Biomacromolecules

pubs.acs.org/Biomac

Article

Accelerated Ten-Gram-Scale Synthesis of One-Component Multifunctional Sequence-Defined Ionizable Amphiphilic Janus Dendrimer 97

Mahwish Arshad,[‖] Elena N. Atochina-Vasserman,[‖] Srijay S. Chenna, Devendra S. Maurya, Muhammad Irhash Shalihin, Dipankar Sahoo, Alec C. Lewis, Jordan J. Lewis, Nathan Ona, Jessica A. Vasserman, Houping Ni, Wook-Jin Park, Drew Weissman,* and Virgil Percec*

Cite This: *Biomacromolecules* 2024, 25, 6871−6882

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: One-component multifunctional sequence-defined ionizable amphiphilic Janus dendrimers (IAJDs) were discovered in our laboratories in 2021 to represent a new class of synthetic vectors for the targeted delivery of messenger RNA (mRNA). They coassemble with mRNA by simple injection of their ethanol solution into a pH 4 acetate buffer containing the nucleic acid into monodisperse dendrimersome nanoparticles (DNPs) with predictable dimensions. DNPs are competitive with 4-component lipid nanoparticles (LNPs), which are used in commercial COVID-19 vaccines, except that IAJDs are prepared in fewer reaction steps than each individual component of the LNPs. This simple methodology for the synthesis of IAJDs and their coassembly with mRNA into DNPs, together with the precise placement of their individual components and indefinite stability at room temperature in air, make them attractive candidates for the development of nanomedicine-based targeted mRNA delivery. Access to the large-scale synthesis of IAJDs without the need for sophisticated technologies, instrumentation, and synthetic skills is expected to open numerous new opportunities worldwide in nanomedicine. The goal of this publication is to report an accelerated ten-gram-scale synthesis of IAJD97 from inexpensive food additives obtained from renewable plant phenolic acid starting materials by methodologies accessible to any laboratory. This accelerated synthesis can be accomplished in 4 days. We expect that the work reported here will impact the field of nanomedicine in both developed and less developed countries.

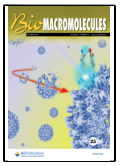
INTRODUCTION

The field of nanomedicine relies on the targeted delivery of nucleic acids into cells or their nucleus by viral and nonviral vectors. Each of these delivery systems has its own advantages and disadvantages, which have been discussed in previous publications[1−6] and, therefore, will not be repeated here. Nonviral vectors have as their main advantage an infinite number of synthetic capabilities.

The extraordinary success of COVID-19 vaccines[7−32] promoted the four-component-based lipid nanoparticles (LNPs) (Figure 1a,b) to the forefront of messenger RNA (mRNA) delivery with synthetic rather than with viral vectors.[8,12−14,16] In 2021, our laboratories elaborated a one-component multifunctional sequence-defined ionizable amphiphilic Janus dendrimer delivery system for mRNA (Figure 1c). This system, known for short as IAJD, self-assembles into vesicles that we named dendrimersome nanoparticles (DNPs) and is complementary to the 4-component-based LNPs except that it contains the ionizable amine in a precise location in the IAJD (Figure 1c) rather than in a nondefined distribution of its 4-components (Figure 1b). According to current knowledge, there seems to be little difference in the delivery of mRNA with LNPs versus DNPs (Figure 1a). However, IAJDs provide a simpler approach to mechanistic and design studies for the targeted delivery process with synthetic vectors (Figure 1b,c). IAJDs were inspired by amphiphilic Janus dendrimers (JDs) that we discovered in 2010[33−39] to self-assemble by simple injection in water and buffer into monodisperse vesicles of predictable dimensions known as dendrimersomes (DSs).[40] The sequence-defined part of the IAJD concept was inspired by our work on amphiphilic Janus glycodendrimers (JGDs), which were employed by our laboratory to self-assemble glycodendrimersomes (GDSs), which mimic the glycans of cell membranes.[1,41−48]

Sequence-defined, combined with suitable concentration and primary structure of JGDs, was discovered to provide the highest activity of the GDSs to agglutinating sugar-binding pro-

Received: August 9, 2024
Revised: September 26, 2024
Accepted: September 26, 2024
Published: October 3, 2024



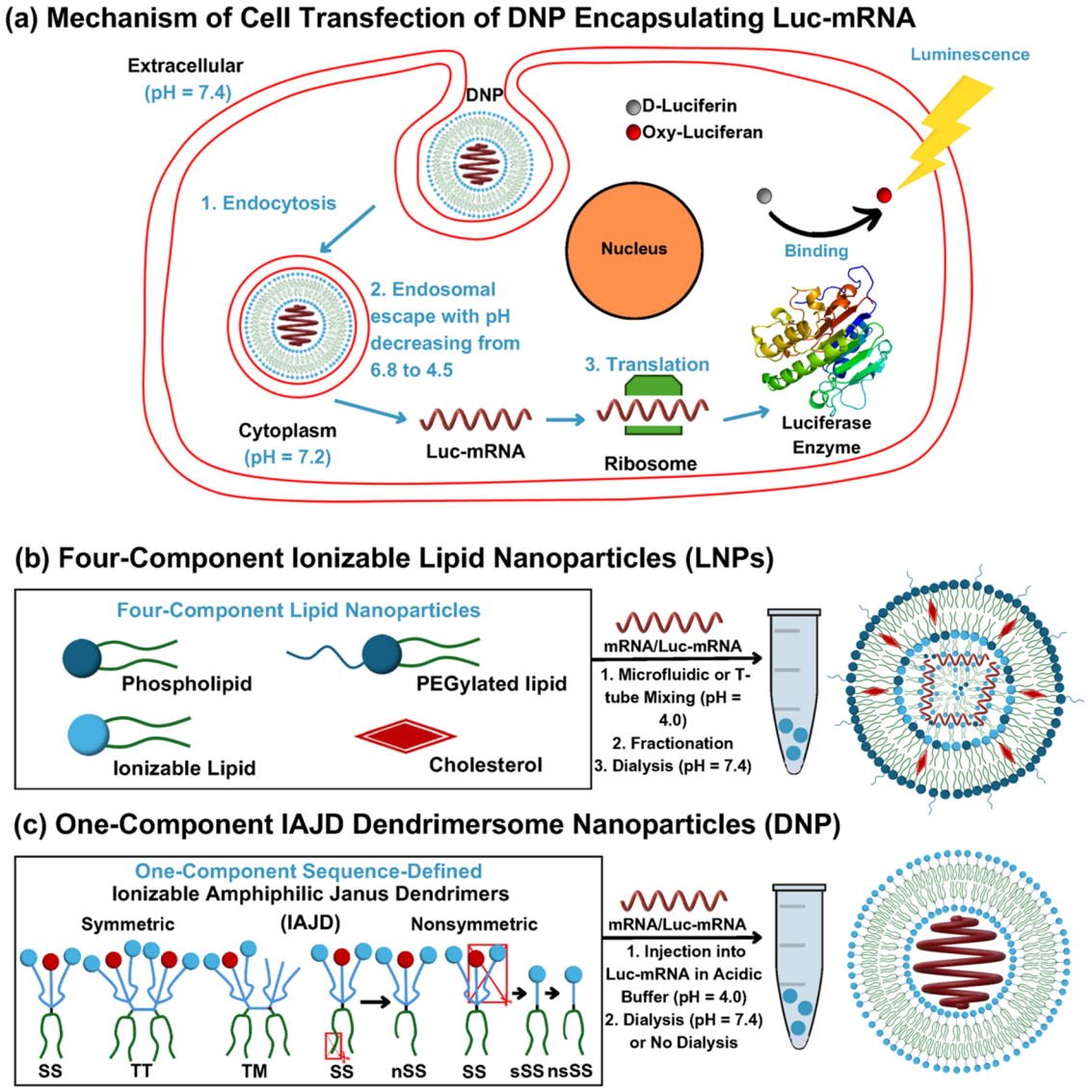


Figure 1. Simplified comparison of the delivery of RNA with four-component LNPs and with one-component DNPs, including the transfection mechanism for DNPs encapsulating Luc-mRNA (a); structure of the 4-components and assembly of LNPs (b) and structure of 1-component IAJDs and their assembly into DNPs (c). Color-code: red circle: benzyl ether; blue circle: ionizable amine; green: hydrophobic; and blue: hydrophilic.

teins.[1,42,49−51] The evolution of the original IAJDs has a variety of parent structures, known as single−single (SS), twin−twin (TT), twin-mixed (TM), as well as simpler variants such as nonsymmetric SS (nSS), simplified SS (sSS), and nonsymmetric simplified SS (nsSS), which are outlined in Figure 1c. The preparation of sSS requires a 3 or 4-step synthesis,[2] while the synthesis of more active nsSS requires a 7-step synthesis, including a multistep construction of the hydrophobic fragment.[3] The synthesis of pentaerytrithol-based sSS IAJDs involves a 3-step synthesis consisting of a low conversion for the trialkylation of pentaerytrithol.[5] This simplification in the structure of IAJDs is accompanied by a dramatic reduction in the number of synthetic steps and, unexpectedly, by an increase of the *in vivo* activity as well as by a larger number of organs targeted by the corresponding DNPs. Most important for the research activity on targeted delivery with synthetic vectors is the number of steps required during the synthesis of IAJDs compared to the 4-components of LNPs, as well as the methodology used to assemble DNPs and LNPs. We would like to mention that the number of steps required for an IAJD synthesis is comparable or lower to the number of steps employed in the preparation of only one of the 4-components of the LNP, the ionizable lipid. The assembly of the LNPs by microfluidic or T-tube technologies, followed by fractionation and dialysis, requires several days, while the preparation of the DNPs requires less than 1 min. One additional factor to consider that will be addressed in this report is the large-scale synthesis of active IAJDs. For numerous systematic delivery investigations, including larger animals and humans, a substantial amount of inexpensive and easily accessible delivery material is required. Easily attainable and accelerated synthetic methods that do not demand a lot of expertise and complex instrumentation would be ideal in the synthesis of IAJDs. It is the goal of this paper to report such a methodology for the synthesis of IAJD97, which predominantly targets the spleen and lymph nodes.[4]

EXPERIMENTAL SECTION

Materials. Propyl gallate (Acros Organics, 98%), 2-ethylhexyl bromide (TCI, > 97%), lithium aluminum hydride ($LiAlH_4$) (Oakwood Chemical), 4-bromobutyric acid (Acros Organics, 98%), thionyl

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

chloride ($SOCl_2$) (Thermo Scientific, 99%), triethylamine (NEt_3) (TCI, > 99%), 4-dimethylaminopyridine (DMAP) (TCI, > 99%), *N*-(2-hydroxyethyl)piperazine (98.5%, Acros Organics), anhydrous potassium carbonate (K_2CO_3) (Fisher Chemical), sodium sulfate (Na_2SO_4) (Fisher Chemical), sodium bicarbonate ($NaHCO_3$) (Fisher Chemical, Certified ACS), and anhydrous magnesium sulfate ($MgSO_4$) (Fisher Chemical) were used as received. Dimethylformamide (DMF), ethyl acetate (EtOAc), hexane (Hex), tetrahydrofuran (THF), dichloromethane (DCM), acetonitrile (MeCN), and methanol (MeOH) were all ACS grade solvents from Fisher. DCM and NEt_3 were dried over CaH_2 and distilled before use. THF was dried over sodium benzophenone and distilled before use.

General Methods. ^{1}H spectra were recorded at 400 MHz and ^{13}C NMR spectra were recorded at 101 MHz on a Bruker NEO NMR spectrometer. All NMR characterizations were carried out at 23.5 °C in $CDCl_3$ with 0.05% TMS. NMR spectra were analyzed using an MNova 14. Chemical shifts (δ) are reported in ppm. The resonance multiplicities for the ^{1}H NMR spectra are labeled as "s" for singlet, "d" for doublet, "t" for triplet, "m" for multiplet, and "br" for broad resonance. Residual protonic solvent, $CHCl_3$ (^{1}H, δ 7.26 ppm; ^{13}C, δ 77.16 ppm) and tetramethyl silane (TMS, δ 0 ppm) were used as internal reference in all NMR spectra. TLC was used to monitor reactions and evaluate compound purity using silica gel 60 F_{254} precoated plates (E. Merck). Individual aromatic compounds were visualized with UV light (λ = 254 nm). Purification by column chromatography on SiO_2 was performed using silica gel from Silicycle (60 Å, 40−63 μm). High-pressure liquid chromatography (HPLC) analysis was performed using a Shimadzu LC-20AD high-performance liquid chromatograph pump, a PE Nelson Analytical 900 Series integration data station, a Shimadzu SPD-10A VP (UV−vis, λ = 254 nm), a Shimadzu RID-10A refractive index (RI) detector, and three AM gel columns (guard column, two 500 Å, 10 μm columns). THF with 5% triethylamine was used as the solvent for all HPLC characterizations, which were carried out at 23.5 °C. Molar masses of all compounds were determined by MALDI-TOF mass spectrometry using a PerSeptive Biosystem-Voyager-DE (Framingham, MA) mass spectrometer equipped with a nitrogen laser (337 nm) operating in linear mode. Angiotensin II and Bombesin were used as the standards for calibration. To prepare the sample solution, the compound for characterization was first dissolved in THF (5−10 mg/mL). The matrix (2,5-dihydroxybenzoic acid, 99%, Thermo Scientific) was also dissolved in THF 10 mg/mL, and the two solutions were mixed in a 1/5 (*v*/*v*, compound solution/matrix solution) ratio. One drop of solution was dried on the MALDI-TOF plate at 23.5 °C, the plate was inserted into the vacuum chamber for analysis, and laser intensity/voltages applied were adjusted for the molar mass and nature of each compound.

Synthesis. A modular-orthogonal methodology elaborated on and used routinely in our laboratory was employed. Structural analysis of all compounds was performed by a combination of ^{1}H and ^{13}C NMR spectroscopy, TLC, HPLC, and MALDI-TOF. ^{1}H NMR spectra are available in Figures S1−S4, ^{13}C NMR spectra are in Figures S5−S8, HPLC traces are in Figures S9 and S10, and MALDI-TOF spectra are in Figure S11.

General Procedure for the Two-Gram-Scale Synthesis of Propyl 3,4,5-Tris((2-ethylhexyl)oxy)benzoate, 3. Compound **3** was obtained by the alkylation of propyl gallate, **1**, with 2-ethylhexyl bromide, **2**. Propyl gallate, **1** (2 g, 9.4 mmol) and K_2CO_3 (9.1 g, 65 mmol) were stirred in 25 mL of DMF. 2-Ethylhexyl bromide (8.15 g, 42 mmol) was added to the reaction mixture under stirring. The reaction mixture was bubbled with nitrogen for at least 30 min. The flask equipped with a reflux condenser was then introduced into an oil bath preheated to 130 °C under stirring and the extent of the reaction was monitored by TLC. After 30 min, TLC analysis indicated complete conversion, showing only product **3** at R_f = 0.80. At this time, the reaction mixture was quenched in ice−water and extracted three times with 50 mL of EtOAc. The organic layer was isolated, dried over anhydrous $MgSO_4$, and vacuum filtered. The filtrate was concentrated on a rotary evaporator and subsequently purified by column chromatography using 95/5:Hex/EtOAc as an eluent to yield **3** as a colorless oil (4.80 g, 93.1%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 2H, PhH), 4.26 (t, 2H, H × $PhCOOCH_2$), 3.93−3.86 (m, 6H, 3 × $PhOCH_2$−), 1.87−1.78 (m, 3H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_{32}$), 1.64−1.30 (m, 26H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$,−$OCH_2CH_2CH_3$), 1.05 (t, 3H, −$OCH_2CH_2CH_3$), 0.98−0.91 (m, 18H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.76, 153.13, 142.44, 125.07, 107.58, 76.10, 71.40, 66.67, 40.78, 39.73, 30.69, 30.59, 29.44, 29.27, 23.99, 23.81, 23.28, 23.23, 22.33, 14.27, 14.23, 11.34, 11.32, 11.30, 11.27, 10.64. Purity by HPLC: 99+%. MALDI-TOF MS *m*/*z* of [M + Na]+ calculated for $C_{34}H_{60}NaO_5$+: 571.43; found: 571.05.

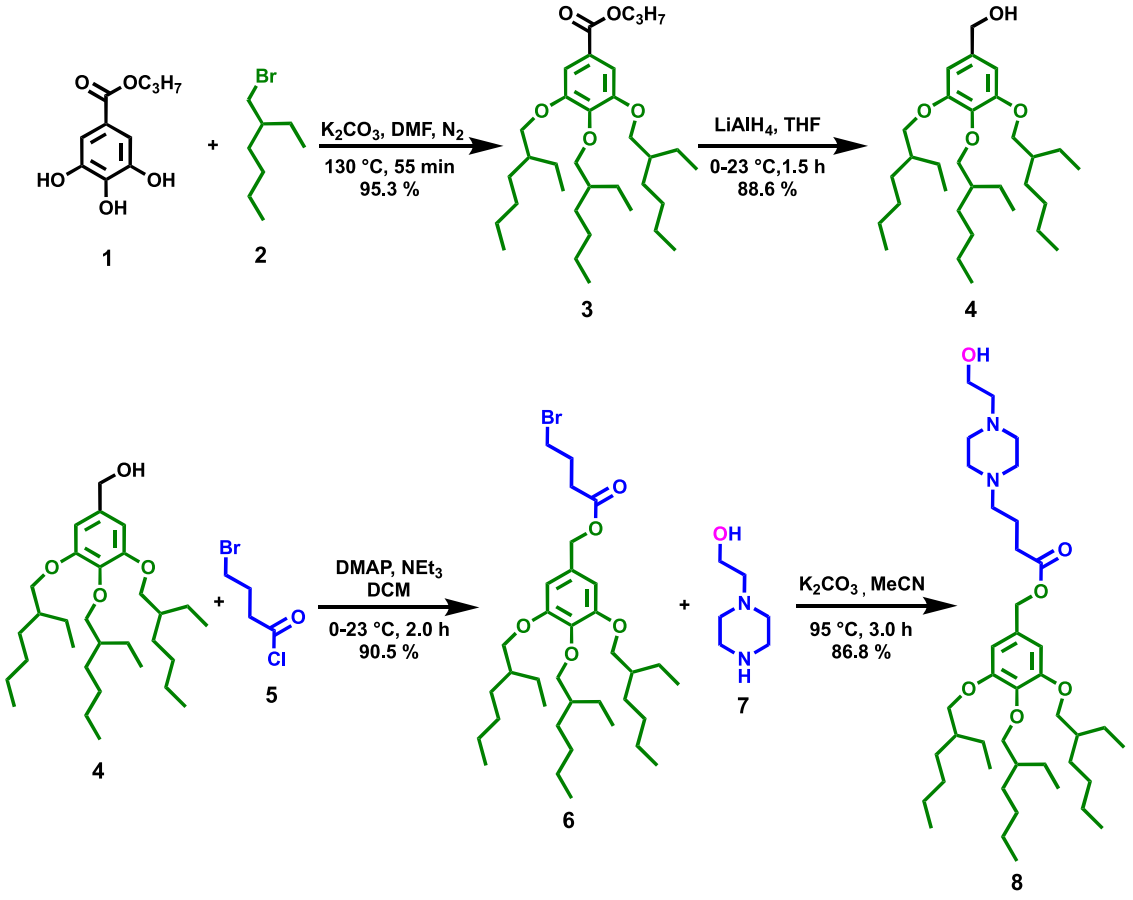
General Procedure for the Ten-Gram-Scale Synthesis of Propyl 3,4,5-Tris((2-ethylhexyl)oxy)benzoate, 3. Compound **3** was obtained by the alkylation of propyl gallate, **1**, with 2-ethylhexyl bromide, **2**. Propyl gallate, **1** (15.0 g, 70.7 mmol) and K_2CO_3 (58.0 g, 424 mmol) were stirred in 200 mL of DMF. 2-Ethylhexyl bromide (61.1 g, 318 mmol) was added to the reaction mixture under stirring. The solution was bubbled with nitrogen for at least 30 min. The flask equipped with a reflux condenser was then introduced into an oil bath preheated to 130 °C under stirring for 55 min, at which time TLC analysis indicated complete conversion, showing only product **3** at R_f = 0.80. The reaction mixture was allowed to cool to room temperature, and DMF was removed in a rotary evaporator. The residue was mixed with 100 mL of water and extracted 5 times with 100 mL of EtOAc. The organic layer was isolated, dried over anhydrous $MgSO_4$, and vacuum filtered. The filtrate was concentrated on a rotary evaporator and further purified by column chromatography using 95/5:Hex/EtOAc as an eluent to yield the title compound as a colorless oil (36.3 g, 95.3%). TLC (95/5:Hex/EtOAc): R_f = 0.8. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 (s, 2H, PhH), 4.26 (t, 2H, H × $PhCOOCH_2$), 3.93−3.86 (m, 6H, 3 × $PhOCH_2$−), 1.87−1.78 (m, 3H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_{32}$), 1.64−1.30 (m, 26H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$,−$OCH_2CH_2CH_3$), 1.05 (t, 3H, −$OCH_2CH_2CH_3$), 0.98−0.91 (m, 18H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.76, 153.13, 142.44, 125.07, 107.58, 76.10, 71.40, 66.67, 40.78, 39.73, 30.69, 30.59, 29.44, 29.27, 23.99, 23.81, 23.28, 23.23, 22.33, 14.27, 14.23, 11.34, 11.32, 11.30, 11.27, 10.64. Purity by HPLC: 99+%. MALDI-TOF MS *m*/*z* of [M + Na]+ calculated for $C_{34}H_{60}NaO_5$+: 571.43; found: 571.05.

General Procedure for the Synthesis of (3,4,5-Tris((2-ethylhexyl)oxy)phenyl)methanol (4). Compound **4** was obtained by the reduction of propyl 3,4,5-tris((2-ethylhexyl)oxy)benzoate, **3**, with $LiAlH_4$. Propyl 3,4,5-tris((2-ethylhexyl)oxy)benzoate, **3** (12 g, 21.8 mmol) was dissolved in 100 mL of anhydrous THF. $LiAlH_4$ (1.08 g, 20.1 mmol) was added slowly to the reaction mixture maintained at 0 °C with an ice bath. The ice bath was subsequently removed, and the reaction was allowed to proceed at 23.5 °C for 1.5 h, at which time TLC analysis indicated a complete conversion of **3** into **4**. The reaction was quenched by adding a saturated aqueous solution of Na_2SO_4 dropwise until the bubbling stopped. The reaction mixture was filtered, the solid and the flask were rinsed with THF and DCM. The filtrate was dried over anhydrous $MgSO_4$, vacuum filtered, and the filtrate was concentrated in a rotary evaporator to yield compound **4** as a colorless oil (10.4 g, 88.6%). TLC (50/50:Hex/DCM): R_f = 0.39. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.58 (s, 2H, PhH), 4.62 (d, 2H, $PhCH_2OH$), 3.91−3.81 (m, 6H, 3 × $PhOCH_2$−), 1.80−1.69 (m, 3H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$), 1.60−1.29 (m, 24H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$), 0.98−0.90 (m, 18H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 153.68, 137.54, 136.08, 104.82, 76.12, 71.34, 65.92, 40.74, 39.78, 30.66, 29.48, 29.26, 23.96, 23.87, 23.31, 23.24, 14.29, 14.25, 11.36, 11.34, 11.32, 11.24. Purity by HPLC: 99+%. MALDI-TOF MS *m*/*z* of [M + H]+ calculated for $C_{31}H_{57}O_4$+: 492.4; found: 492.78.

General Procedure for the Synthesis of 3,4,5-Tris((2-ethylhexyl)oxy)benzyl 4-Bromobutanoate, 6. Compound **6** was obtained by the esterification of **(3,4,5-tris((2-ethylhexyl)oxy)-phenyl)methanol, 4**, with 4-bromobutanoyl chloride freshly prepared just before use. 4-Bromobutyric acid was dissolved in a mixture containing 6 mL of anhydrous DCM and approximately 10 mL of $SOCl_2$ (16.89 g, 142.1 mmol). A drop of DMF was added, and the reaction was stirred at 23.5 °C for 1 h to form 4-bromobutanoyl

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

Scheme 1. Accelerated 10-Gram-Synthesis of IAJD97



chloride. DCM and excess $SOCl_2$ were distilled in a rotary evaporator under reduced pressure. 3,4,5-Tris((2-ethylhexyl)oxy)phenyl)-methanol, **4** (10 g, 20.3 mmol) was dissolved in 20 mL of dry DCM to which dry NEt_3 (2.45 g, 24.24 mmol) and a catalytic amount of DMAP (0.46 g, 4.06 mmol) were added. A small amount of anhydrous DCM was used to dissolve 4-bromobutanoyl chloride, and the solution was added dropwise to an ice−water bath of 3,4,5-tris((2-ethylhexyl)-oxy)phenyl)methanol, **4**, solution. The ice bath was subsequently removed, and the reaction was allowed to proceed under stirring at 23.5 °C for 2 h, at which time TLC analysis indicated complete conversion. The reaction was quenched by adding the reaction mixture to 50 mL of water. This mixture was extracted 3 times with 50 mL of DCM. The organic layer was dried over anhydrous $MgSO_4$ and vacuum filtered. The filtrate was concentrated on a rotary evaporator and purified by column chromatography using 50/50:Hex/DCM as the eluent to yield compound **6** as a colorless oil (11.8 g, 90.5%). TLC (50/50:Hex/DCM): R_f = 0.76. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.53 (s, 2H, PhH), 5.03 (s, 2H, $-CH_2Ph$), 3.87−3.78 (m, 6H, 3 × $PhOCH_2-$), 3.47 (t, 2H, $-CH_2Br$), 2.56 (t, 2H, $BrCH_2CH_2CH_2COO-$), 2.24−2.16 (m, 2H, $BrCH_2CH_2CH_2COO-$), 1.78−1.65 (m, 3H, 3 × $PhOCH_2CH(CH_2CH_3)-$), 1.59−1.29 (m, 24H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$), 0.95−0.85 (m, 18H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 172.50, 153.57, 153.53, 138.13, 130.69, 106.37, 76.05, 71.31, 67.02, 44.13, 40.69, 39.75, 32.75, 32.63, 31.38, 30.63, 29.46, 29.44, 29.42, 29.24, 27.88, 27.77, 23.93, 23.83, 23.28, 23.22, 14.27, 14.23, 11.33, 11.31, 11.29, 11.25, 8.70. Purity by HPLC: 99+%. MALDI-TOF MS m/z of $[M + Na]+$ calculated for $C_{35}H_{61}BrNaO_5+$: 663.36; found: 664.55.

General Procedure for the Synthesis of IAJD97. IAJD97, **8**, was obtained by reacting N-(2-hydroxyethyl)piperazine with 3,4,5-tris((2-ethylhexyl)oxy)benzyl 4-bromobutanoate, **6**. 3,4,5-Tris((2-ethylhexyl)oxy)benzyl 4-bromobutanoate, **6** (11 g, 17.2 mmol), K_2CO_3 (2.9 g, 21.1 mmol), and N-(2-hydroxyethyl)piperazine, **7** (2.74 g, 21.1 mmol) were stirred in 100 mL of MeCN at 95 °C for 3 h, at which time TLC analysis indicated complete conversion. The reaction was quenched by mixing the reaction mixture with 50 mL of water. This mixture was extracted 3 times with 20 mL of DCM. The organic layer was dried over anhydrous $MgSO_4$ and vacuum filtered. The filtrate was concentrated in a rotary evaporator and purified by column chromatography using 40/1:DCM/MeOH and 50/1: DCM/MeOH as eluents. The resulting oil was dissolved in DCM (25 mL) and washed with $NaHCO_3$ (2%, 25 mL). The aqueous phase was extracted with DCM (25 mL) two additional times. The organic phase was combined and dried over anhydrous $MgSO_4$. Filtration, evaporation of the solvent, and vacuum drying yielded IAJD97, **8**, as a colorless oil (10.2 g, 86.8%). TLC (95/5:DCM/MeOH): R_f = 0.32. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.52 (s, 2H, PhH), 5.00 (s, 2H, $-CH_2Ph$), 3.84−3.78 (m, 6H, 3 × $PhOCH_2-$), 3.59 (t, 2H, $-CH_2CH_2OH$), 2.83 (br, 1 H, $-CH_2CH_2OH$), 2.62−2.29 (m, 14 H, $N(CH_2CH_2)_2$, $-OCOCH_2CH_2CH_2-$ and $-CH_2CH_2OH$), 1.88−1.77 (m, 2H, $-OCOCH_2CH_2CH_2-$), 1.77−1.64 (m, 3H, 3 × $PhOCH_2CH(CH_2CH_3)-$), 1.62−1.25 (m, 24H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$), 0.95−0.86 (m, 18H, 3 × $PhOCH_2CH(CH_2CH_3)(CH_2)_3CH_3$). ^{13}C NMR (101 MHz, $CDCl_3$) δ: 173.5, 153.5, 138.1, 130.9, 106.5, 76.1, 71.3, 66.8, 59.3, 57.8, 57.7, 53.3, 53.0, 40.7, 39.8, 32.4, 30.6, 30.6, 29.5, 29.4, 29.4, 29.2, 23.9, 23.8, 23.3, 23.2, 22.3, 14.3, 14.2, 11.3, 11.3, 11.3, 11.3. Purity by HPLC: 99+%. MALDI-TOF MS m/z of $[M + H]^+$ calculated for $C_{41}H_{75}N_2O_{6+}$: 691.6; found: 691.1 and $[M + Na]^+$ calculated for $C_{41}H_{74}N_2NaO_{6+}$: 713.5; found: 713.3.

RESULTS AND DISCUSSION

Accelerated Synthesis of Propyl 3,4,5-Tris((2-ethylhexyl)oxy)benzoate, 3. The preparation of the gallic acid-based sSS IAJD97 starts from the inexpensive food additive propyl gallate,[52−535455] which, under suitable conditions, should

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

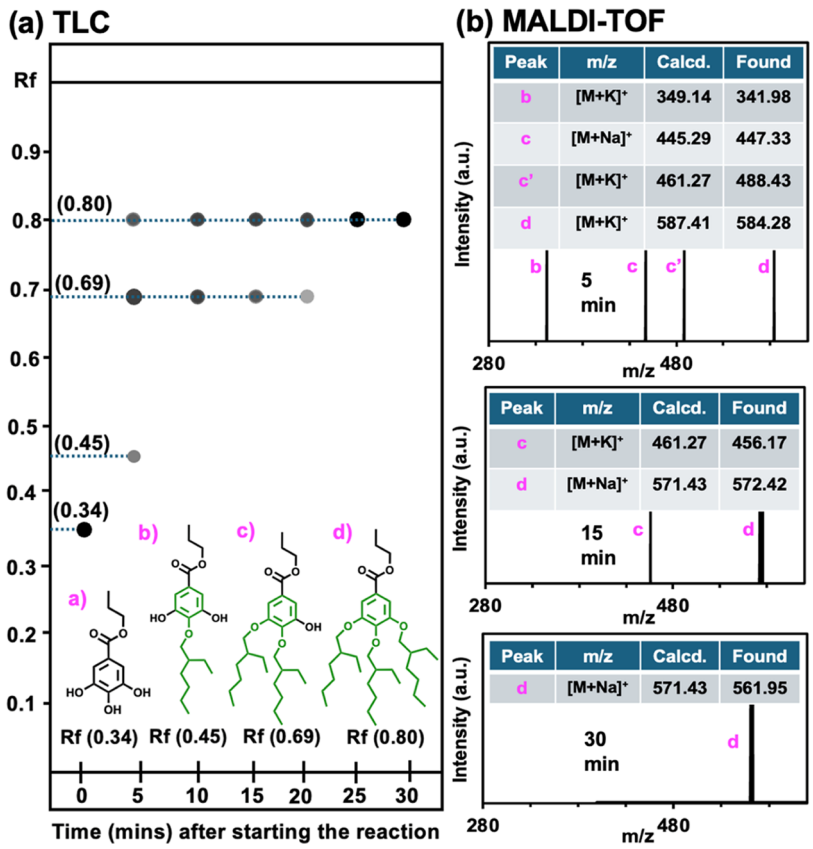


Figure 2. TLC (a) and MALDI-TOF (b) analysis as a function of time during the alkylation of propyl gallate.

Table 1. Accelerated Synthesis of Propyl 3,4,5-Tris((2-ethylhexyl)oxy)benzoate

No.	propyl gallate (g/mmol/molar equiv)	ethylhexyl bromide (g/mmol/molar equiv)	K_2CO_3 (g/mmol/molar equiv)	solvent (type/bp °C/mL)	reaction mixture (color)	temp (°C)	time (h/min)	conversion (% by TLC)	yield (%)
1	2/9.4/1	9.02/47/5	7.81/56/6	DMF/153/25	Orange-Brown	80	5/--	<100	54.3
2	2/9.4/1	9.02/47/5	7.81/56/6	DMF/153/25	Orange-Brown	100	4/--	<100	57.4
3	2/9.4/1	9.02/47/5	7.81/56/6	DMF/153/25	Orange-Brown	120	5/--	<100	75.6
4	2/9.4/1	9.02/47/5	7.81/56/6	DMF/153/25	Orange-Brown	140	4/--	100	91.1
5	2/9.4/1	9.02/47/5	7.81/56/6	DMF/153/25	Orange-Brown	150	3/--	100	89.6
6	2/9.4/1	9.02/47/5	9.1/65/7	DMF/153/25	Orange-Brown	120	--/40	100	93.6
7	2/9.4/1	8.15/42/4.5	9.1/65/7	DMF/153/25	Orange-Brown	130	--/30	100	93.1
8	2/9.4/1	8.15/42/4.5	9.1/65/7	DMF/153/25	Orange-Brown	135	--/20	100	93.1
9	2/9.4/1	8.15/42/4.5	9.1/65/7	DMF/153/25	Orange-Brown	140	--/30	100	93.0
10	2/9.4/1	8.15/42/4.5	9.1/65/7	DMF/153/25	Orange-Brown	150	--/30	100	92.9
11	2/9.4/1	7.24/37/4	9.1/65/7	DMF/153/25	Orange-Brown	130	16/--	<100[a]	88.1
12	2/9.4/1	7.24/37/4	9.1/65/7	DMF/153/25	Orange-Brown	130	17/--	100	86.4
13	2/9.4/1	8.15/42/4.5	7.81/56/6	DMF/153/25	Orange-Brown	130	--/30	100	94.2
14	2/9.4/1	8.15/42/4.5	6.51/47/5	DMF/153/25	Orange-Brown	130	16/--	<100[a]	89.3
15	2/9.4/1	8.15/42/4.5	6.51/47/5	DMF/153/25	Orange-Brown	130	17/--	100	86.1
16	2/9.4/1	8.15/42/4.5	7.81/56/6	DMSO/189/25	Pink	130	--/50	100	89.3
17	2/9.4/1	8.15/42/4.5	7.81/56/6	DMAC/165/25	Pink-Yellow	130	--/70	100	96.9
18	2/9.4/1	8.15/42/4.5	7.81/56/6	NMP/202/25	Violet-Dark Blue	130	2/--	100	91.2
19	2/9.4/1	8.15/42/4.5	7.81/56/6	MeCN/82/25	Colorless	80	2/--	<100[a]	87.3
20	2/9.4/1	8.15/42/4.5	7.81/56/6	CyO/156/25	Colorless	130	20/--	<100[b]	84.3
21	2/9.4/1	8.15/42/4.5	7.81/56/6	MIBK/117/25	Colorless	130	2.5/--	<100[a]	83.1
22	15/70.7/1	61.1/318/4.5	58/424/6	DMF/153/200	Orange-Brown	130	--/55	100	95.3

[a]2 spots by TLC. [b]3 spots by TLC.

be alkylated with 100% conversion in order to construct the hydrophobic domain of the IAJD (Scheme 1).[2] Therefore, the rate-determining step for the synthesis of both sSS and nsSS IAJDs is the alkylation reaction employed to construct the

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

Figure 3. Colorless propyl 3,4,5-tris((2-ethylhexyl)oxy)benzoate, **3**, obtained after purification by column chromatography followed by Kulgelrohr distillation and stored at room temperature, 23.5 °C, in air in the organic chemistry lab for 6 months.

hydrophobic region of IAJDs. For IAJD97, the propyl gallate precursor, **1**, has three phenol groups that must be quantitatively substituted with 2-ethylhexyl bromide, **2**. The pathway to complete trialkylation occurs through monoalkylation of the most reactive 4-position followed by dialkylation in the 3- and/or 5-positions. All of these intermediary compounds of IAJD97 can be monitored by a combination of thin-layer chromatography (TLC), HPLC, and MALDI-TOF (Figure 2).

Our inspiration for the trialkylation of gallic acid derivatives was provided by experiments from our own laboratory with more reactive *n*-alkylhalides.[33,56−65] The first trialkylation of gallic acid derivatives with the less reactive 2-ethylhexyl bromide was reported by us[2,41,66] and was employed later by other laboratories.[67] Our first attempts to increase the rate of reaction under closely related reaction conditions began with an increased reaction temperature (Table 1). It is important to notice the low boiling point of the alkylating reagent 2-ethylhexyl bromide (pb = 75−77 °C). This boiling point limits the reaction temperature of the alkylation to 70 or 80 °C. However, we discovered that 2-ethylhexyl bromide makes an azeotropic mixture with the dipolar aprotic solvent DMF, which was not known to us during the original series of alkylation experiments.[41,66] Therefore, during the experiments reported in Table 1, the reaction temperature of the alkylation step was increased to 150 °C (see entries 1 to 10 from Table 1). At 120 to 130 °C, the reaction time to complete conversion determined by TLC was 30 min, which is a substantial increase in rate compared to the 5 h required for less than 100% conversion and low isolated yields at lower temperatures (see entries 1 and 2 in Table 1). The number of equivalents of the alkylating agent and K_2CO_3 base plays a very important role in maintaining a low reaction time. For example, decreasing the number of equivalents of 2-ethylhexyl bromide from 4.5 to 4 (entry 11, Table 1) increased the reaction time from 30 min to 16 h with only incomplete conversion by TLC and to 17 h with complete

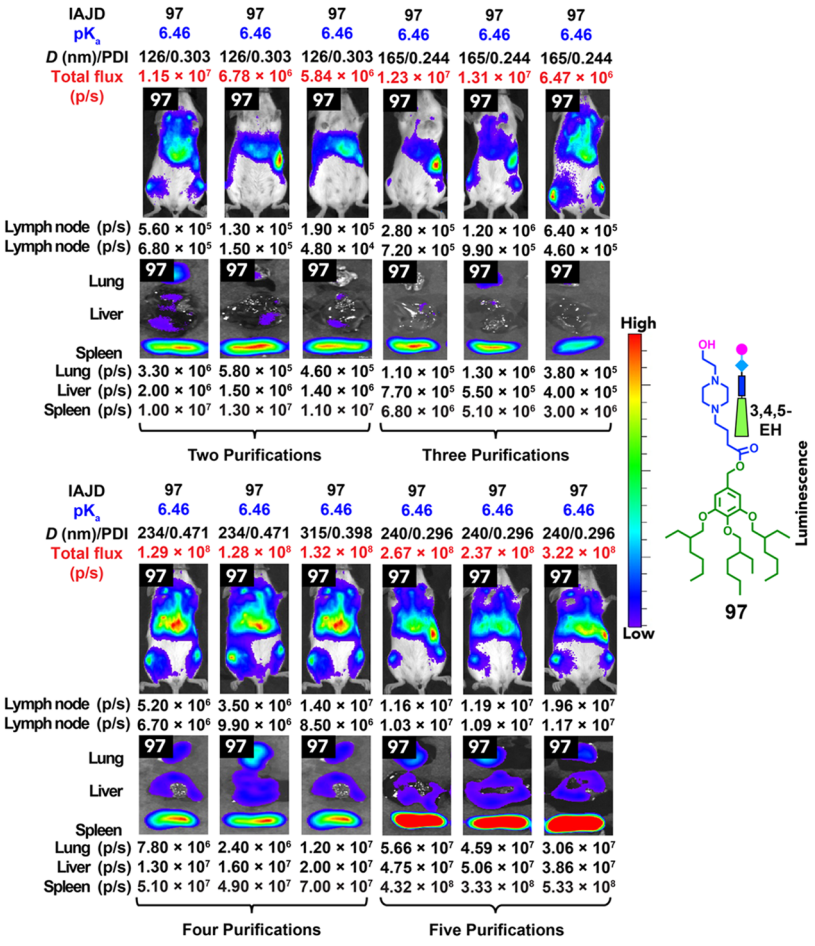


Figure 4. *In vivo* Luc activity of IAJD97 as a function of the number of purifications performed for the first series of experiments.

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

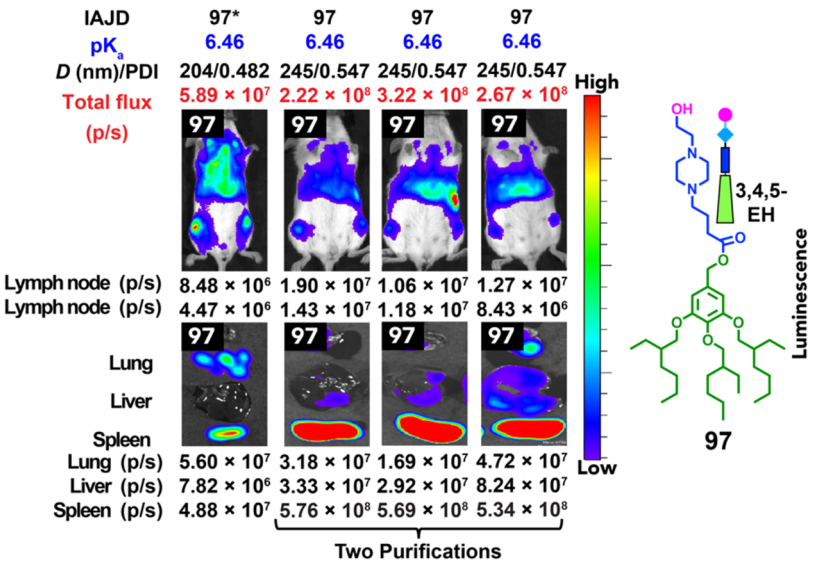


Figure 5. *In vivo* Luc activity of IAJD97 as a function of the number of purifications performed for the second time by the same person who did the experiments from Figure 3. IAJD97* experiments are from published data.[2]

conversion by TLC (entry 12, Table 1). Therefore, an optimum number of 2-ethylhexyl bromide equivalents of 4.5 was maintained in all other experiments. Decreasing the equivalents of K_2CO_3 base from 7 to 6 (entry 13, Table 1) does not affect the reaction time. However, only 5 equiv of base increased the reaction time from 30 min to 16 h with incomplete conversion by TLC (entry 14, Table 1), while a reaction time of 17 h provided complete conversion by TLC (entry 15, Table 1). Therefore, for 2 g or one equivalent of propyl gallate, we decided that the shorter reaction time of 30 min to complete conversion requires 4.5 equiv of 2-ethylhexyl bromide and 6 equiv of base in 25 mL of DMF at 130 °C (entry 13, Table 1) to yield 94.2% of colorless trialkylated product after column chromatography purification (Figure 3). This product was also distilled in a Kulgelrohr apparatus at 200 °C and remains colorless after storage in our laboratory at rt (23.5 °C) in air for as long as required.

We finally screened for this reaction several additional dipolar aprotic solvents (entries 16, 17, and 18, Table 1) as well as other polar solvents (entries 19, 20, and 21, Table 1). Longer reaction times to complete conversion by TLC were required for the dipolar aprotic solvents DMSO, DMAC, and NMP. Only incomplete conversions were obtained in acetonitrile, cyclohexanone, and methyl isobutyl ketone. The polar reaction solvents, which did not provide complete conversion, maintained a clear reaction mixture (entries 19, 20, and 21, Table 1). Without complete conversion, after column chromatography, the final product turns yellow from a clear reaction mixture, while in solvents like DMF, it exhibits a darkly colored reaction mixture but yields a clear colorless product after purification (Figure 3). The optimized reaction conditions mentioned above were scaled to 15 g of propyl gallate to provide in 55 min 36.3 g of entirely colorless product after column chromatography and Kulgelrohr distillation.

Synthesis of IAJD97. The next step of this reaction involves the reduction of propyl 3,4,5-tris((2-ethylhexyl)oxy)benzoate, **3**, with $LiAlH_4$ in dry THF at 0 to 23.5 °C. This classic reduction proceeds to 100% conversion in 1.5 h when 12 g of compound **3** was used in the reduction reaction. The resulting benzyl alcohol **4** (10.4 g, 88.6%) was esterified with 4-bromobutanoyl chloride, **5**, in dry methylene chloride at 0 to 23.5 °C in the presence of dry NEt_3 and a catalytic amount of the supernucleophilic catalyst DMAP.[68−70] **5** was freshly prepared from the corresponding acid in dry methylene chloride with thionyl chloride in the presence of one drop of DMF as a catalyst followed by the distillation of the excess thionyl chloride and methylene chloride to yield 83.7% of **6** after column chromatography. **6** was reacted with N-(2-hydroxyethyl)piperazine, **7**, in the presence of K_2CO_3 as a base in MeCN at 95 °C[2,71] to produce compound **8** known as IAJD97[2] in 86.8% isolated yield after 3 h, purification by column chromatography, washing with dilute $NaHCO_3$ followed by drying on $MgSO_4$, evaporation of the solvent, and vacuum drying.

Potential Side Reactions of IAJD97. IAJD97 synthesized by alkylation of the gallic acid derivative at 80 °C under the conditions reported previously[2] was unstable at room temperature. Originally, we believed that this was a property of the pure IAJD97 and, therefore, investigated the structure of the degradation product and its potential mechanism of degradation quite extensively.[72] However, to our disappointment, we discovered that this was a side reaction mediated by a concentration of free phenol that cannot be detected by the combination of NMR, TLC, HPLC, and MALDI-TOF instrumentation used in the current experiments. This undetectable free phenol is generated during the first step of this synthesis. Quantitative alkylation during the first step eliminated the instability of IAJD97. Since this side reaction influences the total and targeted activity in vivo, we established a method to detect this side reaction by targeted delivery experiments mediated by IAJD97. These experiments are more sensitive to sample purity than any of the analytical methods employed during the synthesis process.

Estimating the Purity of IAJD97 by Targeted Delivery of Luc-mRNA with its DNPs. Targeted delivery experiments of firefly luciferase (Luc)-mRNA mediated by IAJD97 as a function of the number of purification experiments were performed, as reported previously.[2] The accuracy of the IAJD purification depends on the expertise of the experimentalist. These experiments were performed by a scientist doing for the first time the synthesis and purification of IAJD97. We would

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

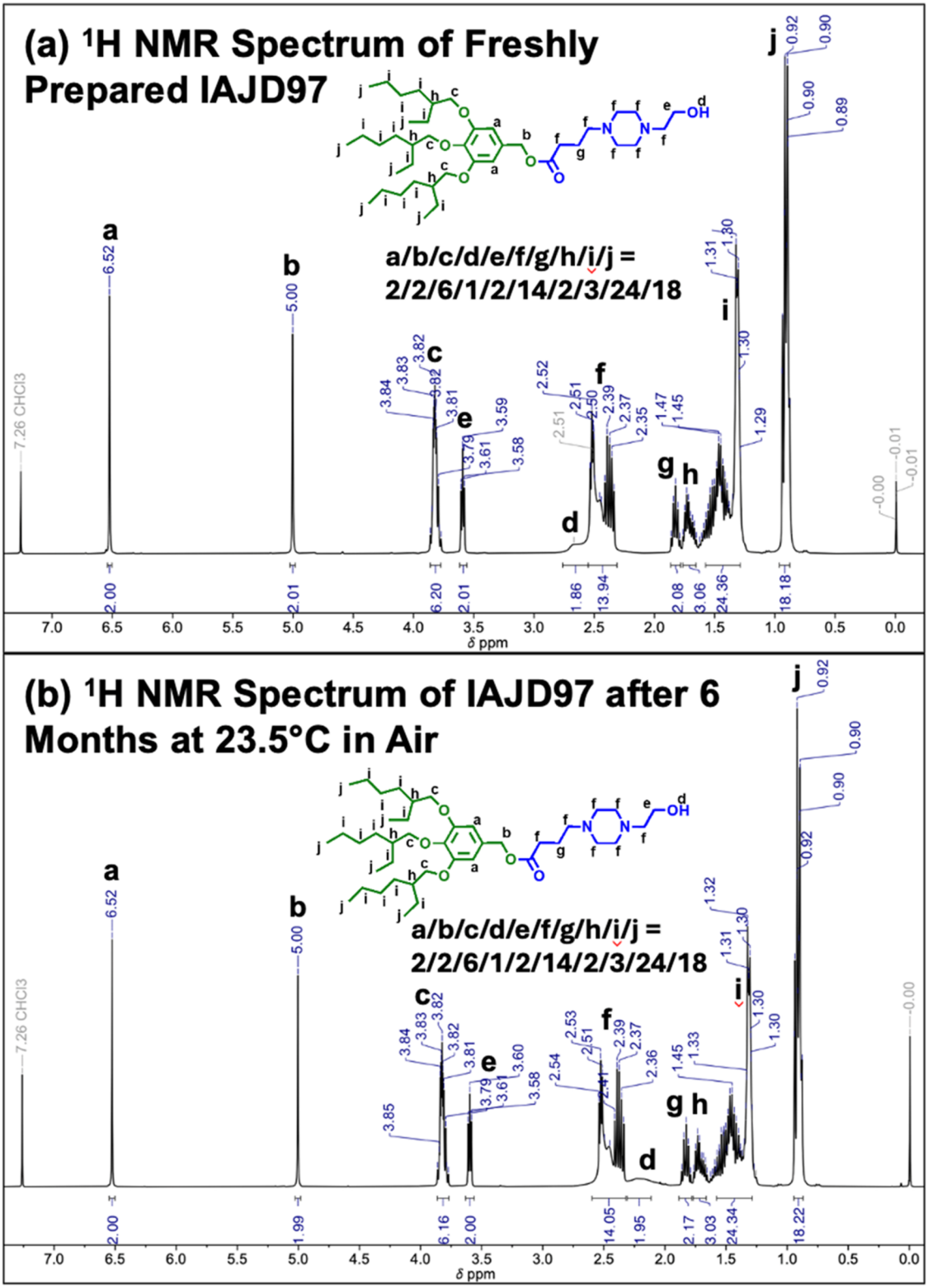


Figure 6. [1]H NMR spectra of freshly prepared IAJD97 (a) and IAJD97 after storing for six months at 23.5 °C in the chemistry laboratory air (b), demonstrating the stability of IAJD97 in time under these conditions.

like to state that regarding the level of training, reproducible results with IAJD97, as well as with other IAJDs, require the synthesis and purification of the corresponding IAJD combined with *in vivo* experiments to be performed several times. However, the *in vivo* delivery was performed by the most experienced experimentalists doing these experiments on a routine basis. Therefore, the change in activity *in vivo* can be only due to the purification and not due to the *in vivo* delivery. The results from Figure 4 show the dependence of total activity as well as of targeted activities as a function of purity. pK_a values of all IAJD97 are constant and are shown in the upper part of Figure 4. DNPs assembled from IAJD97 and Luc-mRNA were

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

generated by simple injection in acetate buffer at pH 4. Under neutral or slightly basic conditions, IAJD97 does not self-assemble into vesicles. However, at pH 4, in the absence of Luc-mRNA, it self-assembles into empty vesicles. These vesicles can be considered as being supramolecular polymers with narrow polydispersity. In the presence of Luc-mRNA, vesicles containing encapsulated Luc-mRNA, known as DNPs, are coassembled. These DNPs can also be considered as supramolecular polymers encapsulating a monodisperse Luc-mRNA natural polymer. The dimensions in nanometers and the polydispersity (PDI) of the DNPs are reported in the third line from the top of Figure 4. The fourth line reports the total flux in p/s. The images of the whole mouse body are shown in line five of Figure 4. Lines six and seven report the activities for the delivery to lymph nodes, while lines eight, nine, and ten show the lung, liver, and spleen with their luminescence. Lines 11, 12, and 13 provide the values for the delivery to the lung, liver, and spleen, all in p/s. Each purification was analyzed *in vivo*, with three mice per condition.

Let us follow the results from Figure 4. After two purifications, both the total activity (1.15×10^7, 6.78×10^6, 5.84×10^6 p/s) and the activity for the delivery to the spleen (1.00×10^7, 1.30×10^7, 1.10×10^7 p/s) show similar values (10^7 p/s) with the results reported previously for IAJD97.[2] After three purifications, the total activity increases to 1.23×10^7, 1.31×10^7, and 6.47×10^6, while the activity to the spleen and lymph nodes are minimally affected. Four purifications provide an increase in the total activity to 1.29×10^8, 1.28×10^8, and 1.32×10^8, with a corresponding activity for delivery to the spleen to 5.10×10^7, 4.90×10^7, and 7.00×10^7. Unexpectedly, the activity to the liver and lung increases after four purifications from 10^5 to 10^7, while the activity to lymph nodes increases from 10^5−10^6 and 10^6−10^7. Five purifications provided a remarkable result. Total activity is 2.67×10^8, 2.38×10^8, and 3.22×10^8, activity to the spleen is 4.32×10^8, 3.33×10^8, and 5.33×10^8, activity to the liver is 4.75×10^7, 5.06×10^7, and 3.86×10^7, activity to the lung is 5.66×10^7, 4.59×10^7, and 3.06×10^7, and activity to lymph nodes is 1.16×10^7, 1.03×10^7, 1.19×10^7, 1.09×10^7, 1.96×10^7, and 1.17×10^7. These results demonstrate that although by conventional analytical methods, in all cases, the purity of IAJD97 was higher than 99% after 2 purifications, *in vivo* activities continue to increase as the number of purifications by column chromatography is higher. After this number of experiments and practice, we expected that the expertise of the experimentalist must have increased, and therefore, we decided to duplicate the *in vivo* activity as a function of a number of purifications of the IAJD. The remarkable progress was demonstrated by the experiments shown in Figure 5. Previously published data on IAJD97, including *in vivo* targeted delivery data, which were not reported before,[2] are shown in the first column marked with a star.

Indeed, with an experienced experimentalist who performed these experiments several times, after only two purifications, all activities are equal or higher than after the five purifications illustrated in Figure 4 and are with 1 order of magnitude higher both as total activity and as targeted activity to the spleen than the data previously reported for alternate synthesis methods for IAJD97.[2] These experiments demonstrated what is well-known from sports and other activities. When working at the interface between several unrelated disciplines, including organic, supramolecular, macromolecular, immunology, and nanomedicine, excess practice is required by even the most experienced scientists in order to generate perfectly reproducible results. These details were provided here at the recommendation of the ACS concerning the reproducibility of the experimental results. Column chromatography experiments on silica gel were performed with no more than 2.5% methanol in DCM, which is widely accepted to not dissolve silica gel. These experiments also demonstrated for the first time that impurities that affect the *in vivo* activity are not detectable by conventional analytical methods and that repeated purifications of IAJDs combined with the determination of activity *in vivo* establish their maximum activity.

Stability of IAJD97 Investigated as a Function of Time by ^{1}H NMR Spectroscopy. Finally, ^{1}H NMR analysis as a function of time combined with TLC, HPLC, and MALDI-TOF demonstrated the stability of IAJD97 at the room temperature of the laboratory (23.5 °C) and in air for more than six months. The ^{1}H NMR spectra demonstrating this stability are shown in Figure 6, while HPLC and MALDI-TOF data are available in the Supporting Information.

CONCLUSIONS

An accelerated ten-gram-scale synthesis of the IAJD97 that delivers Luc-mRNA predominantly to the spleen and lymph nodes is presented. Propyl gallate, an inexpensive food additive derived from renewable plants' phenolic acid, gallic acid, was employed to generate this synthesis in 4 days with instrumentation, technology, and synthetic expertise available in any laboratory from around the world. Coassembly of IAJD97 with Luc-mRNA is generated by simple injection of an ethanol solution of the synthetic vector IAJD97 into a pH 4 acetate buffer containing Luc-mRNA. Total and targeted Luc activities *in vivo* were more than 1 order of magnitude higher than previously reported.[2] IAJD97 is stable for at least 7 months in an organic chemistry laboratory at room temperature (23.5 °C) in air. We believe that the experiments reported here will help to provide worldwide access to nanomedicine mediated by targeted delivery of mRNA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.4c01107.

^{1}H NMR spectrum of propyl 3,4,5-tris((2-ethylhexyl)-oxy)benzoate; (3,4,5-tris((2-ethylhexyl)oxy)phenyl)-methanol; 3,4,5-tris((2-ethylhexyl)oxy)benzyl 4-bromobutanoate; IAJD97; and propyl 3,4,5-tris((2-ethylhexyl)-oxy)benzoate; HPLC traces (UV, RI) of intermediates and final product of the synthesis of IAJD97; MALDI-TOF spectra; and color of the reaction mixtures and products (PDF)

AUTHOR INFORMATION

Corresponding Authors

Drew Weissman − *Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States;* orcid.org/0000-0002-1501-6510; Email: dreww@pennmedicine.upenn.edu

Virgil Percec − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States;* orcid.org/0000-0001-5926-0489; Email: percec@sas.upenn.edu

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

Authors

Mahwish Arshad − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States; Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Elena N. Atochina-Vasserman − *Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States;* orcid.org/0000-0001-7950-7004

Srijay S. Chenna − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States; Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Devendra S. Maurya − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States;* orcid.org/0000-0002-4488-9927

Muhammad Irhash Shalihin − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States;* Present Address: Department of Chemistry, Faculty of Science and Technology, University of Jambi, Jambi, Indonesia, 36361

Dipankar Sahoo − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States;* orcid.org/0000-0002-7646-6419

Alec C. Lewis − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States*

Jordan J. Lewis − *Roy & Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6323, United States*

Nathan Ona − *Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Jessica A. Vasserman − *Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Houping Ni − *Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Wook-Jin Park − *Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.biomac.4c01107

Author Contributions

[‖]M.A. and E.N.A.-V. contributed equally to this work.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the National Science Foundation Grants DMR-2104554 and DMR-1720530, the P. Roy Vagelos Chair at the University of Pennsylvania, the Alexander von Humboldt Foundation (all to V.P.), and the Wellcome Leap R3 Program (to D.W. and V.P.). NMR Instrumentation (NEO400) was supported by the NSF Major Research Instrumentation Program (award NSF CHE-1827457) and the Vagelos Institute for Energy Science and Technology.

■ REFERENCES

(1) Zhang, D.; Atochina-Vasserman, E. N.; Maurya, D. S.; Huang, N.; Xiao, Q.; Ona, N.; Liu, M.; Shahnawaz, H.; Ni, H.; Kim, K.; Billingsley, M. M.; Pochan, D. J.; Mitchell, M. J.; Weissman, D.; Percec, V. One-Component Multifunctional Sequence-Defined Ionizable Amphiphilic Janus Dendrimer Delivery Systems for mRNA. *J. Am. Chem. Soc.* **2021**, *143* (31), 12315−12327.

(2) Zhang, D.; Atochina-Vasserman, E. N.; Maurya, D. S.; Liu, M.; Xiao, Q.; Lu, J.; Lauri, G.; Ona, N.; Reagan, E. K.; Ni, H.; Weissman, D.; Percec, V. Targeted Delivery of mRNA with One-Component Ionizable Amphiphilic Janus Dendrimers. *J. Am. Chem. Soc.* **2021**, *143* (43), 17975−17982.

(3) Zhang, D.; Atochina-Vasserman, E. N.; Lu, J.; Maurya, D. S.; Xiao, Q.; Liu, M.; Adamson, J.; Ona, N.; Reagan, E. K.; Ni, H.; Weissman, D.; Percec, V. The Unexpected Importance of the Primary Structure of the Hydrophobic Part of One-Component Ionizable Amphiphilic Janus Dendrimers in Targeted mRNA Delivery Activity. *J. Am. Chem. Soc.* **2022**, *144* (11), 4746−4753.

(4) Lu, J.; Atochina-Vasserman, E. N.; Maurya, D. S.; Shalihin, M. I.; Zhang, D.; Chenna, S. S.; Adamson, J.; Liu, M.; Shah, H. U. R.; Shah, H.; Xiao, Q.; Queeley, B.; Ona, N. A.; Reagan, E. K.; Ni, H.; Sahoo, D.; Peterca, M.; Weissman, D.; Percec, V. Screening Libraries to Discover Molecular Design Principles for the Targeted Delivery of mRNA with One-Component Ionizable Amphiphilic Janus Dendrimers Derived from Plant Phenolic Acids. *Pharmaceutics* **2023**, *15* (6), No. 1572.

(5) Lu, J.; Atochina-Vasserman, E. N.; Maurya, D. S.; Sahoo, D.; Ona, N.; Reagan, E. K.; Ni, H.; Weissman, D.; Percec, V. Targeted and Equally Distributed Delivery of mRNA to Organs with Pentaerythritol-Based One-Component Ionizable Amphiphilic Janus Dendrimers. *J. Am. Chem. Soc.* **2023**, *145* (34), 18760−18766.

(6) Sahoo, D.; Atochina-Vasserman, E. N.; Maurya, D. S.; Arshad, M.; Chenna, S. S.; Ona, N.; Vasserman, J. A.; Ni, H.; Weissman, D.; Percec, V. The Constitutional Isomerism of One-Component Ionizable Amphiphilic Janus Dendrimers Orchestrates the Total and Targeted Activities of mRNA Delivery. *J. Am. Chem. Soc.* **2024**, *146* (6), 3627−3634.

(7) Luo, D.; Saltzman, W. M. Synthetic DNA Delivery Systems. *Nat. Biotechnol.* **2000**, *18* (1), 33−37.

(8) Thomas, C. E.; Ehrhardt, A.; Kay, M. A. Progress and Problems with the Use of Viral Vectors for Gene Therapy. *Nat. Rev. Genet.* **2003**, *4* (5), 346−358.

(9) Peer, D.; Karp, J. M.; Hong, S.; Farokhzad, O. C.; Margalit, R.; Langer, R. Nanocarriers as an Emerging Platform for Cancer Therapy. *Nat. Nanotechnol.* **2007**, *2* (12), 751−760.

(10) Whitehead, K. A.; Langer, R.; Anderson, D. G. Knocking down Barriers: Advances in siRNA Delivery. *Nat. Rev. Drug Discovery* **2009**, *8* (2), 129−138.

(11) Cullis, P. R.; Hope, M. J. Lipid Nanoparticle Systems for Enabling Gene Therapies. *Mol. Ther.* **2017**, *25* (7), 1467−1475.

(12) Ramamoorth, M. Non Viral Vectors in Gene Therapy- An Overview. *J. Clin. Diagn. Res.* **2015**, *9* (1), GE01−GE06, DOI: 10.7860/JCDR/2015/10443.5394.

(13) Wickham, T. J. Ligand-Directed Targeting of Genes to the Site of Disease. *Nat. Med.* **2003**, *9* (1), 135−139.

(14) Hajj, K. A.; Whitehead, K. A. Tools for Translation: Non-Viral Materials for Therapeutic mRNA Delivery. *Nat. Rev. Mater.* **2017**, *2* (10), No. 17056.

(15) Reichmuth, A. M.; Oberli, M. A.; Jaklenec, A.; Langer, R.; Blankschtein, D. mRNA Vaccine Delivery Using Lipid Nanoparticles. *Ther. Delivery* **2016**, *7* (5), 319−334.

(16) Kauffman, K. J.; Webber, M. J.; Anderson, D. G. Materials for Non-Viral Intracellular Delivery of Messenger RNA Therapeutics. *J. Controlled Release* **2016**, *240*, 227−234.

(17) Pardi, N.; Hogan, M. J.; Porter, F. W.; Weissman, D. mRNA Vaccines—a New Era in Vaccinology. *Nat. Rev. Drug Discovery* **2018**, *17* (4), 261−279.
(18) Buschmann, M. D.; Carrasco, M. J.; Alishetty, S.; Paige, M.; Alameh, M. G.; Weissman, D. Nanomaterial Delivery Systems for mRNA Vaccines. *Vaccines* **2021**, *9* (1), No. 65.
(19) Wilson, J. M. Genetic Diseases, Immunology, Viruses, and Gene Therapy. *Hum. Gene Ther.* **2014**, *25* (4), 257−261.
(20) Cross, R. The Redemption of James Wilson, Gene Therapy Pioneer. *C&EN* **2019**, *97*, No. 26.
(21) Nguyen, J.; Szoka, F. C. Nucleic Acid Delivery: The Missing Pieces of the Puzzle? *Acc. Chem. Res.* **2012**, *45* (7), 1153−1162.
(22) Reinhard, K.; Rengstl, B.; Oehm, P.; Michel, K.; Billmeier, A.; Hayduk, N.; Klein, O.; Kuna, K.; Ouchan, Y.; Wöll, S.; Christ, E.; Weber, D.; Suchan, M.; Bukur, T.; Birtel, M.; Jahndel, V.; Mroz, K.; Hobohm, K.; Kranz, L.; Diken, M.; Kühlcke, K.; Türeci, Ö.; Sahin, U. An RNA Vaccine Drives Expansion and Efficacy of Claudin-CAR-T Cells against Solid Tumors. *Science* **2020**, *367* (6476), 446−453.
(23) Sabnis, S.; Kumarasinghe, E. S.; Salerno, T.; Mihai, C.; Ketova, T.; Senn, J. J.; Lynn, A.; Bulychev, A.; McFadyen, I.; Chan, J.; Almarsson, Ö.; Stanton, M. G.; Benenato, K. E. A Novel Amino Lipid Series for mRNA Delivery: Improved Endosomal Escape and Sustained Pharmacology and Safety in Non-Human Primates. *Mol. Ther.* **2018**, *26* (6), 1509−1519.
(24) Romão, C. C.; Blättler, W. A.; Seixas, J. D.; Bernardes, G. J. L. Developing Drug Molecules for Therapy with Carbon Monoxide. *Chem. Soc. Rev.* **2012**, *41* (9), No. 3571.
(25) Cabral, H.; Kinoh, H.; Kataoka, K. Tumor-Targeted Nanomedicine for Immunotherapy. *Acc. Chem. Res.* **2020**, *53* (12), 2765−2776.
(26) Li, J.; Kataoka, K. Chemo-Physical Strategies to Advance the *in Vivo* Functionality of Targeted Nanomedicine: The Next Generation. *J. Am. Chem. Soc.* **2021**, *143* (2), 538−559.
(27) Heredia, K. L.; Nguyen, T. H.; Chang, C.-W.; Bulmus, V.; Davis, T. P.; Maynard, H. D. Reversible siRNA−Polymer Conjugates by RAFT Polymerization. *Chem. Commun.* **2008**, No. 28, No. 3245.
(28) Deng, Z. J.; Morton, S. W.; Ben-Akiva, E.; Dreaden, E. C.; Shopsowitz, K. E.; Hammond, P. T. Layer-by-Layer Nanoparticles for Systemic Codelivery of an Anticancer Drug and siRNA for Potential Triple-Negative Breast Cancer Treatment. *ACS Nano* **2013**, *7* (11), 9571−9584.
(29) Lacroix, A.; Sleiman, H. F. DNA Nanostructures: Current Challenges and Opportunities for Cellular Delivery. *ACS Nano* **2021**, *15* (3), 3631−3645.
(30) Billingsley, M. M.; Singh, N.; Ravikumar, P.; Zhang, R.; June, C. H.; Mitchell, M. J. Ionizable Lipid Nanoparticle-Mediated mRNA Delivery for Human CAR T Cell Engineering. *Nano Lett.* **2020**, *20* (3), 1578−1589.
(31) Riley, R. S.; Kashyap, M. V.; Billingsley, M. M.; White, B.; Alameh, M.-G.; Bose, S. K.; Zoltick, P. W.; Li, H.; Zhang, R.; Cheng, A. Y.; Weissman, D.; Peranteau, W. H.; Mitchell, M. J. Ionizable Lipid Nanoparticles for in Utero mRNA Delivery. *Sci. Adv.* **2021**, *7* (3), No. eaba1028.
(32) Chahal, J. S.; Khan, O. F.; Cooper, C. L.; McPartlan, J. S.; Tsosie, J. K.; Tilley, L. D.; Sidik, S. M.; Lourido, S.; Langer, R.; Bavari, S.; Ploegh, H. L.; Anderson, D. G. Dendrimer-RNA Nanoparticles Generate Protective Immunity against Lethal Ebola, H1N1 Influenza, and *Toxoplasma Gondii* Challenges with a Single Dose. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (29), E4133−E4142.
(33) Percec, V.; Wilson, D. A.; Leowanawat, P.; Wilson, C. J.; Hughes, A. D.; Kaucher, M. S.; Hammer, D. A.; Levine, D. H.; Kim, A. J.; Bates, F. S.; Davis, K. P.; Lodge, T. P.; Klein, M. L.; DeVane, R. H.; Aqad, E.; Rosen, B. M.; Argintaru, A. O.; Sienkowska, M. J.; Rissanen, K.; Nummelin, S.; Ropponen, J. Self-Assembly of Janus Dendrimers into Uniform Dendrimersomes and Other Complex Architectures. *Science* **2010**, *328* (5981), 1009−1014.
(34) Sherman, S. E.; Xiao, Q.; Percec, V. Mimicking Complex Biological Membranes and Their Programmable Glycan Ligands with Dendrimersomes and Glycodendrimersomes. *Chem. Rev.* **2017**, *117* (9), 6538−6631.
(35) Klajnert-Maculewicz, B.; Janaszewska, A.; Majecka, A. Dendrimersomes: Biomedical Applications. *Chem. Commun.* **2023**, *59* (99), 14611−14625.
(36) Sun, H.-J.; Zhang, S.; Percec, V. From Structure to Function via Complex Supramolecular Dendrimer Systems. *Chem. Soc. Rev.* **2015**, *44* (12), 3900−3923.
(37) Rosen, B. M.; Wilson, C. J.; Wilson, D. A.; Peterca, M.; Imam, M. R.; Percec, V. Dendron-Mediated Self-Assembly, Disassembly, and Self-Organization of Complex Systems. *Chem. Rev.* **2009**, *109* (11), 6275−6540.
(38) Percec, V.; Sahoo, D. From Frank−Kasper, Quasicrystals, and Biological Membrane Mimics to Reprogramming *In Vivo* the Living Factory to Target the Delivery of mRNA with One-Component Amphiphilic Janus Dendrimers. *Biomacromolecules* **2024**, *25* (3), 1353−1370.
(39) Percec, V.; Xiao, Q. Helical Self-Organizations and Emerging Functions in Architectures, Biological and Synthetic Macromolecules. *Bull. Chem. Soc. Jpn.* **2021**, *94* (3), 900−928.
(40) Peterca, M.; Percec, V.; Leowanawat, P.; Bertin, A. Predicting the Size and Properties of Dendrimersomes from the Lamellar Structure of Their Amphiphilic Janus Dendrimers. *J. Am. Chem. Soc.* **2011**, *133* (50), 20507−20520.
(41) Percec, V.; Leowanawat, P.; Sun, H.-J.; Kulikov, O.; Nusbaum, C. D.; Tran, T. M.; Bertin, A.; Wilson, D. A.; Peterca, M.; Zhang, S.; Kamat, N. P.; Vargo, K.; Moock, D.; Johnston, E. D.; Hammer, D. A.; Pochan, D. J.; Chen, Y.; Chabre, Y. M.; Shiao, T. C.; Bergeron-Brlek, M.; André, S.; Roy, R.; Gabius, H.-J.; Heiney, P. A. Modular Synthesis of Amphiphilic Janus Glycodendrimers and Their Self-Assembly into Glycodendrimersomes and Other Complex Architectures with Bioactivity to Biomedically Relevant Lectins. *J. Am. Chem. Soc.* **2013**, *135* (24), 9055−9077.
(42) Zhang, S.; Xiao, Q.; Sherman, S. E.; Muncan, A.; Ramos Vicente, A. D. M.; Wang, Z.; Hammer, D. A.; Williams, D.; Chen, Y.; Pochan, D. J.; Vértesy, S.; André, S.; Klein, M. L.; Gabius, H.-J.; Percec, V. Glycodendrimersomes from Sequence-Defined Janus Glycodendrimers Reveal High Activity and Sensor Capacity for the Agglutination by Natural Variants of Human Lectins. *J. Am. Chem. Soc.* **2015**, *137* (41), 13334−13344.
(43) Xiao, Q.; Zhang, S.; Wang, Z.; Sherman, S. E.; Moussodia, R.-O.; Peterca, M.; Muncan, A.; Williams, D. R.; Hammer, D. A.; Vértesy, S.; André, S.; Gabius, H.-J.; Klein, M. L.; Percec, V. Onion-like Glycodendrimersomes from Sequence-Defined Janus Glycodendrimers and Influence of Architecture on Reactivity to a Lectin. *Proc. Natl. Acad. Sci. U.S.A.* **2016**, *113* (5), 1162−1167.
(44) Xiao, Q.; Ludwig, A.-K.; Romanò, C.; Buzzacchera, I.; Sherman, S. E.; Vetro, M.; Vértesy, S.; Kaltner, H.; Reed, E. H.; Möller, M.; Wilson, C. J.; Hammer, D. A.; Oscarson, S.; Klein, M. L.; Gabius, H.-J.; Percec, V. Exploring Functional Pairing between Surface Glycoconjugates and Human Galectins Using Programmable Glycodendrimersomes. *Proc. Natl. Acad. Sci. U.S.A.* **2018**, *115* (11), E2509−E2518.
(45) Zhang, D.; Xiao, Q.; Rahimzadeh, M.; Liu, M.; Rodriguez-Emmenegger, C.; Miyazaki, Y.; Shinoda, W.; Percec, V. Self-Assembly of Glycerol-Amphiphilic Janus Dendrimers Amplifies and Indicates Principles for the Selection of Stereochemistry by Biological Membranes. *J. Am. Chem. Soc.* **2023**, *145* (7), 4311−4323.
(46) Zhang, S.; Moussodia, R.-O.; Sun, H.-J.; Leowanawat, P.; Muncan, A.; Nusbaum, C. D.; Chelling, K. M.; Heiney, P. A.; Klein, M. L.; André, S.; Roy, R.; Gabius, H.-J.; Percec, V. Mimicking Biological Membranes with Programmable Glycan Ligands Self-Assembled from Amphiphilic Janus Glycodendrimers. *Angew. Chem., Int. Ed.* **2014**, *53* (41), 10899−10903.
(47) Zhang, S.; Moussodia, R.-O.; Murzeau, C.; Sun, H.-J.; Klein, M. L.; Vértesy, S.; André, S.; Roy, R.; Gabius, H.-J.; Percec, V. Dissecting Molecular Aspects of Cell Interactions Using Glycodendrimersomes with Programmable Glycan Presentation and Engineered Human Lectins. *Angew. Chem., Int. Ed.* **2015**, *54* (13), 4036−4040.

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882

(48) Zhang, S.; Sun, H.-J.; Hughes, A. D.; Draghici, B.; Lejnieks, J.; Leowanawat, P.; Bertin, A.; De Leon, L. O.; Kulikov, O. V.; Chen, Y.; Pochan, D. J.; Heiney, P. A.; Percec, V. "Single−Single" Amphiphilic Janus Dendrimers Self-Assemble into Uniform Dendrimersomes with Predictable Size. *ACS Nano* **2014**, *8* (2), 1554−1565.
(49) Rodriguez-Emmenegger, C.; Xiao, Q.; Kostina, N. Yu.; Sherman, S. E.; Rahimi, K.; Partridge, B. E.; Li, S.; Sahoo, D.; Reveron Perez, A. M.; Buzzacchera, I.; Han, H.; Kerzner, M.; Malhotra, I.; Möller, M.; Wilson, C. J.; Good, M. C.; Goulian, M.; Baumgart, T.; Klein, M. L.; Percec, V. Encoding Biological Recognition in a Bicomponent Cell-Membrane Mimic. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (12), 5376−5382.
(50) Yadavalli, S. S.; Xiao, Q.; Sherman, S. E.; Hasley, W. D.; Klein, M. L.; Goulian, M.; Percec, V. Bioactive Cell-like Hybrids from Dendrimersomes with a Human Cell Membrane and Its Components. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (3), 744−752.
(51) Wagner, A. M.; Kostina, N. Yu.; Xiao, Q.; Klein, M. L.; Percec, V.; Rodriguez-Emmenegger, C. Glycan-Driven Formation of Raft-Like Domains with Hierarchical Periodic Nanoarrays on Dendrimersome Synthetic Cells. *Biomacromolecules* **2024**, *25*, 366−378.
(52) Robbins, R. J. Phenolic Acids in Foods: An Overview of Analytical Methodology. *J. Agric. Food Chem.* **2003**, *51* (10), 2866−2887.
(53) Mandal, S. M.; Chakraborty, D.; Dey, S. Phenolic Acids Act as Signaling Molecules in Plant-Microbe Symbioses. *Plant Signaling Behav.* **2010**, *5* (4), 359−368.
(54) Kumar, N.; Goel, N. Phenolic Acids: Natural Versatile Molecules with Promising Therapeutic Applications. *Biotechnol. Rep.* **2019**, *24*, No. e00370.
(55) Javaheri-Ghezeldizaj, F.; Alizadeh, A. M.; Dehghan, P.; Dolatabadi, J. E. N. Pharmacokinetic and Toxicological Overview of Propyl Gallate Food Additive. *Food Chem.* **2023**, *423*, No. 135219.
(56) Percec, V.; Heck, J. Liquid Crystalline Polymers Containing Mesogenic Units Based on Half-Disc and Rod-like Moieties: 4. Side Chain Liquid Crystalline Polymethylsiloxanes Containing Hemiphasmidic Mesogens Based on 4-[3,4,5-Tri-(Alkan-1-Yloxy)-Benzoate]Biphenyl Groups. *Polym. Bull.* **1991**, *25* (4), 431−438.
(57) Percec, V.; Tomazos, D.; Heck, J.; Blackwell, H.; Ungar, G. Self-Assembly of Taper-Shaped Monoesters of Oligo(Ethylene Oxide) with 3,4,5-Tris(n-Dodecan-1-Yloxy)Benzoic Acid and of Their Polymethacrylates into Tubular Supramolecular Architectures Displaying a Columnar Hexagonal Mesophase. *J. Chem. Soc., Perkin Trans. 2* **1994**, *2* (1), 31−44.
(58) Johansson, G.; Percec, V.; Ungar, G.; Abramic, D. Molecular Recognition Directed Self-Assembly of Tubular Liquid Crystalline and Crystalline Supramolecular Architectures from Taper Shaped (15-Crown-5)Methyl 3,4,5-Tris(p-Alkyloxybenzyloxy)Benzoates and (15-Crown-5)Methyl 3,4,5-Tris(p-Dodecyloxy)Benzoate. *J. Chem. Soc., Perkin Trans. 1* **1994**, No. 4, 447−459.
(59) Percec, V.; Heck, J. A.; Tomazos, D.; Ungar, G. The Influence of the Complexation of Sodium and Lithium Triflate on the Self-Assembly of Tubular-Supramolecular Architectures Displaying a Columnar Mesophase Based on Taper-Shaped Monoesters of Oligoethylene Oxide with 3,4,5-Tris[p-(n-Dodecan-1-Yloxy)Benzyloxy]Benzoic Acid and of Their Polymethacrylates. *J. Chem. Soc., Perkin Trans. 2* **1993**, *2* (12), 2381−2388.
(60) Ungar, G.; Abramic, D.; Percec, V.; Heck, J. A. Self-Assembly of Twin Tapered Bisamides into Supramolecular Columns Exhibiting Hexagonal Columnar Mesophases. Structural Evidence for a Microsegregated Model of the Supramolecular Column. *Liq. Cryst.* **1996**, *21* (1), 73−86.
(61) Percec, V.; Peterca, M.; Tadjiev, T.; Zeng, X.; Ungar, G.; Leowanawat, P.; Aqad, E.; Imam, M. R.; Rosen, B. M.; Akbey, U.; Graf, R.; Sekharan, S.; Sebastiani, D.; Spiess, H. W.; Heiney, P. A.; Hudson, S. D. Self-Assembly of Dendronized Perylene Bisimides into Complex Helical Columns. *J. Am. Chem. Soc.* **2011**, *133* (31), 12197−12219.
(62) Percec, V.; Bera, T. K.; Glodde, M.; Fu, Q.; Balagurusamy, V. S. K.; Heiney, P. A. Hierarchical Self-Assembly, Coassembly, and Self-Organization of Novel Liquid Crystalline Lattices and Superlattices from a Twin-Tapered Dendritic Benzamide and Its Four-Cylinder-Bundle Supramolecular Polymer. *Chem. - Eur. J.* **2003**, *9* (4), 921−935.
(63) Percec, V.; Glodde, M.; Peterca, M.; Rapp, A.; Schnell, I.; Spiess, H. W.; Bera, T. K.; Miura, Y.; Balagurusamy, V. S. K.; Aqad, E.; Heiney, P. A. Self-Assembly of Semifluorinated Dendrons Attached to Electron-Donor Groups Mediates Their π-Stacking via a Helical Pyramidal Column. *Chem. - Eur. J.* **2006**, *12* (24), 6298−6314.
(64) Percec, V.; Holerca, M. N.; Uchida, S.; Cho, W.-D.; Ungar, G.; Lee, Y.; Yeardley, D. J. P. Exploring and Expanding the Three-Dimensional Structural Diversity of Supramolecular Dendrimers with the Aid of Libraries of Alkali Metals of Their AB_3 Minidendritic Carboxylates. *Chem. - Eur. J.* **2002**, *8* (5), 1106−1117.
(65) Percec, V.; Imam, M. R.; Peterca, M.; Leowanawat, P. Self-Organizable Vesicular Columns Assembled from Polymers Dendronized with Semifluorinated Janus Dendrimers Act As Reverse Thermal Actuators. *J. Am. Chem. Soc.* **2012**, *134* (9), 4408−4420.
(66) Buzzacchera, I.; Xiao, Q.; Han, H.; Rahimi, K.; Li, S.; Kostina, N. Yu.; Toebes, B. J.; Wilner, S. E.; Möller, M.; Rodriguez-Emmenegger, C.; Baumgart, T.; Wilson, D. A.; Wilson, C. J.; Klein, M. L.; Percec, V. Screening Libraries of Amphiphilic Janus Dendrimers Based on Natural Phenolic Acids to Discover Monodisperse Unilamellar Dendrimersomes. *Biomacromolecules* **2019**, *20* (2), 712−727.
(67) Ryu, H.; Sung, J.; Kim, G.; Xu, Y.; Grubbs, R. H.; Choi, T. Cascade Cyclopolymerization of 5-Ethynyl-1,8-Nonadiyne Derivatives to Synthesize Low Band Gap Conjugated Polyacetylenes Containing a Fused Bicyclic Structure. *Angew. Chem., Int. Ed.* **2022**, *61* (45), No. e202210244.
(68) Steglich, W.; Höfle, G. *N,N* -Dimethyl-4-pyridinamine, a Very Effective Acylation Catalyst. *Angew. Chem., Int. Ed.* **1969**, *8* (12), 981.
(69) Neises, B.; Steglich, W. Simple Method for the Esterification of Carboxylic Acids. *Angew. Chem., Int. Ed.* **1978**, *17* (7), 522−524.
(70) Höfle, G.; Steglich, W.; Vorbrüggen, H. 4-Dialkylaminopyridines as Highly Active Acylation Catalysts. [New Synthetic Method (25)]. *Angew. Chem., Int. Ed.* **1978**, *17* (8), 569−583.
(71) May, J. P.; Undzys, E.; Roy, A.; Li, S.-D. Synthesis of a Gemcitabine Prodrug for Remote Loading into Liposomes and Improved Therapeutic Effect. *Bioconjugate Chem.* **2016**, *27* (1), 226−237.
(72) Shalihin, M. I. The Role of the Primary Structure of 3,4,5-Trialkoxy Benzyl as the Hydrophobic Part of One-Component Ionizable Amphiphilic Janus Dendrimers in Activity and Selectivity of Targeted mRNA Delivery. , Master of Chemical Sciences Thesis; Department of Chemistry, University of Pennsylvania2023.

https://doi.org/10.1021/acs.biomac.4c01107
Biomacromolecules 2024, 25, 6871−6882