## **Appendix A: Supporting Materials for**

## 2D J-Correlated Proton NMR Experiments for Structural Fingerprinting of Biotherapeutics

Robert G. Brinson and John P. Marino\*

Institute for Bioscience and Biotechnology, National Institute of Standards and Technology and the University of Maryland, 9600 Gudelsky Drive, Rockville, Maryland 20850, United States

\* Corresponding author, e-mail: john.marino@nist.gov



**Fig. S1.** 2D <sup>1</sup>H COSY spectral fingerprints of <sup>1</sup>H<sub>N</sub> - <sup>1</sup>H<sub>aliphatic</sub> region of HEWL collected at 600 MHz and 25 °C. (A) 2D <sup>1</sup>H DQF-COSY; (B) 2D IP-COSY. Positive contours are shown in black and negative contours in red. The upfield aliphatic region is included for comparative purposes. While setting 3.0 ppm as the upfield cut-off for the fingerprint region, only a few upfield H $\alpha$  resonances are lost for subsequent analysis. However, as can be seen from the TOCSY spectra (Figure S2A, B), many other side chain <sup>1</sup>H resonances appear  $\leq$ 3.0 ppm. Choosing this cut-off predominantly selects for <sup>1</sup>H<sub>N</sub>-<sup>1</sup>H<sub> $\alpha</sub> correlations.$ </sub>



**Fig. S2**. 2D <sup>1</sup>H homonuclear spectra of  ${}^{1}H_{N}$  -  ${}^{1}H_{aliphatic}$  region of the NIST-Fab collected at 600 MHz and 50 °C. (A) 2D <sup>1</sup>H IP-COSY; (B) 2D <sup>1</sup>H COIN-TACSY using DIPSI-2rc. Positive contours are shown in black and negative contours in red. Negative artifacts in panel B are from residual water, apodization artifacts, or magnetization leakage in the COIN-TACSY. The IP-COSY was collected with 32 scans per transient and the COIN-TACSY with 16 scans per increment, affording total experimental times of 3 hours 19 minutes and 8 hours 23 minutes, respectively. All other experimental parameters were as described in the Materials and Methods section and Table S2.



**Fig. S3**. 2D <sup>1</sup>H TOCSY-type spectra of <sup>1</sup>H<sub>N</sub> - <sup>1</sup>H<sub>aliphatic</sub> region of lysozyme collected at 600 MHz and 25 °C. (A) 2D <sup>1</sup>H TOCSY using DIPSI-2; (B) 2D <sup>1</sup>H TOCSY using DIPSI-2rc; (C) 2D <sup>1</sup>H COIN-TACSY using DIPSI-2; (D) 2D <sup>1</sup>H COIN-TACSY using DIPSI-2rc. Positive contours are shown in black and negative contours in red. ROE artifacts in panels A and C are removed by the addition of a relaxation compensation delay in the DIPSI-2rc pulse train (panels B and D). Additional positive and negative artifacts in panels C and D are from magnetization leakage in the COIN-TACSY.



**Fig. S4.** Pulse sequence for 2D COIN-TACSY experiment with DIPSI-2rc. Thin and thick bars represent nonselective 90° and 180° pulses, respectively. The selective 90° pulses was achieved with the e-SNOB shape pulse, which provides a 270° rotation [1]. For setting parameters for the e-SNOB pulse, it was empirically determined that suppression of up field resonances was best achieved using a pulse duration of 771 µs, corresponding to an excitation bandwidth of 2,100 Hz, and an offset of -2,000 Hz. The isotropic mixing period,  $\tau$ , is represented by rectangles with diagonal slashes, was achieved with two cycles of the DIPSI-2 [2] or DIPSI-2rc [3] pulse trains. For preferred implementation of the COIN-TACSY using DIPSI-2rc, an rf amplitude of 38.5 kHz with a  $\tau$  period of 8.98 ms was used. The cross relaxation compensation delay was set to 41.6 µs. Phases used are  $\varphi_1 = \{x, -x\}; \varphi_2 = \{x, x, x, x, -x, -x, -x\}; \varphi_3 = \{x, x, -x, -x\}; \varphi_4 = \{x, -x, -x, -x, -x, -x, -x\}$ . Quadrature detection using States-TPPI was achieved by incrementing  $\varphi_1$ . The  $\delta$  delay, pread post TACSY mixing period, was 10 µs. Water suppression was achieved by presaturation and 3-9-19 Watergate. PFG designates the pulse field gradients applied along the z-axis: G1 = G2 = 20 G/cm with a duration of 1.0 ms.

**Note:** It was found that implementation of the COIN-TACSY using DIPSI-2rc required a Bruker AVANCE III or later console vintages due to console memory requirements. In contrast, the original COIN-TACSY using DIPSI-2 could be implemented on any Bruker AVANCE console. The pulse sequence was not tested on an instrument of another manufacturer, such as an Agilent/Varian or a JEOL NMR system.

Table S1: Relative peak intensities: qualitative comparison of the sensitivity of the 2D <sup>1</sup>H, <sup>1</sup>H *J*-correlated spectra

	Relative Peak Height <sup>+</sup>						
	Slice at 5.05 ppm		Slice at 4.07 ppm				
	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5		
Clean TACSY	1.00	1.00	1.00	1.00	1.00		
TACSY	0.63	0.66	0.76	0.77	0.78		
TOCSY	0.89	1.09	0.75	0.65	0.80		
Clean TOCSY	0.33	0.35	0.46	0.27	0.82		
IP-COSY	0.26	0.26	0.08	0.09	0.08		

<sup>+</sup>Each peak is normalized to the intensity in the clean COIN-TACSY experiment. The five peaks are labeled accordingly in Fig. 3.

**Table S2: Selected Experimental parameters and overall experimental times.**See Materials and MethodsSection for complete details.

	Peak Type	F1, total points	F2, total points	Mixing Time	Scans	Experimental Time
Clean TACSY	$H_N$ - $H_\alpha$	2,690	1,024	85 ms	8	3 h
TACSY	$H_N$ - $H_\alpha$	2,690	1,024	83 ms	8	3 h
Clean TOCSY	$H_N$ - $H_{all}$	2,690	1,024	80 ms	8	3 h
TOCSY	$H_N$ - $H_{all}$	2,690	1024	80 ms	8	3 h
IP-COSY <sup>+</sup>	$H_N$ - $H_\alpha$	2,690	208	N/A	8	0.5 h
DQF-COSY	$H_N-H_{\alpha}$	2,690	1,024	N/A	32	12 h

<sup>+</sup>Due to the use of a constant time period for  $t_1$  evolution, the maximum number of total points that could be collected was 208.

## **Supplementary References**

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- 3. Cavanagh, J. and M. Rance, Suppression of cross-relaxation effects in TOCSY spectra via a modified DIPSI-2 mixing sequence. J Magn Reson (1969), 1992. **96**(3): p. 670-678.