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Linear versus Branched poly-Lysine/Arginine as Polarity Enhancer Tags

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The design and synthesis of Lys- and Arg-containing peptide as solubilizing tags were studied to evaluate their influence on polarity. The relevance of spatial arrangement of polar groups, in α- or ε-amino positions, was confirmed by chromatographic analysis of a rational PolyLys-based synthesized structure. The most promising of the solubilizing tags here analyzed was conjugated to a commercial water-insoluble drug (Indomethacin) as prove of concept.

The synthesis, purification, and manipulation of biological active molecules are often jeopardized by the poor polarity of the substrates, which is translated into low solubility for small molecules, peptides, and proteins alike. In the case of small molecules, a clear example is the preparation of antibody drug conjugates (ADCs). For the preparation of these molecules, the conjugation of the antibody and the drug in the final step must be performed in aqueous medium. With respect to peptides, the solubility of these molecules can be increased by introducing temporary chemical modifications such as *N*-(2-acetoxy-4-methoxy)benzyl (acyl-Hmb)[1,2] or *O*-acylisoacylpeptides;[3,4] however, these strategies are not straightforward. Another alternative is the introduction of solubilizing tags, mostly derived from oligoethylenglycol or polycationic peptides. The former do not always render the desired solubility.[5] The groups of Aimoto,[6] Kent,[7] and Brimble[8] used $(Arg)_{5/6}$ as part of the thioester moiety to increase the solubility of peptide fragments in a chemical ligation strategy. Furthermore, the groups of Englebretsen,[9] Wade,[10] and Brimble[11] attached $(Gly\text{-}Arg)_4$ and $(Lys)_5$ via a base linker to the *C*-terminus of the poorly soluble peptide. Although Kuroda[12] and co-workers reported that polyArg confers slightly more solubility than its Lys counterpart to the protein Bovine Pancreatic Trypsin Inhibitor-22 (BPTI-22), which contains 22 Ala residues, we were intrigued whether this was also valid for a peptide and, more importantly, by the contribution of the spatial arrangement disposition (linear *vs.* branched) to enhancing solubility. To evaluate the polarity effect, several tags were assembled stepwise by the solid-phase technique on a di-naphthylalanine [H-$(Nal)_2$-NH_2, **1**] moiety, which was selected as a non-polar molecule. By means of a Fmoc/*t*Bu strategy, all the peptide sequences proposed (Fig. 1) were synthesized onto a Rink amide polystyrene resin and using DIPCDI/OxymaPure® as coupling reagents. All couplings were checked by the Kaiser test. Peptides were analyzed by HPLC to determine the difference in polarity.

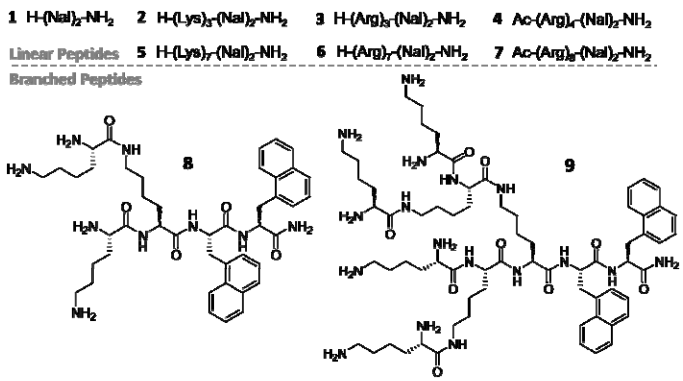

Fig. 1 Chemical structure of the peptides synthesized

First, we performed a comparative study with the incorporation of four amino or four guanidinium groups, which were introduced in four peptides with distinct structures. The amino groups were introduced through Lys, resulting in compounds **2** (linear) and **8** (branched), while guanidinium groups were introduced through Arg,

corresponding to peptides **3** and **4** (both linear). Figure 2a shows the superposed HPLC chromatograms. As expected, all polycationic peptides conferred more polarity to the -(Nal)$_2$ moiety. However, differences were noted depending on the nature of the tag. Thus, Lys-containing peptides induced greater enhancement of polarity than those holding Arg. In addition, branched peptide **8** (t_R = 4.9 min) showed a slightly greater polarity than the linear peptide **2** (t_R = 5.1 min).

In a second experiment, and looking for a confirmation of the first results, we increased the number of cationic groups to **8**. Three linear sequences matching peptides **5** (Lys), **6** and **7** (Arg) and one branched peptide **9** (Lys) were synthesized. Analogously to the first set of peptides, PolyLys conferred higher polarity than PolyArg (Fig. 2b). Most importantly, the branched peptide (**9**, t_R = 4.2 min) gave much more polarity than its equivalent linear peptide (**5**, t_R = 6.8 min), thus confirming the trend observed with the first generation of peptides.

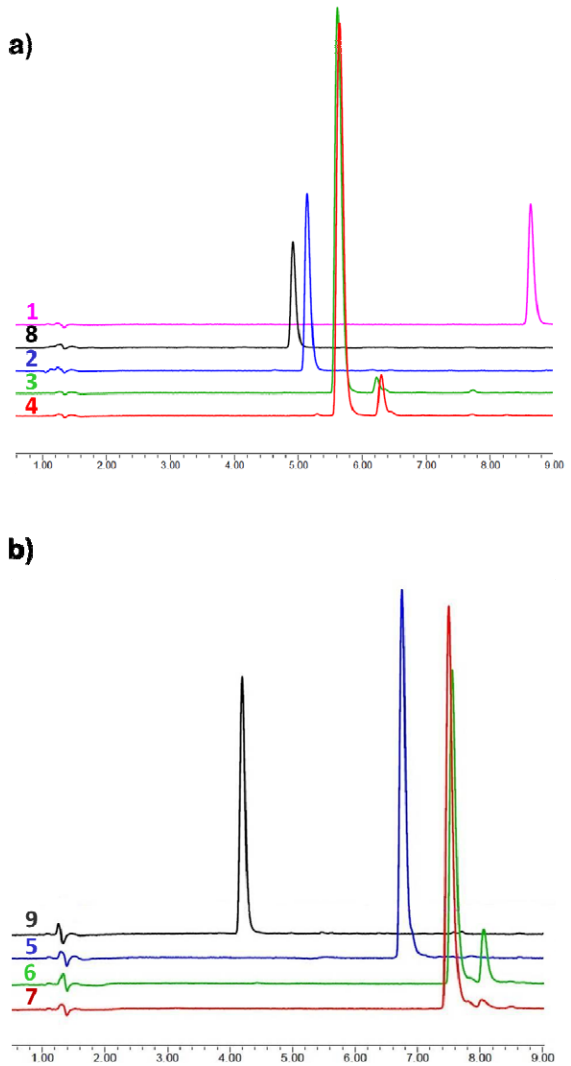

Fig. 2 Superposition of HPLC chromatographic profiles of: (a) first generation peptides (**1**, **2**, **3**, **4**, **8**); and (b) second generation peptides (**5**, **6**, **7**, **9**). Study of polarity influence of amino and guanidinium groups in linear (**2**, **3**, **4**, **5**, **6**, **7**) and branched (**8**, **9**) Poly-AA sequences.

Encouraged and intrigued by the large differences in the behaviour of PolyLys compounds **5** and **9** and taking into account that peptide **5** contains 7 ε- and 1 α-amino groups and **9** bears 4 ε- and 4 α-, we synthesized the linear PolyLys **10** (Fig. 3), which contains 4 ε- and 4 α-amino groups similarly to **9**.

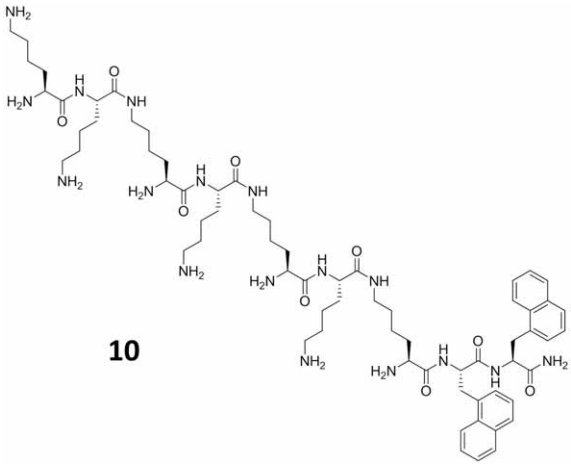

Fig. 3 Chemical structure of a synthesized linear PolyLys peptide.

The synthesis of **10**, which sandwiched α- and ε-amide bonds, was also performed using a Fmoc strategy by sequential addition of Fmoc-Lys(Boc)-OH and Boc-Lys(Fmoc)-OH. Comparative HPLC analysis of **5**, **9**, and **10** (Fig. 4) revealed significant polarity differences depending on the spatial arrangement disposition of the polar moieties. Thus, the two "linear" sequences showed very similar retention times (**5**, t_R = 6.8 min; **10**, t_R = 6.7 min). These times were higher than the most "spherical" structure (**9**, t_R = 4.2 min).

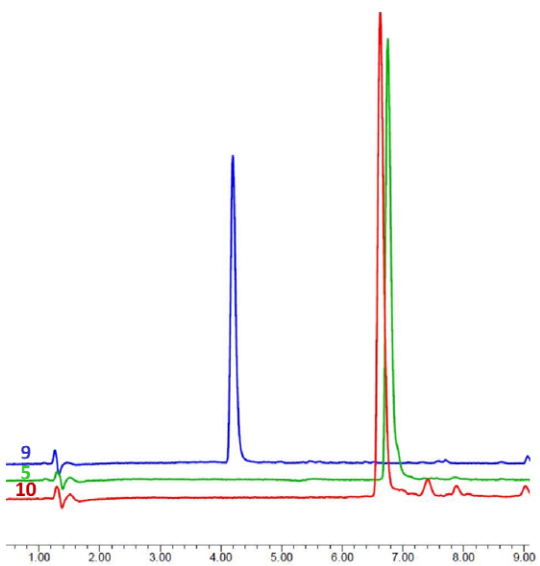

Fig. 4 Superposition of chromatogram analysis by HPLC of three lysine-based peptide sequences of linear- (**5**) and dendron- (**9**, **10**) based structures. Study of both, α/ε type amino groups influence and spatial arrangement disposition.

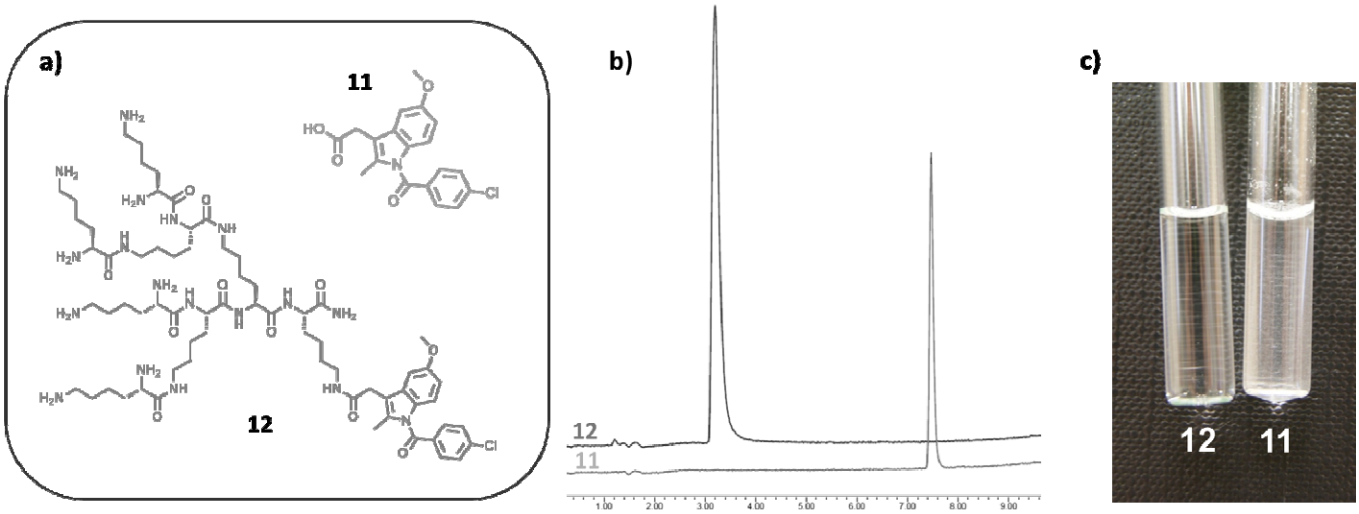

Fig. 5 Indomethacin (**11**) and branched PolyLys peptide conjugated to Indomethacin (**12**) compared by: (a) HPLC chromatography, elution at a linear gradient of 20-90% MeCN containing 0.036% TFA into 0.045% aqueous TFA over 8 min and; (b) solubility in H_2O (0.72 mM) of Indomethacin (dispersion) and conjugated Indomethacin (soluble).

Once the chromatographic results confirmed that the "favorite" PolyLys structure (in terms of substantially increasing the polarity) was the branched shape moiety (**9**), a commercial drug was selected to be conjugated with branched PolyLys. Indomethacin **11** (Fig. 5) is a non-polar drug which provided the water-insolubility feature suitable as a proof of concept to test the polarity/solubility modulation of the PolyLys conjugation. The ligation of both molecules was performed stepwise on solid phase, by incorporating the drug on the side-chain of the *C*-terminal Lys. Once the conjugated PolyLys-Indomethacin was built up on the resin and after its release, **12** (Fig. 5) was purified. HPLC analysis comparison between the standard Indomethacin and the conjugated PolyLys-Indomethacin (Fig. 5a) revealed that the polarity of Indomethacin is impressively increased when it is combined with the branched PolyLys [retention time switches from (**11**, t_R = 7.5 min) to (**12**, t_R = 3.2 min)].

More importantly, the different solubility in water of the two molecules (Fig. 5b) gives the unequivocal evidence of the enhancing polarity power of the branched PolyLys when a non-polar moiety is conjugated to (see ESI† for details).

In summary, linear PolyLys leads to a greater increase in polarity than its Arg counterpart. Moreover, this enhanced effect is much greater when the PolyLys adopts a branched arrangement. The conjugation of branched PolyLys with one commercial drug (Indomethacin) with intrinsic characteristics of insolubility in water media is an example which confirms the capacity of branched PolyLys to increase the polarity and most importantly, the water solubility of a non-polar molecule. We envisage that a branched PolyLys—which in dendrimer chemistry can be considered a dendron—will be crucial as a polarity enhancer tag to facilitate the purification of peptides and/or the manipulation of peptides and small molecules.

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Notes and references

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Electronic Supplementary Information (ESI) available: Materials and methods, characterization of synthesized peptides and solubility analysis. See DOI: 10.1039/c000000x/

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