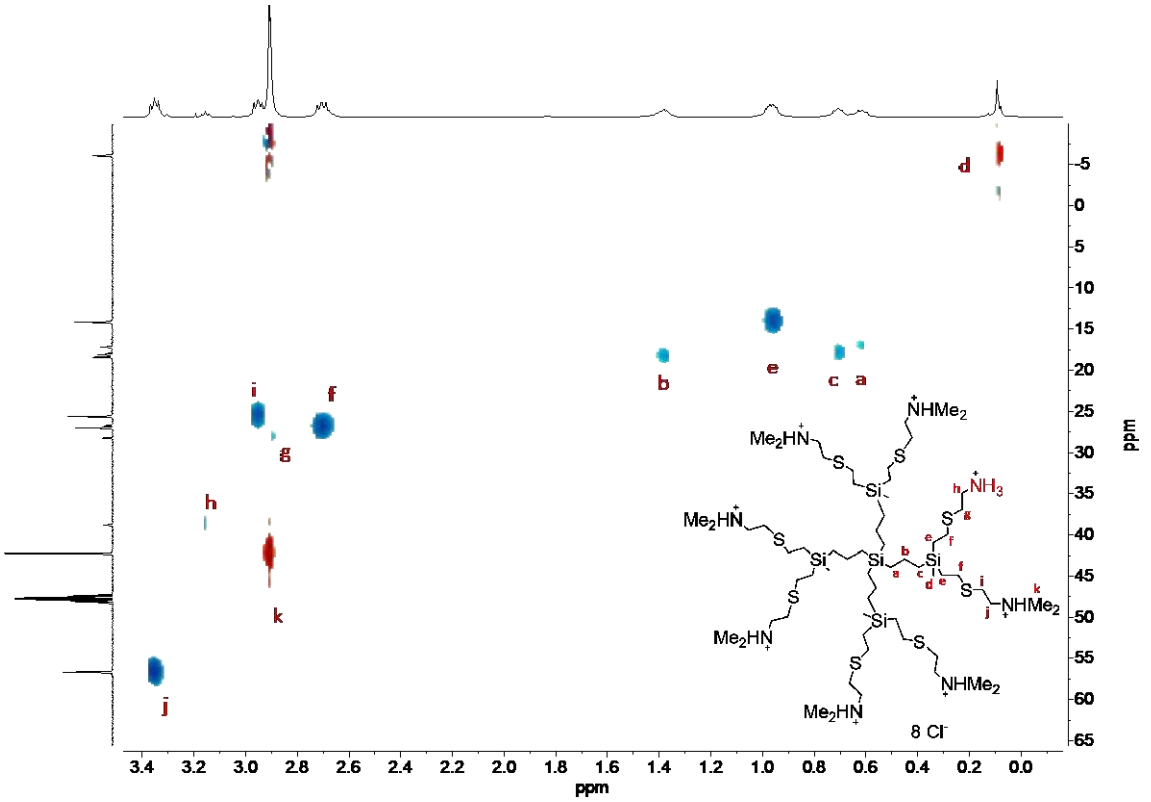


Figure S7. {^{1}H-^{13}C}-HSQC-2D-NMR (500 MHz, CD_3OD) of compound **(3)**

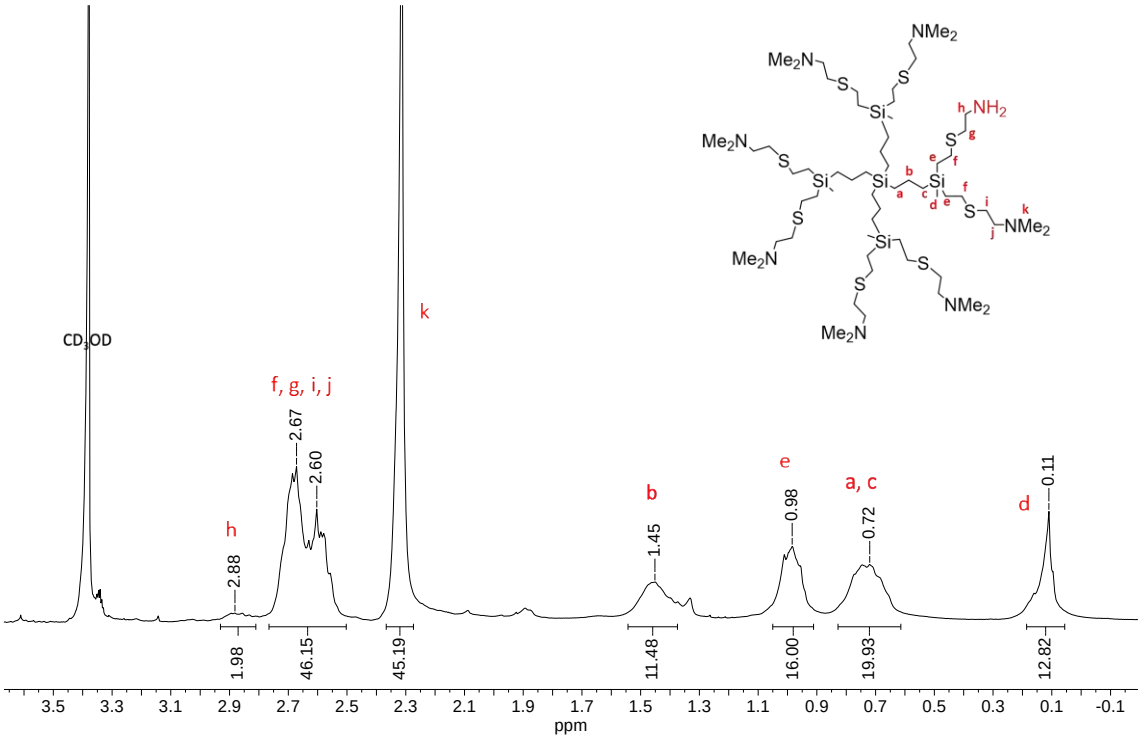


Figure S8. ^{1}H-NMR (500 MHz, CD_3OD) of compound **(7)**

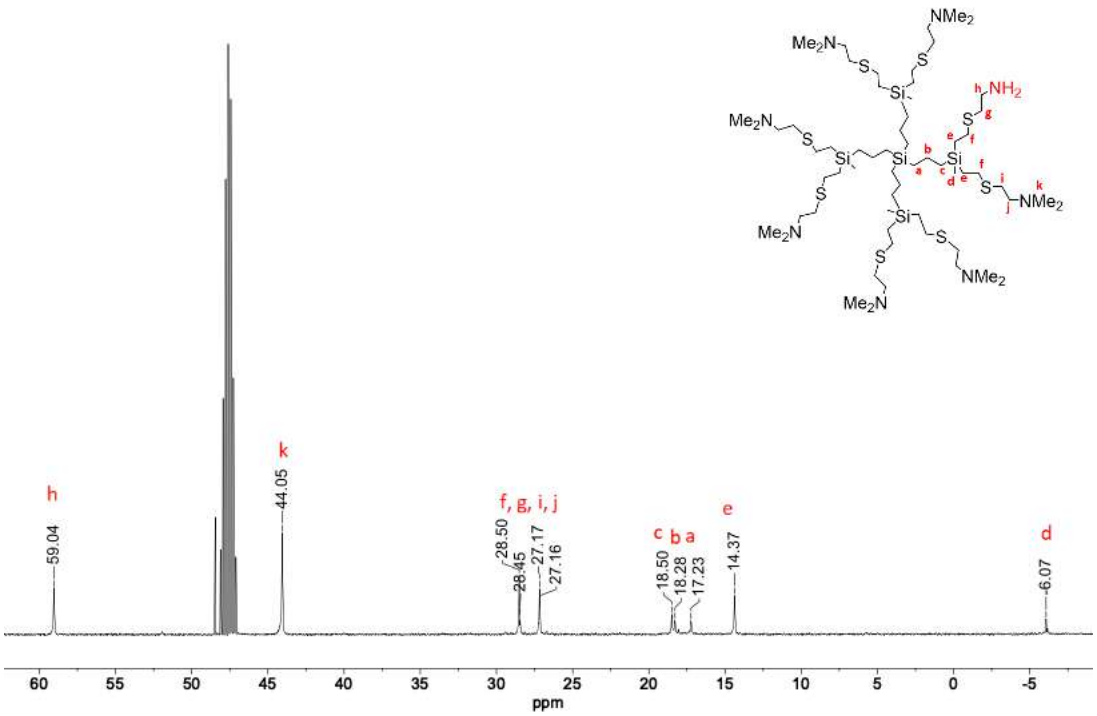


Figure S9. ^{13}C-NMR (500 MHz, CD_3OD) of compound **(7)**

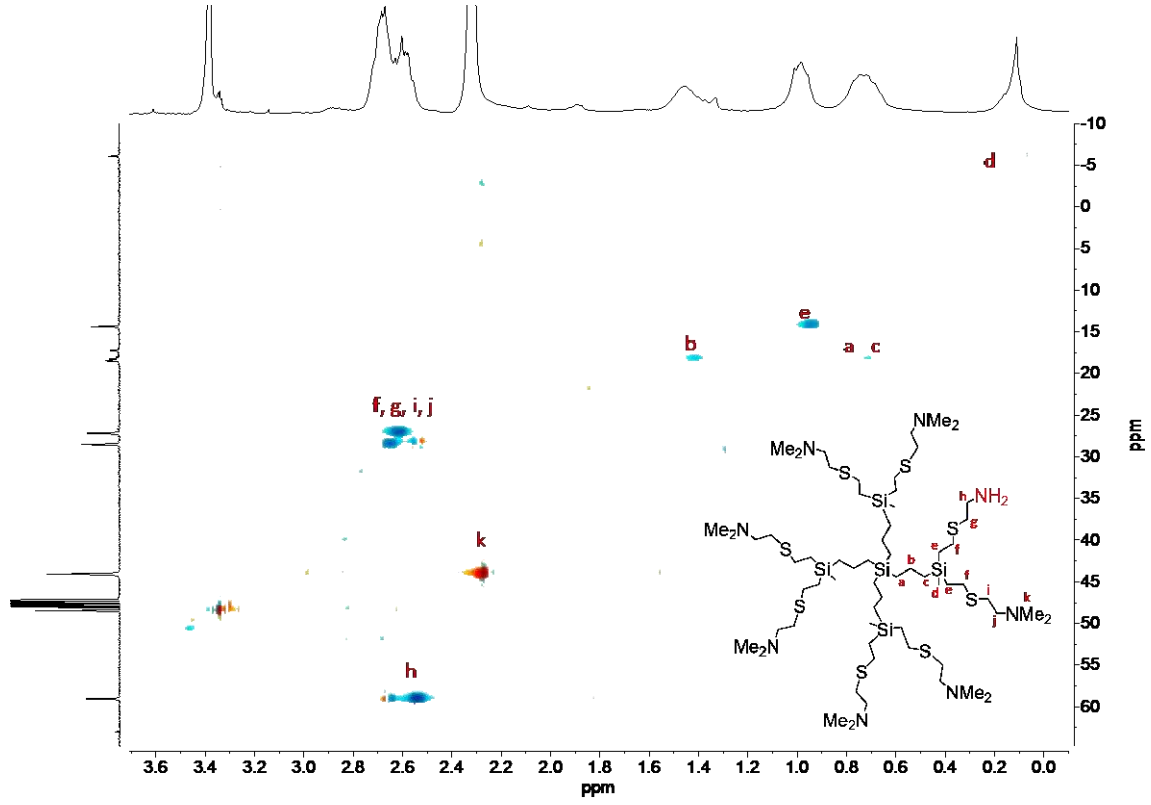


Figure S10. {^{1}H-^{13}C}-HSQC-2D-NMR (500 MHz, CD_3OD) of compound **(7)**

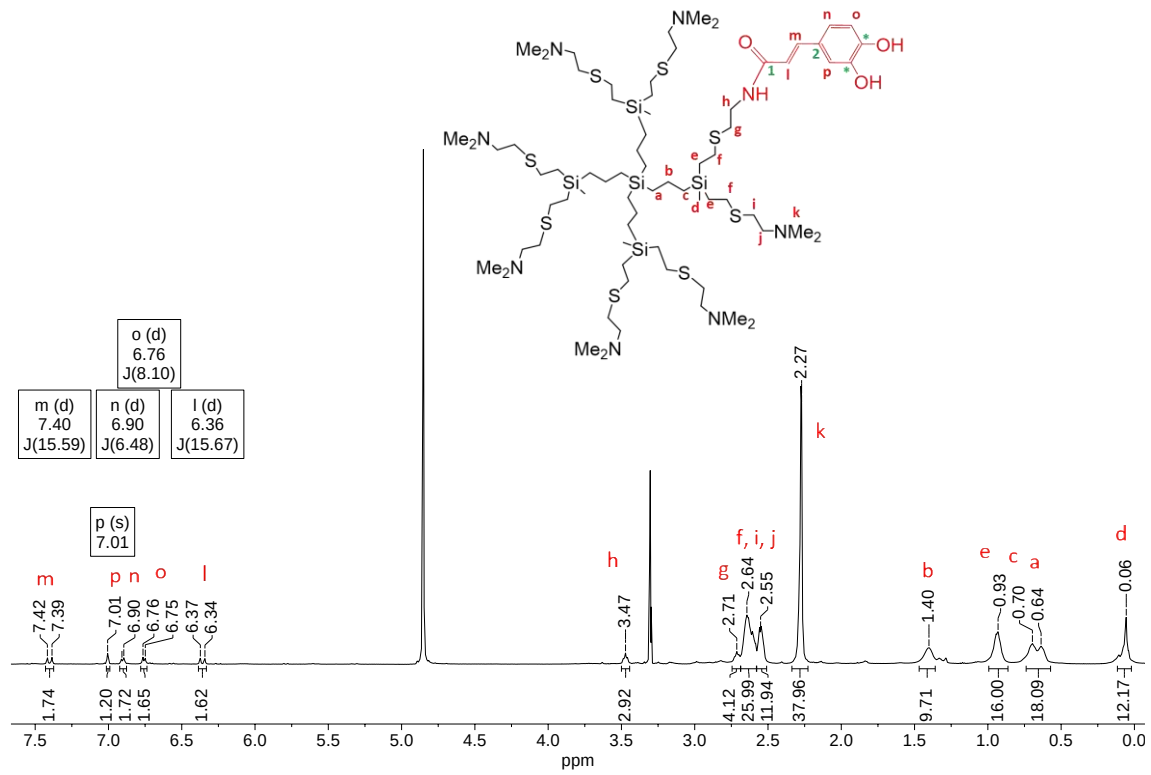


Figure S11. ^{1}H-NMR (500 MHz, CD_3OD) of dendritic polyphenol **(11)**

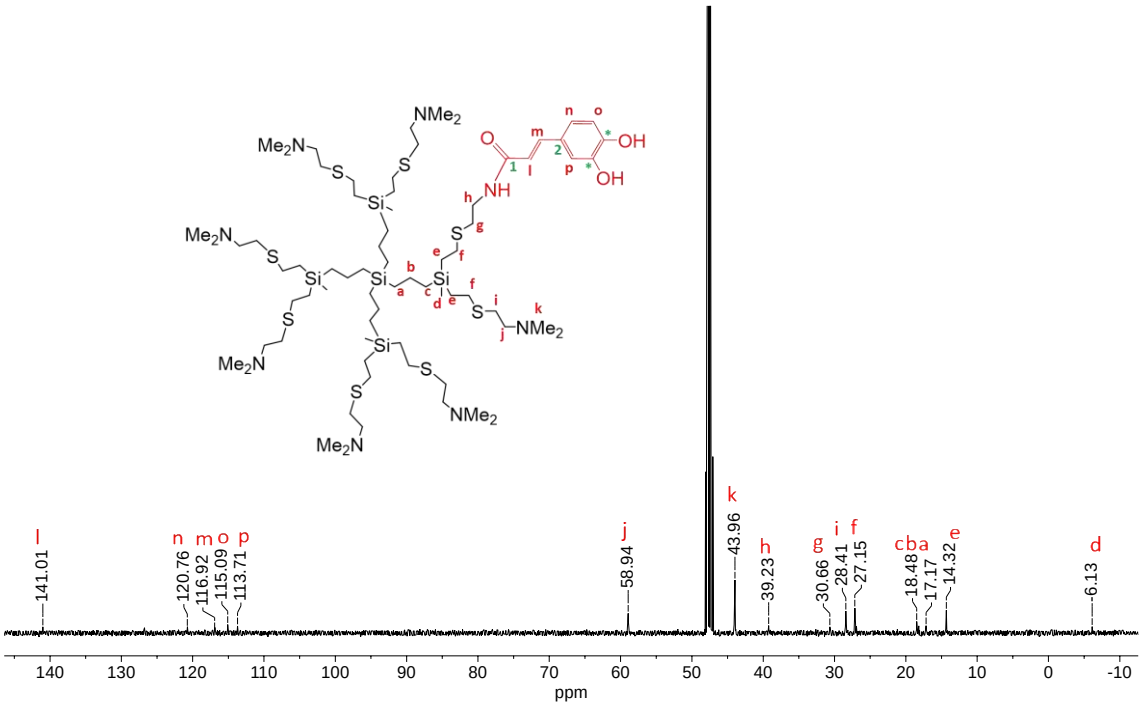


Figure S12. ^{13}C-NMR (500 MHz, CD_3OD) of dendritic polyphenol **(11)**

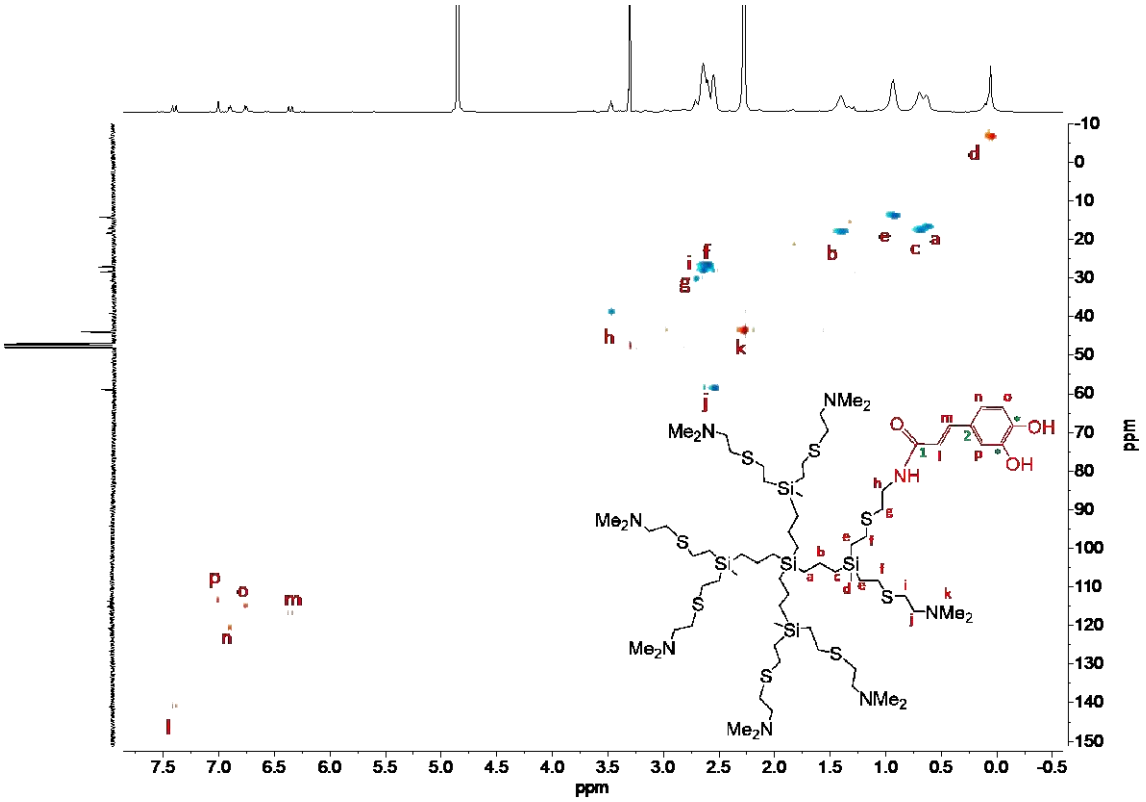


Figure S13. {^{1}H-^{13}C}-HSQC-2D-NMR (500 MHz, CD_3OD) of dendritic polyphenol **(11)**

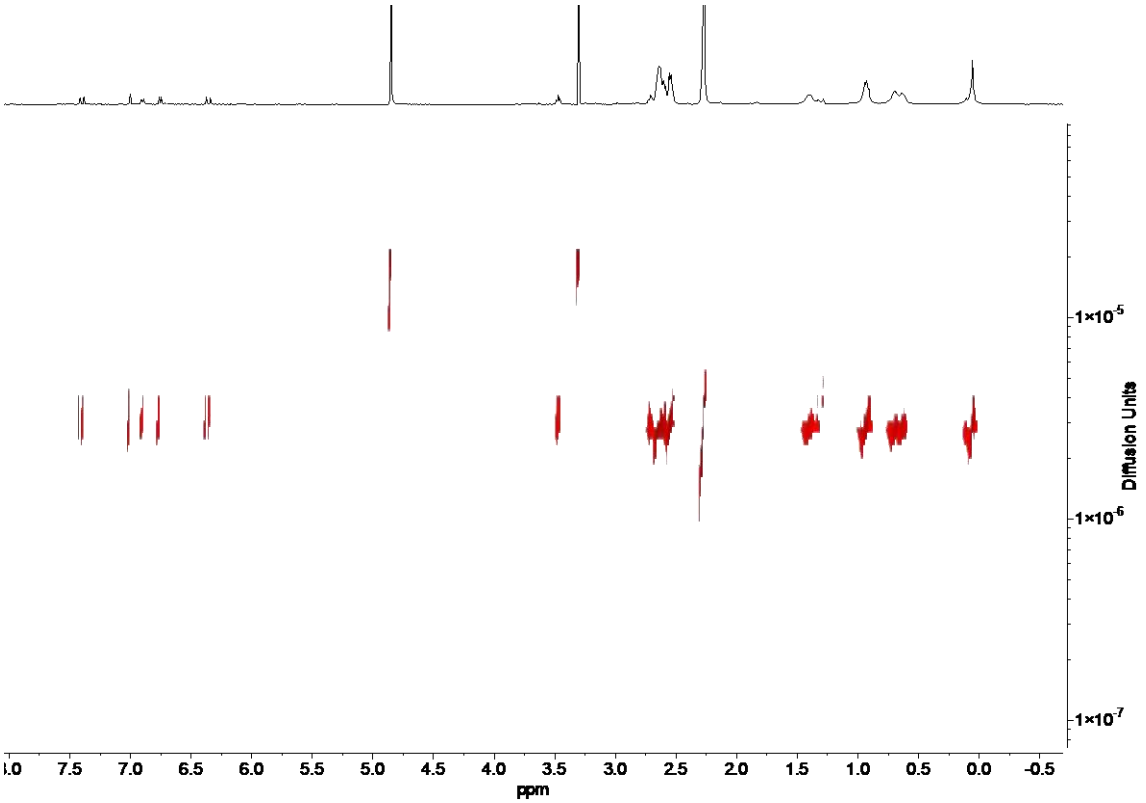


Figure S14. ^{1}H-DOSY-2D-NMR (500 MHz, CD_3OD) of compound **(11)**

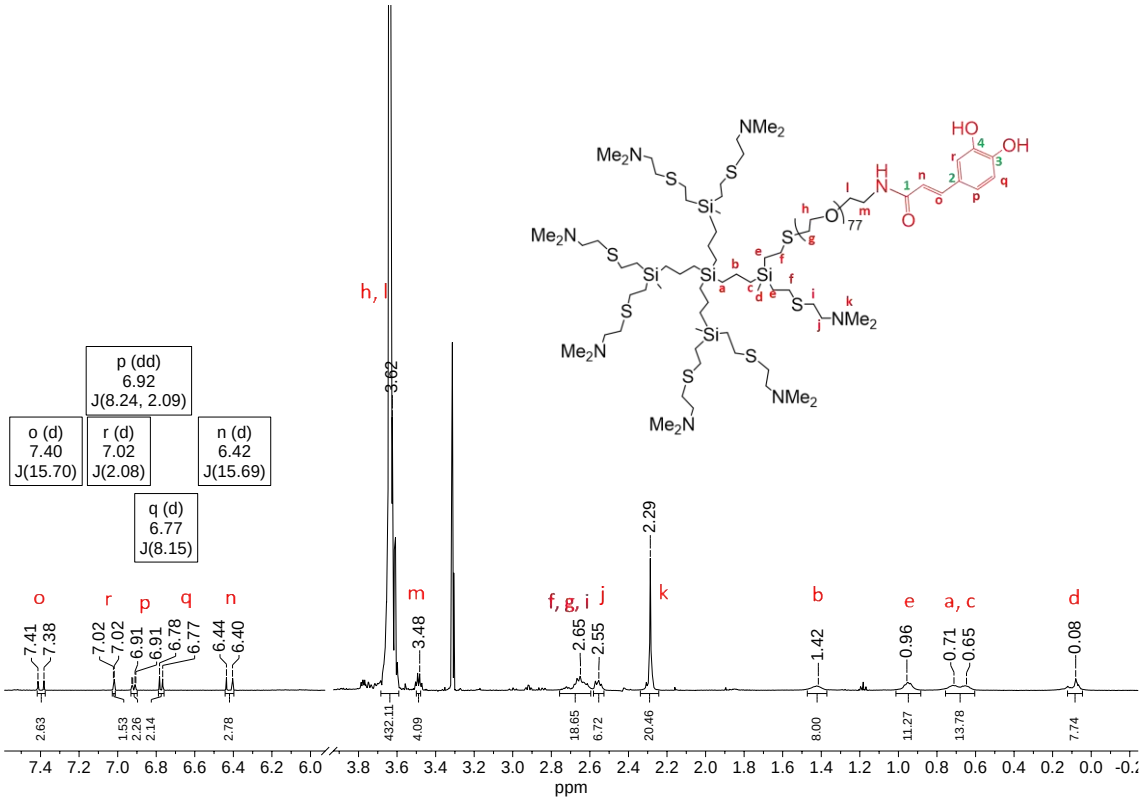


Figure S15. ^{1}H-NMR (500 MHz, CD_3OD) of compound **(13)**

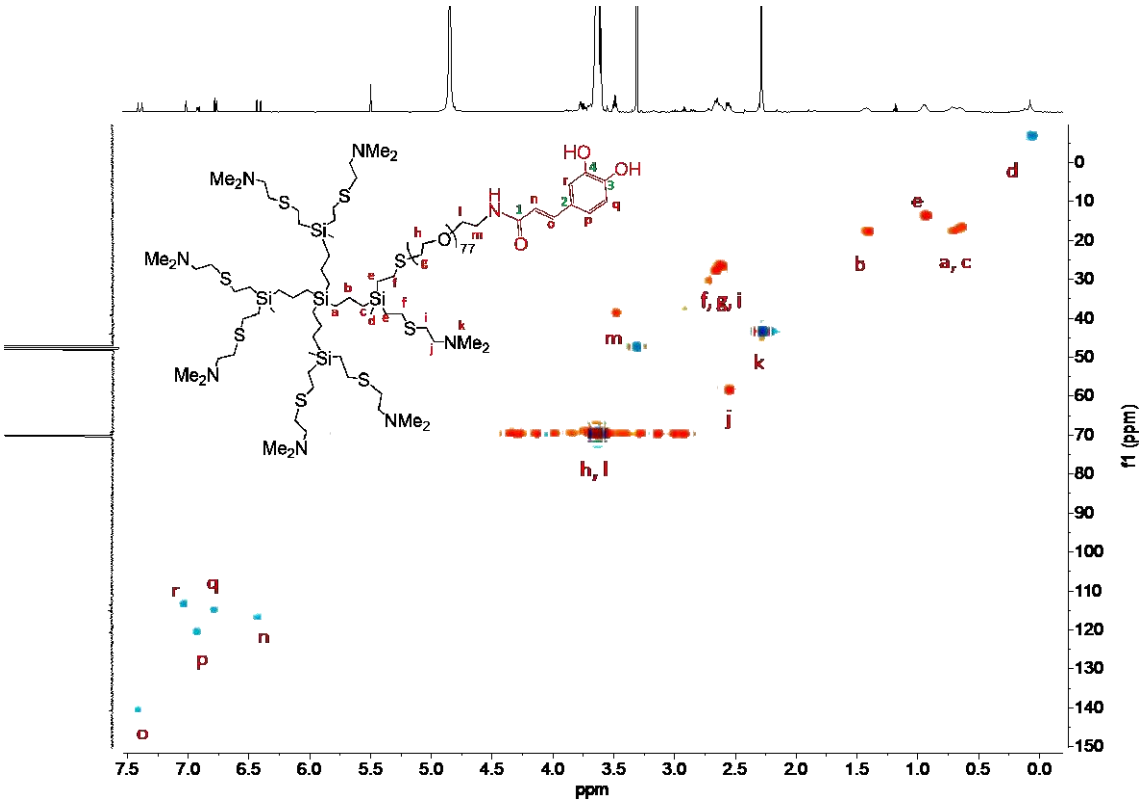


Figure S16. {^{1}H-^{13}C}-HSQC-2D-NMR (500 MHz, CD_3OD) of compound **(13)**

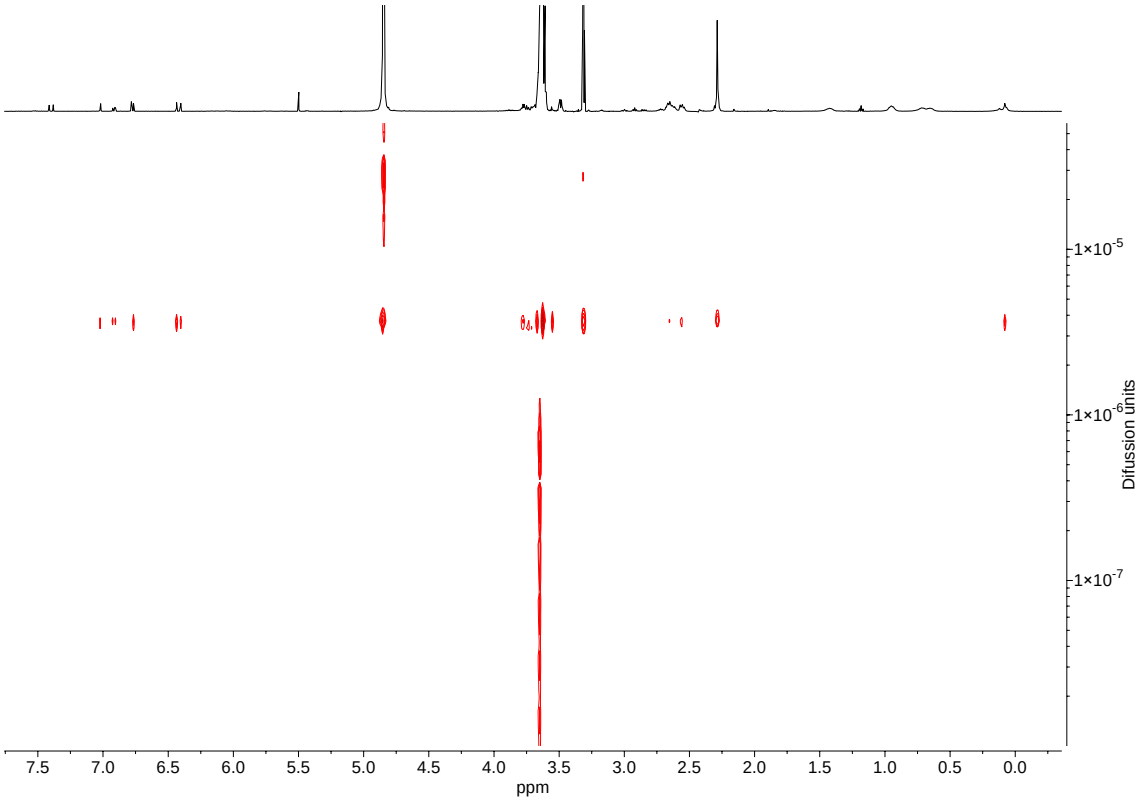


Figure S17. ^{1}H-DOSY-2D-NMR (500 MHz, CD_3OD) of compound **(13)**

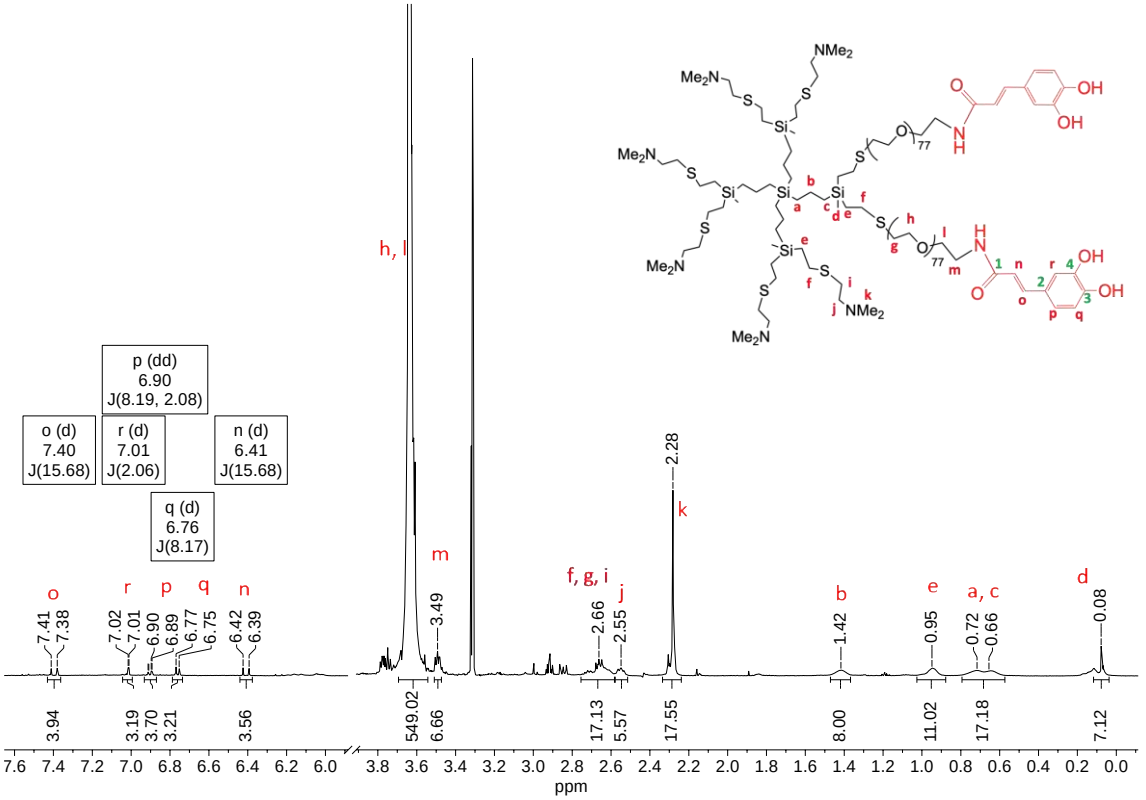


Figure S18. ^{1}H-NMR (500 MHz, CD_3OD) of compound **(14)**

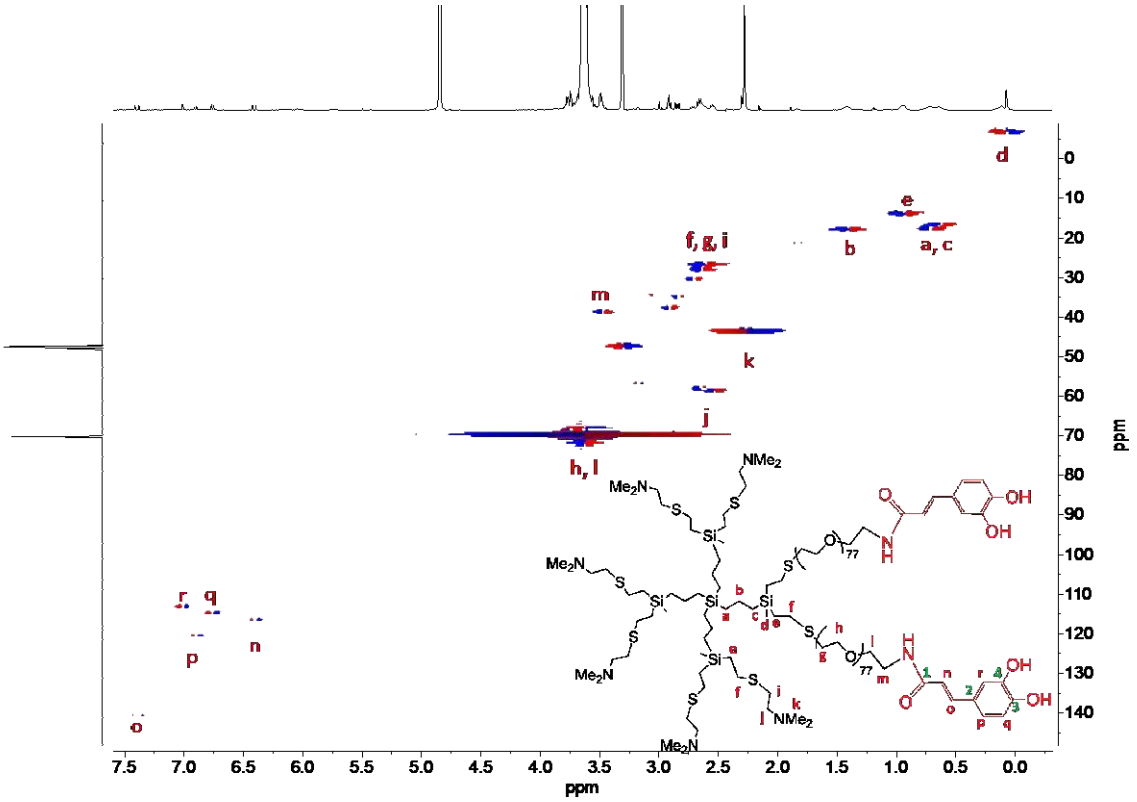


Figure S19. {^{1}H-^{13}C}-HSQC-2D-NMR (500 MHz, CD_3OD) of compound **(14)**

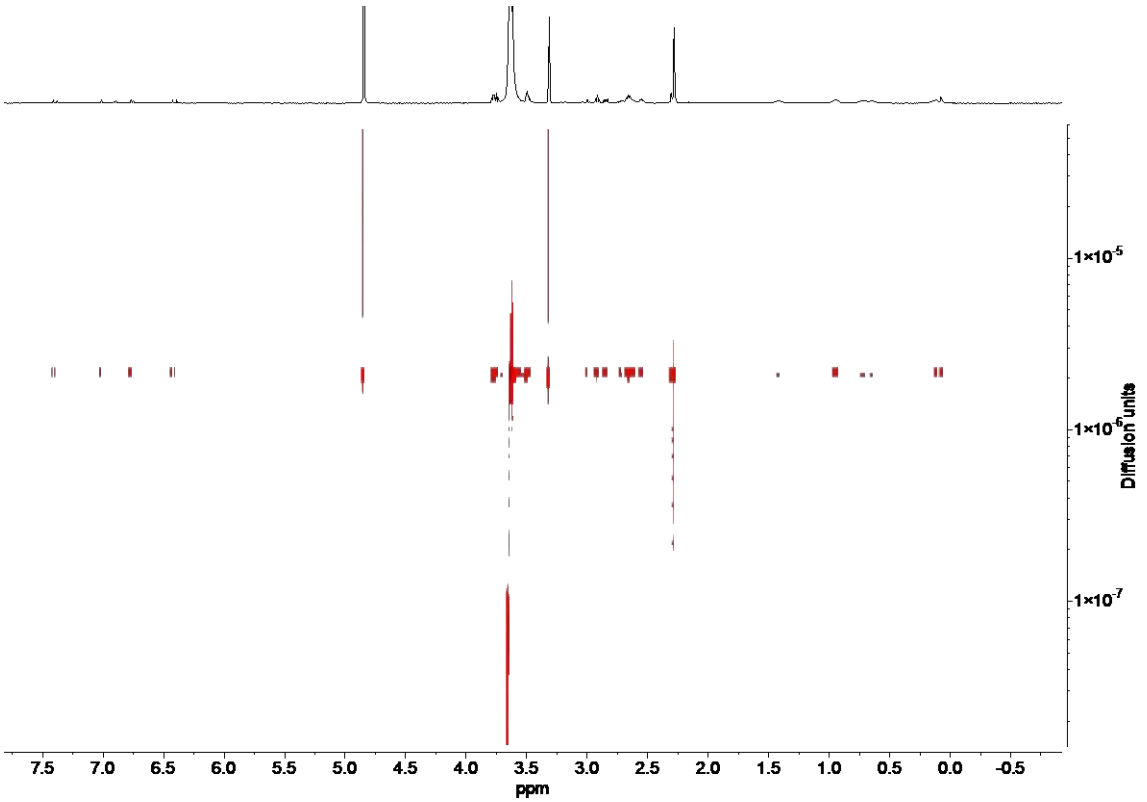


Figure S20. ^{1}H-DOSY-2D-NMR (500 MHz, CD_3OD) of compound **(14)**

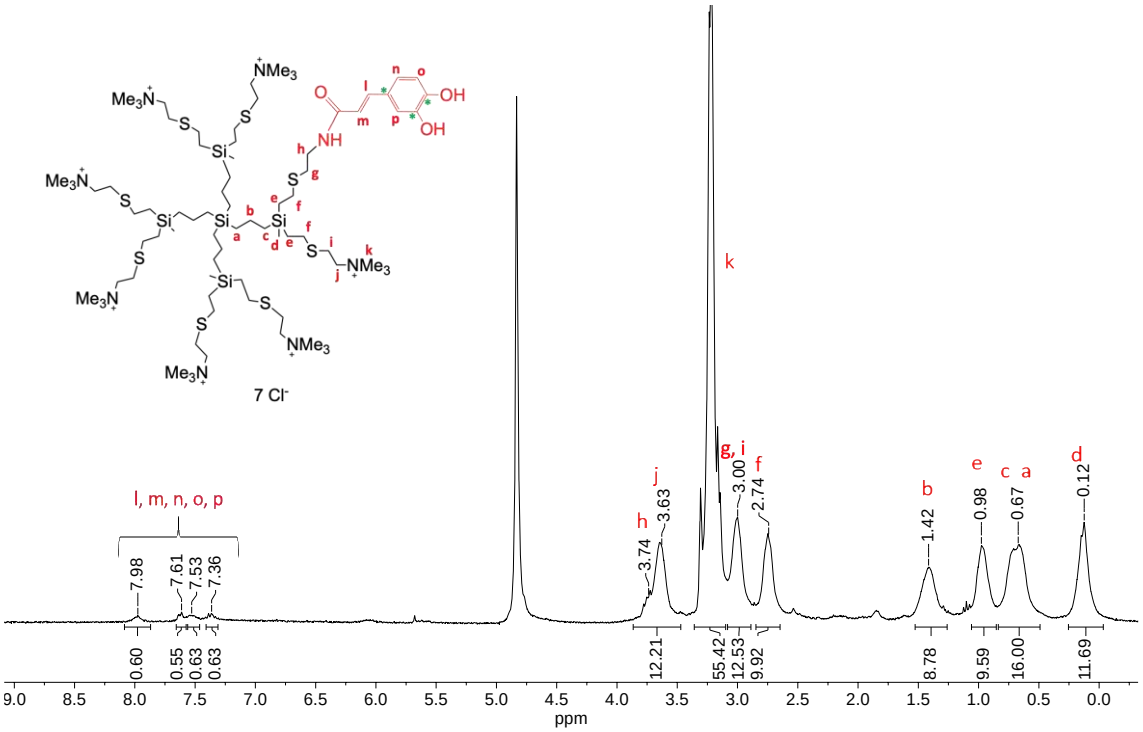


Figure S21. ^{1}H-NMR (500 MHz, CD_3OD) of compound **(15)**

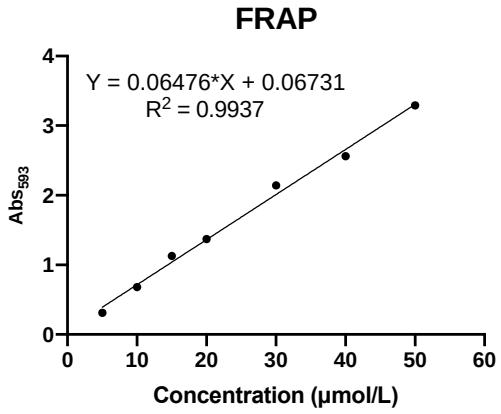


Figure S22. A representative calibration curves of FRAP values by Trolox standards

scientific reports

Check for updates

OPEN

A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion

Marika Grodzicka[1,2], Sylwia Michlewska[3]✉, Adam Buczkowski[4], Szymon Sekowski[5], Cornelia E. Pena-Gonzalez[6], Paula Ortega[6,7], Francisco Javier de la Mata[6,7], Janusz Blasiak[8], Maria Bryszewska[1] & Maksim Ionov[1,8]✉

The use of dendrimers as drug and nucleic acid delivery systems requires knowledge of their interactions with objects on their way to the target. In the present work, we investigated the interaction of a new class of carbosilane dendrimers functionalized with polyphenolic and caffeic acid residues with human serum albumin, which is the most abundant blood protein. The addition of dendrimers to albumin solution decreased the zeta potential of albumin/dendrimer complexes as compared to free albumin, increased density of the fibrillary form of albumin, shifted fluorescence spectrum towards longer wavelengths, induced quenching of tryptophan fluorescence, and decreased ellipticity of circular dichroism resulting from a reduction in the albumin α-helix for random coil structural form. Isothermal titration calorimetry showed that, on average, one molecule of albumin was bound by 6–10 molecules of dendrimers. The zeta size confirmed the binding of the dendrimers to albumin. The interaction of dendrimers and albumin depended on the number of caffeic acid residues and polyethylene glycol modifications in the dendrimer structure. In conclusion, carbosilane polyphenolic dendrimers interact with human albumin changing its structure and electrical properties. However, the consequences of such interaction for the efficacy and side effects of these dendrimers as drug/nucleic acid delivery system requires further research.

Keywords Polyphenolic dendrimers, Serum human albumin, Zeta potential, Circular dichroism, Isothermal titration calorimetry

Carbosilane dendrimers, similarly to their poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) counterparts, are hyperbranched and monodispersed macromolecules explored as drug and nucleic acid carriers in the therapy of many diseases, including cancer and viral infections[1]. Therefore, two aspects of dendrimers as drug/gene carriers must be considered: their efficacy as a carrier and their toxicity. The structure of dendrimers may be modified to increase the former and decrease the latter. The presence of multivalent functional end groups in the dendrimer structure creates ample opportunities for their modification aimed at acquiring specific functions, including the ability to modify the properties of biological macromolecules. Such modifications may result in

[1]Department of General Biophysics, Faculty of Biology and Environmental Protection, University of Lodz, Pomorska 141/143, 90-236 Lodz, Poland. [2]Department of General Biophysics, The Bio-Med-Chem Doctoral School of the University of Lodz and Lodz Institutes of the Polish Academy of Sciences, 21/23 Matejki, 90-237 Lodz, Poland. [3]Laboratory of Microscopic Imaging and Specialized Biological Techniques, Faculty of Biology and Environmental Protection, University of Lodz, Banacha 12/16, 90-237 Lodz, Poland. [4]Division of Biophysical Chemistry, Department of Physical Chemistry, Faculty of Chemistry, University of Lodz, Pomorska 165, 90-236 Lodz, Poland. [5]Laboratory of Molecular Biophysics, Department of Microbiology and Biotechnology, Faculty of Biology, University of Bialystok, Ciolkowskiego 1J, 15-245 Bialystok, Poland. [6]Department of Organic and Inorganic Chemistry, and Research Institute in Chemistry "Andrés M. del Río" (IQAR), Spain and Instituto Ramon y Cajal de Investigacion Sanitaria, IRYCIS, Universidad de Alcalá, Colmenar Viejo Road, Km 9, 100, 28034 Madrid, Spain. [7]Networking Research Center On Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Madrid, Spain. [8]Faculty of Medicine, Collegium Medicum, Mazovian Academy in Plock, Pl. Dabrowskiego 2, 09-402 Plock, Poland. ✉email: sylwia.michlewska@biol.uni.lodz.pl; maksim.ionov@biol.uni.lodz.pl

increased efficacy of the carried drug, which is the case in anticancer therapy when a modified dendrimer has anti-cancer properties[2,3].

Functionalization of dendrimers with polyphenolic residues can give or improve their antioxidant, anti-radical, antiviral, and anti-bacterial properties[4]. The functionalization of carbosilane dendrimers with caffeic acids increased their antioxidant and anti-radical activity[5]. Despite many advantages of positively charged dendrimers, they can form microholes in biological membranes, that may underlie their cellular and organismal toxicity[2]. The incorporation of polyethylene glycol (PEG) residues into the dendrimer structure was reported to have beneficial consequences, increasing its bioavailability and decreasing its toxicity[2]. In addition, PEG is non-immunogenic, water-soluble, nontoxic and can reduce interactions of dendrimers with serum proteins[7,8].

Drug and nucleic acid carriers encounter many objects on their way to the target site. Albumin is the most abundant protein in blood and therefore its interaction with drug and gene carriers should be considered as it may lead to toxicity, change bioavailability, and potential therapeutic effect. Human serum albumin (HSA) is a monomeric protein with one tryptophan residue and three homologous domains. Each domain has 2 subdomains to which different molecules can be attached. Subdomain IIA which contains the only HSA tryptophan residue is the main hydrophobic site for ligand binding and dendrimers interact with this site mainly through hydrophobic interactions and may form "protein corona"[9]. This effect is crucial for the medical application of dendrimers because their absorption on the albumin surface can change their properties, bioavailability, and distribution[10]. In the present work, the interaction of a new class of 1st generation carbosilane dendrimers functionalized with various numbers of caffeic acids and PEG residues with human serum albumin in vitro was investigated with a plethora of biochemical and biophysical techniques.

Materials and methods

Dendrimers

Four water-soluble 1st generation heterofunctionalized polyphenolic carbosilane dendrimers $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$ (**1**); $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$ (**2**); $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$ (**3**) and $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$ (**4**) with ammonium and caffeic acid surface groups were used in this study. The structure and molar mass of dendrimers are presented in Fig. 1. The procedure of the synthesis of these compounds was described elsewhere[5].

Zeta potential and zeta size

The hydrodynamic diameter and the zeta potential of human serum albumin in the presence of the dendrimers were measured using a Zetasizer Nano-ZS photon correlation spectrometer (Malvern Instruments, Malvern, Worcestershire, UK). Measurements were performed in a 10 mmol/L phosphate buffer at pH 7.4 at 25 °C. Zeta potential values were calculated directly from the Helmholtz-Smoluchowski equation and Malvern Zetasizer Nano software v3.30 (Malvern Panalytical Ltd., Malvern, UK) was used for data analysis. At least 3 separate replicates were performed for each experiment.

Transmission electron microscopy

The ultrastructure of the albumin-dendrimer complexes at the molar ratio 1:10 was visualised using a JEOL-1010 (JEOL, Tokyo, Japan) transmission electron microscope. The samples in 10 mmol/L Na-phosphate buffer, pH 7.4, were placed on 200 mesh copper grids with a carbon surface (Ted Pella, Inc, Redding, CA, USA). Samples were stained with 2% uranyl acetate for 2 min, washed with deionized water, and dried at room temperature. Images were taken at a magnification of 100,000 × and analysed with conventional software.

Fluorescence spectroscopy

To analyse the character of interaction between albumin and dendrimers, tryptophan fluorescence was measured using a Perkin-Elmer LS-55B fluorescence spectrometer. Appropriate amounts of dendrimers were added to albumin solution (4 μmol/L) with the final albumin:dendrimer molar ratios of 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10. The excitation wavelength for tryptophan was $\lambda_{exc} = 295$ nm and fluorescence spectra were taken in the 305–450 nm range. Excitation and emission slits were set at 2.5 and 8.0 nm, respectively. Fluorescence spectra of albumin were corrected for the proper baselines. All measurements were performed at 25 °C in 3 independent replicates. Stern–Volmer constant (K_{SV}), quenching constant (k_q), and binding constant ($\log K_b$) were calculated.

Circular dichroism

The circular dichroism (CD) spectra of dendrimer/HSA complexes were measured using a J-815 CD spectrometer (Jasco, Tokyo, Japan). The concentration of albumin was 0.25 μmol/L. Protein/dendrimer complexes were prepared in 10 mmol/L phosphate buffer, pH 7.4, at molar ratios ranging from 1:1 to 1:10. Measurements were made at room temperature, in 5-mm path-length quartz cuvettes. The recording parameters were scan speed 50 nm/min, step resolution 1 nm, response time 4 s, bandwidth – 1.0 nm, slit—auto. CD spectra were obtained in the wavelength range 195–260 nm, as the average of a minimum of 3 independent experiments composed from 3 accumulated scans of each replication. The protein secondary structure and distribution of α-structure and β-sheet percentage under the influence of dendrimers were calculated using the CDNN software available at: https://cdnn-circular-dichroism-spectroscopy-deconvolution.updatestar.com.

Isothermal titration calorimetry

An isothermal titration calorimetric study was performed to measure the thermodynamic properties of the interaction between the dendrimers and albumin. Albumin solution (182 μl; 100 μmol/L in a cell) was titrated

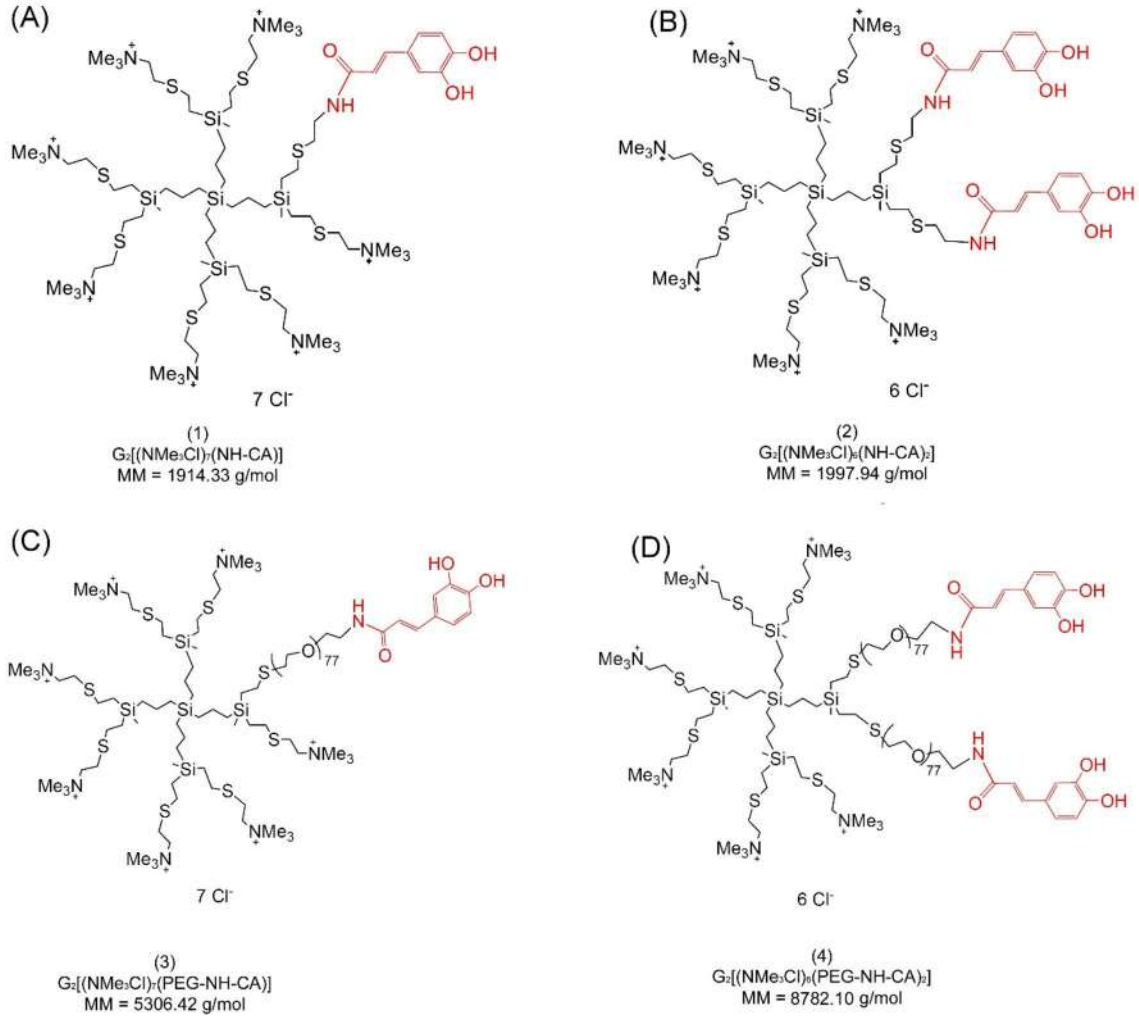


Figure 1. Structure, chemical formula, and molar mass (MM) of polyphenolic carbosilane dendrimers with ammonium surface groups functionalized with caffeic acid and polyethylene glycol (PEG). (**A**) compound (**1**), $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$; (**B**) compound (**2**), $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$; (**C**) compound (**3**), $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$; (**D**) compound (**4**), $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$. Caffeic acid moieties are marked in red.

by adding 40 × 5 µl doses of a 2 mmol/L dendrimer solution in 5-min intervals. Measurements of the thermal effects of the titration were carried out at 25 °C with a stirring rate of 125 RPM in auto-equilibrate mode. During titration, the molar ratio of dendrimer to albumin increased from 1.1:1 to 40:1. The thermal effects of the direct interaction of albumin with dendrimers were calculated by subtracting the effects of dilution of the dendrimer from the corresponding thermal effects of the titration of albumin solution with the dendrimer solution. The binding isotherms were analysed in NanoAnalyze software by a non-linear multiparameter regression using the independent-site model to calculate the stoichiometric parameter n, equilibrium binding constant K, and standard thermodynamic functions of the process of albumin binding with dendrimers: enthalpy ΔH, entropy, ΔS and Gibbs free energy ΔG.

Data analysis

All results were obtained from at least 3 independent experiments and are presented as mean ± SD (standard deviation). Statistical analyses were performed for paired samples using GraphPad Prism version 8.0.1 software (GraphPad Software, Boston, MA, USA). The normality of the study sample was checked using the Shapiro–Wilk test. In the case of a normal distribution of data, the Student's t-test was used, while in other cases the ANOVA post hoc Dunnett's test was used. The 95% confidence interval was set as the confidence interval.

Results

Dendrimers form complexes with albumin as revealed by the zeta potential, zeta size, and transmission electron microscopy

The zeta average size of free albumin was 172.15 ± 15.54 nm and the addition of dendrimers led to an increase in particle size indicating the binding of dendrimer to albumin (Fig. 2B). The highest hydrodynamic diameters

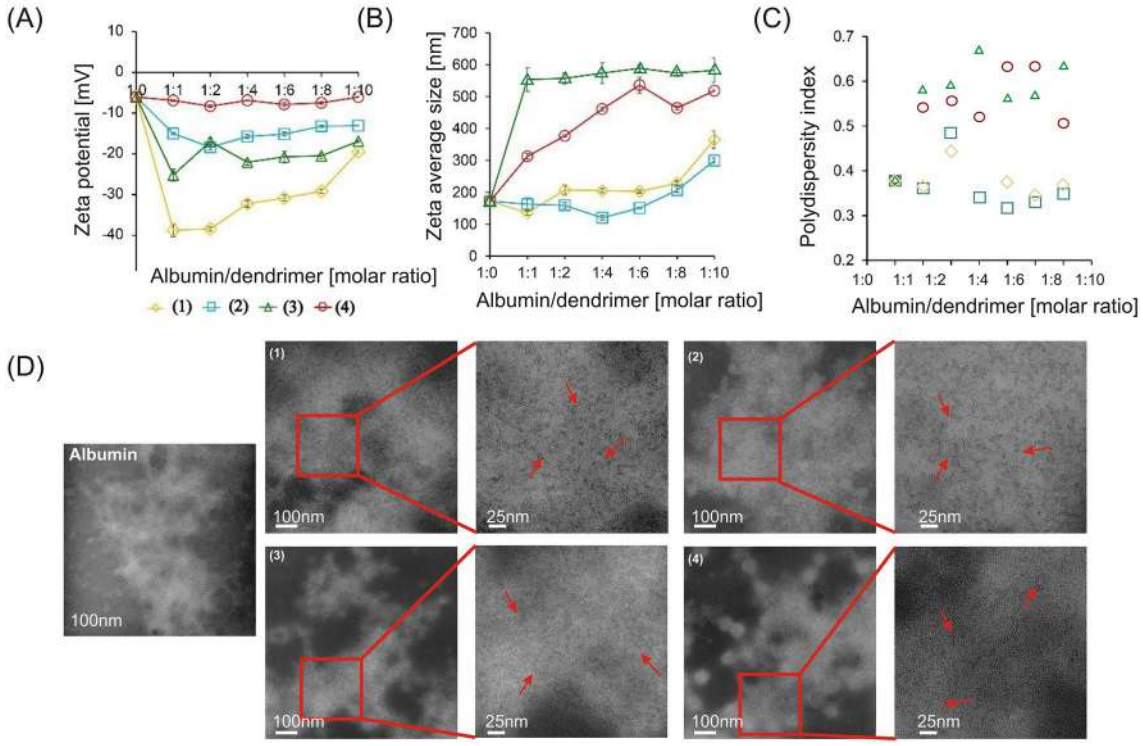


Figure 2. Dose-dependent effects of polyphenolic dendrimers on zeta potential (**A**), zeta size (**B**), and polydispersity index (**C**). Data points represent mean ± SD obtained from a minimum 3 experiments and each experiment was done in 7 replicates for dendrimer (**1**) (yellow line open diamonds), (**2**) (blue line, open squares) (**3**) (green line, open triangles) and (**4**) (red line, and open circles). Panels (**D**) represent the ultrastructure of human serum albumin in the presence of dendrimers. Bars 100 nm and 25 nm, to obtain greater contrast, the color of the micro images has been inverted.

of the dendrimer-albumin complexes were observed in the presence of the dendrimers (**3**) and (**4**). In that case, the size of the complexes at the highest tested molar ratio 1:10 increased to 582.45 ± 38.51 nm (**3**) and 518.33 ± 2.13 nm (**4**). The size of complexes formed with dendrimers (**1**) and (**2**) were smaller, 356.26 ± 26.89 and 299 ± 7.37 respectively, as they did not have any polyethylene glycol chain. The polydispersity index (PDI) of the complexes formed by compounds (**1**) and (**2**) was around 0.4, while in the presence of compounds (**3**) and (**4**) it increased to 0.6 and higher values (Fig. 2C).

The zeta potential of free albumin was − 6.04 ± 0.39 mV and the addition of dendrimers (**1**), (**2**), and (**3**) decreased it. This effect was most pronounced with dendrimers with one caffeic acid residue. Zeta potential values with dendrimers (**1**) and (**3**) were − 38.76 ± 0.62 mV and − 25.17 ± 0.83 mV, respectively, while for (**3**) it was − 18.36 ± 0.28 mV and for (**4**) − 8.30 ± 0.22 mV (Fig. 2A).

Free albumin structure presented a fibrillary form, and the presence of dendrimers made albumin molecules slightly more compact (Fig. 2D). This effect was maximal for the complexes formed by dendrimer (**2**) and minimal for (**4**). All complexes were presented as fibrillar structures with small globular structures (Fig. 2D -arrows).

Fluorescence quenching confirms the formation of albumin-dendrimer complexes

The binding of dendrimers changed the fluorescence spectrum of the tryptophan residue in albumin towards longer wavelengths (Fig. 3). The most pronounced effect was observed for the dendrimer (**4**).

To quantitatively assess the effect of dendrimers on the fluorescence properties of albumin, we calculated the Stern–Volmer constant (K_{SV}), which indicates the efficacy of dendrimers in quenching fluorescence of tryptophan (Table 1). We also calculated the quenching constant k_q to differentiate between the static and dynamic character of quenching by dendrimers and the logarithm of binding constant ($\log K_b$) for all dendrimers (Table 1)[4]. Since k_q for each dendrimer was greater than 2×10^{10} M^{-1} s^{-1}, we concluded that the dendrimers quenched fluorescence via the static mechanism by the formation of complexes with albumin.

The most pronounced quenching effect was observed for dendrimers (**1**) and (**2**), which were characterized by the highest K_{SV}. Consequently, these dendrimers were characterized by the highest $\log K_b$.

Dendrimers slightly change the secondary structure of albumin

We observed two standard negative CD bands for albumin at 208 and 222 nm, which were slightly changed in the presence of the dendrimers (Fig. 4). The changes in the secondary structure of albumin induced by the dendrimers were evaluated with the CDNN software (Table 2). Slight conformational changes in the albumin secondary structure were observed in the presence of the dendrimers.

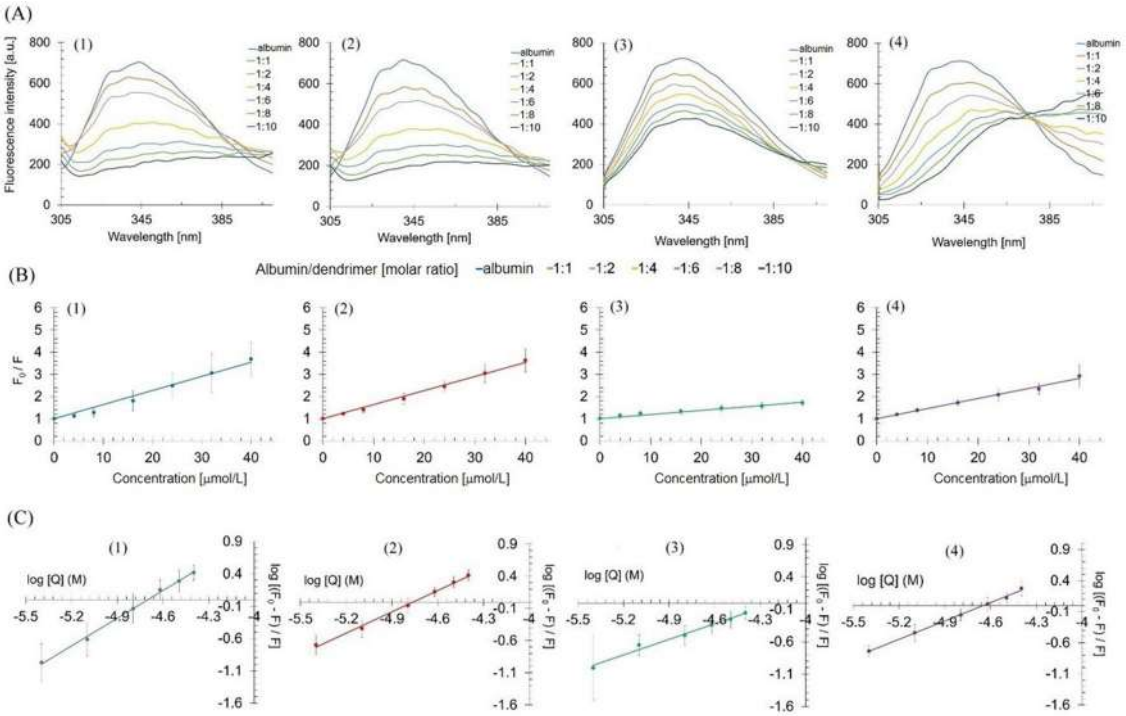


Figure 3. Fluorescence emission spectra of albumin-dendrimer complexes for dendrimers (**1–4**) at the albumin/dendrimer molar ratios 1–10 (**A**). Stern–Volmer plots of tryptophan fluorescence quenching in the presence of increasing concentrations of dendrimers (**B**), double-logarithmic plot of tryptophan fluorescence quenching at albumin concentration 4 μmol/L (**C**). Data points represent mean ± SD obtained from a minimum 3 separate experiments.

Compound	K_{SV} [M^{-1}]	k_q [M^{-1} s^{-1}]	$logK_b$
(1)	$(6.35 \pm 2.26) \times 10^4$	$(1.27 \pm 0.45) \times 10^{13}$	6.72 ± 0.62
(2)	$(6.34 \pm 1.27) \times 10^4$	$(1.27 \pm 0.25) \times 10^{13}$	5.26 ± 0.20
(3)	$(1.86 \pm 0.49) \times 10^4$	$(0.37 \pm 0.10) \times 10^{13}$	3.38 ± 1.39
(4)	$(4.54 \pm 1.05) \times 10^4$	$(0.91 \pm 0.21) \times 10^{13}$	4.57 ± 0.29

Table 1. Protein-polyphenolic dendrimers interaction parameters calculated based on fluorescence results.

On average, one molecule of albumin is bound by 6–10 molecules of dendrimers

Isothermal titration calorimetry analysis (Fig. 5) enabled to calculation some parameters characterizing complexes formed by albumin and dendrimers, namely the stoichiometric parameter n, equilibrium binding constant K, change in enthalpy ΔH, entropy ΔS, and Gibbs free energy ΔG (Table 3).

These results show that one albumin molecule can be associated with approximately 10 molecules of dendrimers. The binding equilibrium constant K and standard thermodynamic functions indicate that the process of dendrimers binding with albumin was thermodynamically spontaneous ($\Delta G < 0$). The driving force behind albumin binding with (**1**), (**2**), and (**3**) dendrimer was a favourable increasing disorder of reagents ($T\Delta S > 0$), which overcame the unfavourable endothermic effects of the direct interactions of the reagent ($\Delta H > 0$). Interactions of HSA with dendrimer (**4**), with 2 caffeic moieties and 2 PEG chains, were favourable, as both the exothermic enthalpy of binding ($\Delta H < 0$) and increasing disorder of reagents ($T\Delta S > 0$), reflecting the role of the 2 polar side PEG chains in the structure.

Discussion

The interaction of dendrimers with serum proteins affects their bioavailability. That interaction may be underlined by various mechanisms, first of all of electrostatic nature, and may not only limit the bioavailability of dendrimers but also underlie their toxicity. In this work, we investigated the interaction of a new class of 1st generation heterofunctional polyphenolic dendrimers modified with caffeic acid with human serum albumin, the most abundant blood protein. We showed that these dendrimers bound albumin in a stochiometric ratio of 5–10 molecules of dendrimers per single molecule of albumin. This binding was confirmed by several methods. Despite the binding, the secondary structure of albumin was not significantly affected.

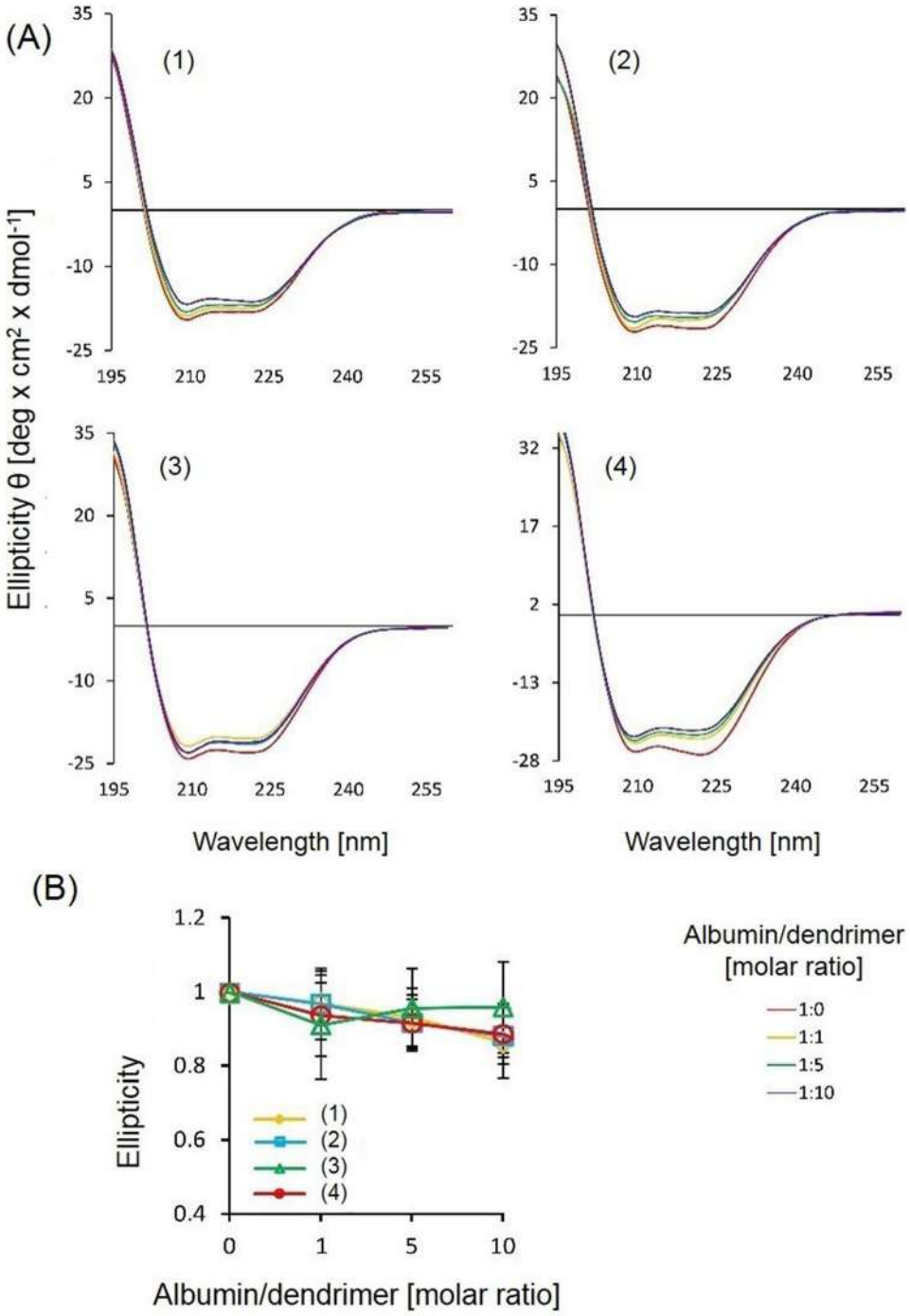


Figure 4. Circular dichroism spectra of free albumin and its complexes with the dendrimers (**A**). Relative ellipticity of the albumin-dendrimer complexes at its different ratios. Data are presented as means ± SD obtained from a minimum of 3 separate experiments.

Dendrimers can bind albumin via electrostatic interactions, hydrogen bonding, hydrophobic interactions, and specific interactions between dendrimer groups and aliphatic acid binding sites of the protein[11–13]. In this study, all dendrimers changed the zeta potential of albumin. Cationic and neutral dendrimers were reported to change the zeta potential of proteins[9]. We showed that the dendrimers (**1**) and (**3**) that were not modified with PEG strongly changed the zeta potential as their inner positive charge was not shielded by PEG as in dendrimers (**3**) and (**4**).

We observed a red-shift effect in our fluorescence experiments, which was the most pronounced for the dendrimer (**4**) that had 2 caffeic acid moieties and PEG. Therefore, that dendrimer might interact with a hydrophilic region of albumin. The formation of complexes of dendrimers and albumin was confirmed in quenching experiments, in which quenching constants were larger than 2×10^{10} M^{-1} s^{-1} for all dendrimers. The Stern–Volmer

Dendrimer	α-helix (%)	β-sheet (%)	Random coil (%)
Free albumin	56.73 ± 2.51	13.26 ± 0.35	17.47 ± 1.28
(1)	53.40 ± 5.65	13.63 ± 0.85	21.07 ± 1.77
(2)	55.53 ± 7.59	13.36 ± 1.09	19.60 ± 3.32
(3)	57.00 ± 1.83	13.23 ± 0.28	18.23 ± 0.67
(4)	57.45 ± 10.1	13.15 ± 1.48	18.60 ± 4.24

Table 2. Contribution of α-helix, β-sheet, and random coil to the overall structure of free albumin and albumin-dendrimer complexes at 1:10 molar ratio calculated with CDNN Software.

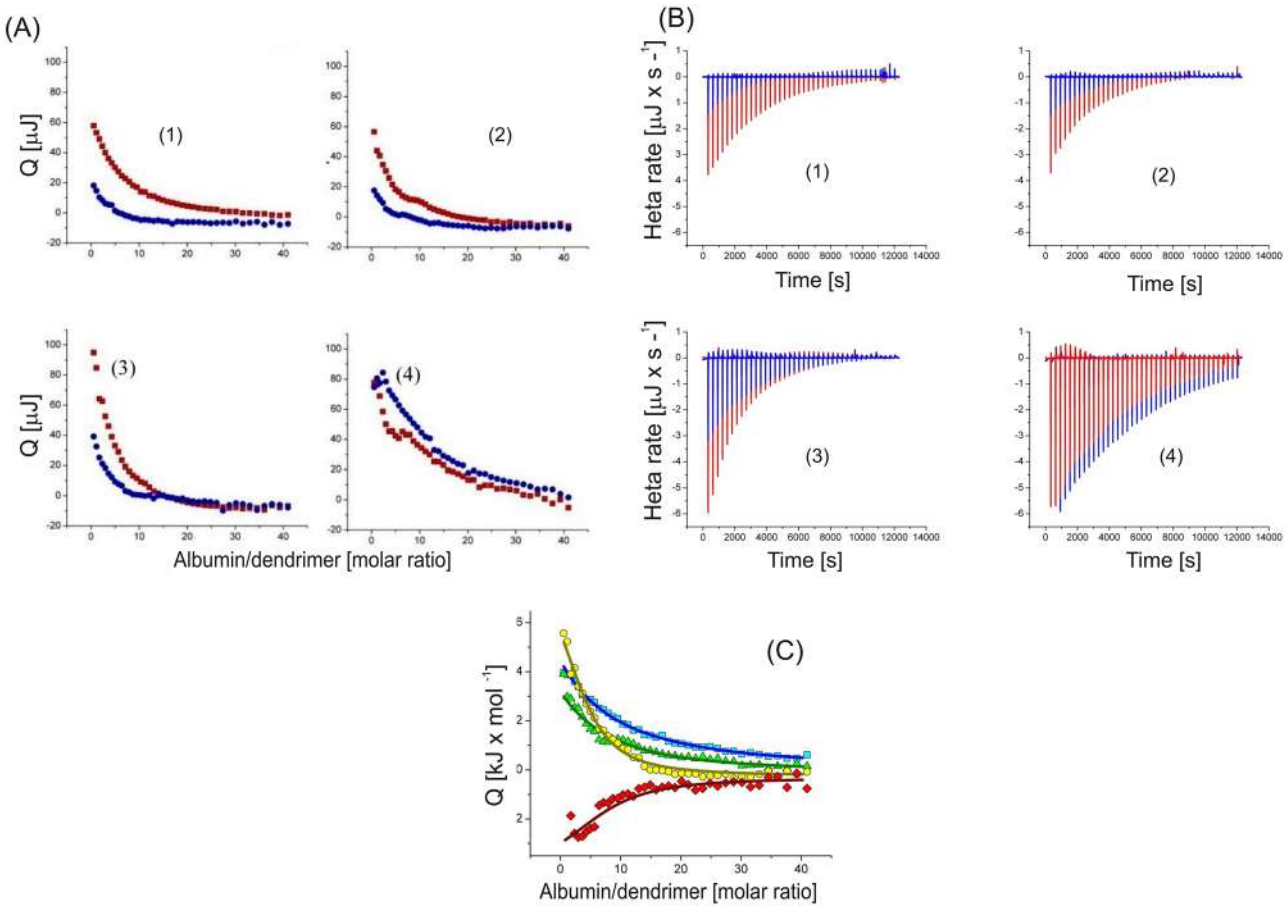


Figure 5. Integrated thermal effects of the isothermal titration calorimetry titration of 100 µmol/L HSA solution with 2 mmol/L solution of each dendrimer (red squares) and corresponding effects of the dilution of dendrimer (blue circles) (**A**). Curves of thermal power as a function of time during titration of 100 µmol/L albumin solution with 2 mmol/L dendrimer solution (red peaks) and dilution of 2 mmol/L dendrimer solution into buffer without albumin (blue peaks). Downward peaks correspond to endothermic heat effects (**B**). Thermal effects of direct interactions (per mole of injectant) between albumin and polyphenolic carbosilane dendrimers as a function of dendrimer to albumin mole ratio. All titrations were carried out at 25 °C in aqueous 10 mmol/L phosphorous buffer solution pH 7.4.

analysis revealed that the dendrimers (**1**) and (**2**), had the largest K_{SV} values, and that corresponded with the magnitude of the quenching effect induced by these compounds. The presence of PEG in the dendrimer structure causes charge masking and an increase in surface density. Therefore, there is a reduced availability of the inner positive charges in any interactions with dendrimers (**3**) and (**4**). On the contrary, the attachment of the caffeic acid residue by the addition of a PEG molecule may result in an easier detachment of this residue from the carbosilane skeleton. Therefore, it is likely that the presence of two PEG molecules results in an easier release of caffeic acid residues, and therefore a higher availability to the carbosilane skeleton.

Our CD study showed that the dendrimers did not change the secondary structure of albumin and observed slight changes in the proportion of α-helix, β-sheet, and random coil in the overall structure of albumin were non-significant. Similar effects were described for cationic PAMAM g3 and g4 dendrimers modified by polyethylene

Dendrimer	*n*	*logK*	ΔH (kJ mol^{-1})	$T\Delta S$(kJ mol^{-1})	ΔG(kJ mol^{-1})
(1)	10 ± 3	3.49 ± 0.57	25 ± 14	45 ± 3	-20 ± 17
(2)	9 ± 4	3.81 ± 0.47	15 ± 8	37 ± 9	-22 ± 17
(3)	6 ± 4	3.61 ± 0.94	8.3 ± 6.8	29 ± 13	-21 ± 20
(4)	7 ± 5	3.76 ± 0.46	-4.0 ± 3.2	17 ± 14	-21 ± 17

Table 3. Stoichiometric parameter *n*, equilibrium binding constant *K*, change in enthalpy ΔH, entropy ΔS, and Gibbs free energy ΔG of the formation of albumin-dendrimer complexes evaluated by isothermal titration calorimetry.

glycol (PEG), and sugar-persubstituted PAMAM dendrimers of the 3rd and 5th generations did not change the secondary structure of albumin[14]. The size and flexibility of dendrimers are crucial in their interaction with proteins, as rigid nanoparticles can change protein structure from α-helix to β-sheets, more flexible particles can rather change the protein structure from α helix to random coil[15]. The weak effect of polyphenolic dendrimers on the albumin secondary structure can be explained by the fact that less hydrophobic dendrimers would exert a smaller effect on the hydrophobic amino acids in the protein composition. While the changes in the percentage of α-helices in the presence of dendrimers provided information about weak changes in the overall protein secondary structure, Trp fluorescence quenching reflected the local changes in the vicinity of the tryptophan residue. This may underline apparent discrepancies in the results we obtained. A similar apparent inconsistency in these 2 methods in the study of dendrimer interactions with thrombin has also been observed[15]. The same studies also showed that the presence of PEG reduced interactions with that serum protein. While dendrimers with reactive aldehyde terminal groups quenched albumin fluorescence and changed the secondary structure of albumin, modification of the terminal groups with phosphonate or PEG quenched Trp fluorescence, but they did not change the conformation of the protein[13]. A similar situation was observed for interactions of other dendrimers with other proteins[16].

Isothermal titration calorimetry analysis indicated that 6–10 molecules of polyphenolic dendrimers might associated with one molecule of albumin. This is in line with our previous study showing that a single molecule of HSA could bind on average 6 particles of dendrimers[17]. The differences between the logarithm of K_b received from fluorescence quenching and ITC analyses can be explained, similarly to CD, by the difference in local (Trp fluorescence) and global (affinity to the whole of albumin) interactions of dendrimers.

As we stated in the introductory section, dendrimers might interact with HSA through its main hydrophobic site encompassing the tryptophan residue, but the precise nature of this interaction remains unknown. However, some dendrimers can adopt a directional three-dimensional structure exposing their hydrophobic inner core and enabling hydrophobic interactions with suitable domains of a protein[18].

On of the limitations of our work is associated with the actual structure of the dendrimers. The process of the functionalization of the dendrimers with polyphenolic moieties is stochastic, excluding the determination of the exact position of polyphenolic groups in the dendritic structure. Therefore, the layout of the branches functionalized with polyphenolic residues cannot be unequivocally determined, potentially affecting their interaction with HSA. The question remains whether such affected interaction was of biological significance and whether it was measurable with the techniques that were applied in this work. Further studies with a more controlled procedure of dendrimer synthesis and functionalization may shed light on these problems.

Conclusions

Carbosilane polyphenolic dendrimers can interact with human serum albumin and this interaction depends on the presence of polyethylene glycol and caffeic acid residues. Therefore, albumin may decrease the bioavailability of these dendrimers, which should be considered in strategies for the use of these dendrimers as drug/gene carriers. Furthermore, the interaction of these dendrimers with albumin may lead to their toxicity and require further research.

Data availability

Data are available upon reasonable request from corresponding authors: Sylwia Michlewska Tel.: + 48 42 635 44 31, e-mail: sylwia.michlewska@biol.uni.lodz.pl. Maksim Ionov Tel.: + 48 42 635 43 80, e-mail: maksim.ionov@biol.uni.lodz.pl.

Received: 28 December 2023; Accepted: 7 March 2024
Published online: 11 March 2024

References

1. Sepúlveda-Crespo, D., Gómez, R., De La Mata, F. J., Jiménez, J. L. & Muñoz-Fernández, M. Polyanionic carbosilane dendrimer-conjugated antiviral drugs as efficient microbicides: Recent trends and developments in HIV treatment/therapy. *Nanomedicine* **11**, 1481–1498. https://doi.org/10.1016/j.nano.2015.03.008 (2015).
2. Michlewska, S. *et al.* Lipid-coated ruthenium dendrimer conjugated with doxorubicin in anti-cancer drug delivery: Introducing protocols. *Colloids Surf. B Biointerfaces* **227**, 113371. https://doi.org/10.1016/j.colsurfb.2023.113371 (2023).
3. Michlewska, S. *et al.* Ruthenium metallodendrimer against triple-negative breast cancer in mice. *Nanomed. Nanotechnol. Biol. Med.* **53**, 102703. https://doi.org/10.1016/j.nano.2023.102703 (2023).

4. Sanz Del Olmo, N. *et al.* Antioxidant and antibacterial properties of carbosilane dendrimers functionalized with polyphenolic moieties. *Pharmaceutics* **12**, 1. https://doi.org/10.3390/pharmaceutics12080698 (2020).
5. Grodzicka, M. *et al.* Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity. *Sustain. Mater. Technol.* **33**, e00497. https://doi.org/10.1016/j.susmat.2022.e00497 (2022).
6. Somani, S. *et al.* PEGylation of polypropylenimine dendrimers: effects on cytotoxicity, DNA condensation, gene delivery and expression in cancer cells. *Sci. Rep.* **8**, 9410. https://doi.org/10.1038/s41598-018-27400-6 (2018).
7. Jain, K., Kesharwani, P., Gupta, U. & Jain, N. K. Dendrimer toxicity: Let's meet the challenge. *Int. J. Pharm.* **394**, 122–142. https://doi.org/10.1016/j.ijpharm.2010.04.027 (2010).
8. Thakur, S., Kesharwani, P., Tekade, R. & Jain, N. Impact of pegylation on biopharmaceutical properties of dendrimers. *Polymer* https://doi.org/10.1016/j.polymer.2014.12.051 (2015).
9. Shcharbin, D. *et al.* Nanoparticle corona for proteins: Mechanisms of interaction between dendrimers and proteins. *Colloids Surf. B Biointerfaces* **134**, 377–383. https://doi.org/10.1016/j.colsurfb.2015.07.017 (2015).
10. Kubczak, M. *et al.* The effect of novel tyrosine-modified polyethyleneimines on human albumin structure—Thermodynamic and spectroscopic study. *Colloids Surf. B Biointerfaces* **227**, 113359. https://doi.org/10.1016/j.colsurfb.2023.113359 (2023).
11. Klajnert, B. & Bryszewska, M. Fluorescence studies on PAMAM dendrimers interactions with bovine serum albumin. *Bioelectrochemistry* **55**, 33–35. https://doi.org/10.1016/s1567-5394(01)00170-0 (2002).
12. Klajnert, B., Stanisławska, L., Bryszewska, M. & Pałecz, B. Interactions between PAMAM dendrimers and bovine serum albumin. *Biochim. Biophys. Acta* **1648**, 115–126. https://doi.org/10.1016/s1570-9639(03)00117-1 (2003).
13. Moreno, S. *et al.* Synthesis, characterization and biological properties of new hybrid carbosilane–viologen–phosphorus dendrimers. *RSC Adv.* **5**, 25942–25958. https://doi.org/10.1039/C5RA00960J (2015).
14. Froehlich, E., Mandeville, J. S., Jennings, C. J., Sedaghat-Herati, R. & Tajmir-Riahi, H. A. Dendrimers bind human serum albumin. *J. Phys. Chem. B* **113**, 6986–6993. https://doi.org/10.1021/jp9011119 (2009).
15. Shcharbin, D. *et al.* Binding of poly(amidoamine), carbosilane, phosphorus and hybrid dendrimers to thrombin-Constants and mechanisms. *Colloids Surf. B Biointerfaces* **155**, 11–16. https://doi.org/10.1016/j.colsurfb.2017.03.053 (2017).
16. Ionov, M. *et al.* Effect of dendrimers on selected enzymes–Evaluation of nano carriers. *Int. J. Pharm.* **499**, 247–254. https://doi.org/10.1016/j.ijpharm.2015.12.056 (2016).
17. Sekowski, S., Buczkowski, A., Palecz, B. & Gabryelak, T. Interaction of polyamidoamine (PAMAM) succinamic acid dendrimers generation 4 with human serum albumin. *Spectrochim. Acta Part A Mol. Biomol. Spectrosc.* **81**, 706–710. https://doi.org/10.1016/j.saa.2011.07.009 (2011).
18. Caminade, A. M. *et al.* The key role of the scaffold on the efficiency of dendrimer nanodrugs. *Nat. Commun.* **6**, 7722. https://doi.org/10.1038/ncomms8722 (2015).

Acknowledgements

The authors wish to thank Sława Glińska from the Laboratory of Microscopic Imaging & Specialized Biological Techniques, Faculty of Biology & Environmental Protection, University of Lodz for her technical assistance.

Author contributions

M.G. investigation, writing—original draft, S.M. data curation, formal analysis, methodology, investigation, visualization, writing—original draft, writing—review and editing, A.B. investigation, methodology, writing—original draft, software, S.S. investigation, methodology, writing—original draft, software, C.E.P.G. writing—review and editing, sources, P.O. writing—review and editing, sources, F.J.M. writing—review and editing, sources, J.B. writing—review and editing, data curation, M.B. funding acquisition, data curation, writing—review and editing, M.I. conceptualization, writing—review and editing, project administration, data curation, formal analysis, supervision.

Funding

This work was supported by grant 2018/30/Z/NZ1/00911 of the Project "NanoTendo" and by grant 2023/07/X/NZ7/00026 of the Project "MINIATURA7" financed by the National Science Centre, Poland under the M-ERA.NET 2, which has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no 685451, co-financed by grant of the National Science Centre of Poland under "Beethoven Life 1" program, project "NP-HALE" no: 2018/ 31/F/NZ5/03454, and grant PID2020- 112924RBI00 (MINECO).

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to S.M. or M.I.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Chemistry and Physics of Lipids 265 (2024) 105444

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Effect of polyphenolic dendrimers on biological and artificial lipid membranes

Marika Grodzicka [a,b,c,*], Sylwia Michlewska [c,**], Adam Buczkowski [d], Paula Ortega [e,f], Francisco Javier de la Mata [e,f], Maria Bryszewska [a], Maksim Ionov [a,g]

[a] University of Lodz, Faculty of Biology and Environmental Protection, Department of General Biophysics, Pomorska 141/143, Lodz 90-236, Poland
[b] The Bio-Med-Chem Doctoral School of the University of Lodz and Lodz Institutes of the Polish Academy of Sciences, Department of General Biophysics, Pomorska 141/143, Lodz 90-236, Poland
[c] University of Lodz, Faculty of Biology and Environmental Protection, Laboratory of Microscopic Imaging and Specialized Biological Techniques, Banacha 12/16, Lodz 90-237, Poland
[d] University of Lodz, Faculty of Chemistry, Department of Physical Chemistry, Division of Biophysical Chemistry, Pomorska 165, Lodz 90-236, Poland
[e] Universidad de Alcalá. Department of Organic and Inorganic Chemistry, and Research Institute in Chemistry "Andrés M. del Río" (IQAR), Spain and Instituto Ramon y Cajal de Investigacion Sanitaria, IRYCIS, Colmenar Viejo Road, Km 9, 100, Madrid 28034, Spain
[f] Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain
[g] Mazovian Academy in Plock, Collegium Medicum, Faculty of Medicine, Pl. Dabrowskiego 2, Plock 09-402, Poland

ARTICLE INFO

Keywords:
Polyphenolic dendrimers
DMPC/DPPG liposomes
Erythrocyte membrane
Haemotoxicity
Differential scanning calorimetry

ABSTRACT

The use of dendrimers as nanovectors for nucleic acids or drugs requires the understanding of their interaction with biological membranes. This study investigates the impact of 1st generation polyphenolic carbosilane dendrimers on biological and model lipid membranes using several biophysical methods. While the increase in the z-average size of DMPC/DPPG liposomes correlated with the number of caffeic acid residues included in the dendrimer structure, dendrimers that contained polyethylene glycol chains generated lower zeta potential when interacting with a liposomal membrane. The increase in the fluorescence anisotropy of DPH and TMA-DPH probes incorporated into erythrocyte membranes predicted the ability of dendrimers to affect membrane fluidity in the hydrophobic interior and hydrophilic/polar region of a lipid bilayer. The presence of caffeic acid and polyethylene glycol chains in the dendrimer structure affected the thermodynamical properties of the membrane lipid matrix.

1. Introduction

The results of recent studies indicate that various cationic polymers can be effective as transfection agents for cells and even tissues (Karimov et al., 2021; Rai et al., 2019; Schulze et al., 2018; Svenson and Tomalia, 2012). Among these polymers, dendrimers are especially useful (Caminade et al., 2005; Svenson and Tomalia, 2012; Wang et al., 2022). Dendrimers are branched nanoparticles characterized by their small size and unique properties such as monodispersity, relatively low immunogenicity and high biocompatibility (Jain et al., 2010; Wang et al., 2022). Some of the best-studied dendrimers are the poly(amidoamine) PAMAM dendrimers (Kesharwani et al., 2014; Lyu et al., 2019; Tupally et al., 2015; Xu et al., 2016). Studies from the late 20th century describe their high efficacy in DNA transfection and plasmid expression studies (Abedi-Gaballu et al., 2018; Pandi et al., 2018). In recent years, carbosilane dendrimers have also been intensively explored as alternative effective carriers of therapeutic biomolecules (Białkowska et al., 2022; Michlewska et al., 2023a, 2023b, 2021; Zhang et al., 2020). It is worth noting that drug vehicles interact with different biological systems such as enzymatic and structural proteins and lipid membranes (Fowler et al., 2016; Fox et al., 2018; Grodzicka et al., 2024b; Kubczak et al., 2023; Nakhaei et al., 2021; Tu et al., 2013). In particular, cationic dendrimers, due to their interaction with the lipid bilayer, can cause micro-holes in the cell membrane affecting their high haemo/cyto- toxicity. They can also be incorporated into the lipid structure (Gardikis et al., 2013; Hong et al., 2006; Ionov et al., 2015; Michlewska et al., 2017; Shcharbin et al.,

* Corresponding author at: University of Lodz, Faculty of Biology and Environmental Protection, Department of General Biophysics, Pomorska 141/143, Lodz 90-236, Poland.
** Corresponding author.
E-mail addresses: marika.grodzicka@biol.uni.lodz.pl (M. Grodzicka), sylwia.michlewska@biol.uni.lodz.pl (S. Michlewska).

https://doi.org/10.1016/j.chemphyslip.2024.105444
Received 26 June 2024; Received in revised form 3 September 2024; Accepted 9 September 2024
Available online 10 September 2024

2006). Therefore, the interaction of dendrimers with biological membranes and control of their permeability seems to be a crucial step in the transfection process and an area worth investigating (Gardikis et al., 2013; Michlewska et al., 2017). Several studies have additionally shown that membrane permeability is affected not only by the size and charge, but also by the concentration of dendrimers (Aurelia Chis et al., 2020; Fox et al., 2018; Mignani et al., 2019; Tu et al., 2013). To increase transfection efficiency while reducing cytotoxic effects, attempts have been made to mask the surface charge by attaching polyethylene glycol (PEG) chains (Germershaus et al., 2008; Thakur et al., 2015). The ease of modifying dendrimer structure opens the way for attaching, e.g., metals with anticancer properties or polyphenols to their surface (Hołota et al., 2019), providing an opportunity to create new improved tools for the treatment various types of diseases, including cancer and neurodegenerative diseases.

Previous studies have shown that novel polyphenolic dendrimers with caffeic acid moieties have highly antioxidant and antiradical properties (Grodzicka et al., 2022). Increased antioxidant activity may also be related to the interactions with various biomolecules (Sekowski et al., 2016). It has been shown that carbosilane polyphenolic dendrimers, depending on the number of caffeic acid residues and polyethylene glycol modifications, interact with human albumin changing its structure and electrical properties (Grodzicka et al., 2024b). Therefore, it is important to evaluate the specific interaction mechanisms of the lipid membranes of these compounds. It has been previously suggested that liposomes can mimic a cell membrane and can be used to simplify experiments that are related to much more complex biological membranes (Akbarzadeh et al., 2013; Michlewska et al., 2023a).

In this study, we have demonstrated that 1st generation polyphenolic dendrimers modified with PEG interact with lipid membranes, and have elucidated how dendrimers behave in membranes and how PEG influences the ordering of membranes.

2. Materials and methods

2.1. Materials

Lipids: DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine), DPPG (1,2-dipalmitoyl-sn-glycero-3-phosphoglycerol); DPH (1,6-diphenyl-1,3,5-hexatriene), TMA DPH (N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatriene-1-yl) phenylammonium p-toluenesulfonate; Phosphate buffer components and HS-PEG-NH_2·HCl with a number average molecular weight (Mn) of 3.5 kDa were purchased from Sigma Aldrich Chemical Company. The water-soluble 1st generation heterofunctionalized polyphenolic carbosilane dendrimers modified with caffeic acid moieties and PEG, were synthesized in the Department of Organic and Inorganic Chemistry of Universidad de Alcalá de Henares, Madrid, Spain. The procedure of the synthesis of these compounds was described elsewhere (Grodzicka et al., 2022). The chemical structure and properties of dendrimers are present in (Fig. 1) and (Table 1).

2.2. Preparation of vesicles

The interaction of the polyphenolic carbosilane dendrimers with lipid membranes was investigated with large unilamellar vesicles (LUVs) obtained by extrusion. For this purpose, a mixture of DMPC and DPPG phospholipids (97:3 molar ratio) were prepared. DMPC and DPPG were separately dissolved in chloroform in a concentration 5 mmol/L and mixed to obtain the DMPC/DPPG molar ratio 97:3. The obtained solution was placed in a round bottom flask under vacuum conditions to evaporate the solvent. The resultant lipid film was washed with 10 mmol/L phosphate buffer, pH 7.4 and incubated for 30 min. above the lipid phase-transition temperature. The lipid suspension was then extruded through a 100 nm polycarbonate membrane using a mini-extruder of Avanti Polar Lipids (Alabaster, Alabama, USA) equipped with two 1000 µL Hamilton gastight syringes. The lipid suspension was forced to pass through a polycarbonate membrane at least 21 times. The

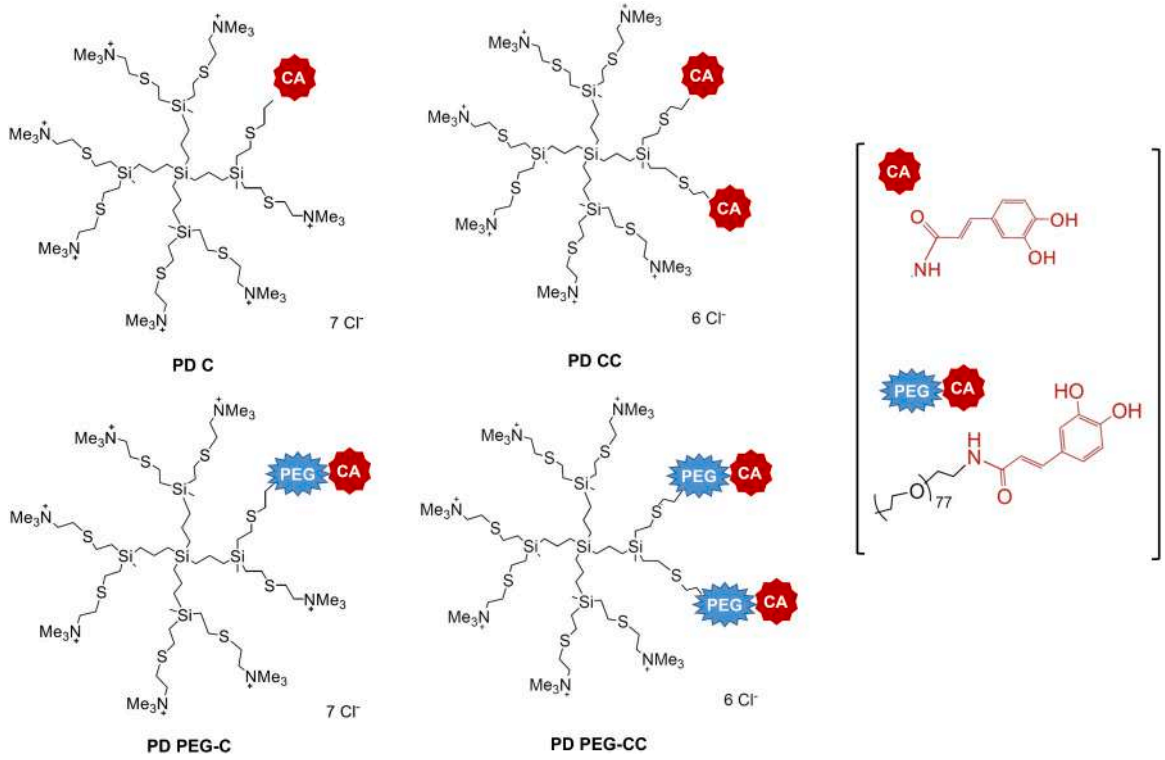


Fig. 1. The structure of the 1st generation polyphenolic carbosilane dendrimers with ammonium surface groups functionalized with caffeic acid (CA) and polyethylene glycol (PEG). PD C – $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$; PD CC – $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$; PD PEG-C – $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$; PD PEG-CC – $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$. PEG chains are marked in blue, caffeic acid moieties in red.

Table 1
Properties of the 1st generation polyphenolic carbosilane dendrimers.

Dendrimer	Chemical formula	Number of caffeic acid moieties	Number of PEG chains	Molecular weight [g/mol]	Number of cationic end groups
PD C	$G_2[(NMe_3Cl)_7(NH\text{-}CA)]$	1	–	1914.33	$[NMe_3]_7^+$
PD CC	$G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$	2	–	1997.94	$[NMe_3]_6^+$
PD PEG-C	$G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$	1	1	5306.42	$[NMe_3]_7^+$
PD PEG-CC	$G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$	2	2	8782.10	$[NMe_3]_6^+$

final concentration of the phospholipid was 5 mmol/L. Obtained liposomes were stored in a fridge at 3 °C and used for further experiments.

2.3. Zeta potential and z-average size

To characterize the interaction between dendrimers and the LUVs, a Zetasizer Nano-ZS, (equipped with 633 nm laser, module at a detection angle of 173°), Malvern Instruments Ltd., Malvern (Worcestershire, UK) was applied. Zeta potential was analyzed from the Helmholtz-Smoluchowski equation after measuring the velocity of samples containing a mixture of dendrimers and LUVs during electrophoresis by Laser Doppler Velocimetry (LDV). The size of the samples was detected by measuring Brownian motions directly depending on the particle size. Measurements were carried out in 10 mmol/L Na-phosphate buffer, pH 7.4. The lipid/dendrimer molar ratios were: 1:1, 1:2, 1:4, 1:6, 1:8, 1:10. Malvern software was used for data analysis. At least 3 separate replicates were performed for each experiment.

2.4. Transmission electron microscopy (TEM)

The ultrastructure of the liposomes and liposome/dendrimer formulations were evaluated by transmission electron microscopy technique using the JEOL-1010 (JEOL, Tokyo, Japan) transmission electron microscope. The samples were suspended in 10 mmol/L Na-phosphate buffer, pH 7.4, placed on 200 mesh copper grids with a carbon surface (Ted Pella, Inc, Redding, CA, USA) and stained with 2 % uranyl acetate. After washing with deionized water, the grids were dried at room temperature for 30 min. Images were taken at a magnification of 100,000 x.

2.5. Differential scanning calorimetry

The impact of polyphenolic dendrimers on the structure of a model phospholipid membrane composed with DMPC (97 %) and DPPG (3 %) was investigated by using differential scanning calorimetry (Setaram DSC III microcalorimeter). The lipids were initially dissolved in chloroform and subsequently dried at 38°C and resuspended in a 10 mmol/L phosphate buffer, pH 7.4. The final concentrations of lipids were the same in all studied samples: 4.85 mmol/L DMPC and 0.15 mmol/L DPPG. DSC measurements were carried out for the studied 5 mmol/L DMPC/DPPG membrane at a constant dendrimer concentration of 0.37 mmol/L. Each studied sample (700 µl) was scanned 3 times from 5°C to 40°C with a scan rate of 0.5 °C/min. From each recorded DSC curve the reference scan (700 µl 10 mmol/L phosphate buffer pH 7.4) was subtracted. Enthalpies of transitions and characteristic temperatures were calculated using Setaram software.

2.6. Steady-state fluorescence spectroscopy

The nature of the interaction between polyphenolic carbosilane dendrimers and biological membranes was assessed by using erythrocyte membranes. The membranes were obtained from erythrocytes isolated from human blood as described below, collected after washing in hypotonic 10 mmol/L Na-phosphate buffer, pH 7.4 diluted with deionized water (1:1) and through a set of centrifugations (15 min., 15 000 rpm) at 4°C. The membrane protein concentration was estimated by the Bradford method (Bradford, 1976). To monitor the changes in fluorescence anisotropy of the fluorescent dyes DPH (1,6-diphenyl-1,3,5-hexatriene) and TMA-DPH (N,N,N-trimethyl-(4–6-phenyl-1,3,5-hexatrien-1-yl)phenyl-ammonium p-toluenesulfonate) was incorporated into the erythrocyte membrane, and the LS-50B (Perkin-Elmer, U.K.) spectrofluorometer equipped for fluorescence polarization measurements was used. The excitation and emission parameters of the DPH and TMA-DPH probes were 348 and 426 nm wavelengths and 358 and 428 nm wavelengths, respectively. The slit-widths of the excitation and emission monochromator were 6 nm and 8 nm, respectively, for both probes. Erythrocyte membranes were suspended in 10 mmol/L phosphate buffer, pH 7.4 and the samples were incubated for 10 min at 37°C. Dendrimers were added to the samples to reach the required concentrations ranging from 5 to 40 µmol/L. Fluorescence anisotropy values (r) were calculated using Perkin-Elmer software from the equation:

$$r = (I_{VV} - GI_{VH}) / (I_{VV} + 2GI_{VH})$$

where Ivv - vertical excitation, vertical emission; Ivh - vertical excitation, horizontal emission, G is a grating correction factor that corrects the polarising effects of the monochromator.

2.7. Haemolysis assay

Plasma-free blood samples of healthy volunteers were purchased from the Regional Centre for Blood Donation and Haemotherapy in Lodz, Poland. This blood was centrifuged at 3500 rpm for 10 min. at 4°C, and washed with cold phosphate buffer saline (PBS), pH 7.4. Isolated erythrocytes (haematocrit 7 %) were incubated with polyphenolic dendrimers at the concentrations ranging from 12.5 µmol/L to 100 µmol/L for 3 h and 24 h at 37°C in FBS-free PBS or in PBS contained 10 % FBS. After incubation, the samples were centrifuged at 3000 rpm for 10 min. at room temperature and the absorbance of the supernatant was measured at λ=535 nm with a Jasco V-650 UV/VIS spectrophotometer (Jasco International Co. Ltd., Tokyo, Japan). The samples with positive and negative controls were measured at the presence of 10 % Triton or free PBS respectively. The percentage of haemolysis at the presence of dendrimers was calculated from the formula:

$$\text{Haemolysis (\%)} = (A_{\text{sample}}\ 540\ \text{nm} / A_{\text{control positve}}\ 540\ \text{nm}) \times 100\%$$

2.8. Data analysis

The Shapiro-Wilk test was used to check whether the distribution of differences was normal. In the case of a normal distribution of differences, the Student's t-test was applied, while in other cases the ANOVA 2-way (Dunnett's multiple comparisons test) was used. All presented results were obtained from at least 3 independent experiments and are presented as mean ± SD. Statistical analyses were performed for paired samples using GraphPad Prism software, version 8.0.1. The 95 % confidence interval was set as the confidence interval. Significance levels were defined as $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$.

3. Results

3.1. Z-average size, zeta potential and transmission electron microscopy

To analyse the interaction between polyphenolic dendrimers and artificial lipid membranes, transmission electron microscopy, z-average size and zeta potential methods were used. For these analyses, 100 nm in size liposomes were prepared using the mixture of DMPC/DPPG phospholipids at 97:3 molar ratio. DMPC is composed of 2 myristoyl acid molecules, whereas DPPG contains 2 palmitic acid molecules. These fatty acids have alkyl chains of different lengths; myristic acid has 14 carbon atoms, whereas palmitoyl acid has 16. The aim of this part of the study was to investigate the interaction between dendrimers and large unilamellar vesicles (LUV) that mimic the cell surface. The presence of DPPG in the lipid mixture caused a change in liposomal surface potential from neutral to negative. Fig. 2A shows that z-average size of dendrimers suspended in buffer ranged between 98.82 ± 2.07 nm and 106.9 ± 0.6 nm. Upon addition of the dendrimer PD PEG-C (containing one caffeic acid and one PEG chain), the z-size just slightly changed. Similarly, in liposomal suspension the dendrimer PD C containing one caffeic acid residue there was a weak growth of hydrodynamic diameter value to 154.26 ± 1.43 nm. However, the addition of the dendrimers PD CC and PD PEG-CC containing 2 caffeic acid residues resulted in a significant increase in nanoparticle size, 291.61 ± 7.32 nm for dendrimer PD CC and 306.78 ± 2.86 nm for dendrimer PD PEG-CC. The values of the polydispersity index (PDI) of liposomal suspension contained dendrimers in the highest tested concentration which varied from ~ 0.4 -dendrimer PD PEG-C to ~ 0.6 -dendrimer PD PEG-CC (Fig. 2C).

The results presented in Fig. 2B showed that zeta potential of dendrimers suspended in buffer ranged from −15 to −18 mV. The addition of dendrimers into LUVs caused a change in their surface charge from negative to positive values. This effect was dependent on the presence of PEG in the dendrimer structure. In the case of dendrimers PD PEG-C and PD PEG-CC, the zeta potential increased to 4.21 ± 0.09 mV and 3.12 ± 0.16 mV, respectively. In contrast, both the nanoparticles without PEG chains caused a significant increase in the zeta potential to up to 15 mV. The results indicated that dendrimer-lipid membrane interaction depended on the number of caffeic acid residues and polyethylene glycol chains presented in the dendrimer structure. In derivatives containing polyethylene glycol (PEG), the configuration of the ethylene glycol chains impeded the exposure of the positive charges from the peripheral ammonium groups. This steric hindrance reduced the interaction with the lipid membrane, thus explaining the comparatively smaller increase in zeta potential towards more positive values.

The microphotographs of liposomes and their mixtures with polyphenolic dendrimers were obtained using transmission electron microscopy. Noncomplexed liposomes were visible as regular vesicles with a size of 100 nm or less (Fig. 2 D). Obtained images show that dendrimers added to the liposome suspension were able to interact with their surface. DMPC/DPPG liposomes mixed with polyphenolic dendrimers revealed visible morphological variations that were of different shape and structure compared with noncomplexed liposomes. All dendrimers were complexed with liposomes, that confirmed the results obtained by zeta techniques and was evidence of the interaction of dendrimers with the negatively charged membrane surface, suggesting an electrostatic nature of interaction between the considered components.

3.2. Differential scanning calorimetry

The DSC heating profiles (Fig. 3) of DMPC/DPPG bilayer in 10 mmol/L phosphorous buffer pH 7.4 for pure lipids and lipid suspension with constant dendrimer concentration exhibit upon heating, endothermic (main) transitions from rippled phase ($P_{\beta'}$) to liquid crystalline phase (L_{α}), due to the cooperative melting of the hydrocarbon chains associated with the changes of the C-C single bonds conformation (Gardikis et al., 2006; Losada-Pérez et al., 2015) from trans to gauche (Sun and Böckmann, 2018; Yellin and Levin, 1977). The thermotropic parameters of transition peaks are given in Table 2. The impact of 4 polyphenolic carbosilane dendrimers PD C; PD CC, PD PEG-C and PD PEG-CC on the structure of DMPC/DPPG membrane was reflected by changes of thermotropic parameters for the transition peak (T_m ~23°C).

With the addition of studied dendrimers at a molar ratio dendrimer/lipid of 0.074:1, the dendrimer interaction with the DMPC/DPPG lipid bilayer was confirmed by the change of phase transition parameters: the

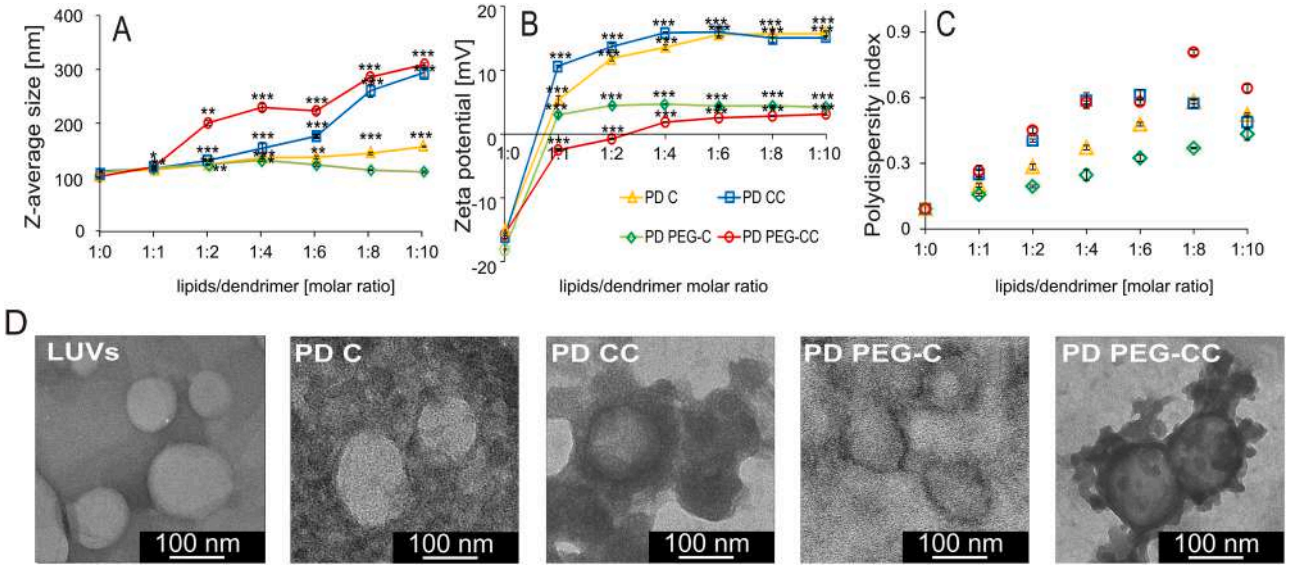


Fig. 2. Z-average size (A), zeta potential (B) and polydispersity index (C) of the vesicles composed with DMPC/DPPG (97:3 molar ratio) upon addition of polyphenolic dendrimers in increased concentrations. The concentration of lipids, 25 μmol/L. Applied lipid/dendrimer molar ratios: 1:1, 1:2, 1:4, 1:6, 1:8, 1:10. Data points represent means ± SD obtained from 3 separate experiments and each experiment was done in 7 replicates. *p < 0.05; **p < 0.01 and ***p < 0.001 vs. control (liposomes suspended in buffer). (D) – Transmission electron microscopy images of DMPC/DPPG (97:3 molar ratio) lipid vesicles and their mixtures with polyphenolic dendrimers ~~(1)~~ PD C; ~~(2)~~ PD CC, ~~(3)~~ PD PEG-C and ~~(4)~~ PD PEG-CC. The liposomes and dendrimers (1:10 molar ratio) were dissolved in 10 mmol/L Na-phosphate buffer, pH-7.4, t- 25 °C. Lipid concentration, 25 μmol/L. Magnification 100,000, bar = 100 nm. To obtain greater contrast, the colour of the images has been inverted.

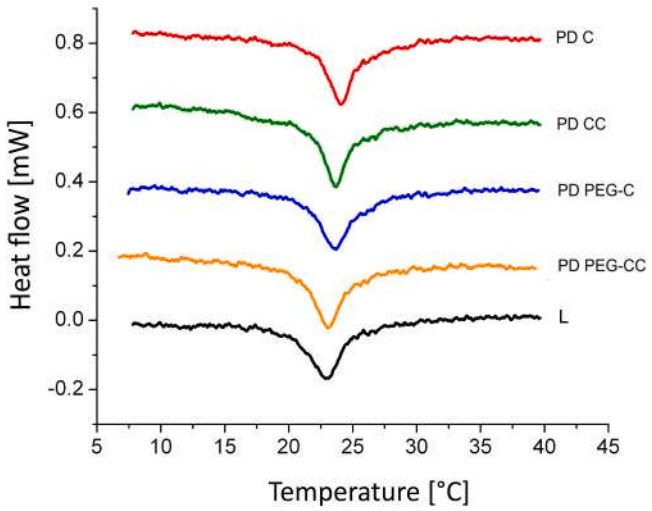


Fig. 3. DSC heating scans of pure DMPC/DPPG membrane and the DMPC/DPPG membrane in the presence of polyphenolic carbosilane dendrimers at constant dendrimer to lipid molar ratio 0.074/1 in 10 mmol/L phosphorous buffer solution pH 7.4. Curve L presents the DSC transition of 5 mmol/L DMPC/DPPG liposomes in the absence of dendrimers.

Table 2
DSC parameters of pure 5 mmol/L DMPC/DPPG lipids and 5 mmol/L DMPC/DPPG lipids with 0.37 mol/L polyphenolic carbosilane dendrimers in 10 mmol/L phosphate buffer, pH 7.4.

System	T_{onset}/°C	T_m/°C	T_{offset}/°C	ΔH/kJ mol^{-1}	$T_{1/2}$/°C
Lipids	20.85 ± 0.55	22.98 ± 0.15	24.40 ± 0.20	17.23 ± 1.70	3.03 ± 0.21
Lipids + PD C	22.19 ± 0.36	23.74 ± 0.04	25.41 ± 0.09	16.05 ± 1.67	2.47 ± 0.14
Lipids + PD CC	22.16 ± 0.06	23.90 ± 0.08	25.54 ± 0.08	15.05 ± 0.83	2.71± 0.26
Lipids + PD PEG-C	22.24 ± 0.19	23.71 ± 0.06	25.03 ± 0.05	14.49 ± 1.00	2.07± 0.08
Lipids + PD PEG-CC	22.39 ± 0.13	24.05 ± 0.04	25.42 ± 0.03	15.60 ± 0.77	2.25 ± 0.05

increase in onset, offset and chain melting transition temperatures (T_{onset}, T_{offset} and T_m) that showed the thermal stabilizing impact of dendrimer association with the studied lipid DMPC/DPPG membrane. Polyphenolic carbosilane dendrimers caused sharpening of the lipid melting peak (reduction of $T_{1/2}$), indicating a higher melting cooperativity, i.e. more phospholipids molecules were melting simultaneously after dendrimer addition in comparison to the pure DMPC/DPPG membrane. In the limit of uncertainties, no changes in the peak areas were observed.

Changes of thermotropic parameters for the DMPC/DPPG membrane transition revealed the impact of introducing a second PEG chain to the macromolecule of the phenolic dendrimer (PD PEG-CC vs PD PEG-C). The increase of chain melting temperature T_m and offset temperature T_{offset} (Table 2) for dendrimer PD PEG-CC in comparison with dendrimer PD PEG-C, indicated that second PEG chain in PD PEG-CC enhanced the thermal stability of the DMPC/DPPG membrane. This perhaps reflected the shielding effect of an additional PEG chain on the membrane surface after an association with the dendrimer PD PEG-CC. At the same time the increase of the half width of the peak transition $T_{1/2}$ at the presence of PD PEG-CC when compared to PD PEG-C, indicated that the inner phospholipid structure of the membrane is more disordered (with less cooperativity of the melting transition) after association with the more steric dendrimer PD PEG-CC.

Calorimetric parameters: T_{onset} – temperature at which the thermal effect starts; T_m–temperature at which heat capacity at constant pressure is at maximum; $T_{1/2}$– half width of the peak transition; ΔH– transition enthalpy. Errors expressed as SEM, n=3.

3.3. Polyphenolic dendrimers affect erythrocyte membrane fluidity

DPH and TMA DPH fluorescent probes were used to evaluate membrane fluidity in the membrane hydrophobic (inner) and hydrophilic (outer) regions, respectively. The DPH probe was located around the long axis of phospholipid molecule, whereas TMA DPH was in the head group region of the lipid bilayer. Membrane fluidity changes induced by incorporation of the investigated dendrimers were measured by analyzing the changes in DPH and TMA DPH fluorescence anisotropy.

The effect of polyphenolic dendrimers on fluidity of the erythrocyte membrane is shown in Fig. 4. The lipid/dendrimer molar ratio as in the results of z-average size or zeta potential obtained for artificial

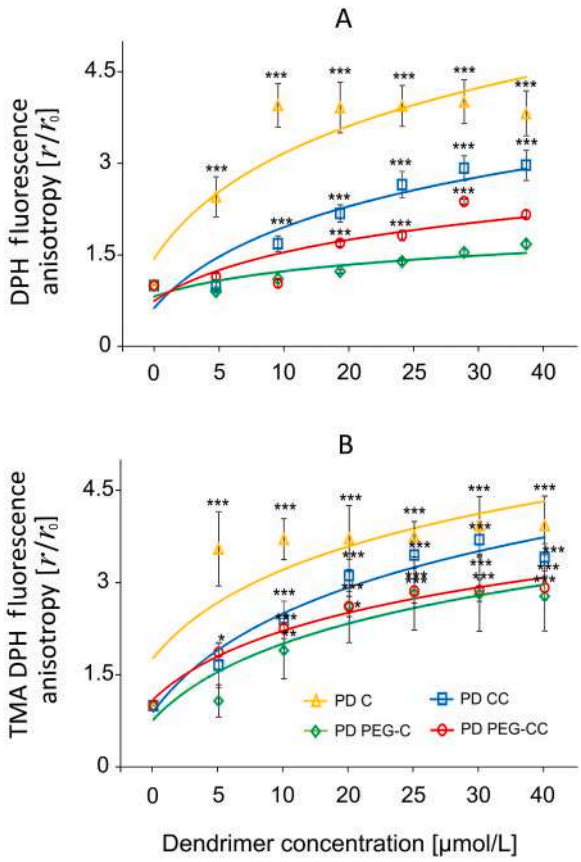


Fig. 4. Fluorescence anisotropy of **DPH** and TMA DPH in erythrocyte membranes at the presence of polyphenolic dendrimers (1) – PD C; (2) – PD CC, (3) – PD PEG-C and (4) – PD PEG-CC. The membranes were isolated from red blood cells by washing isolated erythrocytes with 10 mmol/L phosphate buffer, pH 7.4 diluted with deionised water (1:1). The membrane protein concentration was evaluated by the Bradford method and the final concentration of membrane protein was 25 μmol/L**. Dendrimers were added to the membranes to reach the required concentrations ranging from 5 μmol/L to 40 μmol/L. The DPH or TMA DPH** concentration was **1 μmol/L.** DPH reflects the membrane fluidity in the hydrophobic membrane interior region, whereas TMA DPH anisotropy indicates membrane fluidity in the membrane interface region. r_0 – control fluorescence anisotropy in the absence of dendrimers. r – sample fluorescence anisotropy. The values for the control fluorescence anisotropy (r_0) were as follows: 0.092 ± 0.006 and 0.138 ± 0.011 for DPH and TMA-DPH, respectively. To show the trend of fluorescence anisotropy changes, the trend lines were added in graphs. **Data points represent means ± SD obtained from 3 separate experiments.** *p < 0.05; **p < 0.01 and ***p < 0.001 vs. control (no dendrimers).

membranes are not presented here. Due to difficulties in determining the lipid concentration in the red blood cell membrane, the concentration was evaluated by the presence of membrane protein with the final concentration of protein 25 μmol/L.

The addition of dendrimers to the membrane suspension led to an increase in the DPH and TMA DPH anisotropy that confirmed that the membrane fluidity has been affected and the erythrocyte membranes had become more rigid. Dendrimers with PEG changed the fluidity of both the hydrophobic and hydrophilic regions of membrane more weakly than those without PEG. The strongest effect of decreasing membrane fluidity was found for dendrimer PD C. The effect of membrane stiffening was not dependent on the number of caffeic acid residues presented in the dendrimer structure (Fig. 4).

3.4. Haemolysis assay

The haemotoxicity test was used to analyse the effect of dendrimers on biological membranes. The results showed that studied compounds that were incubated with red blood cells for 3 h in a concentration 12.5 and 25 μmol/L were almost not haemotoxic (**Fig. 5**). Increasing the incubation time and dendrimer concentration led to an increase in the haemolytic effect (**Fig. 5** B). While the most haemotoxic was PD C dendrimer, the PD PEG-CC was completely non-haemotoxic (**Fig. 5** B). Taking into account the presence of numerous proteins in the physiological conditions/liquids and especially in the bloodstream, the presence of FBS in erythrocyte media which could affect the haemotoxic effect of polyphenolic dendrimers, was checked. It was found that after 24 h incubation of erythrocytes with dendrimers in the PBS buffer containing 10 % FBS, there was no haemolysis activity for all tested dendrimers (**Fig. 5** C).

4. Discussion

Among a wide range of nanoparticles, dendrimers are considered to be promising drug carriers. The strength of dendrimer interactions with lipid membranes depends on their size and charge (Huang et al., 2003). Cationic dendrimers can cross negatively charged biological membranes and become useful drug vehicles (Michlewska et al., 2023a; Shcharbin et al., 2020). Biological membranes are complex systems composed mainly of lipids and proteins. In order to explain the mechanism of crossing the membrane barrier by dendrimers, it is necessary to understand the character of the interaction between nanoparticles and the lipid bilayer. For these analyses, liposomes composed with synthetic phospholipids were excellent research material due to their relatively simple lipid composition, ease of synthesis and stability (Ottaviani et al., 2002; Wrobel et al., 2012). In our previous study, it was shown that polyphenolic dendrimers exhibit antioxidant activity and protected erythrocytes against AAPH-induced haemolysis (Grodzicka et al., 2022). This is attributed to the presence of caffeic acid residues in the dendrimers structure (Grodzicka et al., 2022), whereas the presence of PEG chains can reduce cytotoxicity by masking the charge of the nanoparticle. Therefore, the presence of caffeic acid and PEG in dendritic skeleton endowed dendrimers with antiradical activity, reduced toxicity and increased bioavailability (Grodzicka et al., 2024a). Such modification can be useful in designing of new anticancer drug carrier.

In the synthesized model membranes, DMPC (which has no charge), was used. DMPC is a synthetic analogue of lecithin, found as a major component of the lipid membrane matrix of living cells. To simulate the negative charge of a living cell, the DMPC lipid was mixed with DMPG, bringing a negative charge to the liposomal surface (Wrobel et al., 2012).

Changes in the DMPC/DPPG liposome size and morphology in the presence of dendrimers were observed by DLS and TEM assays. The TEM images show vesicular structures of liposomes coated with dendrimers. The curvature of the liposomal membrane seemed to be slightly distorted and was dependent on the number of caffeic acid residues included in dendrimer structure. These results correlated with those obtained by z-average size measurements where the hydrodynamic diameter of liposomes increased more in the presence of dendrimers with 2 polyphenolic residues. The same dendrimers caused a stronger increase in zeta potential and polydispersity index.

Cationic dendrimers are able to bend the anionic lipid membrane and so induce the stresses in the bilayer packing via electrostatic interactions (Zhang and Smith, 2000). The interactions of PAMAM

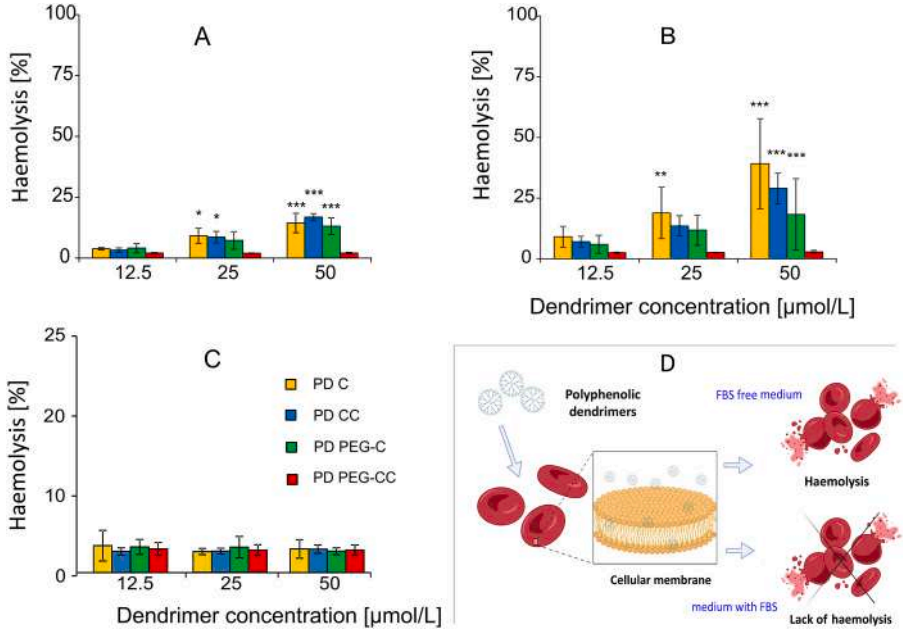


Fig. 5. Haemolytic effect of polyphenolic dendrimers on human erythrocytes. (A) – incubation time 3 h. (B) – incubation time 24 h. (C) - In the presence of 10 % fetal bovine serum (FBS), incubation time 24 h. (D) - Schematic representation of the experiment results showing the haemolytic effect of polyphenolic dendrimers at the absence or presence of FBS. Hematocrit 7 %, PBS buffer 10 mmol/L, pH 7.4. Dendrimer concentrations ranged from 12.5 to 50 μM. Values are expressed as mean ± standard deviation (SD) (n=3). * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs. control (no dendrimers).

dendrimers with lipid membranes have already been described (Fox et al., 2018; Huang et al., 2003). The mechanisms of interaction between PAMAM dendrimers and lipid membranes includes the balance between the electrostatic effect of positive charges of dendrimers and the neutral/negatively charged lipids, leading to the formation of holes in the membrane (Leroueil et al., 2007; Lombardo et al., 2016). A similar mechanism has also been described for carbosilane dendrimers (Wrobel et al., 2012). The hydrophobic effect that occurs between lipid chains and the terminal groups of dendrimers can also take place under such interactions (Åkesson et al., 2010; Wrobel et al., 2012).

Differential scanning calorimetry (DSC) is usually used for determining the thermal effects of a variety of materials, including the analysis of thermodynamic properties of lipid membranes (Wrobel et al., 2011). The DMPC lipid membrane exhibits 2 endothermic transitions upon heating: the pre-transition (PT) and the main transition (MT) (Demetzos, 2008). In our experiments the pre-transition peak was not observed. This could be caused by the presence of DPPG in the lipid mixture, DPPG probably influencing the structural configuration of the additive, engaging with its polar moiety and caused the elimination of a pretransition peak. In the presence of polyphenolic dendrimer DSC, the heating profiles of DMPC/DPPG lipid membrane showed an endothermic main transition peak after heating. Results showed that the number of PEG chains in the dendrimer structure influenced the thermal stability of such lipid membranes. An observed decrease in enthalpy, indicating the relaxation of the hydrocarbon tails of lipids in the presence of dendrimers, suggested their interaction with the hydrophobic region of lipid bilayer (Gennaro et al., 2020).

Lipids and proteins on the biological membrane surface can be interaction targets for dendrimers. The affinity of dendrimers for membrane proteins causes more complex interactions than for model membranes (Wrobel et al., 2012; Ziemba et al., 2012). To check the changes in properties of biological membranes caused by polyphenolic dendrimers, the interaction with erythrocyte membranes was analysed. DPH and TMA-DPH anisotropy assays were applied to investigate the changes in erythrocyte membrane fluidity. The fluorescence anisotropy of DPH and TMA-DPH probes that were incorporated into the lipid bilayer increased in the presence of rising concentrations of dendrimers. This observation indicated that dendrimers are able to decrease the membrane fluidity in the hydrophilic and hydrophobic regions of the lipid bilayer. It was reported that carbosilane dendrimers interacted with the lipid bilayer through electrostatic interactions (Wrobel et al., 2012), with the strength of such interactions relating to the membrane lipid composition, increasing with the increasing concentration of dendrimers (Ionov et al., 2012; Wrobel et al., 2011). Cationic dendrimers can modify membrane fluidity. This effect depends on the number of cationic groups on the dendrimer surface (Wrobel et al., 2012). The presence of PEG chains masks the positive charge of dendrimers (Ziemba et al., 2012). In the dendrimers PD PEG-C (with PEG) and PD C (no PEG), there are same number of cationic end groups. Therefore, the presence of PEG chains can reduce the nanoparticle's effect on membrane fluidity. To confirm the results obtained by the fluorescence anisotropy assay and to try and ascertain if dendrimers are able to damage biological membrane, haemotoxicity tests were performed. The membrane of erythrocytes contains various types of phospholipids, sphingolipids, cholesterol and membrane proteins (Li and Lykotrafitis, 2014). The erythrocyte surface is negatively charged, protecting them from aggregation and adhesion (Ziemba et al., 2012). Determining the haemolytic capacity is important before any biological application of a potential therapeutic agent. Haemolysis tests showed that polyphenolic dendrimers at low concentrations were relatively non-haemotoxic. However, in higher dendrimer concentrations the haemotoxicity increased, (except for the dendrimer PD PEG-CC which, in the highest used concentration, was not haemotoxic). This can be explained by the relatively low surface charge caused by presence of PEG in a dendritic structure. The modification of dendrimers with CA and PEG can lead to reduced disruption on erythrocyte membrane integrity than conventional dendrimers, providing to fewer hemolytic effects.

This effect correlated with previously published results where it was indicated that the presence of PEG can mask the nanoparticle's charge and reduce its ability to interact electrostatically and to cause damage to cell membranes (Klajnert et al., 2004; Ziemba et al., 2012). Interestingly, incubation of erythrocytes in the presence of FBS completely reduced the haemolytic activity of all tested dendrimers. The finding agreed with the data reporting the interactions of the tested nanoparticles with albumin (Grodzicka et al., 2024b). This confirmed the key role of proteins in the interaction of polyphenolic dendrimers with biological systems.

5. Conclusions

Four polyphenolic carbosilane dendrimers containing caffeic acid in their structure interacted with lipid membranes. Dendrimers affected membrane fluidity making the lipid bilayer more rigid. Studied compounds showed a low haemotoxic effect which was completely eliminated by FBS. PEG incorporated in the dendritic scaffold affected the thermal stability of the **DMPC/DPPG lipid membrane**. These results could be useful in determining appropriate drug carriers with antiradical properties (Grodzicka et al., 2022).

Funding

This work was supported by grant 2023/07/X/NZ7/00026 of the Project "MINIATURA7" and by grant 2018/30/Z/NZ1/00911 of the Project "NanoTendo" financed by the National Science Centre, Poland under the M-ERA.NET 2, which has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no 685451, co-financed by grant of the National Science Centre of Poland under "Beethoven Life 1" program, project "NP-HALE" no: 2018/ 31/F/NZ5/03454, and grant PID2020- 112924RBI00 (MINECO), SBPLY/23/180225/000109 Junta de Comunidades de Castilla-La Mancha (JCCM), University of Alcala PIUAH21/CC-041 and EPU-INV-UAH/2021/002. Radioprotect_S2022/BMD/7406 (CAM),

CRediT authorship contribution statement

Marika Grodzicka: Writing – original draft, Visualization, Investigation. **Paula Ortega:** Writing – review & editing, Resources, Data curation. **Francisco Javier de la Mata:** Writing – review & editing, Resources, Data curation. **Sylwia Michlewska:** Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Adam Buczkowski:** Software, Investigation. **Maria Bryszewska:** Writing – review & editing, Funding acquisition. **Maksim Ionov:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Acknowledgments

The authors wish to thank prof. Janusz Błasiak from the Faculty of Medicine, Collegium Medicum, Mazovian Academy in Plock, prof. Lukasz Pulaski and dr. Maciej Studzian from the Department of Oncobiology and Epigenetics, and dr. Łucja Balcerzak from the Laboratory of Microscopic Imaging & Specialized Biological Techniques,

Faculty of Biology & Environmental Protection, University of Lodz for their assistance and valuable effort.

References

Abedi-Gaballu, F., Dehghan, G., Ghaffari, M., Yekta, R., Abbaspour-Ravasjani, S., Baradaran, B., Ezzati Nazhad Dolatabadi, J., Hamblin, M.R., 2018. PAMAM dendrimers as efficient drug and gene delivery nanosystems for cancer therapy. Appl. Mater. Today 12, 177–190. https://doi.org/10.1016/j.apmt.2018.05.002.

Akbarzadeh, A., Rezaei-Sadabady, R., Davaran, S., Joo, S.W., Zarghami, N., Hanifehpour, Y., Samiei, M., Kouhi, M., Nejati-Koshki, K., 2013. Liposome: Classification, preparation, and applications. Nanoscale Res. Lett. 8 (1) https://doi.org/10.1186/1556-276X-8-102.

Åkesson, A., Bendtsen, K.M., Beherens, M.A., Pedersen, J.S., Alfredsson, V., Gómez, M.C., 2010. The effect of PAMAM G6 dendrimers on the structure of lipidvesicles. Phys. Chem. Chem. Phys. 12, 12267–12272. https://doi.org/10.1039/C0CP00172D.

Aurelia Chis, A., Dobrea, C., Morgovan, C., Arseniu, A.M., Rus, L.L., Butuca, A., Juncan, A.M., Totan, M., Vonica-Tincu, A.L., Cormos, G., Muntean, A.C., Muresan, M.L., Gligor, F.G., Frum, A., 2020. Applications and limitations of dendrimers in biomedicine. Molecules. https://doi.org/10.3390/molecules25173982.

Białkowska, K., Komorowski, P., Gomez-ramirez, R., Javier, F., Mata, D., Bryszewska, M., Miłowska, K., 2022. Interaction of Cationic Carbosilane Dendrimers and Their siRNA Complexes with MCF-7 Cells Cultured in 3D Spheroids 1–15.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Caminade, A.-M., Laurent, R., Majoral, J.-P., 2005. Characterization of dendrimers. Adv. Drug Deliv. Rev. 57, 2130–2146. https://doi.org/10.1016/j.addr.2005.09.011.

Demetzos, C., 2008. Differential Scanning Calorimetry (DSC): A tool to study the thermal behavior of lipid bilayers and liposomal stability. J. Liposome Res. 18, 159–173. https://doi.org/10.1080/08982100802310261.

Fowler, P.W., Hélie, J., Duncan, A., Chavent, M., Koldsø, H., Sansom, M.S.P., 2016. Membrane stiffness is modified by integral membrane proteins. Soft Matter 12, 7792–7803. https://doi.org/10.1039/c6sm01186a.

Fox, L.J., Richardson, R.M., Briscoe, W.H., 2018. PAMAM dendrimer - cell membrane interactions. Adv. Colloid Interface Sci. 257, 1–18. https://doi.org/10.1016/j.cis.2018.06.005.

Gardikis, K., Hatziantoniou, S., Viras, K., Wagner, M., Demetzos, C., 2006. Interaction of Dendrimers with Model Lipid Membranes Assessed by DSC and Raman Spectroscopy BT. In: Mozafari, M.R. (Ed.), Nanocarrier Technologies: Frontiers of Nanotherapy. Springer Netherlands, Dordrecht, pp. 207–220. https://doi.org/10.1007/978-1-4020-5041-1_12.

Gardikis, K., Mourelatou, E.A., Ionov, M., Aserin, A., Libster, D., Klajnert, B., Bryszewska, M., Garti, N., Majoral, J. -P., Dimas, K., Demetzos, C., 2013. Natural and Synthetic Biomaterials as Composites of Advanced Drug Delivery Nano Systems (ADDNSS). Biomedical Applications. In: Dendrimers in Biomedical Applications. The Royal Society of Chemistry, pp. 30–39. https://doi.org/10.1039/9781849737296-00030.

Gennaro, A., Deschaume, O., Pfeiffer, H., Bartic, C., Wagner, P., Wübbenhorst, M., 2020. Understanding the Dehydration Stress in Lipid Vesicles by a Combined Quartz Crystal Microbalance and Dielectric Spectroscopy Study. Phys. Status Solidi 217, 1900986. https://doi.org/10.1002/pssa.201900986.

Germershaus, O., Mao, S., Sitterberg, J., Bakowsky, U., Kissel, T., 2008. Gene delivery using chitosan, trimethyl chitosan or polyethylenglycol-graft-trimethyl chitosan block copolymers: Establishment of structure-activity relationships in vitro. J. Control. Release 125, 145–154. https://doi.org/10.1016/j.jconrel.2007.10.013.

Grodzicka, M., Michlewska, S., Blasiak, J., Ortega, P., de la Mata, F.J., Bryszewska, M., Ionov, M., 2024a. Polyphenolic dendrimers as carriers of anticancer siRNA. Int. J. Pharm. 658, 124199 https://doi.org/10.1016/j.ijpharm.2024.124199.

Grodzicka, M., Michlewska, S., Buczkowski, A., Sekowski, S., Gonzalez, C.E.P., Ortega, P., Javier, F., Mata, D., Blasiak, J., Bryszewska, M., Ionov, M., 2024b. OPEN A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure - dependent fashion. Sci. Rep. 1–9. https://doi.org/10.1038/s41598-024-56509-0.

Grodzicka, M., Pena-Gonzalez, C.E., Ortega, P., Michlewska, S., Lozano, R., Bryszewska, M., de la Mata, F.J., Ionov, M., 2022. Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity. Sustain. Mater. Technol. 33, e00497 https://doi.org/10.1016/j.susmat.2022.e00497.

Hołota, M., Magiera, J., Michlewska, S., Kubczak, M., Olmo, N.S.D., García-Gallego, S., Ortega, P., De La Mata, F.J., Ionov, M., Bryszewska, M., 2019. In vitro anticancer properties of copper metallodendrimers. Biomolecules 9. https://doi.org/10.3390/biom9040155.

Hong, S., Leroueil, P.R., Janus, E.K., Peters, J.L., Kober, M.-M., Islam, M.T., Orr, B.G., Baker, J.R.J., Banaszak Holl, M.M., 2006. Interaction of Polycationic Polymers with Supported Lipid Bilayers and Cells: Nanoscale Hole Formation and Enhanced Membrane Permeability. Bioconjug. Chem. 17, 728–734. https://doi.org/10.1021/bc060077y.

Huang, W., Han, X., Wang, E., 2003. Defect Formation Induced by PAMAM Dendrimers on Pt-Supported Bilayer Lipid Membranes Investigated by Electrochemistry. J. Electrochem. Soc. 150, E218 https://doi.org/10.1149/1.1554920.

Ionov, M., Ciepluch, K., Garaiova, Z., Melikishvili, S., Michlewska, S., Balcerzak, Ł., Glińska, S., Miłowska, K., Gomez-Ramirez, R., De La Mata, F.J.F.J., Shcharbin, D., Waczulikova, I., Bryszewska, M., Hianik, T., 2015. Dendrimers complexed with HIV-1 peptides interact with liposomes and lipid monolayers. Biochim. Biophys. Acta - Biomembr. 1848, 907–915. https://doi.org/10.1016/j.bbamem.2014.12.025.

Ionov, M., Garaiova, Z., Waczulikova, I., Wróbel, D., Pędziwiatr-Werbicka, E., Gomez-Ramirez, R., De La Mata, F.J., Klajnert, B., Hianik, T., Bryszewska, M., 2012. siRNA carriers based on carbosilane dendrimers affect zeta potential and size of phospholipid vesicles. Biochim. Biophys. Acta - Biomembr. 1818, 2209–2216. https://doi.org/10.1016/j.bbamem.2012.04.019.

Jain, K., Kesharwani, P., Gupta, U., Jain, N.K., 2010. Dendrimer toxicity: Let's meet the challenge. Int. J. Pharm. 394, 122–142. https://doi.org/10.1016/j.ijpharm.2010.04.027.

Karimov, M., Schulz, M., Kahl, T., Noske, S., Kubczak, M., Gockel, I., Thieme, R., Büch, T., Reinert, A., Ionov, M., Bryszewska, M., Franke, H., Krügel, U., Ewe, A., Aigner, A., 2021. Tyrosine-modified linear PEIs for highly efficacious and biocompatible siRNA delivery in vitro and in vivo: LP10Y for therapeutic siRNA delivery. Nanomed. Nanotechnol., Biol. Med. 36 https://doi.org/10.1016/j.nano.2021.102403.

Kesharwani, P., Jain, K., Jain, N.K., 2014. Dendrimer as nanocarrier for drug delivery. Prog. Polym. Sci. 39, 268–307. https://doi.org/10.1016/j.progpolymsci.2013.07.005.

Klajnert, B., Sadowska, M., Bryszewska, M., 2004. The effect of polyamidoamine dendrimers on human erythrocyte membrane acetylcholinesterase activity. Bioelectrochemistry 65, 23–26. https://doi.org/10.1016/j.bioelechem.2004.06.004.

Kubczak, M., Grodzicka, M., Michlewska, S., Karimov, M., Ewe, A., Aigner, A., Bryszewska, M., Ionov, M., 2023. The effect of novel tyrosine-modified polyethyleneimines on human albumin structure – Thermodynamic and spectroscopic study. Colloids Surf. B Biointerfaces 227, 113359. https://doi.org/10.1016/j.colsurfb.2023.113359.

Leroueil, P.R., Hong, S., Mecke, A., Baker, J.R.J., Orr, B.G., Banaszak Holl, M.M., 2007. Nanoparticle Interaction with Biological Membranes: Does Nanotechnology Present a Janus Face? Acc. Chem. Res. 40, 335–342. https://doi.org/10.1021/ar600012y.

Li, H., Lykotrafitis, G., 2014. Erythrocyte membrane model with explicit description of the lipid bilayer and the spectrin network. Biophys. J. 107, 642–653. https://doi.org/10.1016/j.bpj.2014.06.031.

Lombardo, D., Calandra, P., Bellocco, E., Laganà, G., Barreca, D., Magazù, S., Wanderlingh, U., Kiselev, M.A., 2016. Effect of anionic and cationic polyamidoamine (PAMAM) dendrimers on a model lipid membrane. Biochim. Biophys. Acta - Biomembr. 1858, 2769–2777. https://doi.org/10.1016/j.bbamem.2016.08.001.

Losada-Pérez, P., Mertens, N., de Medio-Vasconcelos, B., Slenders, E., Leys, J., Peeters, M., van Grinsven, B., Gruber, J., Glorieux, C., Pfeiffer, H., Wagner, P., Thoen, J., 2015. Phase Transitions of Binary Lipid Mixtures: A Combined Study by Adiabatic Scanning Calorimetry and Quartz Crystal Microbalance with Dissipation Monitoring. Adv. Condens. Matter Phys. 2015, 479318 https://doi.org/10.1155/2015/479318.

Lyu, Z., Ding, L., Huang, A.Y.-T., Kao, C.-L., Peng, L., 2019. Poly(amidoamine) dendrimers: covalent and supramolecular synthesis. Mater. Today Chem. 13, 34–48. https://doi.org/10.1016/j.mtchem.2019.04.004.

Michlewska, S., Garaiova, Z., Šubjakova, V., Hołota, M., Kubczak, M., Grodzicka, M., Okła, E., Naziris, N., Balcerzak, Ł., Ortega, P., de la Mata, F.J., Hianik, T., Waczulikova, I., Bryszewska, M., Ionov, M., 2023a. Lipid-coated ruthenium dendrimer conjugated with doxorubicin in anti-cancer drug delivery: Introducing protocols. Colloids Surf. B Biointerfaces 227, 113371. https://doi.org/10.1016/j.colsurfb.2023.113371.

Michlewska, S., Ionov, M., Shcharbin, D., Maroto-Díaz, M., Gomez Ramirez, R., Javier de la Mata, F., Bryszewska, M., 2017. Ruthenium metallodendrimers with anticancer potential in an acute promyelocytic leukemia cell line (HL60). Eur. Polym. J. 87 https://doi.org/10.1016/j.eurpolymj.2016.12.011.

Michlewska, S., Maly, M., Wójkowska, D., Karolczak, K., Skiba, E., Hołota, M., Kubczak, M., Ortega, P., Watala, C., Javier de la Mata, F., Bryszewska, M., Ionov, M., 2023b. Carbosilane ruthenium metallodendrimer as alternative anti-cancer drug carrier in triple negative breast cancer mouse model: a preliminary study. Int. J. Pharm. 636, 122784 https://doi.org/10.1016/j.ijpharm.2023.122784.

Michlewska, S., Maroto, M., Hołota, M., Kubczak, M., Sanz Del Olmo, N., Ortega, P., Shcharbin, D., De la Mata, F.J., Bryszewska, M., Ionov, M., 2021. Combined therapy of ruthenium dendrimers and anti-cancer drugs against human leukemic cells. Dalt. Trans. 50, 9500–9511. https://doi.org/10.1039/d1dt01388b.

Mignani, S., Rodrigues, J., Roy, R., Shi, X., Ceña, V., El Kazzouli, S., Majoral, J.P., 2019. Exploration of biomedical dendrimer space based on in-vivo physicochemical parameters: Key factor analysis (Part 2). Drug Discov. Today 24, 1184–1192. https://doi.org/10.1016/j.drudis.2019.03.001.

Nakhaei, P., Margiana, R., Bokov, D.O., Abdelbasset, W.K., Jadidi Kouhbanani, M.A., Varma, R.S., Marofi, F., Jarahian, M., Beheshtkhoo, N., 2021. Liposomes: Structure, Biomedical Applications, and Stability Parameters With Emphasis on Cholesterol. Front. Bioeng. Biotechnol. 9, 1–23. https://doi.org/10.3389/fbioe.2021.705886.

Ottaviani, M.F., Favuzza, P., Sacchi, B., Turro, N.J., Jockusch, S., Tomalia, D.A., 2002. Interactions between starburst dendrimers and mixed DMPC/DMPA-Na vesicles studied by the spin label and the spin probe techniques, supported by transmission electron microscopy. Langmuir 18, 2347–2357. https://doi.org/10.1021/la010771w.

Pandi, P., Jain, A., Kommineni, N., Ionov, M., Bryszewska, M., Khan, W., 2018. Dendrimer as a new potential carrier for topical delivery of siRNA: A comparative study of dendriplex vs. lipoplex for delivery of TNF-α siRNA. Int. J. Pharm. 550, 240–250. https://doi.org/10.1016/j.ijpharm.2018.08.024.

Rai, R., Alwani, S., Badea, I., 2019. Polymeric nanoparticles in gene therapy: New avenues of design and optimization for delivery applications. Polym. (Basel) 11, 1–35. https://doi.org/10.3390/polym11040745.

Schulze, J., Kuhn, S., Hendrikx, S., Schulz-Siegmund, M., Polte, T., Aigner, A., 2018. Spray-Dried Nanoparticle-in-Microparticle Delivery Systems (NiMDS) for Gene Delivery, Comprising Polyethylenimine (PEI)-Based Nanoparticles in a Poly(Vinyl Alcohol) Matrix. Small 14, 1–8. https://doi.org/10.1002/smll.201701810.

Sekowski, S., Ionov, M., Dubis, A., Mavlyanov, S., Bryszewska, M., Zamaraeva, M., 2016. Biomolecular Interactions of Tannin Isolated from Oenothera gigas with Liposomes. J. Membr. Biol. 249, 171–179. https://doi.org/10.1007/s00232-015-9858-x.

Shcharbin, D., Bryszewska, M., Mignani, S., Shi, X., Majoral, J.-P., 2020. Phosphorus dendrimers as powerful nanoplatforms for drug delivery, as fluorescent probes and for liposome interaction studies: A concise overview. Eur. J. Med. Chem. 208, 112788 https://doi.org/10.1016/j.ejmech.2020.112788.

Shcharbin, D., Drapeza, A., Loban, V., Lisichenok, A., Bryszewska, M., 2006. The breakdown of bilayer lipid membranes by dendrimers. Cell. Mol. Biol. Lett. 11, 242–248. https://doi.org/10.2478/s11658-006-0018-2.

Sun, L., Böckmann, R.A., 2018. Membrane phase transition during heating and cooling: molecular insight into reversible melting. Eur. Biophys. J. 47, 151–164. https://doi.org/10.1007/s00249-017-1237-3.

Svenson, S., Tomalia, D.A., 2012. Dendrimers in biomedical applications—reflections on the field. Adv. Drug Deliv. Rev. 64, 102–115. https://doi.org/10.1016/j.addr.2012.09.030.

Thakur, S., Kesharwani, P., Tekade, R.K., Jain, N.K., 2015. Impact of pegylation on biopharmaceutical properties of dendrimers. Polym. (Guildf.) 59, 67–92. https://doi.org/10.1016/j.polymer.2014.12.051.

Tu, C., Chen, K., Tian, W., Ma, Y., 2013. Computational investigations of a peptide-modified dendrimer interacting with lipid membranes. Macromol. Rapid Commun. 34, 1237–1242. https://doi.org/10.1002/marc.201300360.

Tupally, K.R., Kokil, G.R., Thakur, S.S., Singh, P., Parekh, H.S., 2015. Dendrimers. Control. Release Syst. Adv. Nanobottles Act. Nanoparticles 48, 259–285. https://doi.org/10.4032/9789814613224.

Wang, J., Li, B., Qiu, L., Qiao, X., Yang, H., 2022. Dendrimer-based drug delivery systems: history, challenges, and latest developments. J. Biol. Eng. 16, 1–12. https://doi.org/10.1186/s13036-022-00298-5.

Wrobel, D., Ionov, M., Gardikis, K., Demetzos, C., Majoral, J.-P., Palecz, B., Klajnert, B., Bryszewska, M., 2011. Interactions of phosphorus-containing dendrimers with liposomes. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1811, 221–226. https://doi.org/10.1016/j.bbalip.2010.11.007.

Wrobel, D., Kłys, A., Ionov, M., Vitovic, P., Waczulikowa, I., Hianik, T., Gomez-Ramirez, R., de la Mata, J., Klajnert, B., Bryszewska, M., 2012. Cationic carbosilane dendrimers–lipid membrane interactions. Chem. Phys. Lipids 165, 401–407. https://doi.org/10.1016/j.chemphyslip.2012.01.008.

Xu, S., Wu, J., Jiang, W., Tian, R., 2016. Synthesis and characterization of a Poly(amido amine) modified magnetic nanocarrier for controlled delivery of doxorubicin. J. Nanosci. Nanotechnol. 16, 1363–1369. https://doi.org/10.1166/jnn.2016.10697.

Yellin, N., Levin, I.W., 1977. Hydrocarbon chain trans-gauche isomerization in phospholipid bilayer gel assemblies. Biochemistry 16, 642–647. https://doi.org/10.1021/bi00623a014.

Zhang, C., Pan, D., Luo, K., Li, N., Guo, C., Zheng, X., Gu, Z., Turker, A., Güler, N., Fujita, T.C., Sousa-Pereira, N., Amarante, M.K., Watanabe, M.A.E., Inaba, H., Mullighan, C.G., Ciolkowski, M., Rozanek, M., Bryszewska, M., Klajnert, B., Abid, M., Shamsi, F., Azam, A., Pagliara, V., Saide, A., Mitidieri, E., d'Emmanuele di Villa Bianca, R., Sorrentino, R., Russo, G., Russo, A., Zuba, I., Zuba, M., Piotrowski, M., Pawlukojć, A., Alimgazinova, B.S., Yessimbekova, M.A., Chen, K.T.J., Gilabert-Oriol, R., Bally, M.B., Leung, A.W.Y., Conneely, S.E., Stevens, A.M., Długosz-Pokorska, A., Pięta, M., Janecki, T., Janecka, A., Jedrzejczyk, M., Wisniewska, K., Kania, K.D., Marczak, A., Szwed, M., Kenny, R.G., Marmion, C.J., Kim, Y.D., Park, T. E., Singh, B., Maharjan, S., Choi, Y.J., Choung, P.H., Arote, R.B., Cho, C.S., Widomska Justyna, O'Hare, T., Walters, D.K., Stoffregen, E.P., Sherbenou, D.W., Heinrich, M.C., Deininger, M.W.N., Druker, B.J., Ortega, M.Á., Merino, A.G., Fraile-Martínez, O., Recio-Ruiz, J., Pekarek, L., Guijarro, L.G., García-Honduvilla, N., Álvarez-Mon, M., Buján, J., García-Gallego, S., Turk, S., Turk, C., Akbar, M.W., Kucukkaraduman, B., Isbilen, M., Canli, S.D., Malkan, U.Y., Okay, M., Ucar, G., Sayinalp, N., Haznedaroglu, I.C., Osmay Gure, A., DAFM, Ganter, C., Sherje, A.P., Jadhav, M., Dravyakar, B.R., Kadam, D., Sanz del Olmo, N., Maroto-Diaz, M., Quintana, S., Gómez, R., Holota, M., Ionov, M., Bryszewska, M., Carmena, M.J., Ortega, P., Javier de la Mata, F., Lopez-Lopez, E., Evans, W.E., Patel, V., Rajani, C., Paul, D., Borisa, P., Rajpoot, K., Youngren-Ortiz, S.R., Tekade, R.K., 2020. Dendrimers: A versatile nanocarrier for drug delivery and targeting. Mol. Biol. Rep. 48, 1–17. https://doi.org/10.1080/23723556.2020.1865086.

Zhang, Z.-Y., Smith, B.D., 2000. High-generation polycationic dendrimers are unusually effective at disrupting anionic vesicles: membrane bending model. Bioconjug. Chem. 11, 805–814. https://doi.org/10.1021/bc000018z.

Ziemba, B., Matuszko, G., Bryszewska, M., Klajnert, B., 2012. Influence of dendrimers on red blood cells. Cell. Mol. Biol. Lett. 17, 21–35. https://doi.org/10.2478/s11658-011-0033-9.

International Journal of Pharmaceutics 658 (2024) 124199

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Polyphenolic dendrimers as carriers of anticancer siRNA

Marika Grodzicka [a,b,c], Sylwia Michlewska [c,*], Janusz Blasiak [d], Paula Ortega [e,f], Francisco Javier de la Mata [e,f], Maria Bryszewska [a], Maksim Ionov [a,d]

[a] University of Lodz, Faculty of Biology and Environmental Protection, Department of General Biophysics, Pomorska St. 141/143, Lodz 90–236, Poland
[b] The Bio-Med-Chem Doctoral School of the University of Lodz and Lodz Institutes of the Polish Academy of Sciences, Matejki St. 21/23, Lodz 90–237, Poland
[c] University of Lodz, Faculty of Biology and Environmental Protection, Laboratory of Microscopic Imaging and Specialized Biological Techniques, Banacha St. 12/16, Lodz 90–237, Poland
[d] Mazovian Academy in Plock, Collegium Medicum, Faculty of Medicine, Pl. Dabrowskiego 2, 09-402 Plock, Poland
[e] Universidad de Alcalá. Department of Organic and Inorganic Chemistry, and Research Institute in Chemistry "Andrés M. del Río" (IQAR), Spain, and Instituto Ramon y Cajal de Investigacion Sanitaria, IRYCIS, Colmenar Viejo Road, Km 9, 100, 28034 Madrid, Spain
[f] Networking Research Center on Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain

ARTICLE INFO

Keywords:
Polyphenolic dendrimers
Drug carrier
siRNA
Apoptosis
A549 cells
2D & 3D cell culture

ABSTRACT

Dendrimers have emerged as an important group of nanoparticles to transport drugs, DNA, or RNA into target cells in cancer and other diseases. Various functional modifications can be imposed on dendrimers to increase the efficacy and specificity in delivering their cargo to the target cells and decrease their toxicity. In the present work, we evaluated the potential of carbosilane polyphenolic dendrimers modified with caffeic acid (CA) and polyethylene glycol (PEG) to deliver proapoptotic Mcl-1 and Bcl-2 siRNAs to A549 cancer cells. Dendrimers formed stable complexes with siRNAs as assessed by transmission electron microscopy and gel electrophoresis. Modification of dendrimers with PEG reduced the size and the zeta potential of dendrimer/siRNA complexes. The presence of PEG caused a red shift of the CD spectrum, and this effect was the more pronounced, the higher the dendrimer/siRNA ratio was. The nanocomplexes were internalized by A549. All studied dendrimer/siRNA formulations inhibited tumor cell migration and adhesion and caused an increase in the population of early apoptotic cells. Among four tested dendrimers, the polyphenolic compound containing two caffeic acid moieties complexed with siRNA demonstrated the lowest polydispersity index and showed an excellent transfection profile. In conclusion, this dendrimer are a promising candidate for the delivery of siRNA into cancer cells in further *in vivo* studies.

1. Introduction

Dendrimers are nanoparticle polymers that can be used for the delivery of a pharmaceutical compound or a DNA/RNA construct to their target site to achieve a therapeutic effect. In general, dendrimers may transport their cargo in non-covalent or covalent interaction (Kesharwani et al., 2014; Somani et al., 2018). Although dendrimers are polymers, they are different from classical linear polymers as they are monodispersed, highly symmetrical, and surface polyvalent (Michlewska et al., 2023c, 2023b). Repeated dendrimer synthesis reactions result in higher multi-branched generations and may lead to the formation of three-dimensional structure (Hołota et al., 2023; Svenson and Tomalia, 2005). In light of some pitfalls in liposome-based drug delivery and the tendency to avoid viral vectors, the role of dendrimers as drug and gene delivery vehicles is emerging (Biswas and Torchilin, 2013; Dufe et al., 2005).

Abbreviations: **A549**, non-small lung cancer cells (human alveolar basal epithelial cells); **BJ**, human fibroblasts; **CD**, circular dichroism; **DAPI**, 4',6-diamidyno-2-fenyloindol; **DiOC6(3)**, 3'-Dihexyloxacarbocyanine Iodide; **DNA**, deoxyribonucleic acid; **DMEM**, Dulbecco's Modified Eagle Medium; **FBS**, Fetal Bovine Serum; **FITC**, fluorescein isothiocyanate; **HeLa**, human cervical cancer cell; **HEPES**, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; **HL-60**, promyelocytic leukemia cell line; **LDH**, Lactate dehydrogenase; **MCF**, breast cancer cell; **MTT**, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide: Methylthiazolyldiphenyl-tetrazolium bromide; **NaOH**, Sodium hydroxide; **NSCLC**, Non-small-cell lung cancer; **PAMAM**, Poly(amidoamine); **PBS**, Phosphate Buffered Saline; **PDI**, Polydispersity index; **PEG**, Polyethylene glycol; **PI**, propidium iodide; **RNA**, ribonucleic acid; **ROS**, Reactive oxygen species; **RONS**, reactive oxygen and nitrogen species; **TEM**, Transmission electron microscopy.

* Corresponding author.
E-mail address: sylwia.michlewska@biol.uni.lodz.pl (S. Michlewska).

https://doi.org/10.1016/j.ijpharm.2024.124199
Received 29 February 2024; Received in revised form 18 April 2024; Accepted 1 May 2024
Available online 3 May 2024

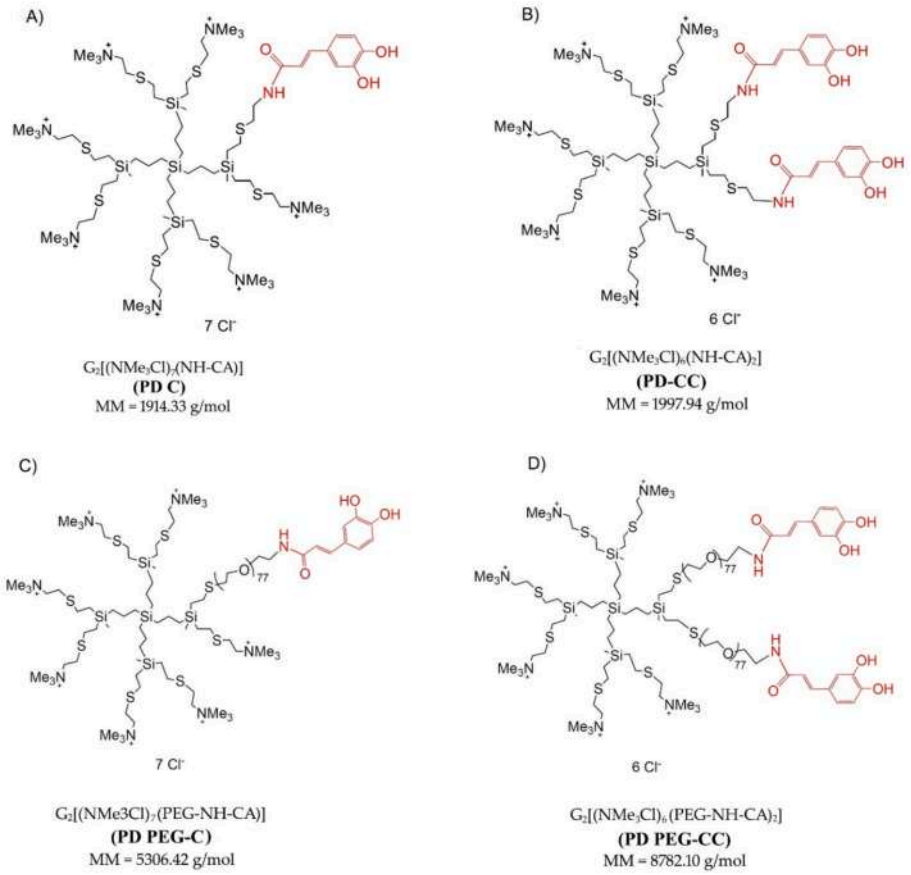


Fig. 1. The structure, chemical formula, abbreviation and molecular weight of carbosilane polyphenolic dendrimers used in this study (**A**) – PD C, $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$; (**B**) – PD CC, $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$; (**C**) – PD PEG-C, $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$; (**D**) – PD PEG-CC, $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$. Caffeic acid moieties are marked in red.

The use of dendrimers as drug/DNA/RNA delivery vehicles should consider not only the action of their cargo in the target sites but also their bioavailability, and as on the way to their target they can meet many biological macromolecules. The interaction of dendrimers with these macromolecules may limit their bioavailability in the target site and induce toxicity (Michlewska et al., 2023c, 2023b, 2023a; Svenson and Tomalia, 2005). Therefore, it is important to evaluate the potential of specific dendrimers to be used as transporting vehicles to interact with not only their cargo but also with biological objects on their way to the target site (Jain et al., 2010; Mendes et al., 2017; Svenson and Tomalia, 2005).

Common cancer treatments are usually associated with unwanted side effects, sometimes as serious as the induction of secondary cancers. Therefore, targeted therapy, affecting only specific genes or proteins responsible for cancer phenotype, is an emerging strategy of cancer treatment (Gandhi et al., 2014; Naghizadeh et al., 2019). Targeted therapy may be also considered as a kind of adjuvant treatment to facilitate the efficacy of traditional strategies to fight cancer (Gandhi et al., 2014; Michlewska et al., 2023c; Naghizadeh et al., 2019). Cancer cells are often characterized by resistance to apoptosis, which is often underlined by the upregulation of genes encoding antiapoptotic proteins. The expression of these proteins is regulated by many proteins and RNAs on the transcriptional and translational levels. Therefore, short interfering RNAs targeting mRNAs of proapoptotic genes (antiapoptotic siRNAs) may increase the susceptibility of cancer cells to a specific drug and break their drug resistance (Campbell et al., 2018; Song et al., 2005; Wesarg et al., 2007; Yang et al., 2009).

As some carbosilane dendrimers display proapoptotic properties and are positively charged, they are suited to carry antiapoptotic siRNAs in a cancer treatment targeting resistance to apoptosis (Del Olmo et al., 2020a; Singh et al., 2018). Moreover, dendrimers, due to modifications to their chemical structure, can acquire various functionalities, including scavenging of reactive oxygen and nitrogen species (RONS), which play an important role in many aspects of the functioning of cancer cells, significantly contributing to their genomic instability, a feature typical for most if not all cancer cells (Cizmarova et al., 2020; De Sá Junior et al., 2017; Del Olmo et al., 2020a; Grodzicka et al., 2022; Mitra et al., 2019; NavaneethaKrishnan et al., 2019). Polyethylene glycol (PEG) is often attached to the dendrimer structure to commit antioxidant properties. Caffeic acid is a hydroxycinnamic acid containing phenolic and acrylic functional groups and it is the most abundant phenolic acid in plants displaying antioxidant, antiproliferative and anti-inflammatory properties and it is reported to have anticancer potential (Cizmarova et al., 2020; Grodzicka et al., 2022).

In the present work, we evaluated the potential of carbosilane dendrimers modified by PEG molecules and residues of caffeic acid to form stable complexes with proapoptotic Mcl-1 and Bcl-2 siRNAs. Anticancer properties of such complexes were checked in A549 human non-small cell lung carcinoma cells.

2. Methods

2.1. Dendrimers and siRNAs

First-generation cationic carbosilane polyphenolic dendrimers $G_2[(NMe_3Cl)_7(NH\text{-}CA)]$ (**PD C**); $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$ (**PD CC**); $G_2[(NMe_3Cl)_7(PEG\text{-}NH\text{-}CA)]$ (**PD PEG-C**) and $G_2[(NMe_3Cl)_6(PEG\text{-}NH\text{-}CA)_2]$ (**PD PEG-C**) with ammonium and caffeic acid surface groups were

Table 1
The sequences of siRNAs used in this study.

	Mcl-1	Bcl-2
Sense	5′ GGACUUUUAUACCUGUUAU 3′	5′ GCUGCACCUGACGCCCUUC 3′
Antisense	5′ AUAACAGGUAUAAAAGUCC 3′	5′ GAAGGGCGUCAGGUGCAGC 3′

used in in this study (Grodzicka et al., 2022). Their chemical structure and properties are shown in Fig. 1.

Mcl-1 and Bcl-2 siRNAs labeled or not with fluorescein isothiocyanate were purchased from Dharmacon Inc. (Lafayette, CO, USA). Their sequences are shown in Table 1.

Dendrimers and siRNAs were dissolved in 10 mmol/L Na-phosphate buffer, pH 7.4, mixed at the appropriate dendrimer/siRNA molar ratio, and used after incubation at the room temperature for a minimum of 30 min.

2.2. Cells

BJ human fibroblasts and A549 human non-small lung cancer cells were grown at 37 °C, 5 % CO_2 in DMEM containing 10 % heat-inactivated fetal bovine serum (FBS) and 1 % antibiotic (streptomycin, penicillin). To evaluate the cytotoxicity of dendrimers, siRNAs, and their complexes, the MTT test was used (Sigma-Aldrich, Darmstadt, Germany). To form spheroids (3D cell cultures), A549 cells were seeded at 20,000 per well in a 96-well plate and cultured under standard conditions. Viability of A549 cells in 3D cultures was determined with the CyQUANT LDH Cytotoxicity Assay Kit Thermofisher Scientific, (Waltham, MA, USA).

2.3. Zeta size and zeta potential

The hydrodynamic diameter and the zeta potential of polyphenolic dendrimers and their complexes with siRNAs were measured by laser Doppler velocimetry technique using Zetasizer Nano ZS-90 spectrometer (Malvern Instruments, Malvern, Worcestershire, UK). Zeta potential values were calculated directly from the Helmholtz-Smoluchowski equation and Malvern software was used for data analysis. At least 3 separate replicates were performed (minimum 7 runs) for each experiment. The dynamic light scattering technique and Helmholtz-Smoluchowski equation were applied to analyze and calculate the hydrodynamic diameter nanoparticles.

2.4. Transmission electron microscopy

The morphology and size of dendrimer/siRNA complexes were examined by transmission electron microscopy technique. A 10 μl aliquot of dendrimer/siRNA complex solutions was placed on 200 mesh copper grids with a carbon surface (Ted Pella, Inc, Redding, CA, USA). Samples were stained with a 2 % uranyl acetate for 20 min. The grids were washed at least two times with deionized water and dried at room temperature Images were obtained using a JEOL1010 (JEOL, Tokyo, Japan) transmission electron microscope.

2.5. Circular dichroism

Changes in siRNA (1 μmol/L) structure upon the addition of the dendrimers at a molar ration from the range 1:1–1:50 were evaluated by circular dichroism (CD). Measurements were performed in 10 mmol/L phosphate buffer, pH 7.4 in Hellma quartz cells using a J-815CD spectrometer (Jasco, Tokyo, Japan). The wavelength was set from 200 to 300 nm and the CD parameters were set to step resolution, 1 nm; bandwidth, 1.0 nm; response time, 4 s; scan speed, 50 nm/min. The mean residue ellipticity Θ expressed in cm^2dmol^{-1}, was calculated using the software provided by Jasco. The results represent the mean ± standard deviation (SD) derived from a minimum of three independent experiments.

2.6. Fluorescence polarisation

The fluorescence polarization (P) assay was used to monitor the biding of polyphenolic dendrimers to FITC-labelled siRNAs with a PerkinElmer LS-50B spectrofluorometer (Perkin-Elmer, Waltham, MA, USA). The siRNA at the concentration 0.35 μmol/L in 10 mmol/L phosphate buffer, pH 7.4 was mixed with appropriate volumes of dendrimers to obtain dendrimer/siRNA molar ratios ranging from 1:1 to 1:70. The emission wavelength was set at 516 nm, and the excitation at 485 nm, the slits were set at 4 and 2.5 nm respectively. The results are shown as the ratio between sample and control values (P/P_0).

2.7. Gel electrophoresis

To choose the appropriate concentrations of the dendrimers and siRNAs needed for stable complex formation, 3 % agarose gel electrophoresis was performed. FITC-labelled siRNA at 2 μmol/L was mixed with an appropriate volume of dendrimers to obtain specific dendrimer/siRNA molar ratios. Before the samples were loaded onto the gel, 1 μl of orange-blue was added to each well. Electrophoresis parameters were 48 V, 40 mA, 2 W, and 45 min. To check the protective effect of dendrimers against siRNA degradation in the presence of RNase, the dendrimer/siRNA complexes were incubated with 3 μg/mL of RNase A at 30 °C. After 30 min incubation samples were placed on ice. To initiate the release of siRNA from the dendrimer/siRNA complex an aliquot of 0.082 mg/ml of heparin was added to in each well.

2.8. Cellular uptake (internalization)

Cellular uptake (internalization) of the dendrimer/siRNA complexes by A549 cells using confocal microscopy and flow cytometry techniques. The nanocomplexes quantification was analysed by measuring of the fluorescence intensity using flow cytometry technique. Additionally, confocal microscopy was applied to confirm the presence of nanocomplexes inside the cells.

The cells were incubated with dendrimer/siRNA complexes for 3 or 24 h. For flow cytometry, the cells were seeded in 24-well plates at the concentration of 100 000 cells per well, in 0.5 ml DMEM medium and siRNA and dendrimer/siRNA complexes were added. After 3 or 24 h incubation cells were centrifuged, suspended in 500 μl PBS, pH-7.4 and kept in the dark on ice. Fluorescence intensity of FITC-labelled siRNA was measured using a Becton Dickinson LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with excitation/emission wavelength set at 488/530 nm. Data were analyzed using FlowJo software. For confocal microscopy assay, the cells were seeded in a concentration 20 000 per well in μ-Slide 18 Well Glass Bottom plate (Ibidi, Gräfelfing, Germany), treated with free siRNA or dendrimer/siRNA complexes, and incubated for 3 h. The cells were fixed with 4 % formaldehyde and stained with Texas Red-X Phalloidin (40 × diluted) for 20 min and DAPI (4′,6-diamidino-2-phenylindole) at 0.5 μg/ml for 5 min. Samples were imaged using Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany), equipped with 63×/1.40 (HC PL APO CS2, Leica Microsystems, Germany) with the following excitation and emission wavelengths: 405 nm and 430–470 nm for DAPI, 489 and 500–530 nm for FITC, and 595 and 610–640 nm for Texas Red-X Phalloidin. The images were analyzed with Leica LAS2.0.215022 software.

2.9. Cell adhesion

To evaluate the impact of dendrimers/siRNA complexes on the adhesion properties of A549 cells they were seeded in a 24-well plate at a density of 25,000 cells per well. After treatment of the cells with siRNA

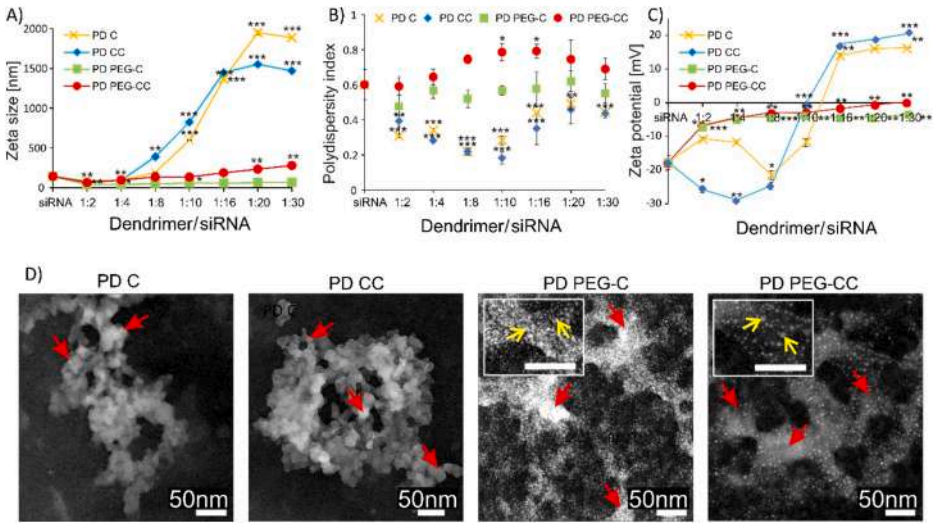


Fig. 2. (**A**) – Zeta average size, (**B**) – Polydispersity index, (**C**) – Zeta potential of dendrimer/siRNA complexes at different molar ratios in 10 mmol/L Na-phosphate buffer, pH 7.4, siRNA concentration was 0.5 μmol/L. (**D**) – Representation of the ultrastructure of dendrimer/siRNA complexes visualized by transmission electron microscopy technique. Red arrows – formed complexes, yellow arrows – dendrimers visible in siRNA structure. The final concentration of siRNA (siBcl-2) was 100 nmol/L, the final concentration of dendrimers was as follows: PD C and PD CC 1.6 μmol/L, PD PEG-C 4 μmol/L and PD PEG-CC 3 μmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. Bar – 50 nm. The values on the graphs represent mean ± SD of n > 3. Effects of complexes vs, free RNA were compared. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

(nonlabelled) and dendrimer/siRNA complexes and incubation in standard conditions for 72 h, cells were treated with trypsin (0.05 %) and washed in PBS. Next, the cells were reseeded in the "μ-Slide 18 Well Glass Bottom plate" (Ibidi) at a concentration of 10,000 per well. After 1 h the cells were washed in PBS, fixed in 4 % FA, and stained with DAPI (0.5 μg/ml). The Microhub Imaging System MICA (Leica Microsystems, Wetzlar, Germany) with 1,6×/0.05 (PLAN FLUOTAR Leica Microsystems, Germany) was used for cell imaging. A 365 nm LED was used to excite DAPI fluorescence. Cells were counted from 16 fields of view using Leica Application Suite X.

2.10. Cell migration assay

The scratch (wound healing) assay was used to evaluate the impact of dendrimer/siRNA complexes on the migration of A549 cells. The cells were seeded onto a 24-well plate at 150,000 cells per well and after the formation of cell monolayer, it was mechanically disrupted with a pipette tip. The cells were then incubated for 72 h with free siRNA or dendrimer/siRNA complexes, fixed in 4 % formaldehyde, and stained with 0.5 μg/ml DAPI and 0.6 μg/ml 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)). The Leica confocal scanning microscopy platform TCS SP8 with a Leica objective 10×/0.40 DRY was used for the analysis of cell migration to heal the scratch. Samples were imaged using the following excitation and emission wavelengths: 405 and 430–480 nm for DAPI, 485 and 530–595 nm for DIOC6(3). Images were examined with Leica Application Suite X (LASX) software.

2.11. Apoptosis

To determine the apoptotic and dead cells treated with siRNA (nonlabelled) and dendrimer/siRNA complexes using Annexin V/Propidium iodide staining, the A549 cells were seeded in a 12-well plate at a density of 200 000 cells per well. Annexin V-FITC binds phosphatidylserine exposed outside apoptotic cells, while propidium iodide (PI) enters necrotic cells, which enabled the detection and quantification of apoptotic and necrotic cells using flow cytometry and imaging of these cells by confocal microscopy. After treatment of the cells with considered compounds and incubation in standard conditions for 72 h, cells were washed twice in cold PBS and resuspended with a binding buffer (HEPES/NaOH, pH-7.4). For the flow cytometry assay cell suspension was adjusted to a concentration of 1 000 000 cells/ml. Next, 100 μL of the cell suspension was placed in a measuring tube, and 5 μL of Annexin V-FITC + 10 μL of propidium iodide (PI) was added and mixed. Compensating/alternative controls: 1) apoptosis induction – cells treated with 80 μmol/L camptothecin (4 h), green fluorescence; 2) necrosis induction – cells treated with frozen 75 % ethanol (1.5 h), red fluorescence. The cells prepared in this way were analyzed using LSRII Flow Cytometer (Becton Dickinson, NJ, USA).

For apoptotic cell imaging by confocal microscopy, cells were seeded in "μ-Slide 18 Well Glass Bottom plate" (Ibidi) at a concentration of 15,000 per well. Cell treatment and incubation time were the same as for flow cytometry measurements. Next, the cells were stained with Annexin V-FITC, PI, and DAPI, and fixed in 4 % FA. After washing with PBS the confocal laser scanning microscopy platform TCS SP8 (Leica Microsystems, Wetzlar, Germany) with 63×/1.40 (HC PL APO CS2, Leica Microsystems, Germany) was used to perform microscopic imaging. Samples were imaged with the following excitation and emission wavelengths: 405 and 430–480 nm for DAPI, 489 and 530–595 nm for Annexin V-FITC. Leica Application Suite X was used for cell visualization.

2.12. Statistical analysis

GraphPad Prism software version 8.0.1 (244) was used for statistical analysis. All results were obtained from at least 3 independent experiments and presented as mean ± SD. The 95 % confidence interval was used as the confidence interval. The normality of the distribution was checked with the Shapiro-Wilk test. In the case of a normal distribution of differences, the Student's *t*-test was used, while in other cases the ANOVA post hoc Bonferroni multiple comparisons test was used.

3. Results

3.1. Polyethylene glycol in the dendrimer structure decreased the size and zeta potential of dendrimer/siRNA complexes

Dendrimers formed stable complexes with the siRNAs (dendriplexes) as revealed by TEM. Dendrimers that were not modified with PEG

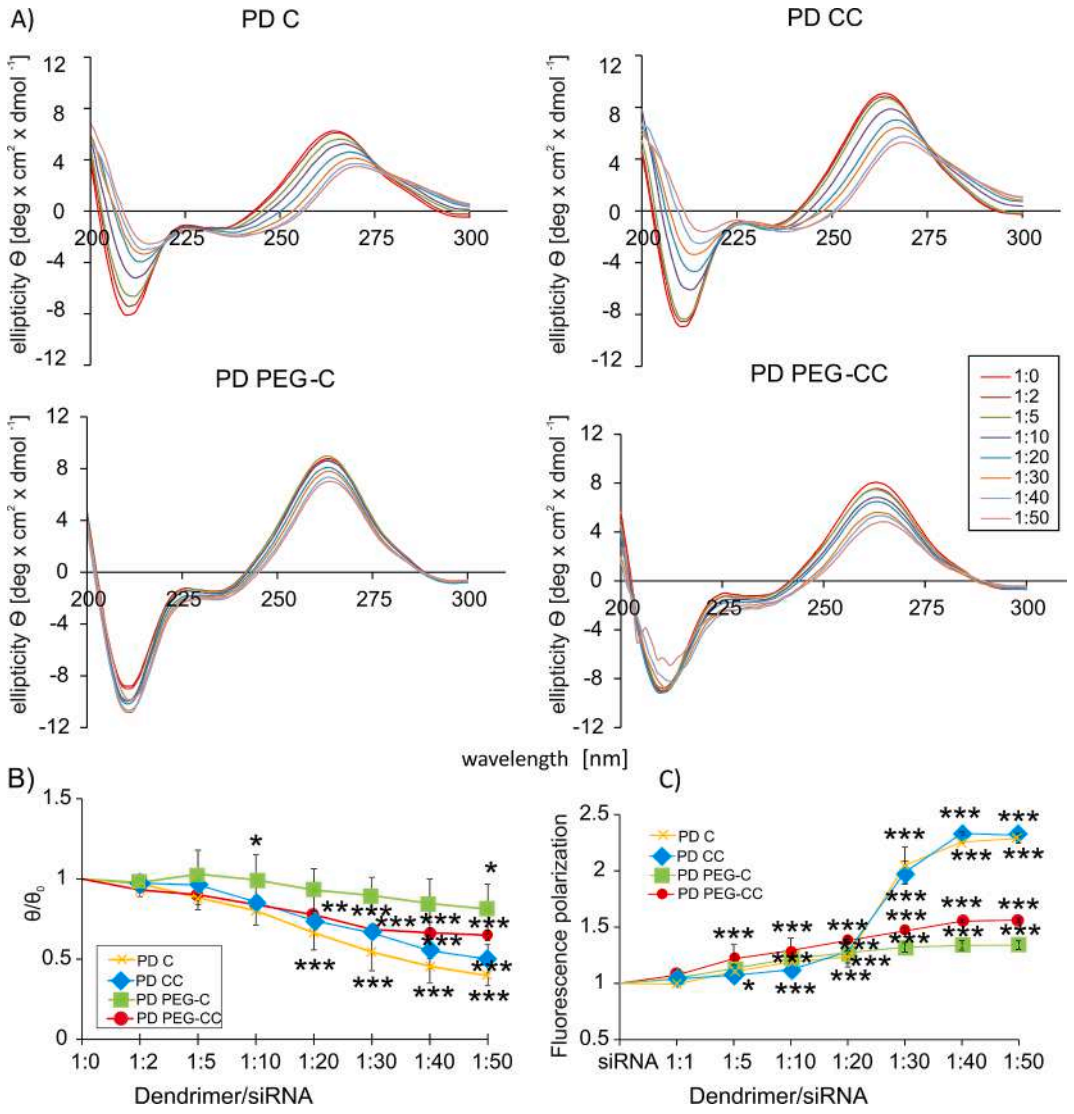


Fig. 3. (**A**) – Ellipticity changes of siRNA measured by circular dichroism in the presence of polyphenolic dendrimers under increasing dendrimer/siRNA molar ratios in 10 mmol/L Na-phosphate buffer, pH 7.4, siRNA concentration, 1 μmol/L. (**B**) – Changes in the mean residue ellipticity of siRNA in the presence of varying ratios of dendrimers at λ = 264 nm. (**C**) – Alternations in fluorescence polarisation in siRNA-FITC solution upon addition of polyphenolic dendrimers in 10 mmol/L Na-phosphate buffer, siRNA concentration, 0.35 μmol/L. Results are represented as mean standard deviation (SD), n = 3. The values on the graphs represent mean ± SD of n > 3. Effects of complexes vs, free RNA were compared. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

formed compact aggregates with different sizes from 1000 to over 1500 nm (Fig. 2D), but their counterpart modified with PEG created spherical complexes surrounded by a fiber-like, electron-dense structure of size not exceeding 300 nm. To characterize the interaction between dendrimers and siRNAs, measurements of dynamic light scattering based on photon correlation spectroscopy were performed. The results of the size distribution of free siRNA or complexed with dendrimers are shown in Fig. 2A. The zeta-size of the dendriplexes formed by dendrimers without PEG was higher than the size of complexes formed by PEG-modified dendrimers. Moreover, the ζ −size of dendriplexes containing a single CA residue (1887.47 ± 10.43 nm) was greater than their counterpart with two CA residues (1470.97 ± 15.80 nm). Such difference was more pronounced for dendrimers modified with PEG – 279.83 ± 17.67 nm and 69.53 ± 5.77 nm respectively (Fig. 2A). This difference may be, at least in part, explained by the reduction of the potential of dendrimers to form aggregates by the PEG chain. Also, the polydispersity index (PDI) was lower for the complexes formed with dendrimers without (0.4) than with PEG modification (0.6) (Fig. 2B). Addition of dendrimers to siRNAs increased their zeta-potential (Fig. 2C). This increase depended on the number of CA residues and PEG modification.

3.2. PEG modifications and caffeic acid residues affect the circular dichroism spectrum of dendriplexes and FTIC fluorescence polarization

The modification imposed on dendrimers differentially affected the CD spectra of their complexes with siRNAs taken in the wavelength range 200–300 nm. Complexes of dendrimers with two CA residues displayed a higher amplitude of the positive bands than dendrimers with single CA (Fig. 3). The presence of PEG modification in the structure of dendrimers caused a red shift of the CD spectrum and this effect was the more pronounced, the higher the dendrimer/siRNA ratio. Complexes containing dendrimers with PEG showed lower fluorescence polarization values at dendrimer/siRNA molar ratios 1:30, 1:40, and 1:50 (Fig. 3C). This effect indicates the influence of PEG chains, on the ability of dendrimers to form stable complexes with siRNA.

3.3. Gel electrophoresis

To analyze complexes of siRNA and dendrimers, we ran agarose gel electrophoresis. Complexes saturation was evaluated by the charge neutralization of the complexes as the retarded migration of siRNA in

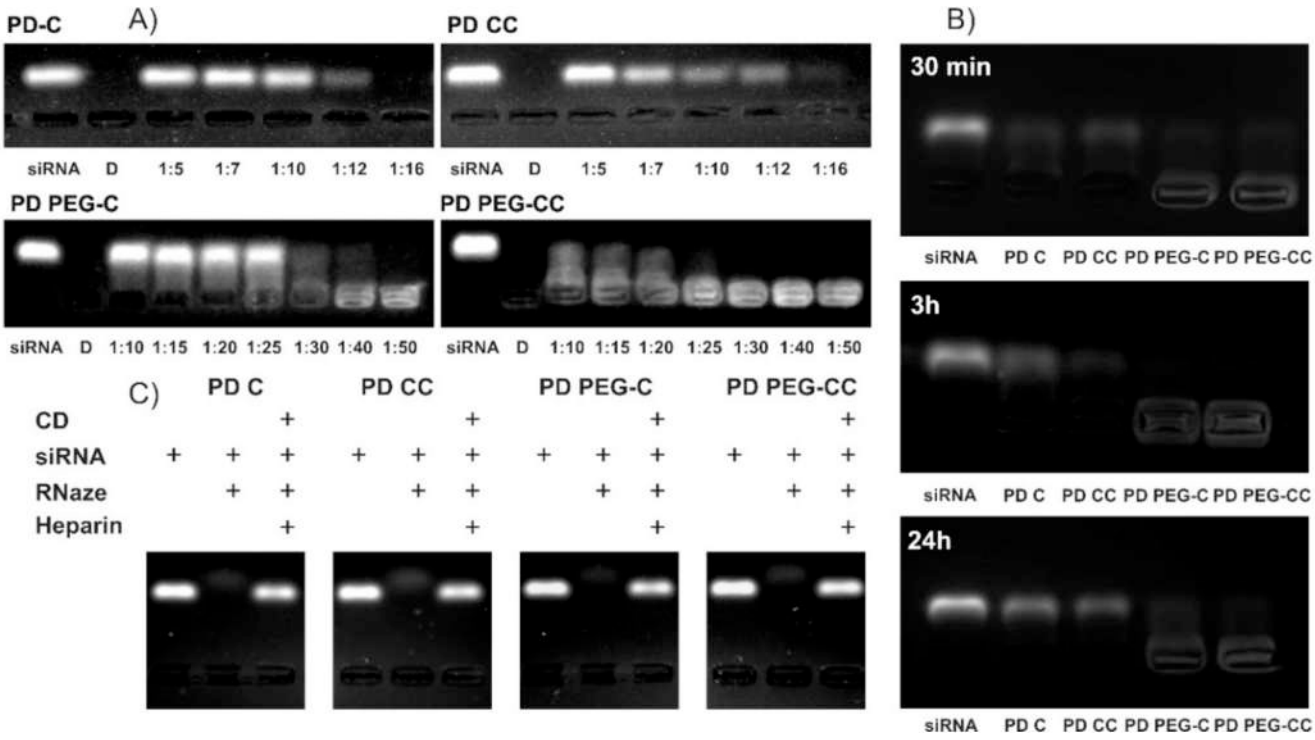


Fig. 4. (**A**) – Gel electrophoresis images representing the formation of complexes between dendrimers and siRNA (Bcl-2). The first lane demonstrates the migration of free siRNA at the concentration of 2 μmol/L. The second lane shows the migration of free dendrimer. The next lanes represent the migration of siRNA/dendrimer complexes prepared at different molar ratios. (**B**) – Gel electrophoresis images representing complexes stability in different time intervals, 30 min, 3 h, and 24 h. (**C**) – Gel electrophoresis images demonstrating the protective effect of dendrimers against degradation of siRNA in the presence of RNase, the concentration of siRNA, 2 μmo/L, RNase A, 3 μg/mL, heparin, 0.082 mg/mL, incubation time 30 min., t = 37 °C.

agarose gels. Obtained results indicate that the polyphenolic dendrimers were able to form complexes with siRNA which was shown as a gradual quenching of siRNA fluorescence.

The quenching effect was observed in the presence of dendrimers at the dendrimer/siRNA molar ratio 1: 16 for dendrimers PD C and PD CC, and at 1:30 and 1: 40 for dendrimer PD PEG-C and PD PEG-CC, respectively (Fig. 4A). Next, we tested that the potential of the dendrimers to protect siRNA from degradation by RNase. Fig. 4B shows that free siRNA was completely digested in the presence of RNase A (middle lane). The siRNA at the presence of dendrimers was also treated with RNase A (third line). The reaction of RNase on siRNA was then stopped by cooling. Next, the heparin at the concentration of 0.082 mg/mL was added to release the siRNA from the dendrimer/siRNA complex. The results of presented in Fig. 4C show the migration of intact siRNA. These findings indicate that all dendrimers were able to protect siRNA against degradation inby RNase. Additionally, the stability in time of formed complexes was assessed. Complexes formed with polyphenolic dendrimers containing PEG were found more stable than dendrimers without PEG (Fig. 4B). PD PEG-CC dendrimer showed a limited ability to complex siRNA. This effect may be related to the presence of two relatively long PEG chains, which can wrap around the dendrimer blocking its binding sites, otherwise available for siRNA.

For further *in vitro* studies the following molar ratios of dendrimer/siRNA complexes were chosen: 1:16 for PD C and PD CC; 1:40 for PD PEG-C, and 1:30 for PD PEG-CC.

3.4. Metabolic activity: MTT and LDH assays

The cytotoxic effect of dendrimer/siRNA complexes presented in the supplementary material section (Fig. 5) was determined toward normal (BJ) and cancer (A549) cells. To form the dendrimer/siRNA complexes for this experiment we used 2 pro-apoptotic siRNAs (Bcl-2 and Mcl-1). The metabolic activity of cells after their treatment with noncomplexed dendrimers or siRNAs was not dropped below 70 % vs. the control. Similarly, dendrimer/siRNA complexes did not negatively affect the viability of normal cells (Fig. 5A,B). In contrast, after incubation of cancer cells (A549) with dendrimer/siRNA complexes, the number of cells significantly decreased. The dendrimer/siMcl-1 complexes were more effective than complexes containing siBcl-2. Among all tested complexes the PD PEG-CC/siMcl-1 was the most effective and decreased the viability of cancer cells to 56.91 ± 3.16 %.

The 3D cell culture LDH assay demonstrated that dendrimer/siRNA complexes reduced the metabolic activity of A549 spheroids to about 30 % vs. untreated spheroids (Fig. 6B). Among all tested complexes the most effective was PD CC/siBcl-2, as in its presence the metabolic activity of A549 cells in 3D culture decreased to 22 %.

Due to the low cytotoxic impact of denriplexes on the normal cells, we then focused only on the effects of studied complexes against the cancer cells.

3.5. Cellular uptake (internalization)

Cellular uptake of the dendriplexes formed with polyphenolic dendrimers and siRNA-FITC was measured by flow cytometry. To visualize the presence of nanocomplexes in the cells we used confocal microscopy. Obtained results show that a 3 h incubation was sufficient for the internalization of complexes (Fig. 7). Moreover, the highest percentage of internalization was observed after 3 h of incubation for dendriplexes formed by dendrimers without PEG, especially for PD CC/siRNA-FITC (Fig. 7). The percentage of internalized complexes was 11.49 ± 3.63 % for the PD C/siRNA and 16.87 ± 3.12 % for the PD CC/siRNA complexes, respectively. The uptake of PEG-contained complexes was much lower and did not exceed 4 %. The micro-images obtained by confocal microscopy confirm the results obtained by flow cytometry and show that 3 h incubation of cells with dendriplexes, led to the internalization of FITC-labelled siRNA into the A549 cells that can be observed on the

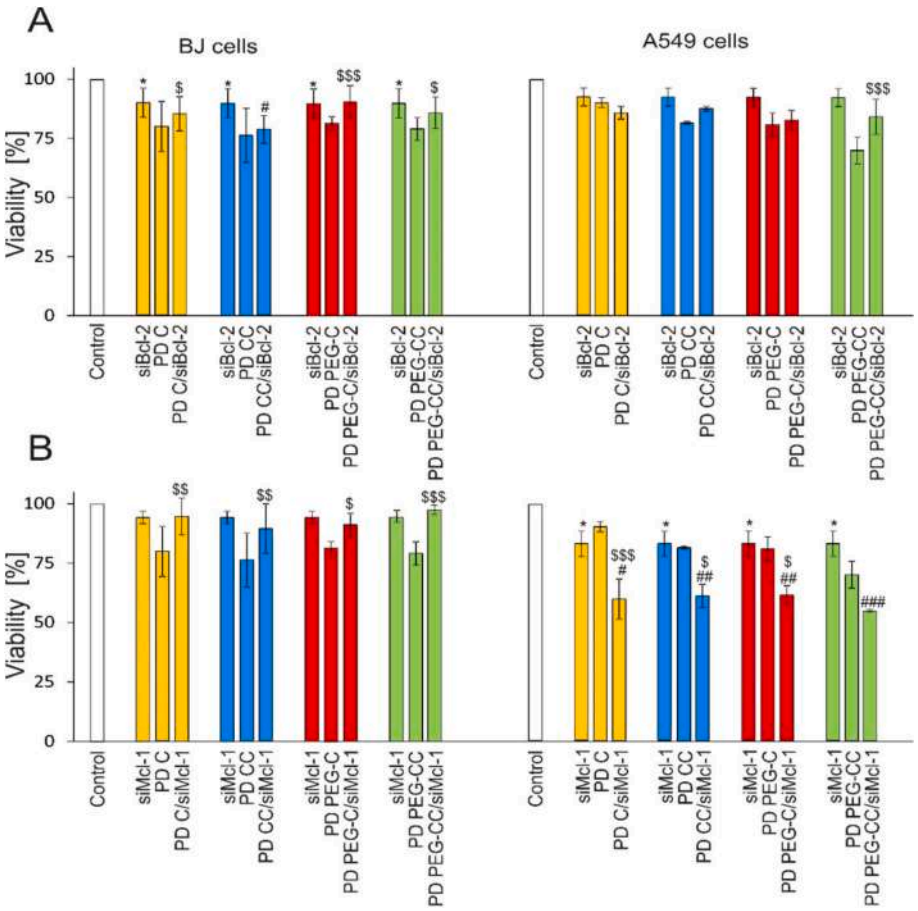


Fig. 5. (**A**) – Percentage of the viability of **BJ** (left panels) and **A549** (right panels) cells incubated with naked siRNA, naked dendrimers or dendrimer/siRNA complexes in PBS 10 mmol/L, pH 7.4, Incubation time was 72 h. To form dendrimer/siRNA complexes, following siRNAs were used: (**A**) – siBcl-2. (**B**) – siMcl-1. In all cases the final concentration of siRNA was 100 nmol/L, the final concentration of dendrimers was as follows: PD C and PD CC 1.6 μmol/L, PD PEG-C 4 μmol/L and PD PEG-CC 3 μmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. Control was untreated cells. The values on the graphs represent mean ± SD of n = 3. Significance of differences, estimated with the ANOVA and the *post-hoc* multiple comparisons Bonferroni test, were: (a) for the effect of the sample vs control (untreated cells), * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, (b) for the effect of the sample vs effect of naked dendrimer, $ $P < 0.05$, $$ $P < 0.005$, $$$ $P < 0.001$, (c) for the effect of the sample vs effect of naked siRNA, # $P < 0.05$, ## $P < 0.005$, ### $P < 0.001$.

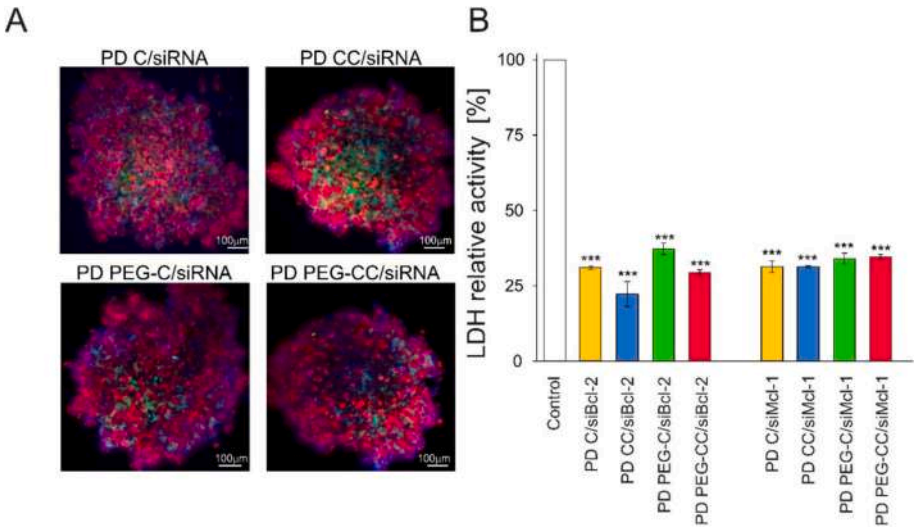


Fig. 6. (**A**) – Confocal microscopy images of 3D cultured A549 cells after 3 h incubation with FITC-labeled siRNA complexed with polyphenolic dendrimers. Bar = 100 μm. (**B**) – Percentage of 3D cultured A549 cells representing their metabolic LDH activity after 72 h exposure to siRNA/dendrimer complexes. In all cases the final concentration of siRNA was 100 nmol/L, the final concentration of dendrimers was as follows: PD C and PD CC 1.6 μmol/L, PD PEG-C 4 μmol/L and PD PEG-CC 3 μmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. Control was untreated cells. The values on the graphs represent mean ± SD of n = 3. Significance of differences, estimated with the ANOVA and the *post-hoc* multiple comparisons Bonferroni test.

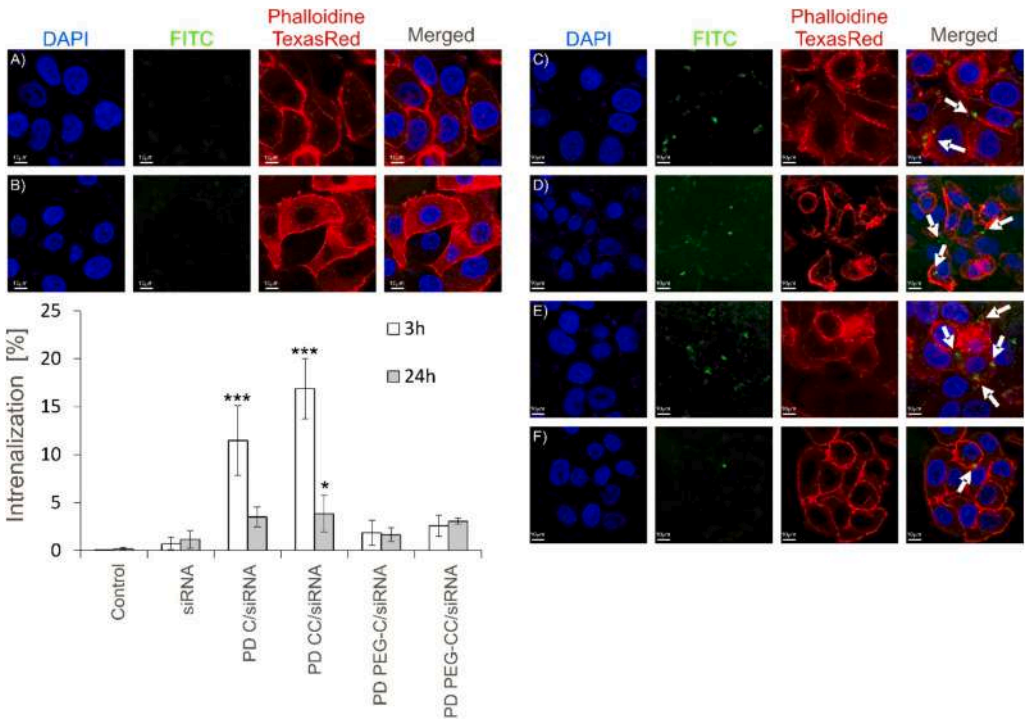


Fig. 7. Confocal microscopy images and flow cytometry values (percentage of internalization) representing the uptake profiles of FITC-labelled siRNA (siBcl-2) by A549 cells. PBS 10 mmol/L, pH 7.4. (**A**) – Cells not treated (control). (**B**) – noncomplexed siRNA. (**C**) – PD C/siRNA complex. (**D**) – PD CC/siRNA complex. (**E**) – PD PEG-C/siRNA complex. (**F**) – PD PEG-CC/siRNA complex. Incubation time, 3 h (confocal microscopy), 3 and 24 h (flow cytometry). In all cases, the final concentration of siRNA was 100 nmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. Control – untreated cells. Bar = 10 μm. Results are represented as mean standard deviation (SD), n = 3. The values on the graphs represent mean ± SD of n > 3. Effects of complexes vs. free RNA were compared. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$.

images as single green dots (Fig. 7). Obtained results for 3D culture show that all complexes were able to internalize 3D spheroids (Fig. 6A). Among all tested dendrimers the most effective were the complexes formed with nanoparticles containing 2 caffeic acid residues, but not PEG.

3.6. Cell adhesion

The impact of 72-hour incubation with dendrimers/siRNA complexes on the A549 cell adhesion properties were determined using semi-confocal microscopy (Fig. 8). The number of attached cells incubated with noncomplexed siRNAs was similar to control cells (Fig. 8A). The same effect was observed when cells were treated with complexes with siBcl-2. In the case of dendriplex PD C/siBcl-2, the number of adhered cells was reduced about 50 % (Fig. 8A,B). The cells treated with all complexes with siMcl-1 exhibited significantly decreased adhesion. The most pronounced effect in the reducing of adhesion properties of A549 exerted PD CC/siMcl-1, reducing the number of adhered cells up to 50 % (Fig. 8A).

3.7. Cell migration (wound healing) assay

To evaluate the impact of dendrimer/siRNA complexes on the migration of cancer cells, the wound healing assay was applied. The images presented in Fig. 9 show that untreated cells within 72 h completely overgrown the scratch surface. A similar effect was observed when cells were treated with noncomplexed siRNAs, indicating re-established cell contacts and unobstructed migration (Fig. 9). In contrast, all studied dendrimer/siRNA complexes inhibited the cell migration process. A549 cells treated with complexes were not able to overgrow the wound, indicating that complexes impeded cell migration. The widest wound was maintained after incubation of cells with PD PEG-C/Mcl-1. Moreover, the presence of PD C/siMcl-1 changed the morphology of cells. In this case, the cells were shrunken and smaller than control cells, which may suggest the onset of apoptosis (Fig. 9E).

3.8. Annexin V/PI

Fig. 10 shows the flow cytometry data of the distribution of different cell fractions after A549 cells treatment with dendrimers, siRNAs, or dendrimer/siRNA complexes. 72 h incubation of A549 cells with dendrimer/siRNA complexes led to a reduction of live cell fraction to about 75–80 %. However, in the presence of free dendrimers or free siRNAs, this effect did not exceed 25–35 %. Approximately only 20–25 % of late apoptotic cells were observed in the pool of A549 cells with trace amounts of early apoptotic and necrotic cells (Fig. 10 A,B). However, the presence of all nanocomplexes led to an increase the number of early apoptotic cells, while the fraction of necrotic cells was still insignificant. The largest fraction of apoptotic cells about 60 %, was observed after cells treatment with PD PEG-CC/siMcl-1 and PD PEG-CC/siBcl-2 complexes (Fig. 10 A,B). A similar effect was noted for PD CC/siBcl-2 complex with a fraction of apoptotic cells over 50 %. Other dendriplexes induced an increase of apoptotic cells up to ~ 45 %.

The obtained effect was confirmed by the observation of the A549 cells with confocal microscopy. The images presented in Fig. 11A-H show the typical morphological features of apoptotic cells such as loss of the structural framework of the nuclei, condensation of chromatin, cell shrinkage, nuclear fragmentation, and detachment of apoptotic bodies.

4. Discussion

Presently, one of the alternative highly effective methods with minimal side effects of cancer treatment is gene therapy (Zhou et al., 2017). The research on targeted anticancer therapy focuses on

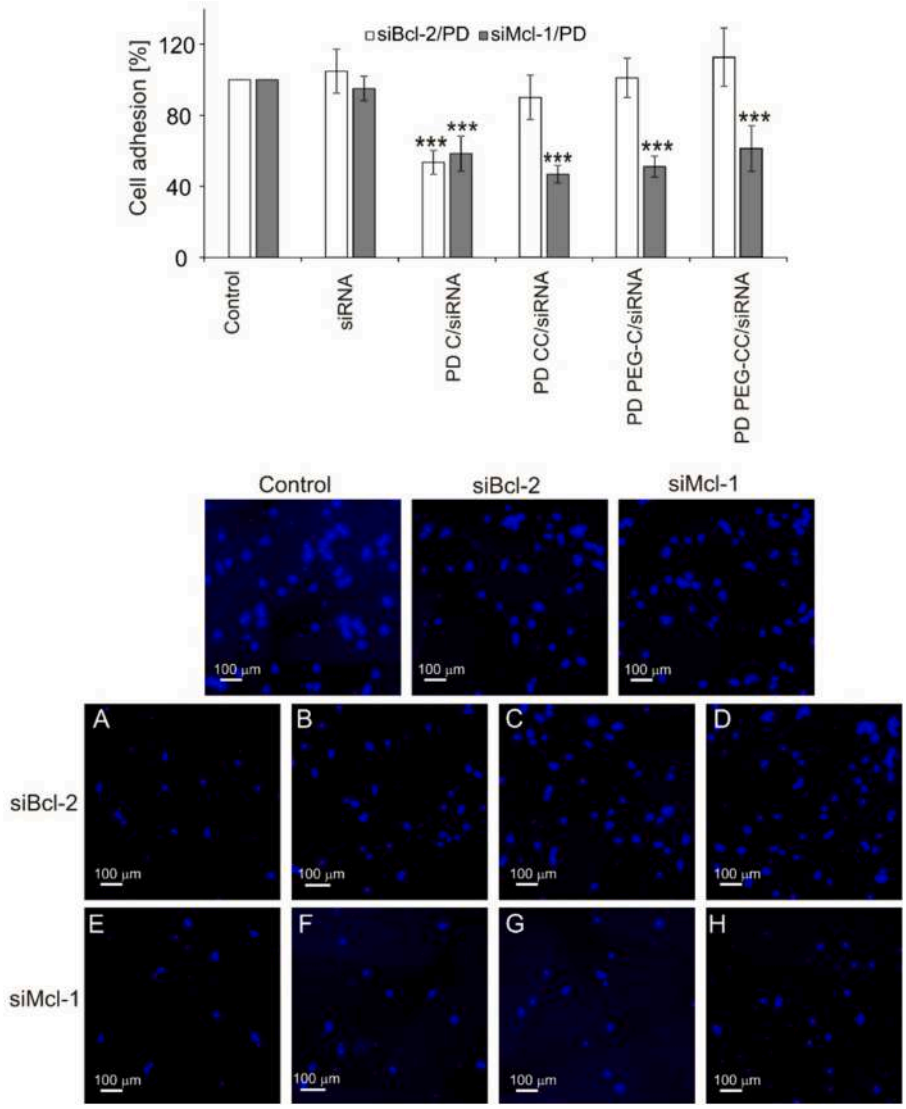


Fig. 8. Adhesion of A549 cells after treatment with naked siRNA or dendrimer/siRNA complexes evaluated by semi-confocal microscopy. (A) – PD C/siBcl-2. (B) – PD CC/siBcl-2. (C) – PD PEG-C/siBcl-2. (D) – PD PEG-CC/ siBcl-2. (E) – PD C/siMcl-1. (F) – PD CC/siMcl-1. (G) – PD PEG-C/siMcl-1. (H) – PD PEG-CC/siMcl-1. Control – untreated cells. The final concentration of siRNA was 100 nmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. Images were captured after 1 h to evaluate the cellular adhesion. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ as compared with free siRNA.

interfering RNA (RNAi), in particular small interfering RNA (siRNA) and microRNA (miRNA) (Gandhi et al., 2014; Singh et al., 2018). However, siRNA interference-based cancer treatment demands the application of a dependable and safe method for delivering genes. Therefore, currently, non-viral methods are being widely investigated (Gridelli et al., 2015). One alternative means of gene delivery is the use of polymeric carriers, including dendrimers. The possibility of formulation complexes by dendrimers with siRNA was verified earlier (Białkowska et al., 2022; Del Olmo et al., 2020b; Ferenc et al., 2013; Krasheninina et al., 2019). Due to the potential of modification of these nanoparticles, there is a chance to endow them with new properties that may positively influence the effectiveness of gene therapy (Rodríguez-Prieto et al., 2021). According to the literature, in cancer cells, long-term oxidative stress, caused by the continuous action of oncogenic signals and active metabolism of cancer cells, requires full mobilization of the cell antioxidant mechanisms. Thus, they are dependent on the action of antioxidant enzymes that enable them to survive under conditions of increased oxidative stress. Exposure of cancer cells to external factors that alter ROS levels can deplete their antioxidant mechanisms. As a result, exceeding a "threshold" concentration of free radicals in cells can lead to cell death (De Sá Junior et al., 2017; Mitra et al., 2019; Nakamura and Takada, 2021; Reczek and Chandel, 2018). According to the literature, polyphenols including caffeic acid, can reduce reactive oxygen species level and inhibit cancer cell proliferation (Del Olmo et al., 2020a; Grodzicka et al., 2022; Mencia et al., 2016; NavaneethaKrishnan et al., 2019). Our previous studies indicate that polyphenolic dendrimers containing caffeic acid in their structure have significant antioxidant properties (Grodzicka et al., 2022). Therefore, in the present study, the polyphenolic dendrimers with caffeic acid were assessed as siRNA carriers against lung cancer, which is one of the most common fatal diseases (Kubczak et al., 2021).

Cancer cells evolved several mechanisms to favor their progression and allow them to avoid elimination by apoptosis. Such mechanisms may be supported by high levels of anti-apoptotic proteins such as Bcl-2 and Mcl-1 (Ambesajir et al., 2012; Campbell et al., 2018; Del Olmo et al., 2020b; Dzmitruk et al., 2015; Ionov et al., 2015). It was reported that Mcl-1 played a pivotal role in resistance to anti-cancer drugs, also in non-small cell lung cancer (NSCLC) (Singh et al., 2013, 2010; Wei et al., 2012; Zhang et al., 2011),(Cetin et al., 2010). Furthermore, it can serve as a novel prognostic biomarker indicating poor outcomes in NSCLC

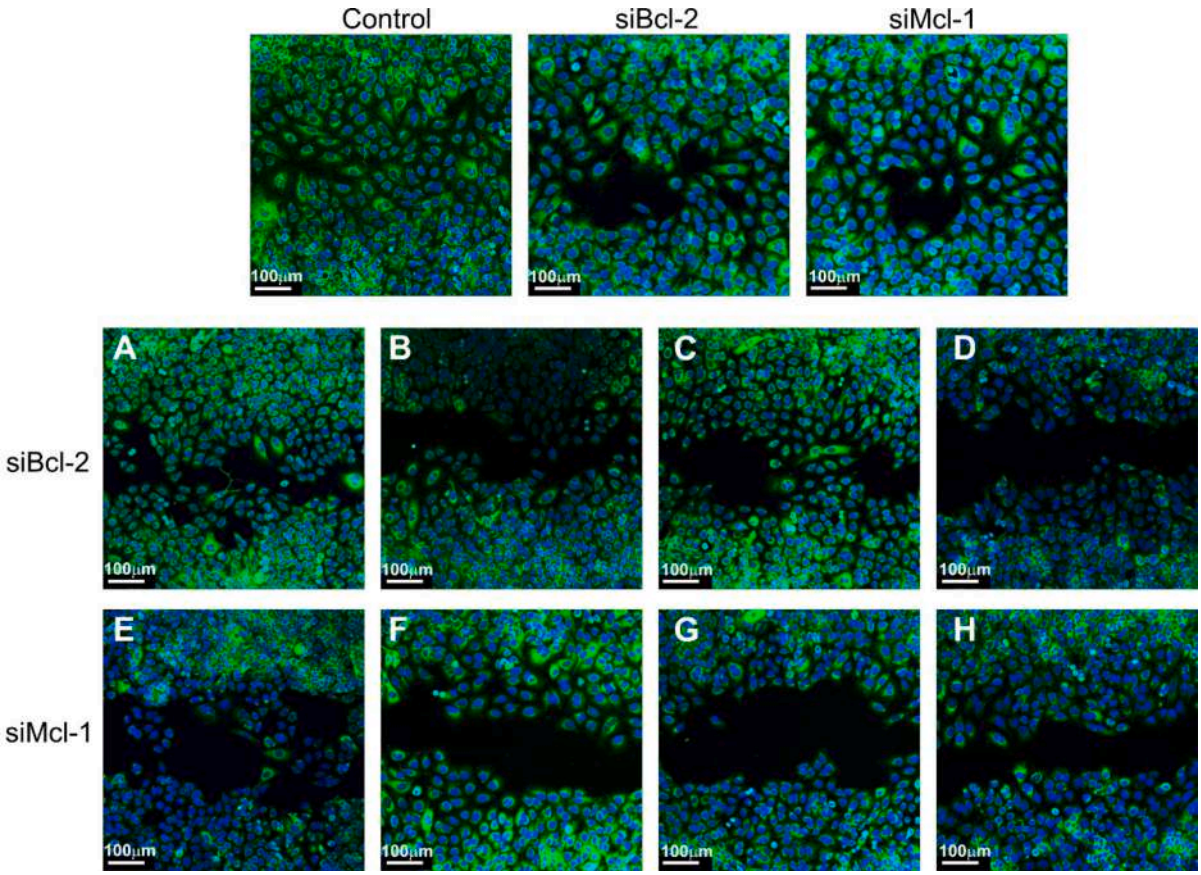
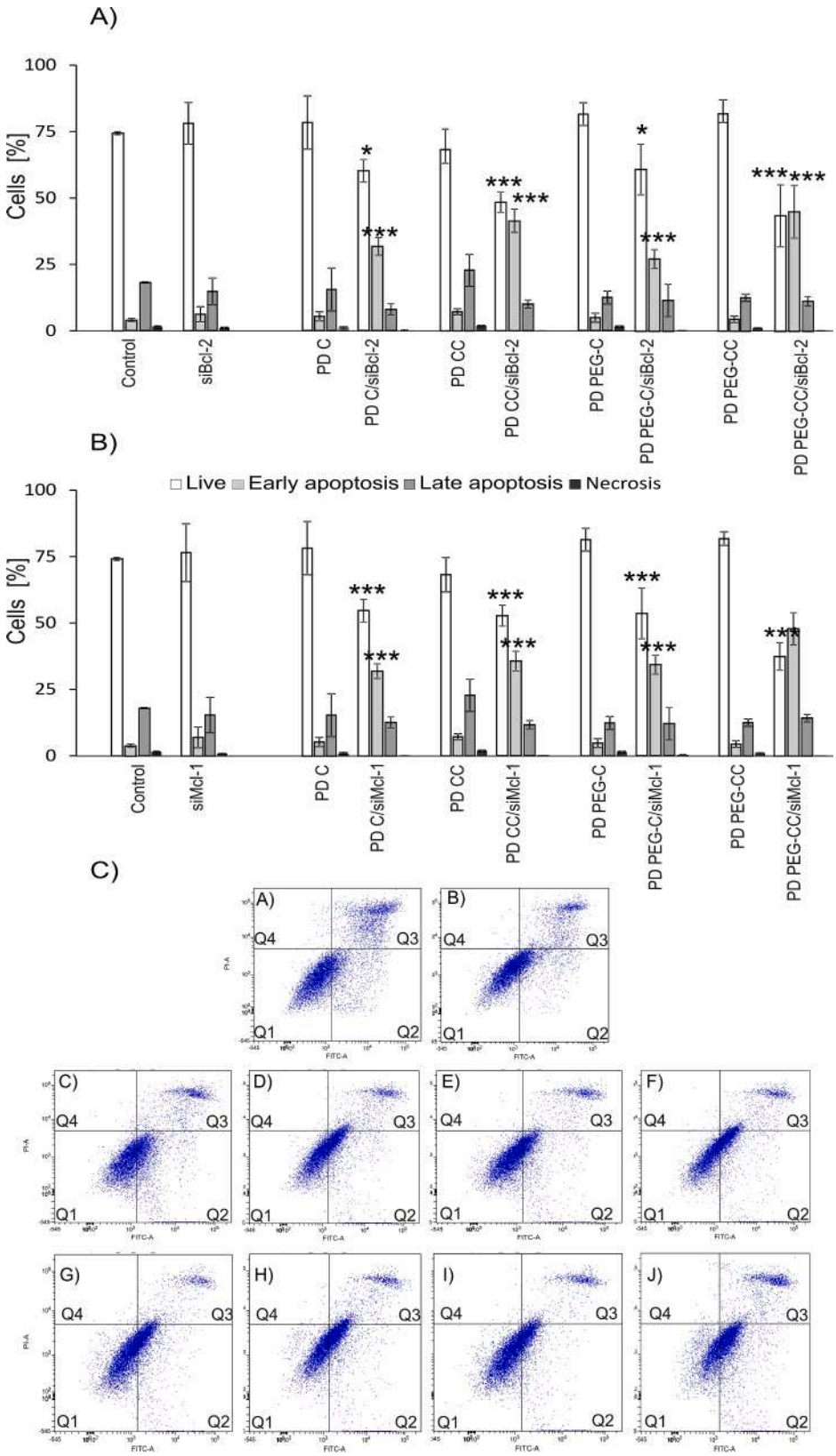


Fig. 9. Cell migration (wound healing assay) was evaluated by confocal microscopy in A549 cells after their treatment with naked siRNA (top panels) or dendrimer/siRNA complexes. (**A**) – PD C/siBcl-2. (**B**) – PD CC/siBcl-2. (**C**) – PD PEG-C/siBcl-2. (**D**) – PD PEG-CC/ siBcl-2. (**E**) – PD C/siMcl-1. (**F**) – PD CC/siMcl-1. (**G**) – PD PEG-C/siMcl-1. (**H**) – PD PEG-CC/siMcl-1. In all cases, the final concentration of siRNA was 100 nmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. Images were captured after 72 h to evaluate the scratch (wound) closure indicative cellular migration. Bar – 100 μm.

patients. Presently, the direct effective inhibitor of Mcl-1 is still unknown. Li et al. [2020] (Li et al., 2020) demonstrated that the application of a Bcl-2 inhibitor for NSCLC cells caused increased Mcl-1 expression (Li et al., 2020). In our study, proapoptotic siRNAs (siMcl-1 and siBcl-2) were complexed with polyphenolic dendrimers and tested for possible decrease the Mcl-1 protein production in A549 cells and direct them on the apoptosis way.

We analyzed firstly the ability of polyphenolic dendrimers to form complexes with siMcl-1 and siBcl-2 siRNAs. Obtained results indicated appropriate dendrimer/siRNA molar ratios needed to form the complexes. Formed nanoconjugates were used in further *in vitro* studies, which allowed us to select the most promising dendrimer out of all four dendrimers. According to the literature, free siRNA has no impact on cells probably due to its negative charge which prevents the interaction with negatively charged cell membranes (Del Olmo et al., 2020b; Dzmitruk et al., 2015; Rodríguez-Prieto et al., 2021). Moreover, siRNA is sensitive to the degradation by nucleases (Del Olmo et al., 2020b; Ferenc et al., 2013). Our experiments confirmed these findings and showed the lack of siRNA effect on both normal and lung cancer cells. It was shown that dendrimers used in this study possess antioxidant properties and, at high concentrations, reduce the viability of both normal and cancer cells (Grodzicka et al., 2022). However, at the low concentrations corresponding we applied, their cytotoxic effect was not significant.

The positive impact of dendriplexes formed with dendrimers and pro-apoptotic siRNA on the cancer cells was observed in other studies (Del Olmo et al., 2020b; Dzmitruk et al., 2015; Rodríguez-Prieto et al., 2021). The same was observed in our studies when polyphenolic dendrimers were applied to transfect the A549 cells. It can be the effect of blocking the synthesis of anti-apoptotic proteins because of gene silencing, making cells more sensitive to the cytotoxic effect of dendrimers applied as siRNA carriers. We have shown additionally that dendrimers can protect the siRNA against RNase activity. This is extremely important, since a major problem in gene therapy is the degradation of siRNA by nucleases before the start of the interference process (Gandhi et al., 2014; Itani and Al Faraj, 2019; Singh et al., 2018). Moreover, formed dendrimer/siRNA complexes were positively charged which facilitated their internalization into cells (Babu et al., 2016; Biswas and Torchilin, 2013; Del Olmo et al., 2020b; Okła et al., 2023). The literature data show that positively charged dendrimers can be cytotoxic as they may cause the formation of micro-holes in plasmalemma (Shcharbin et al., 2010; Wang et al., 2010) but on the other hand can be used as effective transfectants (Conti et al., 2014; Jain et al., 2010; Lazniewska et al., 2013). Our results show that a 3 h incubation was sufficient for a maximal uptake of the dendrimer/siRNA complexes by A549 cells in 2D and 3D cell cultures. Increasing the incubation time from 3 to 24 h did not increase the number of internalized cells. A similar situation was observed for dendriplexes formed by copper dendrimers and gold nanoparticles in monolayers (Abashkin et al., 2021; Del Olmo et al., 2020b) and, in spheroids formed by MCF7 human breast cancer cells (Białkowska et al., 2022). Several factors such as incubation time, kind of dendrimer, and cell line type can affect transfection efficiency (Del Olmo et al., 2020b; Shcharbin et al., 2015). The release of siRNA from the dendrimer/siRNA complex could be a result of an initial release after uptake, or the fact that complexes with siRNA become unstable in time. Surprisingly, complexes formed by polyphenolic dendrimers with PEG, despite their higher stability were not more intensively taken with increasing incubation time. However, the viability experiments show that the metabolic activity of the cancer cells was dependent on the type



(caption on next page)

Fig. 10. The percentages of **A549** cells in different phases and apoptosis profiles evaluated by flow cytometry and measured using annexin V-FITC/propidium iodide staining in PBS 10 mmol/L, pH 7.4, incubation time 72 h. To form dendrimer/siRNA complexes, the following siRNAs were used: (**A**) – siBcl-2. (**B**) – siMcl-1. The final concentration of siRNA was 100 nmol/L, and the final concentration of dendrimers was as follows: PD C and PD CC 1.6 μmol/L, PD PEG-C 4 μmol/L and PD PEG-CC 3 μmol/L. The dendrimer/siRNA molar ratios were: 1:16 for PD C and PD CC; 1:40 for PD PEG-C and 1:30 for PD PEG-CC. (**C**) – Sample dot plot presenting Q1 – Live cells, Q2 – Early apoptotic cells, Q3 – Late apoptotic cells and Q4 – Necrotic cells. A) – not treated (control) A549 cells. (B) – A549 cells incubated with noncomplexed siMcl-1, or: (C) – noncomplexed PD C. (D) – noncomplexed PD CC. (E) – noncomplexed PD PEG-C. (F) – noncomplexed PD PEG-CC. (G) – PD C/siMcl-1 complex. (H) – PD CC/siMcl-1 complex. (I) – PD PEG-C/siMcl-1 complex. (J) – PD PEG-CC/siMcl-1 complex. Bars represent mean ± SD of n = 3. Results are represented as mean standard deviation (SD), n = 3. The values on the graphs represent mean ± SD of n > 3. Effects of complexes vs, free RNA were compared.

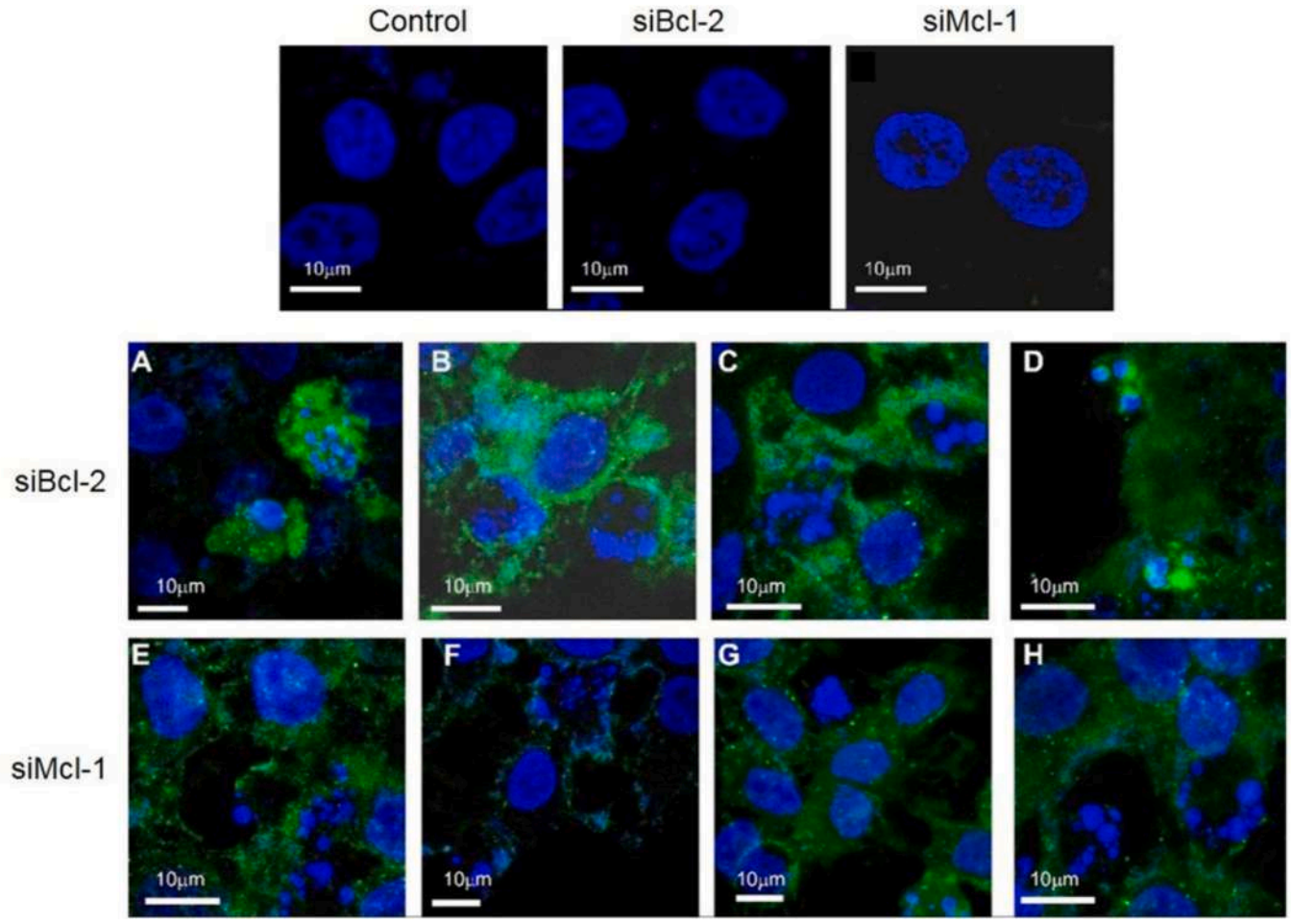


Fig. 11. Confocal microscopy images representing A549 apoptotic cell death were examined by confocal microscopy using AnnexinV kit (green color). The kernels were bleached with DAPI (blue color), in PBS 10 mmol/L, pH 7.4. Incubation time 72 h. The cells were treated with naked siRNA (upper panels) or dendrimer/siRNA complexes (bottom panels). (**A**) – PD C/siMcl-1. (**B**) – PD CC/siMcl-1. (**C**) – PD PEG-C/siMcl-1. (**D**) – PD PEG-CC/siMcl-1. (**E**) – PD C/siBcl-2. (**F**) – PD CC/ siBcl-2. (**G**) – PD PEG-C/ siBcl-2. (**H**) – PD PEG-CC/ siBcl-2. The concentration of siRNA, is 100 nmol/L. The dendrimer/siRNA molar ratios were as follows: 1:16, (PD C and PD CC); 1:40, (PD PEG-C), and 1:30, (PD PEG-CC). The control image shows untreated A549 cells. Bar = 10 μm.

of introduced siRNA rather than the type of used nanoparticle.

Cell migration is an important process for tissue repair and immune function. Disruption of tumor cell movement can lead to uncontrolled migration and invasion in other tissues. The wound healing assay provides a simple way to observe cell migration to some extent by mimicking cell migration *in vivo* (Liang et al., 2007; Valster et al., 2005). The migration and adhesion processes are intimately related. Loss of ability of cell attachment to foreign tissue may limit neoplastic progression, leading to preventing the development of metastases (Gassmann et al., 2004; Paschos et al., 2009). Our results indicate that all tested dendriplexes inhibited the cell migration process and hindered the restoration of intercellular contact regardless of the nature of the dendrimer used or the kind of used siRNA. In turn, the adhesion process was limited only for cells treated by dendriplexes contained siMcl-1 (and PD C/siBcl-2). This may indicate the effect of applied nucleic acid and the decrease in Mcl-1 protein levels. This is consistent with the literature reporting the overexpression of Mcl-1 protein in non-small cell lung cancer (NSCLC) (Song et al., 2005; Wesarg et al., 2007).

The results of the AnnexinV-FITC/PI double staining assay showed that free siRNA or dendrimers caused the appearance of a small fraction of late apoptotic cells. In contrast, all tested nanocomplexes increased the amount of early apoptotic cells. Antiapoptotic proteins such as Bcl-2 and Mcl-1 play a crucial role in cancer survival. These proteins not only protect cancer cells from apoptotic death but are also responsible for multidrug resistance of the tumor (Li et al., 2020; Song et al., 2005; Wesarg et al., 2007; Yang et al., 2009). Protein synthesis depends on the type of cell line (Yang et al., 2009). Bcl-2 protein plays an important role in directing the cancer cell towards survival or death, but in non-small cell lung cancer (NSCLC) mainly Mcl-1 protein is overexpressed (Song et al., 2005; Wesarg et al., 2007). Thus, decreased levels of Mcl-1 may

increase the susceptibility of tumor cells to apoptosis induced by cytotoxic agents, thereby enhancing the effects of chemotherapy (Song et al., 2005). Our results show that the largest fraction of apoptotic cells was obtained regardless of the type of siRNA. Other studies indicated that complexes formed by siBcl-2, siBcl-xL, siMcl-1, and cocktail siRNAs with different dendrimers induced HeLa and HL-60 apoptotic cell death (Dzmitruk et al., 2018, 2015; Ihnatsyeu-Kachan et al., 2017; Ionov et al., 2015).

5. Conclusions

We propose here for the first time that the hetero-functionalized polyphenolic dendrimers can be considered potential nanocarriers for proapoptotic siRNA. Our data suggest that these dendrimers containing the caffeic acid in their structure can bind and protect the siRNA against nuclease degradation. The formed siRNA-dendrimer complexes transfected cancer cells with siRNA. It seems likely that due to a combination of the activity of proapoptotic siRNA, the positive charge of dendrimers, and possibly their ability to influence the cellular level of ROS (Grodzicka et al., 2022), the formed dendrimer/siRNA complexes were able to inhibit the cancer cell migration and induce apoptosis. Obtained results indicate that from 4 studied compounds, the most effective was the, $G_2[(NMe_3Cl)_6(NH\text{-}CA)_2]$ (PD CC) dendrimer contained two caffeic acid residues and ammonium groups on the surface but without polyethylene glycol chains anchored in the dendrimer scaffold.

6. Data availability statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. All data generated or analysed during this study are included in this published article and its supplementary information files.

7. Disclosure of Funding

This work was supported by the National Science Centre of Poland "Beethoven Life 1″ program, project no: 2018/ 31/F/NZ5/03454, grant 2023/07/X/NZ7/00026 of the Project "MINIATURA7", and grant 2018/ 30/Z/NZ1/00911 of Project "NanoTendo" under the NCN "M-ERA.NET 2", which has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement no 685451 and IDUB No. 44/2021 funded under a 2 % increased subsidy for universities in Poland. Grants from PID2020-112924RBI00 (MINECO), SBPLY/17/180501/000358 Junta de Comunidades de Castilla-La Mancha (JCCM), University of Alcala PIUAH21/CC-041 and EPU-INV-UAH/2021/002. Radioprotect_S2022/BMD/7406 (CAM), CIBER-BBN is an initiative funded by the VI National R&D&i Plan 2008–2011, Iniciativa Ingenio 2010, Consolider Program, CIBER Actions, and financed by the Instituto de Salud Carlos III with assistance from the European Regional Development Fund.

Author contributions

M.G. Investigation, Software, Visualization, Writing- Original draft preparation, **S.M.** Conceptualization, Formal analysis, Methodology, Investigation, Visualization, Writing- Original draft preparation, Funding acquisition, **J.B.** Formal analysis, Data curation, Validation, Writing-Reviewing and Editing, **P.O.** Funding acquisition, Sources, Writing-Reviewing and Editing, **F.J.M.** Funding acquisition, Sources, Writing-Reviewing and Editing, **M.B.** Funding acquisition, Writing- Reviewing and Editing, **M.I.** Conceptualization, Project administration, Formal analysis, Writing- Reviewing and Editing, Supervision.

CRediT authorship contribution statement

Marika Grodzicka: Writing – original draft, Visualization, Software, Investigation. **Sylwia Michlewska:** Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Janusz Blasiak:** Writing – review & editing, Validation, Formal analysis, Data curation. **Paula Ortega:** Writing – review & editing, Resources, Funding acquisition, Data curation. **Francisco Javier de la Mata:** Writing – review & editing, Resources, Funding acquisition, Data curation. **Maria Bryszewska:** Writing – review & editing, Funding acquisition. **Maksim Ionov:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgments

The authors are thankful to Dr. Magdalena Gapińska from the Laboratory of Microscopic Imaging & Specialized Biological Techniques, Faculty of Biology & Environmental Protection, University of Lodz, Poland.

References

Abashkin, V., Pędziwiatr-Werbicka, E., Gómez, R., de la Mata, F.J., Dzmitruk, V., Shcharbin, D., Bryszewska, M., 2021. Prospects of Cationic Carbosilane Dendronized Gold Nanoparticles as Non-viral Vectors for Delivery of Anticancer siRNAs siBCL-xL and siMCL-1. Pharmaceutics. https://doi.org/10.3390/pharmaceutics13101549.

Ambesajir, A., Kaushik, A., Kaushik, J.J., Petros, S.T., 2012. RNA interference: A futuristic tool and its therapeutic applications. Saudi J. Biol. Sci. 19, 395–403. https://doi.org/10.1016/j.sjbs.2012.08.001.

Babu, A., Muralidharan, R., Amreddy, N., Mehta, M., Munshi, A., Ramesh, R., 2016. Nanoparticles for siRNA-Based Gene Silencing in Tumor Therapy. IEEE Trans. Nanobioscience 15, 849–863. https://doi.org/10.1109/TNB.2016.2621730.

Białkowska, K., Komorowski, P., Gomez-ramirez, R., Javier, F., Mata, D., Bryszewska, M., Miłowska, K., 2022. Interaction of Cationic Carbosilane Dendrimers and Their siRNA Complexes with MCF-7 Cells Cultured in 3D Spheroids 1–15.

Biswas, S., Torchilin, V.P., 2013. Dendrimers for siRNA Delivery. Pharmaceuticals 6, 161–183. https://doi.org/10.3390/ph6020161.

Campbell, K.J., Dhayade, S., Ferrari, N., Sims, A.H., Johnson, E., Mason, S.M., Dickson, A., Ryan, K.M., Kalna, G., Edwards, J., Tait, S.W.G., Blyth, K., 2018. MCL-1 is a prognostic indicator and drug target in breast cancer. Cell Death Dis. 9, 19. https://doi.org/10.1038/s41419-017-0035-2.

Cetin, Z., Ozbilim, G., Erdogan, A., Luleci, G., Karauzum, S.B., 2010. Evaluation of PTEN and Mcl-1 expressions in NSCLC expressing wild-type or mutated EGFR. Med. Oncol. 27, 853–860. https://doi.org/10.1007/s12032-009-9296-7.

Cizmarova, B., Hubkova, B., Bolerazska, B., Marekova, M., Birkova, A., 2020. Caffeic acid: A brief overview of its presence, metabolism, and bioactivity. Bioact. Compd. Heal. Dis. 3, 74–81. https://doi.org/10.31989/bchd.v3i4.692.

Conti, D.S., Brewer, D., Grashik, J., Avasarala, S., Da Rocha, S.R.P., 2014. Poly (amidoamine) dendrimer nanocarriers and their aerosol formulations for siRNA delivery to the lung epithelium. Mol. Pharm. 11, 1808–1822. https://doi.org/10.1021/mp4006358.

De Sá Junior, P.L., Câmara, D.A.D., Porcacchia, A.S., Fonseca, P.M.M., Jorge, S.D., Araldi, R.P., Ferreira, A.K., 2017. The Roles of ROS in Cancer Heterogeneity and Therapy. Oxid. Med. Cell. Longev. 2017 https://doi.org/10.1155/2017/2467940.

Del Olmo, N.S., González, C.E.P., Rojas, J.D., Gómez, R., Ortega, P., Escarpa, A., de la Mata, F.J., 2020a. Antioxidant and antibacterial properties of carbosilane dendrimers functionalized with polyphenolic moieties. Pharmaceutics 12, 1–16. https://doi.org/10.3390/pharmaceutics12080698.

Del Olmo, N.S., Holota, M., Michlewska, S., Gómez, R., Ortega, P., Ionov, M., de la Mata, F.J., Bryszewska, M., 2020b. Copper (II) metallodendrimers combined with pro-apoptotic sirnas as a promising strategy against breast cancer cells. Pharmaceutics 12, 1–14. https://doi.org/10.3390/pharmaceutics12080727.

Dufe, C., Uchegbu, I.F., Schatzlein, A.G., 2005. Dendrimers in gene delivery. Adv. Drug Deliv. Rev. 57, 2177–2202. https://doi.org/10.1016/j.addr.2005.09.017.

Dzmitruk, V., Szulc, A., Shcharbin, D., Janaszewska, A., Shcharbina, N., Łazniewska, J., Novopashina, D., Buyanova, M., Ionov, M., Klajnert-Maculewicz, B., Gómez-Ramirez, R., Mignani, S., Majoral, J.P., Muñoz-Fernández, M.A., Bryszewska, M., 2015. Anticancer siRNA cocktails as a novel tool to treat cancer cells. Part (B).

Efficiency of pharmacological action. Int. J. Pharm. 485, 288–294. https://doi.org/10.1016/j.ijpharm.2015.03.034.
Dzmitruk, V., Apartsin, E., Ihnatsyeu-Kachan, A., Abashkin, V., Shcharbin, D., Bryszewska, M., 2018. Dendrimers show promise for siRNA and microrna therapeutics. Pharmaceutics 10, 1–25. https://doi.org/10.3390/pharmaceutics10030126.
Ferenc, M., Pedziwiatr-Werbicka, E., Nowak, K.E., Klajnert, B., Majoral, J.P., Bryszewska, M., 2013. Phosphorus dendrimers as carriers of siRNA-characterisation of dendriplexes. Molecules 18, 4451–4466. https://doi.org/10.3390/molecules18044451.
Gandhi, N.S., Tekade, R.K., Chougule, M.B., 2014. Nanocarrier mediated delivery of siRNA/miRNA in combination with chemotherapeutic agents for cancer therapy: Current progress and advances. J. Control. Release 194, 238–256. https://doi.org/10.1016/j.jconrel.2014.09.001.
Gassmann, P., Enns, A., Haier, J., 2004. Role of Tumor Cell Adhesion and Migration in Organ-Specific Metastasis Formation. Onkologie 27, 577–582. https://doi.org/10.1159/000081343.
Gridelli, C., Rossi, A., Carbone, D.P., Guarize, J., Karachaliou, N., Mok, T., Petrella, F., Spaggiari, L., Rosell, R., 2015. Non-small-cell lung cancer. Nat. Rev. Dis. Prim. 1, 15009. https://doi.org/10.1038/nrdp.2015.9.
Grodzicka, M., Pena-Gonzalez, C.E., Ortega, P., Michlewska, S., Lozano, R., Bryszewska, M., de la Mata, F.J., Ionov, M., 2022. Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity. Sustain. Mater. Technol. 33, e00497.
Hołota, M., Michlewska, S., Garcia-gallego, S., Sanz, N., Ortega, P., Bryszewska, M., Javier, F., Mata, D., Ionov, M., 2023. Combination of Copper Metallodendrimers with Conventional Antitumor Drugs to Combat Cancer in In Vitro Models.
Ihnatsyeu-Kachan, A., Dzmitruk, V., Apartsin, E., Krasheninina, O., Ionov, M., Loznikova, S., Venyaminova, A., Miłowska, K., Shcharbin, D., Mignani, S., Muñoz-Fernández, M., Majoral, J.-P., Bryszewska, M., 2017. Multi-Target Inhibition of Cancer Cell Growth by SiRNA Cocktails and 5-Fluorouracil Using Effective Piperidine-Terminated Phosphorus Dendrimers. Colloids and Interfaces 1, 6. https://doi.org/10.3390/colloids1010006.
Ionov, M., Lazniewska, J., Dzmitruk, V., Halets, I., Loznikova, S., Novopashina, D., Apartsin, E., Krasheninina, O., Venyaminova, A., Milowska, K., Nowacka, O., Gomez-Ramirez, R., De La Mata, F.J., Majoral, J.P., Shcharbin, D., Bryszewska, M., 2015. Anticancer siRNA cocktails as a novel tool to treat cancer cells. Part (A). Mechanisms of Interaction. Int. J. Pharm. 485, 261–269. https://doi.org/10.1016/j.ijpharm.2015.03.024.
Itani, R., Al Faraj, A., 2019. siRNA Conjugated Nanoparticles—A Next Generation Strategy to Treat Lung Cancer. Int. J. Mol. Sci. https://doi.org/10.3390/ijms20236088.
Jain, K., Kesharwani, P., Gupta, U., Jain, N.K., 2010. Dendrimer toxicity: Let's meet the challenge. Int. J. Pharm. 394, 122–142. https://doi.org/10.1016/j.ijpharm.2010.04.027.
Kesharwani, P., Jain, K., Jain, N.K., 2014. Dendrimer as nanocarrier for drug delivery. Prog. Polym. Sci. 39, 268–307. https://doi.org/10.1016/j.progpolymsci.2013.07.005.
Krasheninina, O.A., Apartsin, E.K., Fuentes, E., Szulc, A., Ionov, M., Venyaminova, A.G., Shcharbin, D., de la Mata, F.J., Bryszewska, M., Gómez, R., Gómez, R., 2019. Complexes of pro-apoptotic sirnas and carbosilane dendrimers: Formation and effect on cancer cells. Pharmaceutics 11, 25. https://doi.org/10.3390/pharmaceutics11010025.
Kubczak, M., Michlewska, S., Bryszewska, M., Aigner, A., Ionov, M., 2021. Nanoparticles for local delivery of siRNA in lung therapy. Adv. Drug Deliv. Rev. 179, 114038 https://doi.org/10.1016/j.addr.2021.114038.
Lazniewska, J., Milowska, K., Katir, N., El Kadib, A., Bryszewska, M., Majoral, J.P., Gabryelak, T., 2013. Viologen-phosphorus dendrimers exhibit minor toxicity against a murine neuroblastoma cell line. Cell. Mol. Biol. Lett. 18, 459–478. https://doi.org/10.2478/s11658-013-0100-5.
Li, Y., Zhou, D., Xu, S., Rao, M., Zhang, Z., Wu, L., Zhang, C., Lin, N., 2020. DYRK1A suppression restrains Mcl-1 expression and sensitizes NSCLC cells to Bcl-2 inhibitors. Cancer Biol. Med. 17, 387–400. https://doi.org/10.20892/j.issn.2095-3941.2019.0380.
Liang, C.-C., Park, A.Y., Guan, J.-L., 2007. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat. Protoc. 2, 329–333. https://doi.org/10.1038/nprot.2007.30.
Mencia, G., Del Olmo, N.S., Muñoz-Moreno, L., Maroto-Diaz, M., Gomez, R., Ortega, P., José Carmena, M., Javier de la Mata, F., 2016. Polyphenolic carbosilane dendrimers as anticancer agents against prostate cancer. New J. Chem. https://doi.org/10.1039/c6nj02545e.
Mendes, L.P., Pan, J., Torchilin, V.P., 2017. Dendrimers as nanocarriers for nucleic acid and drug delivery in cancer therapy. Molecules 22, 1–21. https://doi.org/10.3390/molecules22091401.
Michlewska, S., Garaiova, Z., Šubjakova, V., Hołota, M., Kubczak, M., Grodzicka, M., Okła, E., Naziris, N., Balcerzak, Ł., Ortega, P., de la Mata, F.J., Hianik, T., Waczulikova, I., Bryszewska, M., Ionov, M., 2023a. Lipid-coated ruthenium dendrimer conjugated with doxorubicin in anti-cancer drug delivery: Introducing protocols. Colloids Surfaces B Biointerfaces 227, 113371. https://doi.org/10.1016/j.colsurfb.2023.113371.
Michlewska, S., Maly, M., Wójkowska, D., Karolczak, K., Skiba, E., Hołota, M., Kubczak, M., Ortega, P., Watala, C., Javier de la Mata, F., Bryszewska, M., Ionov, M., 2023b. Carbosilane ruthenium metallodendrimer as alternative anti-cancer drug carrier in triple negative breast cancer mouse model: a preliminary study. Int. J. Pharm. 122784 https://doi.org/10.1016/j.ijpharm.2023.122784.
Michlewska, S., Wójkowska, D., Watala, C., Skiba, E., Ortega, P., de la Mata, F.J., Bryszewska, M., Ionov, M., 2023c. Ruthenium metallodendrimer against triple-negative breast cancer in mice. Nanomedicine Nanotechnology. Biol. Med. 53 https://doi.org/10.1016/j.nano.2023.102703.
Mitra, S., Nguyen, L.N., Akter, M., Park, G., Choi, E.H., Kaushik, N.K., 2019. Impact of ROS Generated by Chemical, Physical, and Plasma Techniques on Cancer Attenuation. Cancers (basel). https://doi.org/10.3390/cancers11071030.
Naghizadeh, S., Mohammadi, A., Baradaran, B., Mansoori, B., 2019. Overcoming multiple drug resistance in lung cancer using siRNA targeted therapy. Gene 714, 143972. https://doi.org/10.1016/j.gene.2019.143972.
Nakamura, H., Takada, K., 2021. Reactive oxygen species in cancer: Current findings and future directions. Cancer Sci. 112, 3945–3952. https://doi.org/10.1111/cas.15068.
NavaneethaKrishnan, S., Rosales, J.L., Lee, K.Y., 2019. ROS-mediated cancer cell killing through dietary phytochemicals. Oxid. Med. Cell. Longev. 2019 https://doi.org/10.1155/2019/9051542.
Okła, E., Białecki, P., Kędzierska, M., Pędziwiatr-Werbicka, E., Miłowska, K., Takvor, S., Gómez, R., de la Mata, F.J., Bryszewska, M., Ionov, M., 2023. Pegylated Gold Nanoparticles Conjugated with siRNA: Complexes Formation and Cytotoxicity. Int. J. Mol. Sci. https://doi.org/10.3390/ijms24076638.
Paschos, K.A., Canovas, D., Bird, N.C., 2009. The role of cell adhesion molecules in the progression of colorectal cancer and the development of liver metastasis. Cell. Signal. 21, 665–674. https://doi.org/10.1016/j.cellsig.2009.01.006.
Reczek, C.R., Chandel, N.S., 2018. ROS Promotes Cancer Cell Survival through Calcium Signaling. Cancer Cell 33, 949–951. https://doi.org/10.1016/j.ccell.2018.05.010.
Rodríguez-Prieto, T., Michlewska, S., Hołota, M., Ionov, M., de la Mata, F.J., Cano, J., Bryszewska, M., Gómez, R., 2021. Organometallic dendrimers based on Ruthenium (II) N-heterocyclic carbenes and their implication as delivery systems of anticancer small interfering RNA. J. Inorg. Biochem. 223, 111540 https://doi.org/10.1016/j.jinorgbio.2021.111540.
Shcharbin, D., Pedziwiatr, E., Blasiak, J., Bryszewska, M., 2010. How to study dendriplexes II: Transfection and cytotoxicity. J. Control. Release 141, 110–127. https://doi.org/10.1016/j.jconrel.2009.09.030.
Shcharbin, D., Ionov, M., Abashkin, V., Loznikova, S., Dzmitruk, V., Shcharbina, N., Matusevich, L., Milowska, K., Gałecki, K., Wysocki, S., Bryszewska, M., 2015. Nanoparticle corona for proteins: Mechanisms of interaction between dendrimers and proteins. Colloids Surfaces B Biointerfaces 134, 377–383. https://doi.org/10.1016/j.colsurfb.2015.07.017.
Singh, S., Davis, R., Alamanda, V., Pireddu, R., Pernazza, D., Sebti, S., Lawrence, N., Chellappan, S., 2010. Rb-Raf-1 interaction disruptor RRD-251 induces apoptosis in metastatic melanoma cells and synergizes with dacarbazine. Mol. Cancer Ther. 9, 3330–3341. https://doi.org/10.1158/1535-7163.MCT-10-0442.
Singh, S., Bora-Singhal, N., Kroeger, J., Laklai, H., Chellappan, S.P., 2013. βArrestin-1 and Mcl-1 Modulate Self-Renewal Growth of Cancer Stem-Like Side-Population Cells in Non-Small Cell Lung Cancer. PLoS One 8. https://doi.org/10.1371/journal.pone.0055982.
Singh, A., Trivedi, P., Jain, N.K., 2018. Advances in siRNA delivery in cancer therapy. Artif. Cells, Nanomedicine Biotechnol. 46, 274–283. https://doi.org/10.1080/21691401.2017.1307210.
Somani, S., Laskar, P., Altwaijry, N., Kewcharoenvong, P., Irving, C., Robb, G., Pickard, B.S., Dufès, C., 2018. PEGylation of polypropylenimine dendrimers: Effects on cytotoxicity, DNA condensation, gene delivery and expression in cancer cells. Sci. Rep. 8, 1–13. https://doi.org/10.1038/s41598-018-27400-6.
Song, L., Coppola, D., Livingston, S., Cress, W.D., Haura, E.B., 2005. Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. Cancer Biol. Ther. 4, 267–276. https://doi.org/10.4161/cbt.4.3.1496.
Svenson, S., Tomalia, D.A., 2005. Dendrimers in biomedical applications - Reflections on the field. Adv. Drug Deliv. Rev. 57, 2106–2129. https://doi.org/10.1016/j.addr.2005.09.018.
Valster, A., Tran, N.L., Nakada, M., Berens, M.E., Chan, A.Y., Symons, M., 2005. Cell migration and invasion assays. Methods 37, 208–215. https://doi.org/10.1016/j.ymeth.2005.08.001.
Wang, J., Lu, Z., Wientjes, M.G., Au, J.L.S., 2010. Delivery of siRNA therapeutics: Barriers and carriers. AAPS J. 12, 492–503. https://doi.org/10.1208/s12248-010-9210-4.
Wei, G., Margolin, A.A., Haery, L., Brown, E., Cucolo, L., Julian, B., Shehata, S., Kung, A. L., Beroukhim, R., Golub, T.R., 2012. Chemical Genomics Identifies Small-Molecule MCL1 Repressors and BCL-xL as a Predictor of MCL1 Dependency. Cancer Cell 21, 547–562. https://doi.org/10.1016/j.ccr.2012.02.028.
Wesarg, E., Hoffarth, S., Wiewrodt, R., Kröll, M., Biesterfeld, S., Huber, C., Schuler, M., 2007. Targeting BCL-2 family proteins to overcome drug resistance in non-small cell lung cancer. Int. J. Cancer 121, 2387–2394. https://doi.org/10.1002/ijc.22977.
Yang, T.-M., Barbone, D., Fennell, D.A., Broaddus, V.C., 2009. Bcl-2 Family Proteins Contribute to Apoptotic Resistance in Lung Cancer Multicellular Spheroids. Am. J. Respir. Cell Mol. Biol. 41, 14–23. https://doi.org/10.1165/rcmb.2008-0320OC.
Zhang, H., Guttikonda, S., Roberts, L., Uziel, T., Semizarov, D., Elmore, S.W., Leverson, J. D., Lam, L.T., 2011. Mcl-1 is critical for survival in a subgroup of non-small-cell lung cancer cell lines. Oncogene 30, 1963–1968. https://doi.org/10.1038/onc.2010.559.
Zhou, Z., Liu, X., Zhu, D., Wang, Y., Zhang, Z., Zhou, X., Qiu, N., Chen, X., Shen, Y., 2017. Nonviral cancer gene therapy: Delivery cascade and vector nanoproperty integration. Adv. Drug Deliv. Rev. 115, 115–154. https://doi.org/10.1016/j.addr.2017.07.021.

Universidad de Alcalá

UNIVERSIDAD DE ALCALÁ

Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
Alcalá de Henares, Spain

Rebeca Lozano **Alcalá de Henares, 30 de julio de 2024**

09068143W REBECA LOZANO

Digitally signed by 09068143W REBECA LOZANO
Date: 2024.07.30 13:25:37 +02'00'

Statement of contribution

To whom it may concern
I am co-author of following publication:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497, I participated in:

- Validation of results

Overall contribution - 5%.

.....

Universidad de Alcalá

UNIVERSIDAD DE ALCALÁ

Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
Alcalá de Henares, Spain

PhD Cornelia E. Pena-Gonzalez **Alcalá de Henares, date** 15/08/2024

Statement of contribution

To whom it may concern
I am co-author of following publication:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497, I participated in:

- synthesis of the dendrimers used in the work
- making the FRAP test
- co-writing the manuscript

Overall contribution - 10%.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, I participated in:

- synthesis of the dendrimers used in the work
- editing of the final version manuscript

Overall contribution - 4%

Universidad de Alcalá

UNIVERSIDAD DE ALCALÁ

Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
Alcalá de Henares, Spain
e-mail.: javier.delamata@uah.es

Prof. Francisco Javier de la Mata

Alcalá de Henares, August, 16. 2024

Statement of contribution

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497. I declare that in this publication my contribution is 5 % and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion*, Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0. I declare that in this publication my contribution is 5 % and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

3. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA*, International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199. I declare that in this publication (paper under review) my contribution is 5 % and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

4. **M. Grodzicka**, S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry and Physics of Lipids, Elsevier. I declare that in this manuscript prepared for publication (paper under review) my contribution is 5 % and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

DE LA MATA DE LA MATA FRANCISCO JAVIER - DNI 08969382A

Firmado digitalmente por DE LA MATA DE LA MATA FRANCISCO JAVIER - DNI 08969382A
Fecha: 2024.08.16 21:16:14 +02'00'

.....

Universidad de Alcalá

UNIVERSIDAD DE ALCALÁ

Departamento de Química Orgánica y Química Inorgánica
Facultad de Farmacia
Alcalá de Henares, Spain
e-mail.: paula.ortega@uah.es

Prof. Paula Ortega López

Alcalá de Henares, 17.08.2024

Statement of contribution

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497. I declare that in this publication my contribution is 5% and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0. I declare that in this publication my contribution is 5% and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

3. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA,* International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199. I declare that in this publication (paper under review) my contribution is 5% and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

4. **M. Grodzicka,** S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry and Physics of Lipids, Elsevier. I declare that in this manuscript prepared for publication (paper under review) my contribution is 5% and includes: assistance in paper conceptualization, improving the quality of the manuscript, supervision on the compounds synthesis.

ORTEGA LOPEZ PAULA - 52994917L

Firmado digitalmente por ORTEGA LOPEZ PAULA - 52994917L
Fecha: 2024.08.17 16:04:31 +02'00'

UNIVERSITY OF BIALYSTOK
Faculty of Biology

K. Ciołkowskiego 1J, 15-245 Białystok, Poland
tel. 85 738 8383 • e-mail: biologia.dziekanat@uwb.edu.pl • biologia.uwb.edu.pl

Dr hab. Szymon Sękowski, prof. UwB
Laboratorium Biofizyki Molekularnej
Katedra Mikrobiologii i Biotechnologii
Wydział Biologii,
Uniwersytet w Białymstoku
Ul. K. Ciołkowskiego 1J, 15-245, Białystok
e-mail: s.sekowski@uwb.edu.pl

Białystok, 02.09.2024

Oświadczenie

Oświadczam, że mój udział w pracy:

1. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na:

- analizie i opracowaniu danych dotyczących wygaszania fluorescencji białka,
- współudziale w przygotowaniu i edycji manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 4%.

Sękowski Szymon

WYDZIAŁ CHEMII
Uniwersytet Łódzki

UNIC

UNIWERSYTET ŁÓDZKI

Wydział Chemii, Katedra Chemii Fizycznej, Zakład Chemii Biofizycznej
ul. Pomorska165, 90-236 Łódź,
e-mail.: adam.buczkowski@chemia.uni.lodz.pl

Dr hab. Adam Buczkowski, prof. UŁ

Łódź, dnia 13.09.2024

Oświadczenie

Oświadczam, że mój udział w pracy:

1. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na:

- projektowaniu części badań,
- wykonaniu pomiarów i analizie izotermicznej kalorymetrii miareczkowej,
- współudział w przygotowaniu manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 7%.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry and Physics of Lipids, Elsevier, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444, polegał na:

- projektowaniu części badań,
- wykonaniu pomiarów i analizie danych różnicowej kalorymetrii skaningowej,
- współudział w przygotowaniu manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 10%.

ABuczkowski

AKADEMIA MAZOWIECKA
W PŁOCKU

AKADEMIA MAZOWIECKA W PŁOCKU
Collegium Medicum, Wydział Lekarski
Pl. Dąbrowskiego 2, 09-402 Płock
e-mail.: janusz.blasiak@mazowiecka.edu.pl

Prof. dr hab. Janusz Błasiak **14 sierpnia 2024 r.**

Oświadczenie

1. Oświadczam, że mój udział w pracy: M. Grodzicka, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na: analizie i opracowywaniu otrzymanych danych, korekcie ostatecznej wersji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 8%.

2. Oświadczam, że mój udział w pracy: M. Grodzicka, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA,* International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199, polegał na: analizie i opracowywaniu otrzymanych danych, korekcie ostatecznej wersji manuskryptu. Mój wkład w przygotowanie pracy w formie publikacji stanowi 7%.

Łączę wyrazy poważania,

Janusz Błasiak

WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

UNIC

UNIWERSYTET ŁÓDZKI

Wydział Biologii i Ochrony Środowiska, Katedra Biofizyki Ogólnej,
ul. Pomorska 141/143, 90-236 Łódź
e-mail.: maria.bryszewska@biol.uni.lodz.pl

Prof. dr hab. Maria Bryszewska

Łódź, dnia 24.09.2024

Oświadczenie

Oświadczam, że mój udział w pracy:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497, polegał na:

- finansowaniu badań uwzględnionych w pracy,
- korekcie ostatecznej wersji manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 3%.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion*, Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na:

- finansowaniu badań uwzględnionych w pracy,
- korekcie ostatecznej wersji manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 3%.

3. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA*, International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199, polegał na:

- finansowaniu badań uwzględnionych w pracy,
- korekcie ostatecznej wersji manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 5%.

4. **M. Grodzicka**, S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry and Physics of Lipids, Elsevier, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444, polegał na:

- finansowaniu badań uwzględnionych w pracy,
- korekcie ostatecznej wersji manuskryptu

Wkład włożony w przygotowanie publikacji wynosił 3%.

KIEROWNIK
KATEDRY BIOFIZYKI OGÓLNEJ UŁ
prof. zw. dr hab. Maria Bryszewska

.....

WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

SZKOŁA DOKTORSKA
BioMedChem
Uniwersytetu Łódzkiego i Instytutów
Polskiej Akademii Nauk w Łodzi

UNIC

UNIWERSYTET ŁÓDZKI

Wydział Biologii i Ochrony Środowiska, Katedra Biofizyki Ogólnej,
ul. Pomorska 141/143, 90-236 Łódź
e-mail.: marika.grodzicka@biol.uni.lodz.pl

mgr Marika Grodzicka

Łódź, dnia 12.08.2024 r.

Oświadczenie

Oświadczam, że mój udział w pracy:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497, polegał na:

- wykonaniu pomiarów średnicy hydrodynamicznej i potencjale zeta,
- przygotowaniu i obrazowaniu preparatów za pomocą Transmisyjnej Mikroskopii Elektronowej,
- ocenie cytotoksyczności i aktywności antyoksydacyjnej dendrymerów,
- interpretacji i analizie statystycznej otrzymanych wyników,
- przygotowaniu pierwotnej wersji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 64%.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na:

- wykonaniu pomiarów średnicy hydrodynamicznej i potencjale zeta,
- przygotowaniu i obrazowaniu preparatów za pomocą Transmisyjnej Mikroskopii Elektronowej,
- wykonaniu pomiarów wygaszania fluorescencji białka,
- wykonaniu pomiarów widm dichroizmu kołowego,
- interpretacji i analizie statystycznej otrzymanych wyników,
- przygotowaniu pierwotnej wersji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 53%.

3. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA*, International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199, polegał na:

- wykonaniu pomiarów średnicy hydrodynamicznej i potencjale zeta,
- przygotowaniu i obrazowaniu preparatów za pomocą Transmisyjnej Mikroskopii Elektronowej,
- wykonaniu elektroforezy w żelu agarozowym,
- wykonaniu pomiarów polaryzacji fluorescencji,
- wykonaniu pomiarów widm dichroizmu kołowego,
- internalizacji kompleksów do komórek,
- ocenie cytotoksyczności dendrymerów oraz ich kompleksów z siRNA,
- zdolności adhezji i migracji komórek po inkubacji z kompleksami dendrymer/siRNA,
- określeniu mechanizmu śmierci komórkowej linii A549 po inkubacji z kompleksami dendrymer/siRNA za pomocą cytometrii przepływowej oraz mikroskopii konfokalnej,
- interpretacji i analizie statystycznej otrzymanych wyników,
- przygotowaniu pierwotnej wersji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 61%.

4. **M. Grodzicka,** S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry and Physics of Lipids, Elsevier, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444, polegał na:

- wyizolowaniu błon erytrocytarnych i erytrocytów z kożucha leukocytarno-płytkowego,
- syntezie liposomów zawierających DMPC/DPPG,
- wykonaniu pomiarów średnicy hydrodynamicznej i potencjale zeta,
- przygotowaniu i obrazowaniu preparatów za pomocą Transmisyjnej Mikroskopii Elektronowej,
- wykonaniu pomiarów anizotropii fluorescjencji,
- ocenie cytotoksyczności i hemotoksyczności dendrymerów,
- interpretacji i analizie statystycznej otrzymanych wyników,
- przygotowaniu pierwotnej wersji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 62%.

Monika Grodzicka

WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

UNIC

UNIWERSYTET ŁÓDZKI

Wydział Biologii i Ochrony Środowiska, Katedra Biofizyki Ogólnej,
ul. Pomorska 141/143, 90-236 Łódź
e-mail.: maksim.ionov@biol.uni.lodz.pl

Prof. dr hab. Maksim Ionov

Łódź, dnia 14.08.2024 r.

Oświadczenie

Oświadczam, że mój udział w pracy:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497, polegał na:

- projektowaniu i kierowaniu badaniami opisanymi w pracy
- korekcie przygotowanego manuskryptu
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 3%.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na:

- projektowaniu i kierowaniu badaniami opisanymi w pracy
- korekcie przygotowanego manuskryptu
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 3%.

3. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA,* International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199, polegał na:

- projektowaniu i kierowaniu badaniami opisanymi w pracy
- recenzji i redagowaniu powstałego manuskryptu
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 5%.

4. **M. Grodzicka,** S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry

and Physics of Lipids, Elsevier, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444, polegał na:

- projektowaniu i kierowaniu badaniami opisanymi w pracy
- analizie wyników i redagowaniu powstałego manuskryptu
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 5%.

.....

WYDZIAŁ BIOLOGII
i OCHRONY ŚRODOWISKA
Uniwersytet Łódzki

UNIC

UNIWERSYTET ŁÓDZKI

Wydział Biologii i Ochrony Środowiska, Pracownia Obrazowania Mikroskopowego
i Specjalistycznych Technik Biologicznych,
ul. Pomorska 141/143, 90-236 Łódź
e-mail.: sylwia.michlewska@biol.uni.lodz.pl

Dr hab. Sylwia Michlewska

Łódź, dnia 12.03.2024 r.

Oświadczenie

Oświadczam, że mój udział w pracy:

1. **M. Grodzicka**, C. E. Pena-Gonzalez, P. Ortega, S. Michlewska, R. Lozano, M. Bryszewska, F. J. de la Mata, M. Ionov; *Heterofunctionalized polyphenolic dendrimers decorated with caffeic acid: Synthesis, characterization and antioxidant activity*, Sustainable Materials and Technologies, Elsevier, 2022, https://doi.org/10.1016/j.susmat.2022.e00497, polegał na:

- zaplanowaniu części eksperymentów,
- wykonaniu analiz z wykorzystaniem techniki Transmisyjnej Mikroskopii Elektronowej,
- obrazowania za pomocą techniki mikroskopii konfokalnej
- współtworzeniu i edycji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 7%.

2. **M. Grodzicka**, S. Michlewska, A. Buczkowski, S. Sekowski, C. E. Pena-Gonzalez, P. Ortega, F. J. de la Mata, J. Błasiak, M. Bryszewska, M. Ionov; *A new class of polyphenolic carbosilane dendrimers binds human serum albumin in a structure-dependent fashion,* Scientific Reports, Nature, 2024, https://doi.org/10.1038/s41598-024-56509-0, polegał na:

- zaplanowaniu części eksperymentów,
- wykonaniu analiz z wykorzystaniem techniki Transmisyjnej Mikroskopii Elektronowej,
- współtworzeniu i edycji manuskryptu,
- pomocy w analizie wyników,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 8%.

3. **M. Grodzicka**, S. Michlewska, J. Błasiak, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; *Polyphenolic dendrimers as carriers of anticancer siRNA,* International Journal of Pharmaceutics, Elsevier, 2024, https://doi.org/10.1016/j.ijpharm.2024.124199, polegał na:

- zaplanowaniu części eksperymentów,
- wykonaniu analiz z wykorzystaniem techniki Transmisyjnej Mikroskopii Elektronowej,
- obrazowania za pomocą techniki mikroskopii konfokalnej

- pomiarach ilościowych w testach adhezji i migracji komórek
- współtworzeniu i edycji manuskryptu,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 12%.

4. **M. Grodzicka,** S. Michlewska, A. Buczkowski, P. Ortega, F. J. de la Mata, M. Bryszewska, M. Ionov; Effect of polyphenolic dendrimers on biological and artificial lipid membranes, Chemistry and Physics of Lipids, Elsevier, 2024, https://doi.org/10.1016/j.chemphyslip.2024.105444, polegał na:

- zaplanowaniu części eksperymentów,
- wykonaniu analiz z wykorzystaniem techniki Transmisyjnej Mikroskopii Elektronowej,
- współtworzeniu i edycji manuskryptu,
- pomocy w analizie wyników,
- współuczestniczeniu w odpowiedzi na recenzje

Wkład włożony w przygotowanie publikacji wynosił 10%.

Michlewska
.....