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Communication

The Unexpected Importance of the Primary Structure of the Hydrophobic Part of One-Component Ionizable Amphiphilic Janus Dendrimers in Targeted mRNA Delivery Activity

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ABSTRACT: Viral and synthetic vectors for delivery of nucleic acids impacted genetic nanomedicine by aiding the rapid development of the extraordinarily efficient Covid-19 vaccines. Access to targeted delivery of nucleic acids is expected to expand the field of nanomedicine beyond most expectations. Both viral and synthetic vectors have advantages and disadvantages. The major advantage of the synthetic vectors is their unlimited synthetic capability. The four-component lipid nanoparticles (LNPs) are the leading nonviral vector for mRNA used by Pfizer and Moderna in Covid-19 vaccines. Their synthetic capacity inspired us to develop a one-component multifunctional sequence-defined ionizable amphiphilic Janus dendrimer (IAJD) delivery system for mRNA. The first experiments on IAJDs provided, through a rational-library design combined with orthogonal-modular accelerated synthesis and sequence control in their hydrophilic part, some of the most active synthetic vectors for the delivery of mRNA to lung. The second experiments employed a similar strategy, generating, by a less complex hydrophilic structure, a library of IAJDs targeting spleen, liver, and lung. Here, we report preliminary studies designing the hydrophobic region of IAJDs by using dissimilar alkyl lengths and demonstrate the unexpectedly important role of the primary structure of the hydrophobic part of IAJDs by increasing up to 90.2-fold the activity of targeted delivery of mRNA to spleen, lymph nodes, liver, and lung. The principles of the design strategy reported here and in previous publications indicate that IAJDs could have a profound impact on the future of genetic nanomedicine.

Efficient delivery of nucleic acids by viral[1] and synthetic[2] vectors impacted extraordinarily genetic nanomedicine as seen by the success of Covid-19 vaccines.[3] Recent perspectives, reviews[4] and a publication from our laboratory[5] summarize advantages and disadvantages of viral and nonviral vectors. Four-component lipid nanoparticles (LNPs)[6] consisting of ionizable lipids,[6a,7] phospholipids,[6b,c] cholesterol and a PEG-conjugated lipid[6a,b,7] represent the state-of-the-art technology employed by Pfizer[3d] and Moderna[3e] in their vaccines. Statistical distribution of the four components in the LNPs contributes to some of their limitations. The segregation of the neutral ionizable lipid as droplets in the core of LNPs,[8] the "PEG dilemma,"[9] and their optimal stability only at low temperatures (−70 °C) limit their efficacy. One-component ionizable amphiphilic Janus dendrimers (IAJDs) elaborated recently in our laboratory rely on the precise composition and sequence of its components and are stable at 5 °C.[5] One-component IAJDs do not require microfluidic or T-tube technology employed by four-component LNPs to coassemble with mRNA. One-component systems coassemble with mRNA into dendrimersome nanoparticles (DNPs) with 97% nucleic acid encapsulation efficiency by simple injection of their ethanol solution into an acidic buffer containing mRNA rather than by the microfluidic methodology required by LNPs. The original architecture of one-component IAJDs[5] was inspired from the structure of amphiphilic Janus dendrimers (JDs),[10] Janus glycodendrimers (JGDs),[11] and sequence-defined JGDs self-assembling by simple injection of their ethanol solution into water or buffer producing monodisperse vesicles known as dendrimersomes and glycodendrimersomes with predictable dimensions.[10b] Methoxyoligooxyethylene and oligooxyethylene fragments were originally employed to design their multifunctional sequence-defined hydrophilic part and generate single−single (SS, single hydrophilic dendron connected to single hydrophobic dendron), twin−twin (TT, two hydrophilic dendrons connected to two hydrophobic dendrons), and twin-mixed (TM, two different hydrophilic dendrons connected to two hydrophobic dendrons) IAJDs.[5] A simplified SS, sSS architecture, resulting in an efficient IAJD, was obtained by eliminating the oligooxyethylene fragments from the hydrophilic dendron while maintaining only the ionizable amine that becomes hydrophilic and active to mRNA binding upon protonation.[12] Hydrophobic dendrons were constructed either from 3,4-, 3,5-, and 3,4,5-substituted phenolic acids or from trisubstituted pentaerythritol containing identical alkyl groups. Sequence-defined JGDs were demonstrated to be extraordi-

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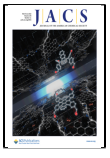

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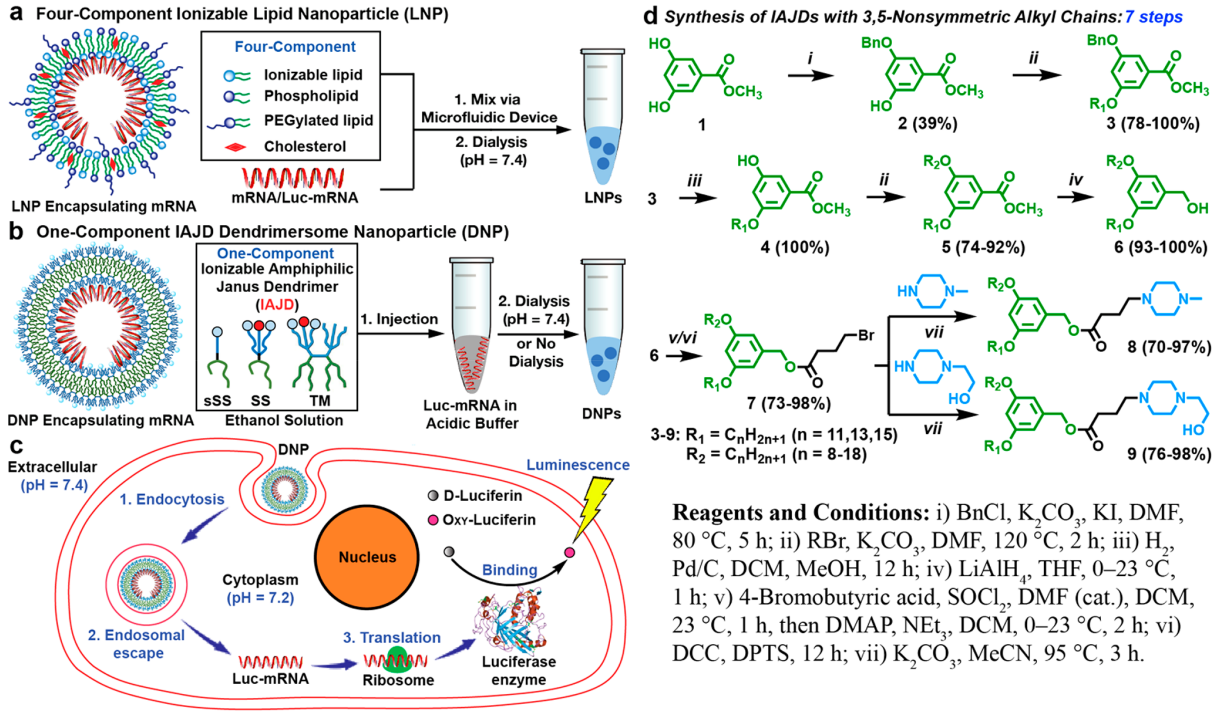

Reagents and Conditions: i) BnCl, K_2CO_3, KI, DMF, 80 °C, 5 h; ii) RBr, K_2CO_3, DMF, 120 °C, 2 h; iii) H_2, Pd/C, DCM, MeOH, 12 h; iv) $LiAlH_4$, THF, 0–23 °C, 1 h; v) 4-Bromobutyric acid, $SOCl_2$, DMF (cat.), DCM, 23 °C, 1 h, then DMAP, NEt_3, DCM, 0–23 °C, 2 h; vi) DCC, DPTS, 12 h; vii) K_2CO_3, MeCN, 95 °C, 3 h.

Figure 1. Schematic representation of four-component LNPs (a), one-component IAJDs based DNPs (b), and the cell transfection mechanism of DNP encapsulating Luc-mRNA (c). Synthesis of IAJDs containing 3,5-nonsymmetric alkyl groups (d).

narily important in providing the highest activity of their glycan when binding to sugar-binding proteins known as lectins and galectins.[11] Sequence-defined self-assembling dendronized perylene bisimides (PBIs) were also demonstrated to accelerate their rate of self-organization proceeding by a cogwheel mechanism accompanied by deracemization in the crystal state.[13] In addition, phospholipids forming the bilayer of the natural cell membranes are generated by a non-symmetric substitution of glycerol with alkyl groups containing double bonds[14,15] which provides a dissimilar chain length in the bilayer even for identical alkyl groups.

The mechanism of self-assembly of JDs into DNPs was shown to be preferred by the 3,5- rather than 3,4- or 3,4,5-substitution since 3,5-substitution favors interdigitation of the alkyl groups in the bilayer.[10b] These literature data prompted us to advance the hypothesis that sequence-defined arrangement in the hydrophobic part of IAJDs could also be influential, as was the hydrophilic part, on the activity of targeted delivery of mRNA mediated by IAJDs.[5] Therefore, we decided to screen selected examples of simplified IAJDs from the second library[12] and of SS IAJDs from the first libraries of IAJDs[5] by replacing their identical alkyl groups with dissimilar groups in their hydrophobic part and test their *in vitro* and *in vivo* delivery of mRNA. Figure 1a outlines the structure and coassembly with mRNA of LNPs.[6] Figure 1b shows the structures of sSS, SS, and TM IAJDs with dissimilar and similar alkyl groups in their hydrophobic parts and their coassembly with mRNA. Figure 1c illustrates the mechanism currently considered for the *in vivo* delivery of mRNA with both four-component LNPs and with one-component IAJDs. This mechanism involves endocytosis of the LNP or DNP, followed by endosomal escape of mRNA and synthesis of a protein in collaboration with ribosomes. Since normal mRNA experiments can require an extended duration of time per experiment especially vaccines, we employed Firefly Luciferase mRNA (Luc-mRNA) that provides important results in several hours.[5,12] In this case, the protein translated in the cell is the Luciferase enzyme that interacts with D-Luciferin to generate oxy-Luciferin emitting light that identifies the organs targeted. Figure 1d shows the synthesis of nonsymmetric IAJDs. In the first step the 3-benzyl ether of 3,5-dihydroxy methyl benzoate was produced in 39% isolated yield in 5 h by etherification of **1** with BnCl at 80 °C in DMF. Subsequently **2** was alkylated with 1-bromoundecane or 1-bromopentadecane in DMF, with K_2CO_3 base at 120 °C to produce a 78−100% isolated yield of **3**. Hydrogenolysis of **3** (H_2/Pd, DCM/MeOH, 12 h) produced **4** in 100% isolated yield. Alkylation of **4** with varying alkyl lengths from 1-bromooctane to 1-bromooctadecane in DMF, with K_2CO_3 base at 120 °C, generated the non-symmetric compounds **5** in 74−92% isolated yield. Reduction of compounds **5** with $LiAlH_4$ in THF (0−23 °C, 1 h) produced the benzyl alcohols **6** in 93−100% isolated yield. Compounds **6** were reacted with 4-bromobutyric acid either via its acid chloride generated with $SOCl_2$ catalyzed by DMF in CH_2Cl_2 at 23 °C followed by esterification in the presence of NEt_3/DMAP (0−23 °C, 2 h) or by direct esterification with DCC/DPTS in 12 h to produce compounds **7** in 73% to 98% isolated yield. Compounds **7** were reacted with methylpiperazine or hydroxyethyl piperazine, selected for synthetic simplicity (K_2CO_3, MeCN, 95 °C, 3 h), to yield IAJDs **8** (70−97% isolated yield) and **9** (76−98% isolated yield). Their purity by a combination of HPLC, MALDI-TOF, and NMR was higher than 99%. Their structures are shown in Figure 2 (IAJDs 113 to 178). IAJD133 has a similar structure with IAJD105 except that the interconnecting ester group of 105 was replaced with an amide in 133. The benzyl amine

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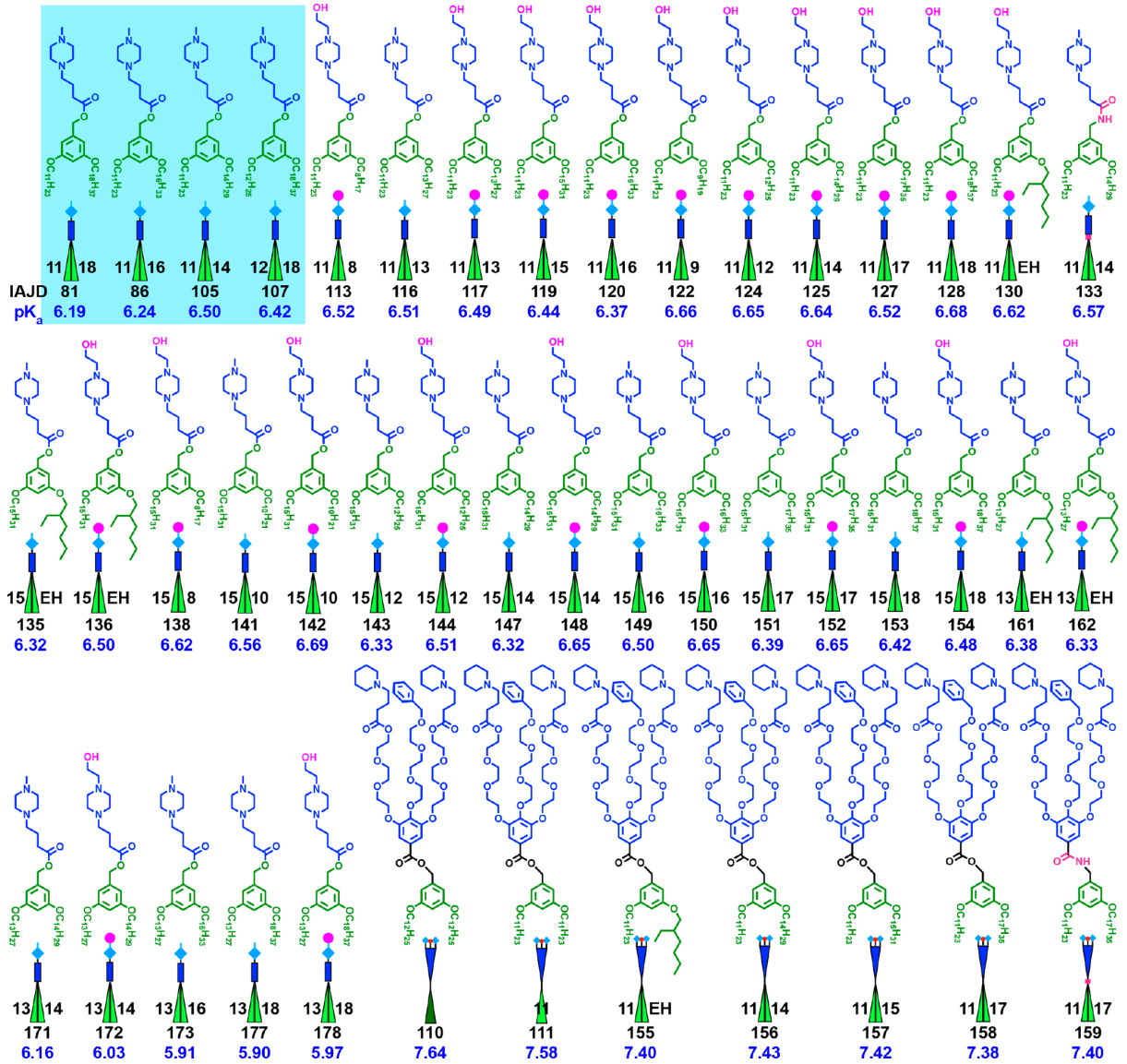

Figure 2. Structures of IAJDs with nonsymmetric alkyl chains. IAJD numbers and pK_a values are shown under corresponding schematic representations of IAJDs.

precursor of 133 was generated from the corresponding benzyl alcohol via its benzyl chloride obtained with $SOCl_2$ followed by reaction with K-phthalimide and subsequently hydrazine as reported[5,12] and shown in Scheme S1. Few single−single (SS) IAJDs reported previously to display very high activity for delivery to lung[5] were also synthesized with nonsymmetric alkyl groups in their hydrophobic part. They are IAJDs 110 to 159 from Figure 2.

Their sequence-defined hydrophilic dendrons were synthesized as reported.[5] The hydrophilic dendrons were reacted with selected nonsymmetric hydrophobic dendrons **6** or their amine as shown in Scheme S4. Within the rest of this report, we will refer to IAJDs by their number followed by the ratio between their two alkyl groups forming their nonsymmetric hydrophobic part. This nomenclature together with their entire and schematic structures shown in Figure 2 will facilitate the discussion on their *in vitro* and *in vivo* activity vs molecular structure. For example, 116(11/13) and 117(11/13) both contain a combination of 11 and 13 carbons in their hydrophobic part but 116 contains a methyl piperazine while 117 a hydroxyethyl piperazine ionizable amine. The large red dot on the top of the cartoon for 117 refers to hydroxyethyl while the blue thin line on 116 indicates the methyl group, both attached to piperazine (Figure 2). A combination of 33 IAJDs sSS with 7 IAJDs SS is shown in Figure 2. IAJDs 81, 86, 105, 106, and 107 marked in blue on top left corner of Figures 2 and S1 were reported previously.[12] *They did not generate lower transfection activities vs their symmetric IAJDs and, therefore, encouraged us to perform the experiments reported here*. Transfection experiments with Luc-mRNA were performed both *in vitro* and *in vivo* by following the methodology reported.[5,12] The overall transfection activity *in vivo* was analyzed according to its target selectivity and organized in Figure 3. The first important result of the

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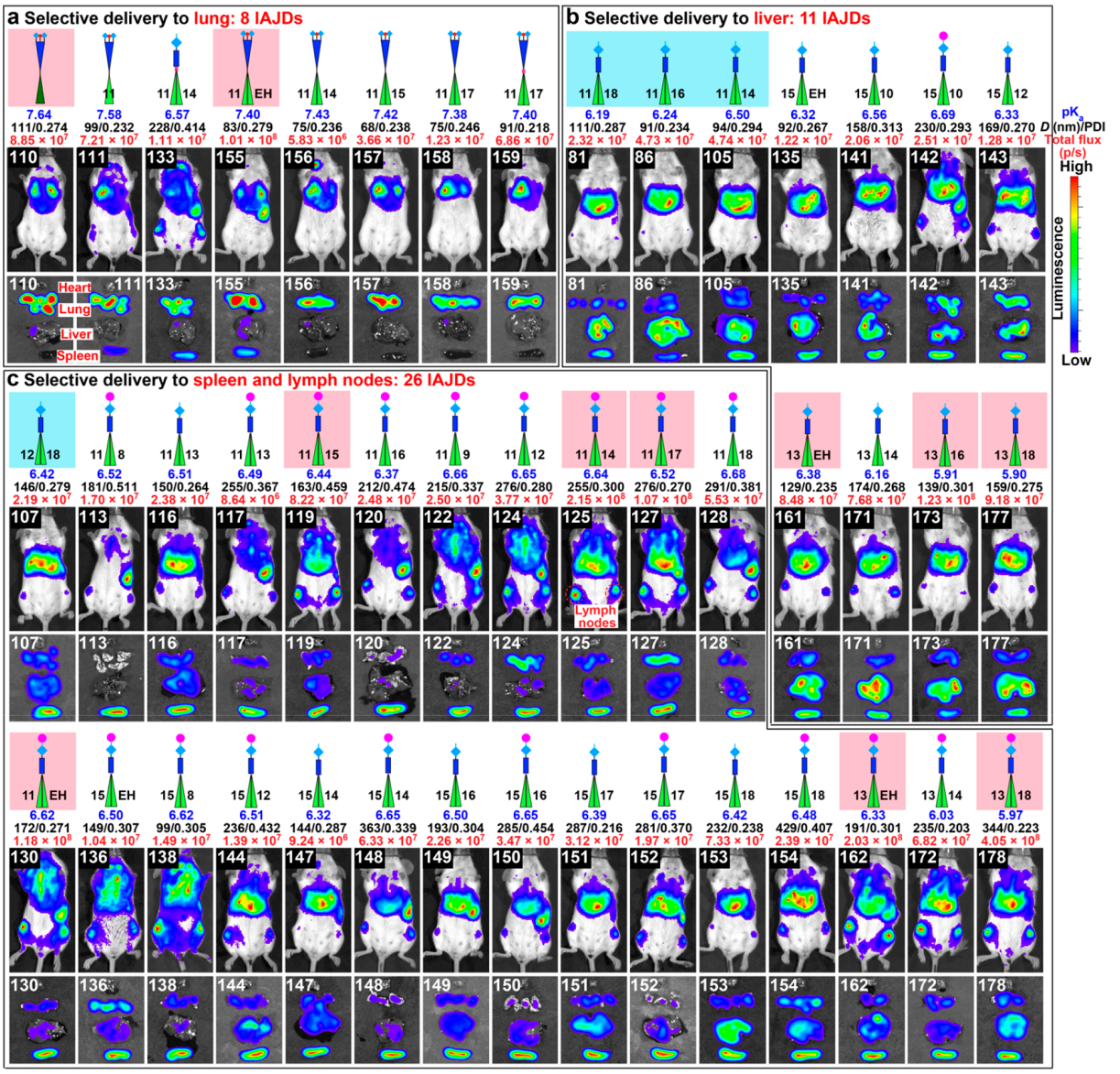

Figure 3. Selective delivery of Luc-mRNA *in vivo* by DNPs to (a) lung, (b) liver, (c) spleen and lymph node organs . Previously published IAJDs[12] are in light blue; IAJDs with about 10^8 activity are in pink.

transfection experiments is that 11 IAJDs show approximately 10^8 activity, 2 for lung, 3 for liver, and 6 for spleen and lymph (Figure 3a–c marked in pink, Figure S1). The symmetric IAJDs 110(12/12) and 111(11/11) were previously reported to show the highest activity to lung as IAJDs 33(12/12) and 34(11/11) from the first publication[5] when they contained an amide interconnecting group. The new IAJDs 110(12/12) and 111(11/11) containing an interconnecting ester rather than an amide group show also very high activity compared to lung.

Nonsymmetric IAJDs are stable in serum and PBS buffer and exhibit very high activity in lung by a mechanism different from aggregation (Tables S7, S8, Figures S9, S10, S17). The highest activity of all IAJDs is for 178(13/18), which displays a total flux luminescence of 4.05×10^8 p/s, which is 90.2 times higher than that of symmetric 99 with the same headgroup (18/18) and only 4.2 times lower than that of MC3 (Figure 4, Table S5). It is also important to mention that the transition from 158(11/17) to 159(11/17), the second being an IAJD containing an amide interconnecting group, while the first was an ester, increased activity about 6-fold. This demonstrates the significance of an amide interconnecting group for the delivery to lung but simultaneously reveals that the presence of oligooxyethylenes in the hydrophilic part is required for targeted delivery to lung. Future investigations on the role of oligooxyethylenes are, therefore, required and ongoing. These experiments demonstrate the important role of the dissimilar alkyl groups from the hydrophobic part of IAJDs. Most probably, this report provides also the largest number of synthetic vectors from the literature producing specific delivery to such a diversity of organs. Research in progress demonstrated that replacing Luc-mRNA with different virus mRNA delivered with IAJDs provided high antibody activity

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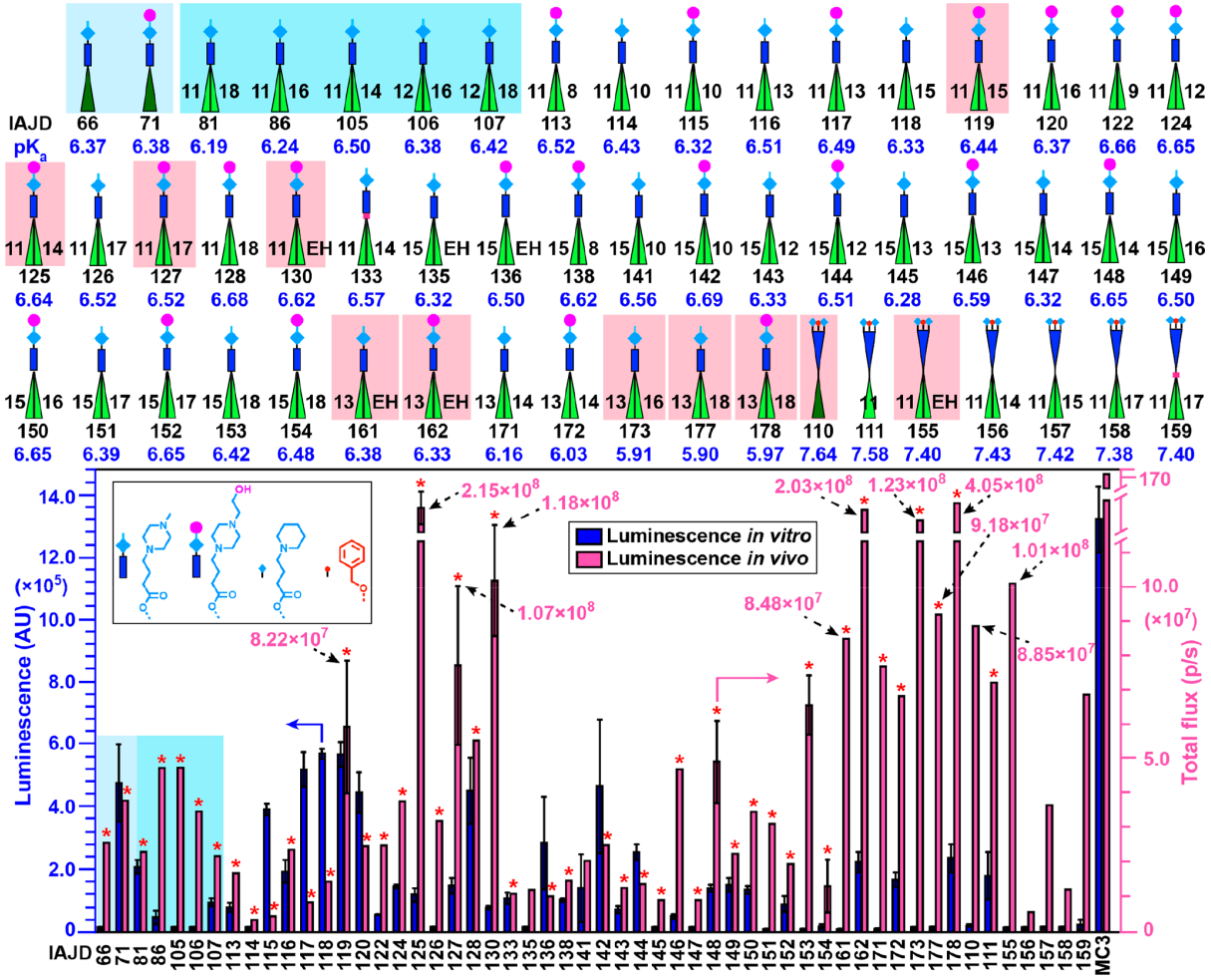

Figure 4. Comparison of the activities of DNPs assembled from the IAJDs, shown schematically in the top of the figure, *in vitro* (in blue) and *in vivo* (in red). Light blue[12] and blue[12] were previously published IAJDs. The red asterisk (*) indicates additional delivery of Luc-mRNA to lymph nodes.

compared to commercial vaccines based on four-component LNPs. Finally, Figure 4 summarizes the activity of all IAJDs reported here and compares it with the very few symmetric (marked in light blue) and nonsymmetric (marked in blue) IAJDs reported previously,[12] which can be used as control experiments.

The results from Figure 4 are remarkable, in that an increase of up to 90.2 times in the activity of the IAJDs was observed by changing their primary structure in the hydrophobic part from symmetric to nonsymmetric (Figure S2). This provides an unexpectedly promising new molecular design principle that must be elucidated with all other combinations of alkyl groups in 3,5-, 3,4-, and 3,4,5-substituted phenolic acids employed in their hydrophobic part. In the previous symmetric sSS IAJDs, the largest *in vivo* activity was in the mid-10^7 total flux, p/s range. In Figures 4 and S2, and Tables S3−S5, we see 11 IAJDs that exhibit a total flux, p/s in the range of 10^8. Ratios between the two-alkyl lengths, preferably from odd−even combinations, equal to or larger than 3 and less than 7 seem to result in the largest increase in activity. Selected examples of IAJDs supporting this conclusion are in Figures 4, S2, Table S5. Exceptions from this rule are also available, and therefore, we intend to develop a Periodic Table of IAJDs similar to those already elaborated for proteins[16a,c] as well as supramolecular[16d] and covalent dendrimers.[16e,f] This will provide a platform to aid the development of complex[17] genetic nanomedicine based on mRNA and will be important also for the fields of cell and synthetic cell biology. The current status of this Periodic Table of IAJDs correlating primary structure to activity is shown in Figure S18.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c00273.

Experimental methods, synthesis and structural characterization of all 47 IAJDs, the dimensions and polydispersities of their DNPs with Luc-mRNA, *in vitro* and *in vivo* transfection data of all DNPs, DLS curves of DNPs, stability characterization of DNPs, pK_a measurements and supporting references (PDF)

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Notes

The authors declare no competing financial interest.

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