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Communication

The Constitutional Isomerism of One-Component Ionizable Amphiphilic Janus Dendrimers Orchestrates the Total and Targeted Activities of mRNA Delivery

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ABSTRACT: Constitutional isomerism has been previously demonstrated by one of our laboratories to represent a powerful design strategy for the elaboration of complex functional self-organizations. Here we report the design, synthesis, and characterization of 14 positional, skeletal, and functional constitutional isomeric one-component, multifunctional, sequence-defined, amphiphilic ionizable Janus dendrimers (IAJDs). Their coassembly by simple injection with luciferase mRNA (Luc-mRNA) to form dendrimersome nanoparticles (DNPs) was studied. Subsequently, the resulting DNPs were employed to investigate, with screening experiments, the delivery of Luc-mRNA in vivo. Constitutional isomerism was shown to produce changes of up to two orders of magnitude of the total-body luciferase activity and targeted luciferase activity to the spleen and liver, of up to three orders of magnitude difference in targeted luciferase activity to the lungs and up to six orders of magnitude to lymph nodes. These results indicate that constitutional isomerism may represent not only a simple but also an important synthetic strategy that most probably may impact the activity of all components of synthetic vectors used in RNA-based nanomedicine, including in mRNA vaccines and therapeutics.

Constitutional isomers possess identical molecular formulas but diverse connectivity between their atoms, causing them to exhibit different physical properties. We previously elaborated on constitutional isomerism as the most powerful molecular design methodology to discover[1] and predict[1f,k] the primary functional structure of self-assembling dendrons, dendrimers, and self-organizable dendronized polymers, including dendritic dipeptides.[1i,j] Positional constitutional isomerism discriminated between periodic helical columnar and spherical-based A15[2] and σ[3] Frank−Kasper phases as well as quasiperiodic liquid quasicrystal (LQC) self-organizations[4] (Figure 1a). Constitutional isomerism also selected between supramolecular helical channels and hollow spheres.[1i,j] The same strategy was successful in the design of cogwheel helical self-organizations that disregard chirality.[5] Positional constitutional isomers were employed in Frank−Kasper and quasicrystal investigations,[1−4] while sequence-defined skeletal constitutional isomers were utilized in cogwheel experiments.[5] However, except for several topological experiments,[6a] constitutional isomerism was not employed to design synthetic vectors delivering RNA vaccines and therapeutics.

The leading industrial vector used for the delivery of mRNA-based Covid-19 vaccines is a 4-component ionizable lipid-based nanoparticle (LNP)[6] consisting of laboriously synthesized phospholipids, cholesterol, a PEG-conjugated lipid, and an ionizable amine. Inspired from our previous work on amphiphilic Janus dendrimers (JDs)[7] and sequence-defined Janus glycodendrimers (JGDs),[8] our laboratories developed a 1-component multifunctional sequence-defined ionizable amphiphilic Janus dendrimer (IAJD) to provide a simple methodology to access targeted delivery of mRNA as dendrimersome nanoparticles (DNPs).[9] Both LNPs and DNPs have advantages and disadvantages already discussed in previous publications.[9a−e] Major advantages of IAJDs are derived from known placement of their functional groups in DNPs, their unlimited synthetic capabilities based on uncomplicated, accelerated, orthogonal-modular synthesis, and their compliant assembly into monodisperse DNPs with predictable dimensions via a simple-injection methodology transplanted from JDs and JGDs[7a−e,i,8c] rather than by complex microfluidic and T-tube technologies required by 4-component LNPs.[6] Our accelerated methodology accesses rapid screening in vivo in less time than any other delivery vector. Since previous constitutional isomeric experiments reported from our laboratory involved self-assembling dendrons[1−5] and very few self-assembling amphiphilic JDs,[7a,i] we decided to study the role of constitutional isomerism of IAJDs for in vivo activity of DNPs during Luc-mRNA delivery.

Here we report our investigation of a library of 14 IAJDs exhibiting positional, functional, and skeletal constitutional isomerism. These experiments demonstrated up to 2 orders of magnitude increase in the total-body luciferase (Luc) expression in vivo and in Luc-delivery to the targeted organs

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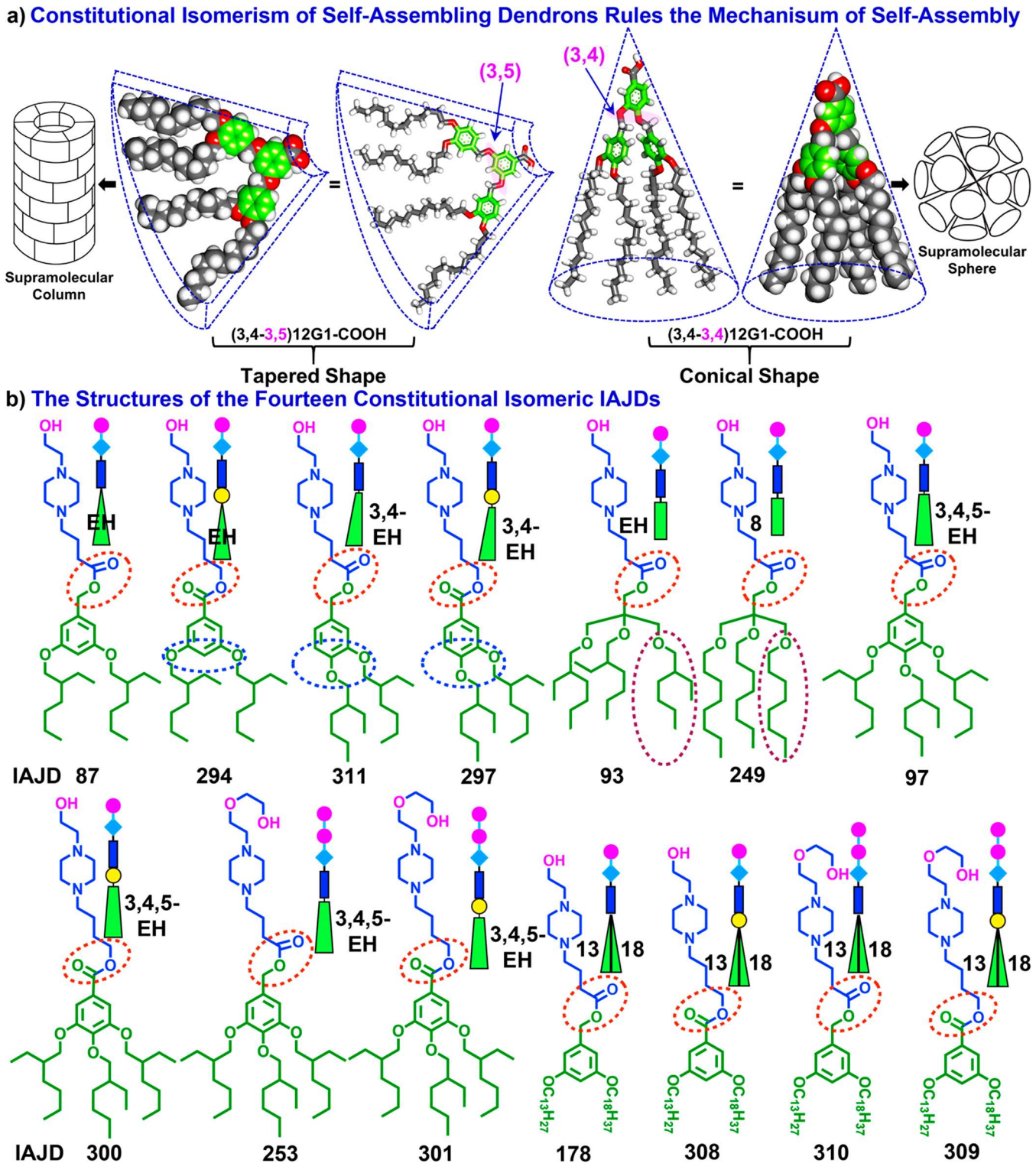

Figure 1. (a) Positional constitutional isomerism in dendron self-organization. (b) Structures of IAJDs containing ionizable amines attached via benzyl and benzoate esters to hydrophobic fragments. IAJD number and schematics are shown under and near structures.

(liver and spleen), up to 3 orders of magnitude increase in the delivery to the lungs, and up to 6 orders of magnitude to lymph nodes (LN). Changes of the targeted organ were also observed. Nine new IAJDs (**294**, **297**, **300**, **253**, **301**, **308**, **309**, **310**, and **311**) were designed to generate pairs of constitutional isomers complementary to previously reported but resynthesized IAJDs (**87**,[9b] **93**,[9e] **249**,[9e] **97**,[9b] and **178**[9c]). All were synthesized by accelerated modular-orthogonal procedures in higher than 99% purity as reported.[9b−e] Therefore, IAJD synthesis is discussed in the Supporting Information.

Figures 1b and 2 portray the chemical structures of the investigated constitutional isomeric IAJDs along with their pK_a values, dimensions and polydispersities of their DNPs coassembled with Luc-mRNA by injection,[9a−e] and their corresponding total-body and targeted-organ Luc expression in

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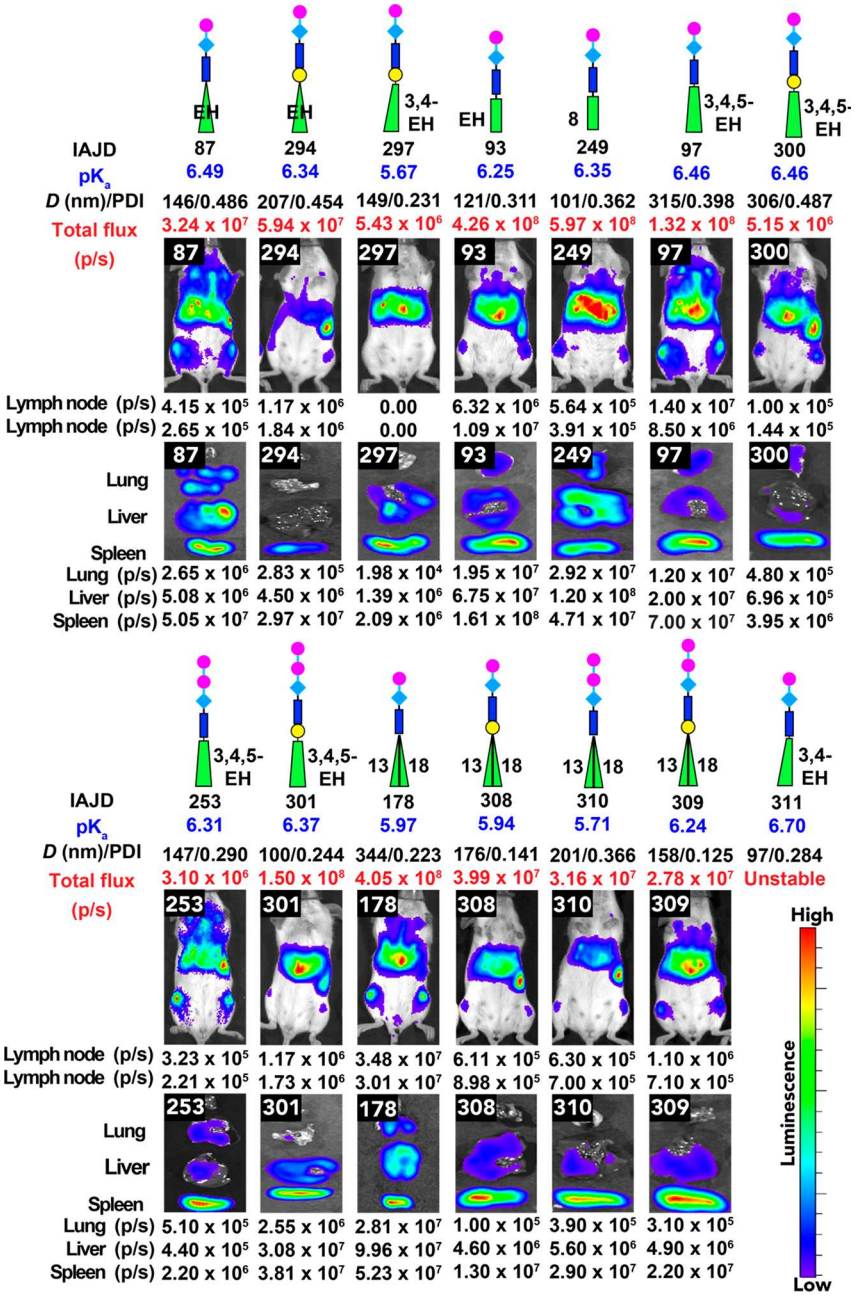

Figure 2. Library of constitutional isomeric IAJDs shown with corresponding pK_a values, along with DNPs coassembled with Luc-mRNA and their dimensions and polydispersities. Total-body and targeted-organ Luc-mRNA activities were determined 4 h post intravenous (iv) injection (SI).

vivo determined as previously described.[9e] Note that Luc activity is determined by whole-body mouse scanning, and individual organ Luc activity is determined after removal. This results in summation of organ Luc activities not equaling the total-body Luc activity (Figures 2 and S1). Functional constitutional isomers are based on benzyl and benzoate ester groups. These groups are marked in dotted red ovoidal contours. **IAJDs 87** with **294**, **97** with **300**, **253** with **301**, **178** with **308**, and **309** with **310** are pairs of functional constitutional isomers. Constitutional positional isomers or regioisomers are **IAJDs 294** and **297**. Their positional isomeric groups are 3,5- and 3,4-disubstituted benzoate esters, marked with dotted blue ovoidal frames. Chain or skeletal constitutional isomers are **IAJDs 93** with **249** and are marked with dotted brown ovoidal patterns.

Reversing the ester group of the functional constitutional isomer **87** from a flexible benzyl to rigid benzoate (**294**) increased total-body Luc activity of **294** from 3.24×10^7 (for **87**) to 5.94×10^7 (for **294**) while maintaining major targeted delivery to the spleen with reverse Luc activity. Both **87** and **294** also deliver to LNs, with higher Luc activity exhibited by **294** (10^6 for **294** vs 10^5 for **87**). Changing the positional constitutional isomerism of **294** from 3,5- to 3,4- for **297** while maintaining benzoate ester and 2-ethylhexyl alkyl groups in the hydrophobic parts decreased total-body Luc activity from 5.94×10^7 for **294** to 5.43×10^6 for **297**. This modification is also reflected by a corresponding reduction of Luc expression in the spleen activity from 2.97×10^7 for **294** to 2.09×10^6 for **297**. This makes Luc activities in the liver and spleen for **297** almost identical (10^6). The Luc expression in LNs of **294** is 10^6, while

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that of **297** is 0.00. A total change of about 1 order of magnitude in Luc expression in the whole body and also in the spleen was observed when comparing **87** with **294** and **294** with **297**. We next compared the chain or skeletal constitutional isomers **93** with **249**. The whole-body Luc expression for **93** and **249** is 10^8. However, for **93**, the spleen exhibits the highest targeted-organ Luc expression (1.61×10^8), whereas for **249**, the liver has the highest Luc expression (1.20×10^8). Additionally, Luc expression in LN is approximately 10^7 for **93**, compared to about 10^5 for **249**, representing a remarkable change in Luc expression among the three targeted organs: spleen, liver, and LN.

To confirm the impact of IAJDs' conformational isomerism on Luc expression and organ targeting, we decided to investigate four additional pairs of functional constitutional isomers. The benzyl ester **97** exhibits a whole-body Luc expression of 1.32×10^8, with significant Luc expression in LN (1.40×10^7 and 8.50×10^6). The isolated spleen, liver, and lung exhibit Luc expression levels of 7.00×10^7, 2.00×10^7, and 1.20×10^7, respectively. Reversing the benzyl ester of **97** into benzoate ester for **300** reduces the whole-body Luc expression to 5.15×10^6, with LN Luc activity dropping to 1.00×10^5 and 1.44×10^5. Isolated spleen also exhibits a reduced Luc expression of 3.95×10^6. Replacing the 1-(2-hydroxyethyl)piperazine ionizable amine of **97** and **300** with 1-2(2-hydroxyethoxy)ethyl piperazine provided the constitutional isomeric **253** and **301**, respectively. The benzyl ester **253** exhibits a total-body Luc activity of 3.10×10^6 with the highest organ Luc activity of 2.20×10^6 for the spleen. Its constitutional isomeric benzoate ester **301** displays a total-body luciferase activity of 1.50×10^8 with the highest organ Luc activity of 3.81×10^7 for spleen and 3.08×10^7 for liver. The LNs of both IAJDs **253** and **301** exhibited 1 order of magnitude higher Luc activity (10^6 vs 10^5) for **301**. This experiment demonstrated that constitutional isomerism of the ester group dramatically affects total-body Luc activity of IAJD regardless of the structure of its ionizable group. In the next experiments, we changed the ionizable amine from 1-2(2-hydroxyethoxy)ethyl piperazine back to 1-(2-hydroxyethyl)-piperazine and the hydrophobic part of the IAJD from symmetric as it was the case for **87**, **294**, **297**, **93**, **249**, **97**, **300**, **253**, and **301** to nonsymmetric by employing a combination of octadecyl and tridecyl alkyl groups to produce **178** and **308**. **IAJD 178** showed a total-body Luc activity of 4.05×10^8, with a highest organ Luc activity of 9.96×10^7, nearly 10^8, observed in liver. In contrast, **308** exhibited a total-body Luc activity of 3.99×10^7, with the highest Luc activity of 1.30×10^7 in spleen. It is interesting to observe that the Luc activities in the spleen, liver, and lung are also in the range of 10^7 (5.23×10^7, 9.96×10^7, and 2.81×10^7) for **178**. The activity of 308 to the lung was only 1.00×10^5, which is a difference of two orders of magnitude. Very high Luc activities in LNs of **178** (3.48×10^7 and 3.01×10^7) were observed, while two orders of magnitude lower LN-Luc activities were detected for **308** (6.11×10^5 and 8.98×10^5).

Reversing back from the 1-(2-hydroxyethyl)piperazine ionizable amine to the 1-2(2-hydroxyethoxy)ethyl piperazine while maintaining the nonsymmetric hydrophobic part, we generated the pair of constitutional isomeric **IAJDs 309** and **310**. **IAJD 309** displayed the maximum total-body Luc activity of 2.78×10^7, with Luc activity of 2.20×10^7 in spleen, 4.90×10^6 in liver, and 3.1×10^5 in lungs, as well as 1.10×10^6 and 7.10×10^5 in LNs. **IAJD 310** exhibits only slightly higher total and targeted activities than **309**, supporting that a more flexible ionizable amine increases the rate of assembly of the benzoate ester close to that of the benzyl ester. DNPs of **311**, the constitutional isomer of **297**, are, as expected from the bilayer assembly discussion that follows, unstable and unable to be investigated in vivo.

Figures 3 and S1 illustrate a quantitative comparison of all data from Figure 2, including the statistical analysis. Excellent

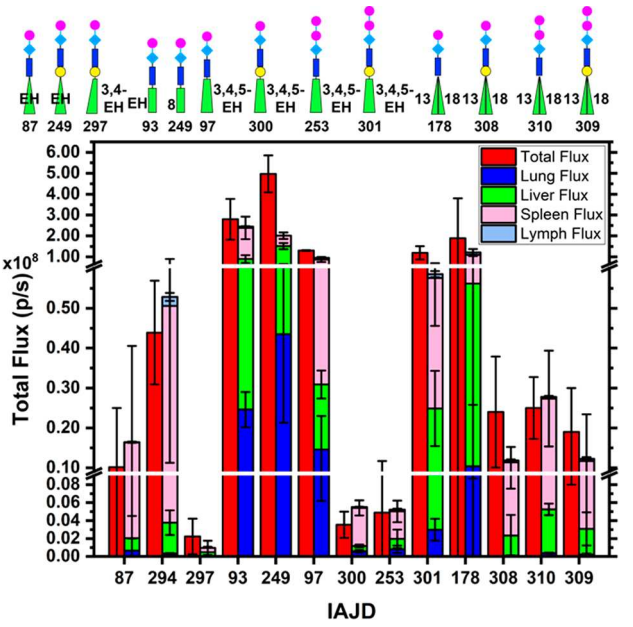

Figure 3. Comparison of total-body flux and targeted-organ Luc activities of DNPs assembled from constitutional-isomers-based IAJDs demonstrating the structure–activity dependence.

reproducibility of in vivo results was observed for most IAJDs (Figure S2), although these are only screening experiments. A brief inspection of Figure 3 indicates that **IAJDs 93**, **97**, **249**, **301**, and **178** exhibit total-body flux values close to the highest current LNPs that are in the range of 10^9 to the liver.[9a−e] However, most efficient delivery vectors for vaccines must deliver to LNs or, preferably, to spleen and LNs, as is the case for **93**, **97**, **249**, **301**, and **178**. It is interesting to remark that **93** and **249** are skeletal constitutional isomers, **301** is the functional isomer of **253**, while **178** and **97** are the functional constitutional isomers of **308** and **300**. Notably, the benzoate ester **301** exhibits higher Luc activity compared to its benzyl ester counterpart, **253**. At the same time, **178** is a benzyl ester that shows higher Luc activity than its constitutional isomeric benzoate **308**. This trend is not statistically significant. All benzoate ester constitutional isomers containing the 1-2(2-hydroxyethoxy)ethyl piperazine as ionizable amine exhibit higher activity than their benzyl esters with the same ionizable amine. However, all IAJDs containing the 1-(2-hydroxyethyl)-piperazine ionizable amine exhibit higher Luc activity as benzyl esters compared to benzoate esters (e.g., **87** vs **294**, **249** vs **93**, **97** vs **300**, and **178** vs **308**). This could be due to increased overall flexibility of the benzoate IAJDs containing the more flexible 1-(2-hydroxyethyl)piperazine ionizable amine group. IAJDs with benzoate ester form more thermodynamically stable but slower self-assembling bilayers than their counterparts with benzyl esters (Figure 4). The complementarity between kinetics and thermodynamics is seen in Figure 4,

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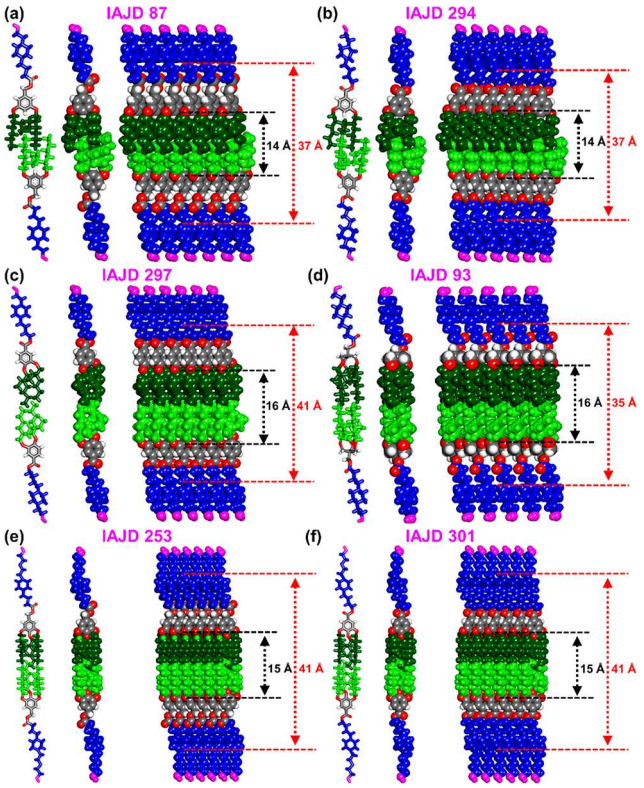

Figure 4. Molecular models of bilayers assembled from (a) IAJD 87, (b) IAJD 294, (c) IAJD 297, (d) IAJD 93, (e) IAJD 253, and (f) IAJD 301 with Supplemental Movies 1−6 in the SI, respectively.

supporting previous models for other IAJDs.[9d,e] The bilayers of DNPs from **87** and **294** are partially interdigitated in the hydrophobic part that leads to higher bilayer stability but slower assembly. In contrast, end-to-end assembly of **297**, **93**, **253**, and **301** eliminates this kinetic requirement, reaching equilibrium faster,[10] although bilayer stability is higher for three and lower for two alkyl groups.[9e] **IAJDs 297**, **308**, and **309** show higher bulk viscosity, likely due to their hydrophobic interdigitation[9d,e] making their assembly slow despite high bilayer stability.

In conclusion, the results reported here demonstrated that constitutional isomerism represents an important synthetic methodology to orchestrate the total-body and targeted-organ activities of 1-component IAJDs coassembled with Luc-mRNA into DNPs (see cryo-TEM in Figure S8) and to endow the stability of the hydrophobic part of their bilayer. Although the results reported here are screening experiments and, therefore, have not been optimized for formulation, they demonstrated access to a simple, if not the simplest, synthetic strategy to engineer total-body and targeted activities to the spleen, liver, lung, and LNs of DNPs with up to 6 orders of magnitude. These results are complementary to experiments with achiral symmetric and chiral nonsymmetic constitutional isomeric JDs based on glyceride,[7i] indicating that constitutional isomerism must be taken into consideration when designing individual parts of 4-component LNPs including phospholipids, lipid-conjugated-PEG, ionizable amines,[6] as well as other vectors.[9f−m]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c13569.

Experimental methods; complete experimental details for the synthesis and structural characterization of 10 IAJDs with ^{1}H NMR, ^{13}C NMR, HPLC, and MALDI-TOFMS data; dimensions and polydispersities of their DNPs with mRNA and original DLS traces; all pK_a measurements and calculations; representative cryo-TEM images; supplemental references (PDF)

Supplemental Movie 1 (MP4)

Supplemental Movie 2 (MP4)

Supplemental Movie 3 (MP4)

Supplemental Movie 4 (MP4)

Supplemental Movie 5 (MP4)

Supplemental Movie 6 (MP4)

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Notes

The authors declare no competing financial interest.

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