Supporting Information for

Investigating Therapeutic Protein Structure with Diethylpyrocarbonate Labeling and Mass Spectrometry

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Supplemental Methods

Proteolytic Digestion

Since β2m and HGH have a single disulfide bond, TCEP (protein:TCEP=1:40 molar ratio) was added to reduce the disulfide bond and iodoacetamide (100 µM) was added simultaneously at room temperature for 30 min in the dark to alkylate the reduced Cys residues. The resulting samples were incubated with 10% (vol/vol) acetonitrile at 50 °C for 45 min prior to digestion with immobilized chymotrypsin (enzyme/substrate ratio of 1:10) for B2m and immobilized trypsin (1:10) for HGH at 37 °C. After 2 hours, the reaction mixture was centrifuged for 2 min at 9000 relative centrifugal force (RCF) to separate the enzyme from the protein. After that, the samples were either immediately analyzed by LC/MS or flash frozen in liquid nitrogen and stored at -80°C until LC/MS analysis.

To achieve complete digestion of IgG1, an initial digestion with activated papain was necessary. To activate papain 0.5 µM of the enzyme was incubated in 1 mM EDTA and 10 mM L-cysteine at 37 °C for 30 min. Once complete the cysteine concentration was reduced to less than 2 µM via four spins with a 3000 MWCO filter. The papain digestion was then performed for 2.5 h using a 1:100 (papain:IgG1) ratio. IgG1 was then denatured and its disulfide bonds were reduced in a 50 mM phosphate buffered solution at pH 7.4 with 1 M urea and 20 mM DTT at 60 °C for 20 min. The reduced disulfides were then alkylated with 40 mM iodoacetamide for 2 min. Immobilized trypsin was then added to achieve a 1:3 (enzyme:substrate) ratio, and the digestion reaction was allowed to proceed overnight at room temperature. After digestion, the samples were spun at 9200 RCF for 5 min, and the supernatant was collected, flash-frozen in liquid nitrogen, and stored at -80 °C until analysis via LC/MS.

HPLC Separation

β2m HPLC separations were conducted using an HP1100 HPLC system (Agilent, Wilmington, DE) with a Discovery C18 column (15 cm × 2.1 mm, 5 µm particle size; Supelco, St. Louis, MO, USA). Peptide fragments from the proteolytic digests were eluted using a linear gradient of methanol containing 0.1% acetic acid that increased from 10% to 100% methanol over 30 min at a flow rate of 0.25 mL/min.

HPLC separations of IgG were performed using a Thermo Scientific Acclaim PepMap RSLC C18 (15 cm x 50 µm, 2 µm particle size) on an Easy-nLC 1000 system (Thermo Scientific, Tewksbury, MA). To achieve sufficient separation of the proteolytic peptides, a shallow gradient was used where %B (0.1% formic acid in acetonitrile) was increased from 0 to 40% over 90 min. The column was then flushed by jumping to 95 % B over 15 min. It was then held at 95% B for the remainder of the separation (i.e. another 20 min). A flow rate of 0.225 µL/min was used.

HPLC analyses of HGH were performed using an Accela LC system (Thermo Scientific, Tewksbury, MA) with a ZORBAX 300SB-C18 MicroBore RR column (1.0 X 150 mm, 3.5 µm particle size, Agilent, Wilmington, DE). Peptides were eluted over a 50-minute gradient where %B was increased from 2% to 45% over the first 35 minutes, then elevated to 80% for an additional 5 minutes. The column was then re-equilibrated with 0% B for 10 minutes. Blank runs were run in between each sample, and were each run at a flow rate of .05 mL/min.

Circular Dichroism

All IgG solutions were diluted to 0.75 µM in 50 mM phosphate buffer at pH 7.4 prior to analysis. For HGH conditions all solutions were diluted to 5 µM in 10 mM phosphate buffer at pH 8.0. Circular dichroism was measured using a J-715 spectropolarimeter (Jasco, Easton, MD). The scan ranged from 250 to 195 nm with a scan resolution of 0.5 nm, a scan rate of 100 nm/min, and a response time of 1 sec. Raw data were converted into mean residue ellipticity using the CD Analysis & Plotting Tool (CAPITO).[45]

Fluorescence

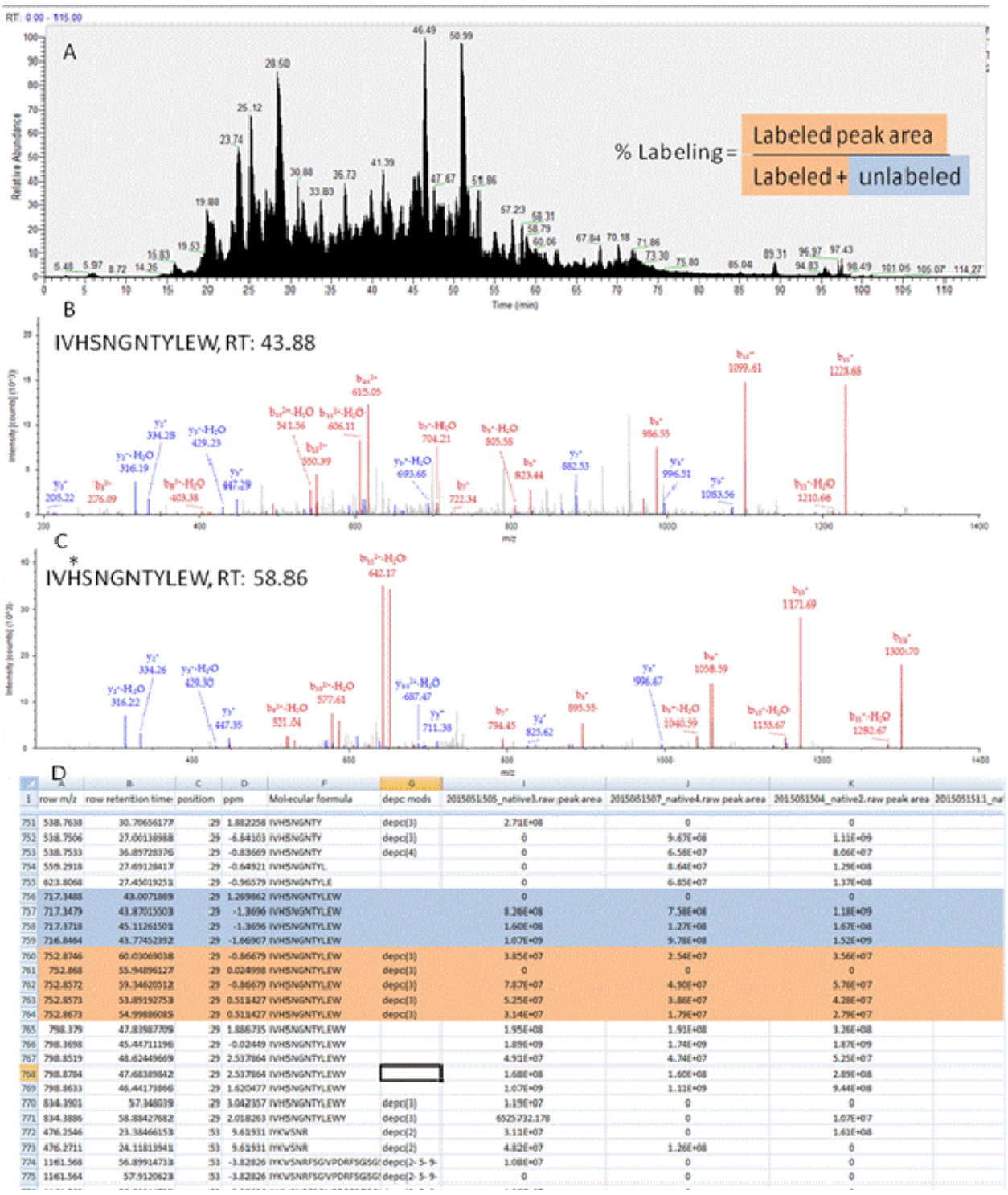
The concentration of IgG was diluted to 0.75 μM in 50 mM phosphate buffer at pH 7.4 prior to analysis. For HGH, the protein was diluted to 5 μM in 10 mM phosphate buffer at pH 8.0. A Photon Technology International Quantamaster-4SE (PTI, Edison, NJ) was used to obtain fluorescence spectra for IgG. Tryptophan fluorescence was collected using an excitation wavelength of 295 nm and slit widths of 1 nm. Emission scans ranged from 310-440 nm. A Synergy H1 multi-mode plate reader (BioTek, Winooski, VT) was used to measure the fluorescence of HGH. Samples were excited at 295 nm and emissions were monitored from 300-440 nm.

Dynamic Light Scattering

A Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, U.K.) was used to measure the hydrodynamic radii of native and heat denatured IgG. A 1 mL solution of 1 μM IgG was used for these experiments. Five runs were conducted for each sample, and volume particle size distribution is reported. For HGH the measurements were carried out using a Zetasizer Nano-S (Malvern Instruments, Worcestershire, U.K.). HGH was measured at 5 μM after treatment by the conditions indicated. Measurement duration was according to preset levels, and intensity/volume distributions of the samples from at least four runs were recorded in each dataset.

Size Exclusion Chromatography

For SEC experiments, the protein was separated using a SuperSW2000 30 cm × 4.6 mm column (GE Healthcare Biosciences, Piscataway, NJ) installed on an Agilent HP1100 series HPLC system (Wilmington, DE). Before injection of the sample, the SEC column was first equilibrated with a 20 mM ammonium acetate mobile phase (pH = 7.4) at a 0.035 mL/min flow rate for 1 h. 20 μL of the protein sample was injected into the sample loop. A variable wavelength detector set at 214 nm was used for detection.



	row m/z	row retention time	position	ppm	Molecular formula	depc mods	2015051505_native3.raw peak area	2015051507_native4.raw peak area	2015051504_native2.raw peak area
751	538.7638	30.70656177	29	1.882258	IVHSNGNTY	depc(3)	2.73E+08	0	0
752	538.7506	27.60118988	29	-6.84103	IVHSNGNTY	depc(3)	0	9.67E+08	1.11E+09
753	538.7533	36.89728376	29	-0.83669	IVHSNGNTY	depc(4)	0	6.58E+07	8.06E+07
754	559.2918	27.69128417	29	-0.64921	IVHSNGNTYL		0	8.64E+07	1.29E+08
755	623.8068	27.45019251	29	-0.96579	IVHSNGNTYLE		0	6.85E+07	1.37E+08
756	717.3488	43.0071869	29	1.269862	IVHSNGNTYLEW		0	0	0
757	717.3479	43.87015503	29	-1.3696	IVHSNGNTYLEW		8.28E+08	7.58E+08	1.18E+09
758	717.3718	45.11261501	29	-1.3696	IVHSNGNTYLEW		1.60E+08	1.27E+08	1.67E+08
759	716.8464	43.77452392	29	-1.66907	IVHSNGNTYLEW		1.07E+09	9.78E+08	1.52E+09
760	752.8746	60.03069038	29	-0.86679	IVHSNGNTYLEW	depc(3)	3.85E+07	2.54E+07	3.56E+07
761	752.868	55.94896127	29	0.024998	IVHSNGNTYLEW	depc(3)	0	0	0
762	752.8572	59.34620512	29	-0.86679	IVHSNGNTYLEW	depc(3)	7.87E+07	4.90E+07	5.76E+07
763	752.8573	53.89192753	29	0.518427	IVHSNGNTYLEW	depc(3)	5.25E+07	3.86E+07	4.28E+07
764	752.8673	54.99886085	29	0.518427	IVHSNGNTYLEW	depc(3)	3.14E+07	1.79E+07	2.79E+07
765	798.379	47.83987709	29	1.886715	IVHSNGNTYLEWY		1.95E+08	1.91E+08	3.26E+08
766	798.3698	45.44711196	29	-0.02449	IVHSNGNTYLEWY		1.89E+09	1.74E+09	1.87E+09
767	798.8519	48.62449669	29	2.537864	IVHSNGNTYLEWY		4.91E+07	4.74E+07	5.25E+07
768	798.8784	47.68389842	29	2.537864	IVHSNGNTYLEWY		1.68E+08	1.60E+08	2.89E+08
769	798.8633	46.44173866	29	1.620477	IVHSNGNTYLEWY		1.07E+09	1.11E+09	9.44E+08
770	834.3901	57.348039	29	3.042357	IVHSNGNTYLEWY	depc(3)	1.15E+07	0	0
771	834.3886	58.88427682	29	2.018263	IVHSNGNTYLEWY	depc(3)	6525732.178	0	1.07E+07
772	476.2546	23.38466153	53	9.612931	IYKWSNR	depc(2)	3.11E+07	0	1.61E+08
773	476.2711	24.11813941	53	9.612931	IYKWSNR	depc(2)	4.82E+07	1.26E+08	0
774	1161.568	56.89914733	53	-3.82826	IYKWSNRFSGVPDRFSGSGS	depc(2- 5- 9-	1.08E+07	0	0
775	1161.564	57.9120623	53	-3.82826	IYKWSNRFSGVPDRFSGSGS	depc(2- 5- 9-	0	0	0

Figure S1: Illustration of the process by which the labeling percent is calculated. (A): HPLC/MS analysis of the digested protein is carried out. All peptides are subjected to MS/MS for identification, and unlabeled (B) and labeled peptides (C) are identified. For peptide identification, SearchGUI takes the raw MS/MS data (e.g. spectra in B and C) and uses five different search engines to compare the data against a database constructed using cRAP. The data from the identified peptides is then used to construct a custom database for peak identification in the program MZmine. MZmine is then used to calculate peak

area for each identified peptide. This peak area information along with peptide identity, m/z ratio, retention time, and mass accuracy is exported into a spreadsheet format for calculation of DEPC labeling percentages (D). Using the exported peak areas, peptide identities, and the % labeling equation shown in A, the percent modification for each peptide can be calculated. All identified peptides containing the residue of interest are used in the calculation.

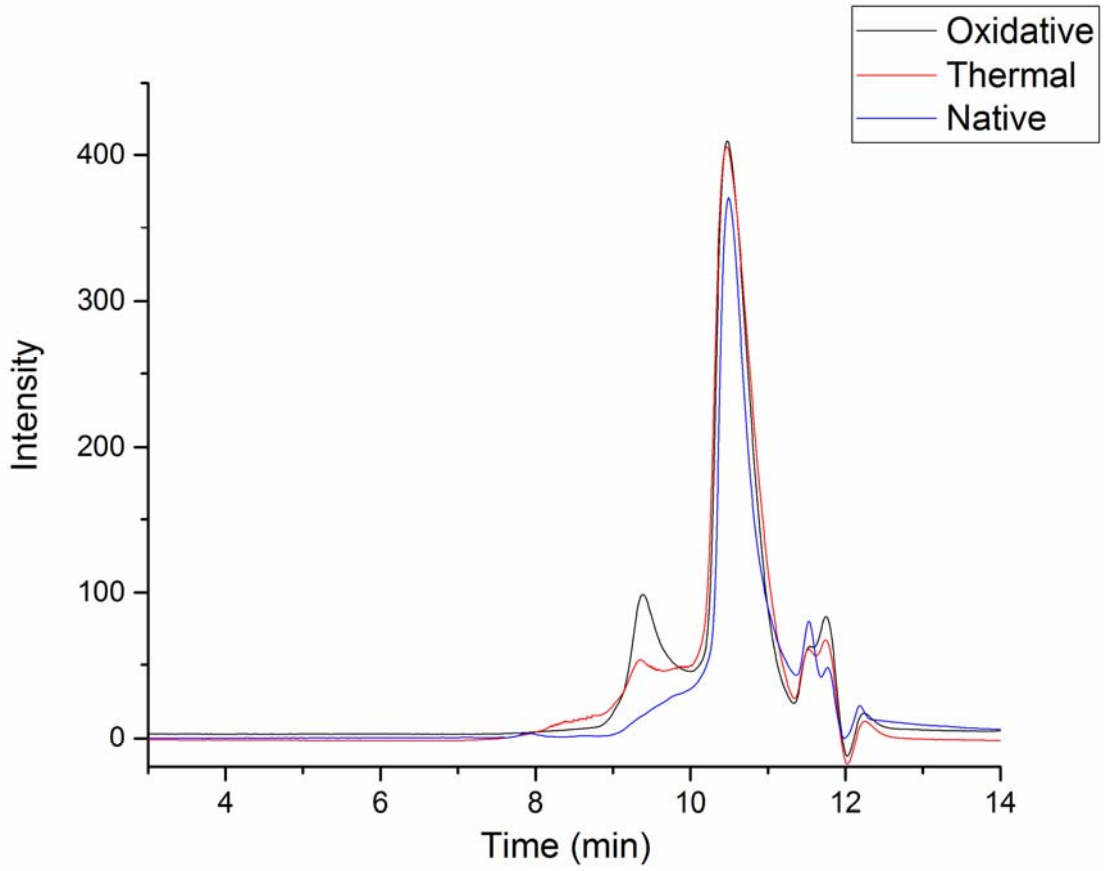


Figure S2: Size exclusion chromatography of β2m before (blue) and after heating (red) and oxidation (black). Both chromatograms demonstrate the presence of aggregated species. These aggregated complexes are evident from the peaks eluting earlier than 10.5 min.

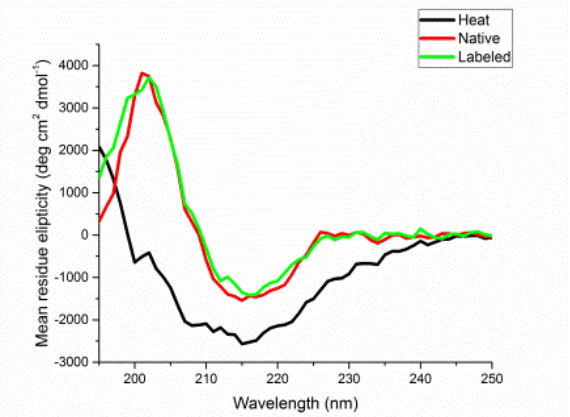


Figure S3: Circular dichroism of IgG1 under normal (red), heated (Black), and DEPC-labeled (green) conditions. The essentially identical overlap between the CD spectra of the normal and DEPC-labeled samples demonstrates that DEPC labeling has little effect on the secondary structure of IgG1.

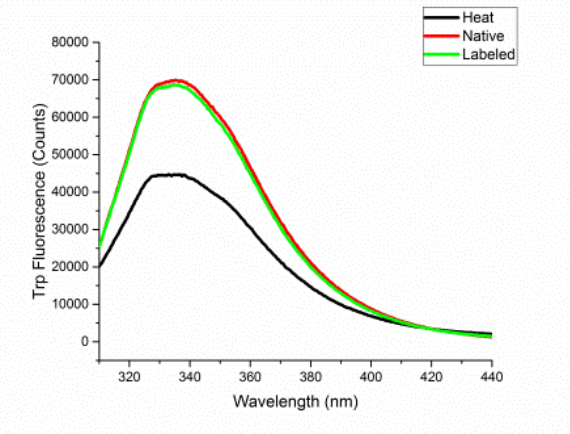


Figure S4: Tryptophan fluorescence of IgG1 under normal (red), heated (Black), and DEPC-labeled (green) conditions. The essentially identical overlap between the spectra of the normal and DEPC-labeled samples demonstrates that covalent labeling has little effect on the structure of IgG1.

Table S1: Modification percentages for individual residues of IgG's light chain before and after heating at 75 °C for 15 minutes.

LC	Native	15 min @ 75°C	Significant*	LC	Native	15 min @ 75°C	Significant*
Thr5	6% ± 3%	0.0% ± 0.0%	yes	Ser127	3.7% ± 0.7%	0.4% ± 0.3%	yes
Thr7	51% ± 7%	14% ± 10%	yes	Thr131	2.4% ± 0.1%	1.0% ± 0.4%	yes
Ser10	21% ± 3%	0.0% ± 0.0%	yes	Ser132	2.3% ± 0.2%	0.3% ± 0.2%	yes
Ser14	15% ± 4%	2% ± 1%	yes	Ser136	0.0% ± 0.0%	14% ± 3%	no
Ser20	1.5% ± 0.3%	0.9% ± 0.2%	yes	Tyr145	8.1% ± 0.9%	2% ± 2%	yes
Ser22	1.3% ± 0.3%	0.3% ± 0.1%	yes	Lys147	0.0% ± 0.0%	30% ± 6%	no
Ser25	9% ± 2%	0.0% ± 0.0%	yes	Lys152	11% ± 1%	7% ± 2%	yes
Ser26	8% ± 1%	1.0% ± 0.3%	yes	Lys154	1.5% ± 0.4%	38% ± 5%	yes
Tyr28	22% ± 3%	10% ± 2%	yes	Ser158	28% ± 4%	1.8% ± 1.0%	yes
His31	14% ± 13%	13% ± 3%	no	Ser167	4% ± 1%	21% ± 16%	yes
Ser32	2.4% ± 0.8%	0.6% ± 0.2%	yes	Thr169	0.6% ± 0.2%	18% ± 16%	yes
Thr36	11% ± 4%	8.3% ± 5%	no	Ser173	1.8% ± 0.2%	1.5% ± 0.2%	yes
Tyr37	11% ± 4%	0.9% ± 0.6%	yes	Lys174	1.1% ± 0.2%	5% ± 1%	yes
Tyr41	11% ± 3%	3.6% ± 0.7%	yes	Ser176	13% ± 2%	23% ± 5%	yes
Lys44	46% ± 4%	19% ± 4%	yes	Thr177	6% ± 2%	19% ± 4%	yes
Ser48	10% ± 3%	9% ± 1%	no	Tyr178	12% ± 1%	8% ± 4%	no
Lys50	2.5% ± 0.5%	73% ± 4%	yes	Ser179	7% ± 2%	20% ± 6%	yes
Tyr54	90% ± 6%	0.0% ± 0.0%	yes	Ser181	6% ± 2%	29% ± 10%	yes
Lys55	0.0% ± 0.0%	0.0% ± 0.0%	no	Ser182	0.8% ± 0.1%	4% ± 1%	yes
Ser57	9% ± 5%	0.0% ± 0.0%	yes	Thr183	9% ± 2%	3% ± 1%	yes
Ser61	4% ± 3%	1% ± 1%	no	Thr185	9% ± 2%	1.7% ± 0.8%	yes
Ser68	18% ± 4%	0.0% ± 0.0%	yes	Thr187	0.34% ± 0.09%	0.8% ± 0.9%	no
Ser70	6% ± 3%	1.1% ± 0.8%	yes	Lys188	0.0% ± 0.0%	6% ± 5%	no
Ser72	0.3% ± 0.2%	0.2% ± 0.1%	no	Tyr191	63% ± 9%	88% ± 4%	yes
Thr74	0.09% ± 0.09%	4% ± 1%	yes	His194	39% ± 5%	0.4% ± 0.4%	yes
Thr77	11% ± 5%	4.3% ± 0.8%	yes	Ser196	32% ± 3%	89% ± 3%	yes
Lys79	0.0% ± 0.0%	24% ± 2%	no	Tyr197	0.12% ± 0.07%	8% ± 5%	yes
Ser81	0.0% ± 0.0%	29% ± 7%	no	Thr198	50% ± 9%	34% ± 2%	yes
Tyr91	6% ± 3%	1.7% ± 0.3%	yes	Thr202	43% ± 11%	1.9% ± 0.5%	yes
Tyr92	5% ± 3%	0.0% ± 0.0%	yes	His203	0.64% ± 0.33%	31% ± 5%	yes
Ser97	2.6% ± 0.9%	14% ± 3%	yes	Lys204	44% ± 7%	39% ± 6%	no
His98	3.2% ± 1.0%	5% ± 3%	no	Thr205	1.2% ± 0.6%	66% ± 4%	yes
Thr102	0.21% ± 0.05%	1.1% ± 0.1%	yes	Ser206	2% ± 1%	12% ± 4%	yes
Thr107	33% ± 28%	0.0% ± 0.0%	yes	Thr207	1.9% ± 0.9%	0.3% ± 0.3%	yes
Lys108	77% ± 3%	82% ± 2%	no	Ser208	10% ± 4%	71% ± 15%	yes
Thr119	76% ± 9%	0.0% ± 0.0%	yes	Lys212	16% ± 5%	45% ± 12%	yes
Ser121	70% ± 11%	4.0% ± 0.9%	yes	Ser213	15.3% ± 3%	73% ± 35%	no
Ser126	3.8% ± 0.9%	0.0% ± 0.0%	yes				

*A difference was considered significant if the p-value, calculated by performing an unpaired T-test, was less than 0.05 (corresponding to a 95% confidence level, n=5).

Table S2: Modification percentages for individual residues of IgG's heavy chain before and after heating at 75 °C for 15 minutes.

HC	Native	15 min @ 75°C	Significant*	HC	Native	15 min @ 75°C	Significant*	HC	Native	15 min @ 75°C	Significant*
Lys5	0.0% ± 0.0%	0.0% ± 0.0%	no	Tyr150	16% ± 7%	3% ± 2%	yes	Ser293	43% ± 3%	9% ± 2%	yes
Ser7	3% ± 2%	0.0% ± 0.0%	yes	Thr156	35% ± 9%	0.0% ± 0.0%	yes	Thr294	28% ± 13%	60% ± 16%	yes
Ser15	1.2% ± 0.5%	46% ± 7%	yes	Thr158	23% ± 8%	0.0% ± 0.0%	yes	Ser297	5% ± 2%	1% ± 1%	yes
Ser17	1.1% ± 0.4%	52% ± 7%	yes	Ser161	1% ± 1%	7.0% ± 0.8%	yes	Ser299	6% ± 4%	0.2% ± 0.2%	yes
Ser19	2.0% ± 0.8%	0.0% ± 0.0%	yes	Ser163	2.8% ± 0.6%	0.0% ± 0.0%	yes	His305	5% ± 2%	10% ± 1%	yes
Thr21	1.2% ± 0.5%	3.0% ± 0.4%	yes	Ser165	2.9% ± 0.5%	7% ± 3%	yes	Lys312	4.2% ± 0.6%	56% ± 2%	yes
Thr23	13% ± 4%	0.0% ± 0.0%	yes	Ser166	0.2% ± 0.0%	1.2% ± 0.6%	yes	Lys315	31% ± 7%	3.0% ± 0.5%	yes
Ser25	1.5% ± 1.2%	0.0% ± 0.0%	yes	His169	0.7% ± 0.2%	3% ± 1%	yes	Ser320	2.1% ± 0.6%	0.0% ± 0.0%	yes
Ser28	12% ± 4%	1.2% ± 0.7%	yes	Thr170	0.8% ± 0.2%	3.1% ± 0.6%	yes	Lys329	1.5% ± 0.7%	38% ± 4%	yes
Tyr32	13% ± 1%	22% ± 1%	yes	Ser177	49% ± 6%	49% ± 14%	no	Thr330	67% ± 10%	8.5% ± 0.6%	yes
Ser56	10% ± 3%	15% ± 4%	no	Tyr180	29% ± 4%	59% ± 17%	yes	Ser332	18% ± 7%	0.0% ± 0.0%	yes
Thr57	24% ± 6%	2% ± 1%	yes	Thr181	1.0% ± 0.1%	51% ± 21%	yes	Lys333	0.0% ± 0.0%	9% ± 5%	no
Tyr59	1.3% ± 0.5%	19% ± 2%	yes	Ser183	12% ± 4%	4% ± 3%	no	Thr334	12% ± 4%	24% ± 1%	yes
Ser61	7% ± 1%	0.0% ± 0.0%	yes	Ser184	7% ± 1%	3% ± 3%	yes	Lys335	28% ± 16%	6% ± 5%	yes
Lys64	1.0% ± 0.6%	17.9% ± 2.0%	yes	Ser185	22% ± 5%	2% ± 2%	yes	Lys339	3% ± 3%	26% ± 7%	yes
Ser65	5% ± 3%	0.8% ± 0.7%	yes	Thr187	0.6% ± 0.3%	9% ± 5%	yes	Tyr344	27% ± 4%	1.0% ± 0.5%	yes
Ser68	59% ± 6%	0.0% ± 0.0%	yes	Ser190	1.4% ± 0.4%	7% ± 3%	yes	Thr345	0.0% ± 0.0%	0.6% ± 0.1%	no
Thr70	44% ± 10%	14% ± 1%	yes	Ser191	16% ± 1%	2.9% ± 0.7%	yes	Lys350	76% ± 2%	27% ± 4%	yes
Lys71	13% ± 6%	68% ± 4%	yes	Thr192	0.6% ± 0.4%	6% ± 6%	no	Lys355	50% ± 5%	74% ± 3%	yes
Ser74	59% ± 3%	11% ± 3%	yes	Ser195	0.8% ± 0.4%	2.8% ± 0.6%	yes	Lys357	18% ± 1%	6.2% ± 0.8%	yes
Lys75	3% ± 2%	32% ± 5%	yes	Thr197	0.6% ± 0.5%	3% ± 1%	yes	Ser359	22% ± 6%	45% ± 24%	no
Ser76	10% ± 5%	0.0% ± 0.0%	yes	Thr199	11% ± 1%	0.3% ± 0.4%	yes	Thr361	6% ± 3%	3% ± 1%	yes
Lys81	15% ± 2%	51% ± 12%	yes	His204	20% ± 2%	42% ± 6%	yes	Thr365	1.3% ± 0.4%	0.8% ± 0.3%	no
Ser84	15% ± 1%	11% ± 3%	yes	Ser207	23% ± 4%	16% ± 5%	yes	Thr373	3.0% ± 1.0%	0.07% ± 0.08%	yes
Thr87	0.7% ± 0.2%	8% ± 3%	yes	Ser208	0.5% ± 0.2%	0.0% ± 0.0%	yes	Tyr386	0.6% ± 0.1%	0.6% ± 0.3%	no
Thr90	2.8% ± 0.7%	8% ± 3%	yes	Thr209	0.4% ± 0.2%	3% ± 3%	no	Lys387	0.1% ± 0.1%	1.5% ± 0.3%	yes
Lys92	2.5% ± 0.7%	14% ± 5%	yes	Lys210	0.0% ± 0.0%	33% ± 17%	no	Thr389	0.1% ± 0.0%	0.2% ± 0.1%	no
Tyr93	9% ± 1%	7% ± 3%	no	Lys213	55% ± 4%	54% ± 4%	no	Thr395	7% ± 1%	4% ± 2%	yes
Tyr94	1.1% ± 0.7%	8% ± 5%	yes	Lys214	52% ± 2%	98% ± 4%	yes	Ser398	1.0% ± 0.2%	1.3% ± 0.5%	no
Thr96	2% ± 1%	34% ± 48%	no	Lys223	4% ± 2%	8% ± 4%	no	Tyr399	0.9% ± 0.3%	2.0% ± 0.4%	yes
Tyr100	13% ± 4%	0.0% ± 0.0%	yes	Thr228	0.0% ± 0.0%	0.0% ± 0.0%	no	Tyr402	1.1% ± 0.9%	0.0% ± 0.0%	yes
Lys102	14% ± 4%	93% ± 9%	yes	Ser233	6% ± 3%	9% ± 8%	no	Ser403	11% ± 9%	3.8% ± 0.9%	no
Tyr104	14% ± 3%	0.0% ± 0.0%	yes	Ser234	21% ± 10%	17% ± 17%	no	Lys404	0.0% ± 0.0%	81% ± 9%	no
Tyr107	0.0% ± 0.0%	1.7% ± 0.5%	no	Lys241	12% ± 2%	24% ± 5%	yes	Lys409	7% ± 4%	4% ± 2%	no
Thr112	0.7% ± 0.4%	4.4% ± 0.8%	yes	Lys243	24% ± 3%	53% ± 6%	yes	Ser410	0.0% ± 0.0%	1.4% ± 0.5%	no
Thr115	19% ± 5%	5% ± 2%	yes	Thr247	8% ± 2%	0.0% ± 0.0%	yes	Thr417	9% ± 4%	15% ± 1%	yes
Ser117	6% ± 4%	6.1% ± 0.3%	no	Thr249	34% ± 12%	0.0% ± 0.0%	yes	Thr419	2.1% ± 0.7%	1% ± 1%	no
Lys120	2% ± 1%	10% ± 6%	yes	Thr251	26% ± 12%	10% ± 3%	yes	Ser421	20% ± 8%	2% ± 1%	yes
Thr121	0.3% ± 0.4%	0.0% ± 0.0%	yes	Lys253	19% ± 2%	54% ± 11%	yes	His424	27% ± 4%	34% ± 3%	yes
Thr122	4.0% ± 0.6%	24% ± 4%	yes	Thr255	0.0% ± 0.0%	0.0% ± 0.0%	no	His428	3.3% ± 0.6%	16% ± 2%	yes
Ser125	5% ± 1%	2% ± 1%	yes	Ser262	2.0% ± 0.3%	1.2% ± 0.2%	yes	His430	6% ± 3%	0.8% ± 0.2%	yes
Tyr127	6% ± 2%	7% ± 3%	no	Lys263	0.2% ± 0.2%	9.3% ± 0.6%	yes	His431	1.8% ± 0.9%	0.4% ± 0.1%	yes
Ser133	5% ± 2%	7% ± 1%	yes	Ser271	1.1% ± 0.3%	0.0% ± 0.0%	yes	Thr432	0.32% ± 0.07%	1.4% ± 0.7%	yes
Thr137	1.5% ± 0.7%	5% ± 1%	yes	His280	2.1% ± 0.6%	7.5% ± 0.9%	yes	Lys434	0.0% ± 0.0%	10% ± 4%	no
Ser139	3% ± 2%	10% ± 5%	yes	Thr281	0.6% ± 0.1%	0.5% ± 0.09%	yes	Ser435	22% ± 4%	5% ± 2%	yes
Thr142	4% ± 2%	9% ± 3%	yes	His283	0.4% ± 0.3%	0.0% ± 0.0%	yes	His438	0.0% ± 0.0%	87% ± 10%	no
Lys148	25% ± 4%	19% ± 9%	no	Thr284	0.0% ± 0.0%	11% ± 3%	no	Ser439	0.0% ± 0.0%	0.0% ± 0.0%	no

*A difference was considered significant if the p-value, calculated by performing an unpaired T-test, was less than 0.05 (corresponding to a 95% confidence level, n=5).

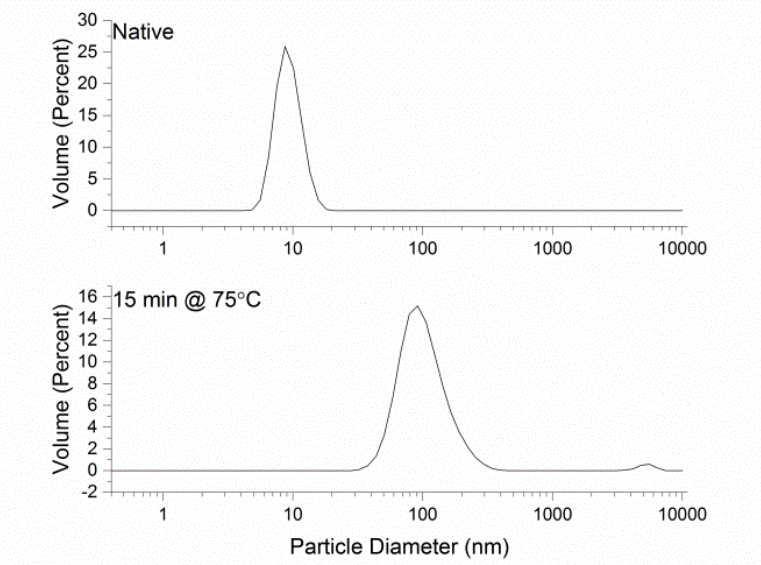


Figure S5: Dynamic light scattering data for IgG1 before (top) and after heating at 75 ^{o}C for 15 minutes (bottom). These data demonstrate that IgG1 aggregates upon heating.

Table S3: Zyggregator calculated Z-scores representing aggregation propensity of each residue for the light chain of IgG.

Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score
Asp1	0.740141	Tyr37	1.51593	Phe76	2.33023	Lys112	-0.50375	Asp148	-0.09626	Leu184	2.2071
Val2	1.26291	Leu38	1.78082	Thr77	2.12179	Arg113	-0.65845	Ile149	-0.23159	Thr185	1.61285
Leu3	0.910169	Glu39	1.86017	Leu78	1.71678	Ala114	-0.66578	Asn150	1.37555	Leu186	1.64172
Met4	1.60023	Trp40	1.13562	Lys79	1.34919	Asp115	-2.2083	Val151	1.81502	Thr187	0.729396
Thr5	0.474526	Tyr41	0.373129	Ile80	1.20088	Ala116	-1.39673	Lys152	2.23183	Lys188	1.0058
Gln6	0.247509	Leu42	-0.99092	Ser81	-0.15091	Ala117	-0.56377	Trp153	1.81502	Asp189	-0.34599
Thr7	0.167981	Gln43	-0.39257	Arg82	-0.30561	Pro118	-0.3009	Lys154	1.46642	Glu190	-1.13963
Pro8	0.105319	Lys44	-1.03472	Val83	-0.84583	Thr119	-0.07178	Ile155	1.15988	Tyr191	-1.56276
Leu9	-1.58422	Pro45	-1.57835	Glu84	-1.26264	Val120	0.458246	Asp156	0.619662	Glu192	-1.15603
Ser10	-1.14584	Gly46	-2.9424	Ala85	-1.18311	Ser121	-0.56341	Gly157	-0.41707	Arg193	-0.99721
Leu11	-1.55085	Gln47	-3.21713	Glu86	-0.37796	Ile122	-0.56341	Ser158	-0.39411	His194	0.053732
Pro12	-0.34586	Ser48	-2.73104	Asp87	-0.37796	Phe123	-1.15612	Glu159	-0.83963	Asn195	0.102814
Val13	-0.33434	Pro49	-1.55468	Leu88	0.485283	Pro124	-1.65036	Arg160	-0.40207	Ser196	1.32821
Ser14	-0.30547	Lys50	-1.57543	Gly89	0.916388	Pro125	-2.15767	Gln161	-0.18657	Tyr197	1.34731
Leu15	-0.51684	Leu51	-1.08765	Val90	1.95408	Ser126	-2.35981	Asn162	-0.29474	Thr198	1.29026
Gly16	0.692517	Leu52	-1.49422	Tyr91	2.56776	Ser127	-2.73513	Gly163	0.025051	Cys199	1.5074
Asp17	0.385973	Ile53	0.096855	Tyr92	2.35639	Glu128	-1.04559	Val164	0.739162	Glu200	1.67698
Gln18	0.643967	Tyr54	0.503417	Cys93	2.16869	Gln129	0.238933	Leu165	1.00592	Ala201	1.10218
Ala19	0.564439	Lys55	0.799455	Phe94	1.86215	Leu130	0.329975	Asn166	1.41076	Thr202	0.914485
Ser20	1.19148	Val56	0.005816	Gln95	1.67579	Thr131	0.421018	Ser167	1.34859	His203	0.008706
Ile21	0.823887	Ser57	0.202672	Gly96	1.6264	Ser132	0.853155	Trp168	0.91021	Lys204	1.10873
Ser22	0.955726	Asn58	-0.15326	Ser97	-0.18851	Gly133	0.984994	Thr169	0.671861	Thr205	1.1839
Cys23	1.0309	Arg59	0.344344	His98	-0.56383	Gly134	1.21201	Asp170	0.859384	Ser206	-0.69334
Arg24	0.89906	Phe60	0.344344	Val99	-0.02698	Ala135	1.11354	Gln171	0.265126	Thr207	-0.41722
Ser25	0.996998	Ser61	-0.75249	Pro100	0.524527	Ser136	1.83162	Asp172	-0.40401	Ser208	0.483583
Ser26	1.25499	Gly62	-0.91148	Leu101	0.61557	Val137	2.38313	Ser173	-0.80902	Pro209	-0.32799
Gln27	0.843454	Val63	-0.91148	Thr102	0.370822	Val138	2.18392	Lys174	-0.24519	Ile210	-0.16893
Tyr28	1.16405	Pro64	-0.91148	Phe103	-0.03238	Cys139	2.44696	Asp175	0.242585	Val211	-0.1191
Ile29	1.35175	Ser68	0.532936	Gly104	1.65716	Phe140	2.44713	Ser176	0.840874	Lys212	-0.11893
Val30	1.35192	Gly69	0.504061	Ala105	1.35877	Leu141	2.59544	Thr177	0.983064	Ser213	0.639186
His31	1.5748	Ser70	1.30921	Gly106	1.03328	Asn142	2.45713	Tyr178	1.57732	Phe214	0.381364
Ser32	1.21904	Gly71	1.25938	Thr107	0.510588	Asn143	0.454523	Ser179	1.54845	Asn215	-0.43249
Asn33	1.36606	Ser72	0.91286	Lys108	0.67754	Phe144	-0.59459	Met180	1.76577	Arg216	0.692154
Gly34	1.22775	Gly73	1.27667	Leu109	0.346151	Tyr145	-0.83294	Ser181	1.62798	Asn217	0.496002
Asn35	1.5131	Thr74	2.30885	Glu110	-0.27131	Pro146	-0.38742	Ser182	1.92883	Glu218	0.09521
Thr36	1.00579	Asp75	2.29733	Ile111	-0.75149	Lys147	0.052048	Thr183	1.94428	Cys219	0.031949

Table S4: Zyggregator calculated Z-scores representing aggregation propensity of each residue for the heavy chain of IgG.

Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score	Residue	Z-Score
Gln1	0.939101	Lys64	-0.08939	Tyr127	-1.50868	Ser190	0.735901	Lys253	0.616636	Cys316	0.432904	Asn379	0.021251
Val2	0.599465	Ser65	-0.02532	Pro128	-1.32098	Ser191	-0.95364	Val254	0.843653	Arg317	0.940215	Gly380	-0.56424
Gln3	0.091299	Arg66	0.307851	Leu129	-1.22994	Thr192	-1.26018	Thr255	0.745184	Val318	0.410191	Gln381	-1.19148
Leu4	0.229224	Ile67	0.633337	Ala130	-1.53648	Trp193	-0.48297	Cys256	2.33625	Asn319	0.553884	Pro382	-1.51392
Lys5	0.452106	Ser68	0.633337	Pro131	-2.15529	Pro194	-0.07795	Val257	2.3963	Ser320	0.290652	Ala383	-1.15816
Glu6	-1.13896	Ile69	0.286819	Gly132	-0.94593	Ser195	0.22859	Val258	2.34775	Ala321	-0.46746	Glu384	-1.84346
Ser7	-1.35555	Thr70	0.813407	Ser133	-1.1573	Glu196	0.22859	Val259	1.94273	Ala322	-0.84918	Asn385	-1.71145
Gly8	-1.35555	Lys71	0.367716	Ala134	-0.67711	Thr197	0.43636	Asp260	0.818089	Phe323	-2.13387	Tyr386	-0.02191
Pro9	-0.64244	Asp72	-0.47831	Ala135	0.44859	Val198	1.72106	Ile261	0.352722	Pro324	-2.06358	Lys387	-0.07857
Gly10	-0.2103	Asn73	-0.73631	Gln136	0.357547	Thr199	2.0276	Ser262	-0.11264	Ala325	-2.68341	Asn388	-0.85579
Leu11	-1.49483	Ser74	-1.27316	Thr137	0.499737	Cys200	2.64744	Lys263	-1.70371	Pro326	-2.8271	Thr389	-0.59797
Val12	-1.58587	Lys75	-0.37236	Asp138	1.06915	Asn201	2.2243	Asp264	-2.2399	Ile327	-3.06464	Gln390	-0.81171
Ala13	-0.43319	Ser76	0.241315	Ser139	1.54934	Val202	0.445535	Asp265	-2.37904	Glu328	-1.33442	Pro391	-0.56397
Pro14	-0.52423	Gln77	0.508366	Met140	1.76071	Ala203	0.153044	Pro266	-2.25911	Lys329	-1.44694	Ile392	-0.15913
Ser15	-0.52423	Val78	0.2895	Val141	1.44673	His204	-0.75273	Glu267	-1.3977	Thr330	-0.56898	Met393	-0.72297
Gln16	-0.579	Phe79	1.02595	Thr142	2.32364	Pro205	-0.75291	Val268	-1.42658	Ile331	-0.42196	Asp394	-0.31239
Ser17	-0.05814	Leu80	1.02612	Leu143	2.21547	Ala206	-0.65444	Gln269	-0.75744	Ser332	-0.32121	Thr395	1.15983
Leu18	1.6314	Lys81	1.15796	Gly144	2.37982	Ser207	-1.17352	Phe270	0.981934	Lys333	0.176393	Asp396	1.25777
Ser19	2.34948	Met82	0.930944	Cys145	1.66672	Ser208	-1.03655	Ser271	1.98349	Thr334	-0.75504	Gly397	1.57043
Ile20	2.88634	Asn83	0.156558	Leu146	1.35275	Thr209	0.276846	Trp272	1.51812	Lys335	-2.29756	Ser398	1.8481
Thr21	3.19288	Ser84	0.482044	Val147	1.62915	Lys210	-0.05454	Phe273	1.05167	Gly336	-2.70412	Tyr399	1.98672
Cys22	3.11335	Leu85	0.729784	Lys148	1.99296	Val211	-0.46111	Val274	0.715668	Arg337	-2.37273	Phe400	2.14554
Thr23	2.76493	Gln86	0.616469	Gly149	0.178047	Asp212	-0.20311	Asp275	0.83552	Pro338	-4.06227	Val401	1.64794
Val24	2.96179	Thr87	0.833613	Tyr150	-0.40879	Lys213	-0.48928	Asp276	0.819449	Lys339	-3.97524	Tyr402	1.72746
Ser25	2.55677	Asp88	0.75844	Phe151	-1.99986	Lys214	-1.36724	Val277	0.534173	Ala340	-3.75974	Ser403	1.62347
Gly26	1.91822	Asp89	0.272351	Pro152	-1.28675	Ile215	-2.2002	Glu278	0.632643	Pro341	-2.6897	Lys404	1.47517
Phe27	1.59273	Thr90	0.760123	Glu153	-0.97278	Val216	-2.2002	Val279	0.528595	Gln342	-1.00016	Leu405	1.22448
Ser28	1.18953	Ala91	0.711041	Pro154	-0.5827	Pro217	-1.07555	His280	0.669296	Val343	-0.3356	Asn406	0.461985
Leu29	1.54546	Lys92	1.58795	Val155	-0.63254	Arg218	-0.57795	Thr281	0.767765	Tyr344	-1.35726	Val407	0.274289
Leu30	1.54546	Tyr93	2.15178	Thr156	1.1623	Asp219	-0.11786	Ala282	0.70377	Thr345	-1.54495	Gln408	0.681024
Gly31	1.39716	Tyr94	1.22035	Val157	1.66978	Cys220	-0.83097	His283	-0.8873	Ile346	-2.32225	Lys409	1.11181
Tyr32	1.39733	Cys95	1.22035	Thr158	2.95431	Gly221	-0.45558	Thr284	-1.39559	Pro347	-3.22305	Ser410	0.164859
Gly33	1.82812	Thr96	0.342389	Trp159	2.55111	Cys222	0.788921	Gln285	-2.30792	Pro348	-3.8986	Asn411	-0.21686
Val34	2.05513	Arg97	0.342389	Asn160	2.14609	Lys223	1.01804	Pro286	-2.36466	Pro349	-4.43545	Trp412	0.006023
Asn35	1.43768	Ala98	0.077499	Ser161	1.47961	Pro224	1.20574	Arg287	-2.29068	Lys350	-4.36356	Glu413	0.412758
Trp36	1.1376	Pro99	-1.23484	Gly162	1.0746	Cys225	1.70741	Glu288	-2.24085	Glu351	-3.1542	Ala414	0.817772
Val37	-0.23797	Tyr100	-1.95939	Ser163	0.564284	Ile226	1.29587	Glu289	-1.92114	Gln352	-2.27624	Gly415	1.27245
Arg38	-1.82904	Gly101	-1.07704	Leu164	0.84285	Cys227	0.417904	Gln290	-0.82431	Met353	-1.15054	Asn416	1.16715
Gln39	-1.73817	Lys102	-0.54702	Ser165	1.14939	Thr228	1.19512	Phe291	0.107118	Ala354	-1.15054	Thr417	2.39254
Pro40	-2.38032	Gln103	0.850028	Ser166	1.04023	Val229	0.595885	Asn292	1.06928	Lys355	-0.33668	Phe418	2.46772
Pro41	-2.59582	Tyr104	0.850028	Gly167	1.44524	Pro230	0.525587	Ser293	1.05017	Asp356	0.422319	Thr419	2.68322
Gly42	-1.98988	Phe105	1.2693	Val168	1.63287	Glu231	-0.38019	Thr294	1.18201	Lys357	0.359657	Cys420	2.57488
Gln43	-2.36535	Ala106	1.7669	His169	0.348346	Val232	-0.47866	Phe295	1.03371	Val358	0.839844	Ser421	2.15174
Gly44	-0.75821	Tyr107	1.7669	Thr170	0.273173	Ser233	-0.51805	Arg296	1.03354	Ser359	1.96449	Val422	1.18958
Leu45	0.605844	Trp108	1.68971	Phe171	0.488675	Ser234	1.39986	Ser297	0.338529	Leu360	2.0778	Leu423	0.687914
Glu46	0.656991	Gly109	1.63987	Pro172	0.261658	Val235	2.36202	Val298	-0.17465	Thr361	2.36697	His424	0.424752
Trp47	0.879873	Gln110	1.60688	Ala173	0.14794	Phe236	0.770952	Ser299	-1.72633	Cys362	2.65313	Glu425	0.40663
Leu48	0.859129	Gly111	1.55749	Val174	-0.25707	Ile237	-0.51357	Glu300	-0.75423	Met363	2.05485	Gly426	0.100259
Met49	1.10222	Thr112	1.45219	Leu175	-0.87075	Phe238	-0.92014	Leu301	-0.61204	Ile364	2.43017	Leu427	0.00261
Gly50	1.70057	Leu113	1.85539	Gln176	0.493306	Pro239	-2.51121	Pro302	-0.74901	Thr365	2.29231	His428	0.00261
Ile51	1.03144	Val114	1.98723	Ser177	0.924412	Pro240	-3.37262	Ile303	-1.06854	Asp366	0.102004	Asn429	0.914934
Trp52	1.04295	Thr115	1.82101	Asp178	1.02288	Lys241	-3.78943	Met304	-0.72006	Phe367	-0.3598	His430	0.316581
Gly53	0.90076	Val116	1.34083	Leu179	1.02288	Pro242	-4.12544	His305	-0.10157	Phe368	-0.96431	His431	-0.16951
Asp54	1.40243	Ser117	0.854736	Tyr180	1.15472	Lys243	-2.94908	Gln306	1.26248	Pro369	-1.11133	Thr432	0.036309
Gly55	0.985612	Ala118	0.953205	Thr181	1.15472	Asp244	-1.25954	Asp307	1.00466	Glu370	-0.10803	Glu433	0.115664
Ser56	1.01893	Ala119	0.953205	Leu182	1.31354	Val245	-0.15552	Trp308	0.95351	Asp371	-0.25634	Lys434	0.133785
Thr57	1.11576	Lys120	-0.63787	Ser183	1.54056	Leu246	1.53402	Leu309	0.565069	Ile372	-0.77903	Ser435	0.133785
Asp58	1.27458	Thr121	-1.92239	Ser184	1.58964	Thr247	2.02011	Asn310	0.189598	Thr373	1.01581	Leu436	-0.27123
Tyr59	1.10836	Thr122	-1.84722	Ser185	1.49117	Ile248	2.58395	Gly311	0.615574	Val374	1.64305	Ser437	-1.04844
Asn60	1.18789	Pro123	-1.4655	Val186	-0.06058	Thr249	0.992878	Lys312	-0.3013	Glu375	2.31218	His438	-0.55084
Ser61	0.188617	Pro124	-0.703	Thr187	-0.06058	Leu250	0.694484	Glu313	0.337255	Trp376	2.05436	Ser439	-0.55092
Ala62	0.535135	Ser125	-2.39254	Val188	-0.06058	Thr251	0.596015	Phe314	-0.18933	Gln377	1.67632	Pro440	-0.86521
Leu63	-0.34721	Val126	-2.71803	Pro189	0.532133	Pro252	0.491264	Lys315	0.026169	Trp378	1.23793	Gly441	-0.89465

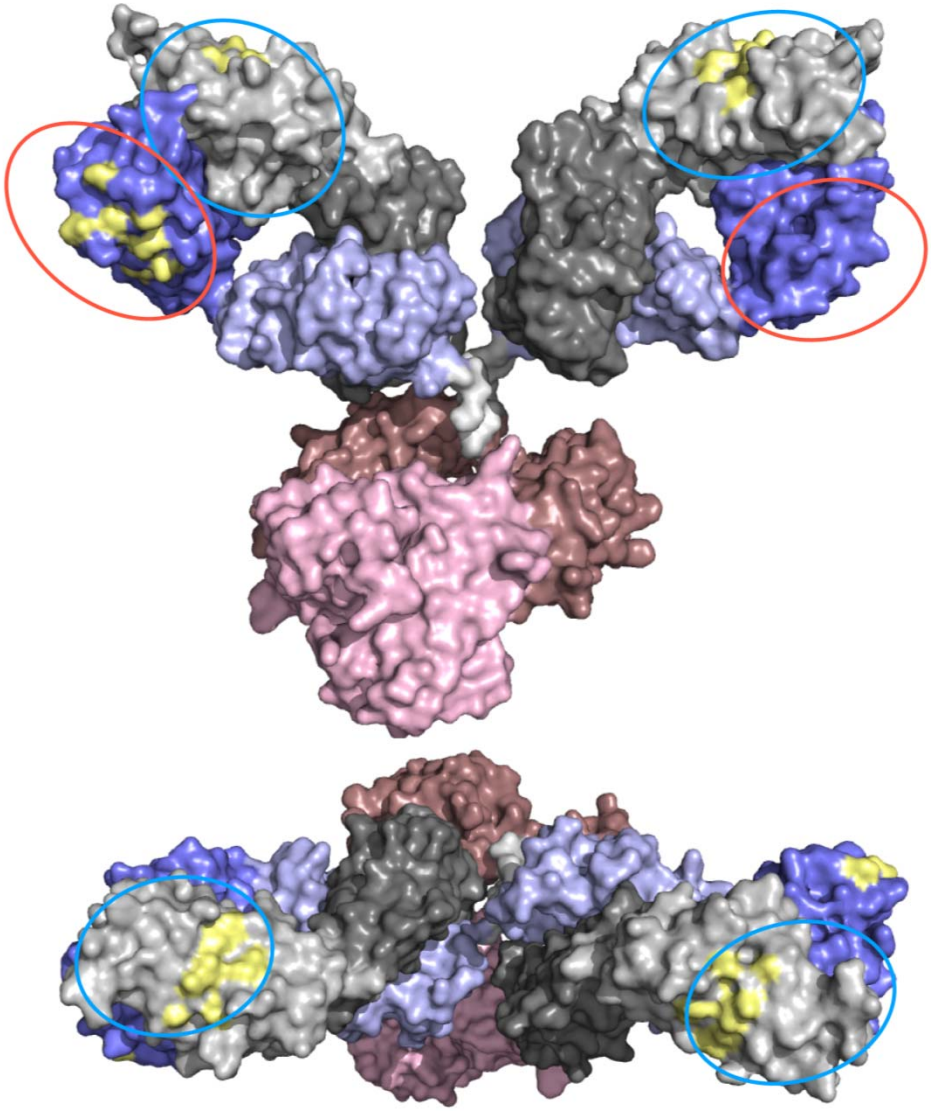


Figure S6 Structure of IgG1 with each domain represented in a different color: V_L (light grey), V_C (dark grey), V_H (dark blue), C_H^1 (light blue), C_H^2 (dark purple), and C_H^3 (pink). The potential interfacial residues are circled and represented in yellow.

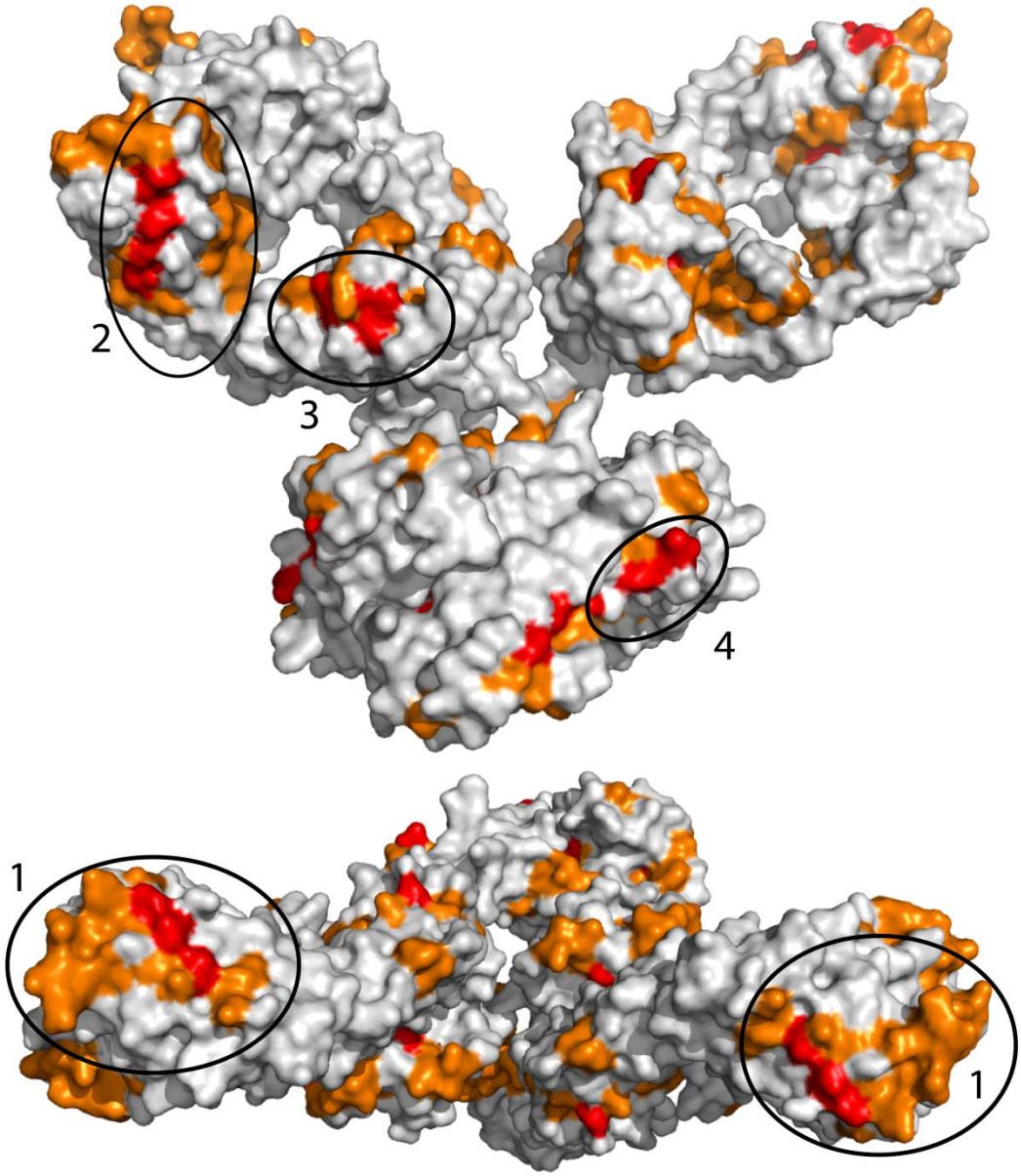


Figure S7: Propensities for aggregation from Zyggregator as indicated by Z-scores. Z-Score > 2 (red) Z-score > 1 (orange). A higher score denotes an increased likelihood of aggregation. Circled sites highlight the regions where there is a clustering of residues with a high calculated aggregation propensity. Labeled sites 1 and 2 are the covalent labeling indicated aggregation sites on the V_L domain and the V_H domain, respectively.

Table S5: Modification percentages for individual residues of HGH before and after heating at 65 °C for 24 hours.

HGH	Native	24 hour	Significant*
N-Term	5% ± 4%	9% ± 5%	no
Thr4	12% ± 3%	10% ± 2%	no
Ser8	5% ± 1%	3.6% ± 0.8%	no
His19	39.9% ± 0.4%	37.7% ± 0.7%	yes
His22	5% ± 2%	6% ± 1%	no
Thr28	0.73% ± 0.01%	0.66% ± 0.03%	yes
Tyr29	30% ± 7%	33% ± 8%	no
Tyr36	6.3% ± 0.1%	5.9% ± 0.3%	no
Lys39	0.91% ± 0.07%	0.93% ± 0.07%	no
Lys42	6.1% ± 0.3%	5.2% ± 0.7%	no
Tyr43	8.0% ± 0.3%	8% ± 1%	no
Ser44	1.2% ± 0.2%	1.14% ± 0.06%	no
Thr51	3.0% ± 0.7%	3.7% ± 0.7%	no
Ser56	12% ± 2%	14% ± 2%	no
Ser58	15% ± 2%	16% ± 2%	no
Thr61	2.7% ± 0.5%	1.8% ± 0.2%	yes
Thr68	71% ± 14%	72% ± 9%	no
Ser86	15% ± 2%	12% ± 1%	no
Ser96	17% ± 4%	14% ± 1%	no
Ser101	15% ± 2%	13% ± 1%	no
Tyr104	0.8% ± 0.4%	0.5% ± 0.1%	no
Ser107	1.5% ± 0.5%	0.9% ± 0.2%	no
Ser109	0.8% ± 0.3%	0.6% ± 0.1%	no
Tyr112	0.8% ± 0.2%	0.5% ± 0.1%	no
Lys116	1.6% ± 0.7%	0.9% ± 0.2%	no
Thr124	1.2% ± 0.3%	1.0% ± 0.1%	no
Ser133	16% ± 1%	14% ± 3%	no
Thr136	42% ± 2%	60% ± 6%	yes
Lys141	42% ± 5%	40% ± 7%	no
Thr143	80% ± 6%	84% ± 4%	no
Ser145	1.6% ± 0.5%	1.4% ± 0.3%	no
Lys146	0.83% ± 0.02%	0.8% ± 0.2%	no
Thr149	7% ± 4%	9% ± 4%	no
Ser151	2.2% ± 0.3%	2.3% ± 0.3%	no
His152	2% ± 1%	3% ± 1%	no
Lys159	0.37% ± 0.05%	0.8% ± 0.2%	yes
Tyr165	64% ± 2%	45% ± 2%	yes
Lys169	10% ± 1%	14% ± 3%	no
Lys173	0.9% ± 0.4%	1.3% ± 0.3%	no
Thr176	0.14% ± 0.01%	0.14% ± 0.01%	no
Ser185	38% ± 8%	51% ± 4%	no

*A difference was considered significant if the p-value, calculated by performing an unpaired T-test, was less than 0.05 (corresponding to a 95% confidence level, n=3).

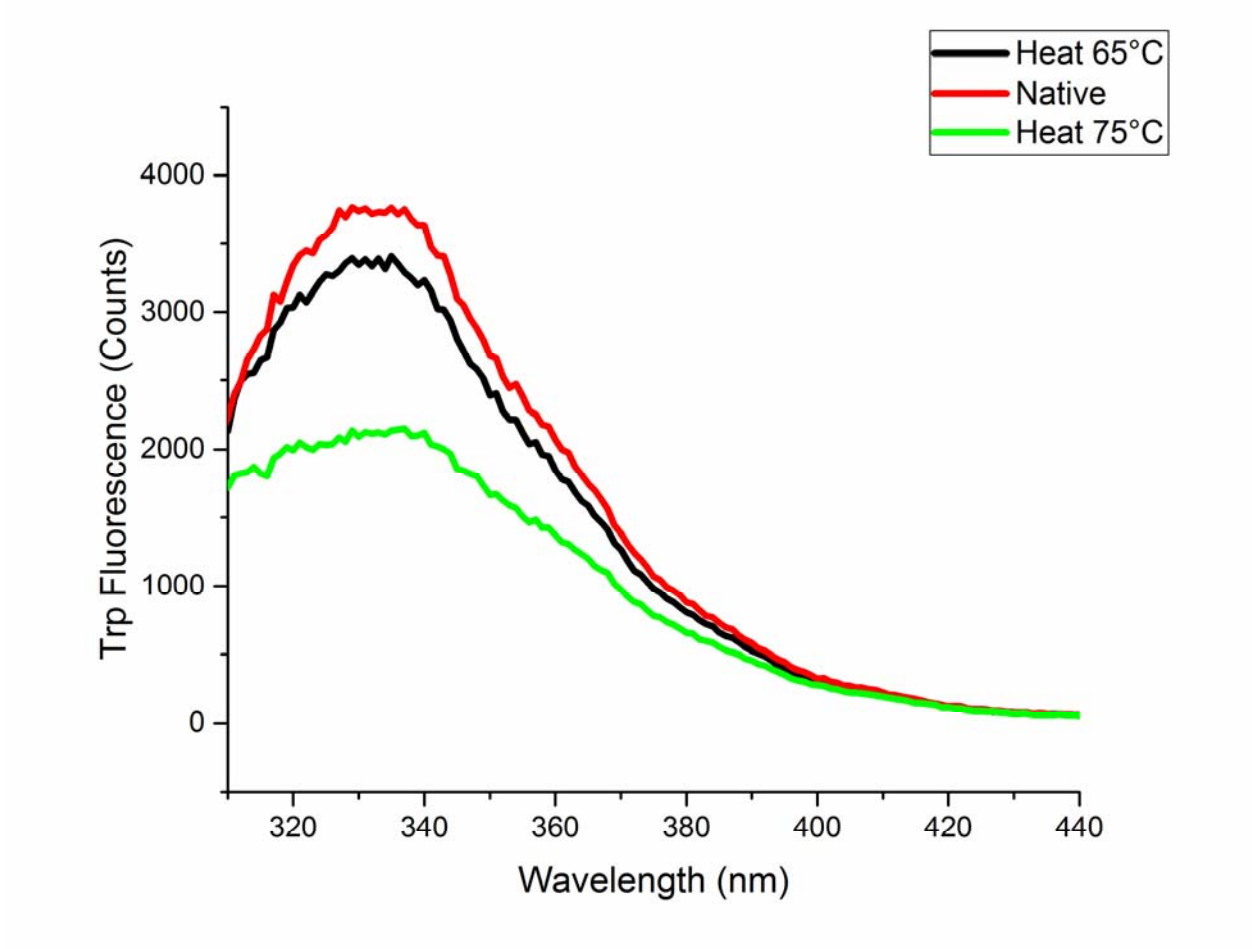


Figure S8: Tryptophan fluorescence of HGH under normal (red), heated to 65 ^{o}C (black), and heated to 75 ^{o}C (green) conditions. The fluorescence spectrum at 75 ^{o}C is included as a positive control to demonstrate that fluorescence can reveal structural perturbations for HGH.

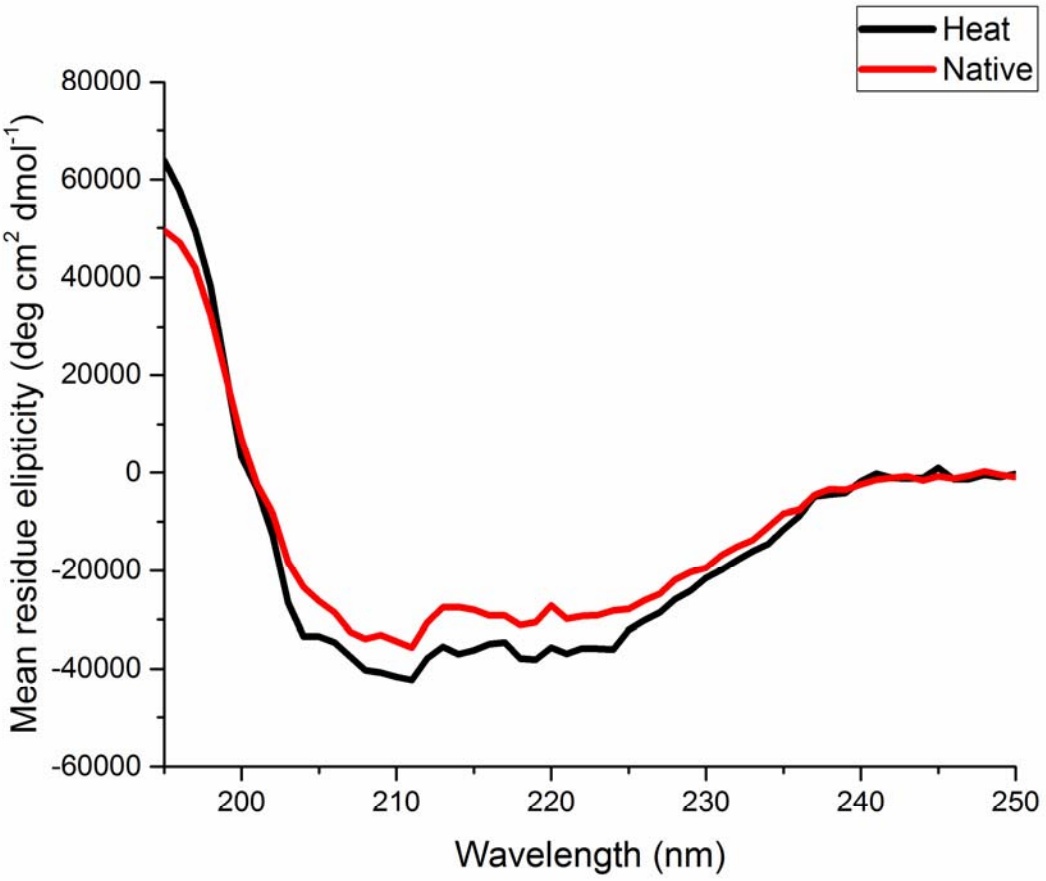


Figure S9: Circular dichroism of HGH under normal (red) and heated (65 ^{o}C, black) conditions. CD does not reveal any significant changes in HGH structure.

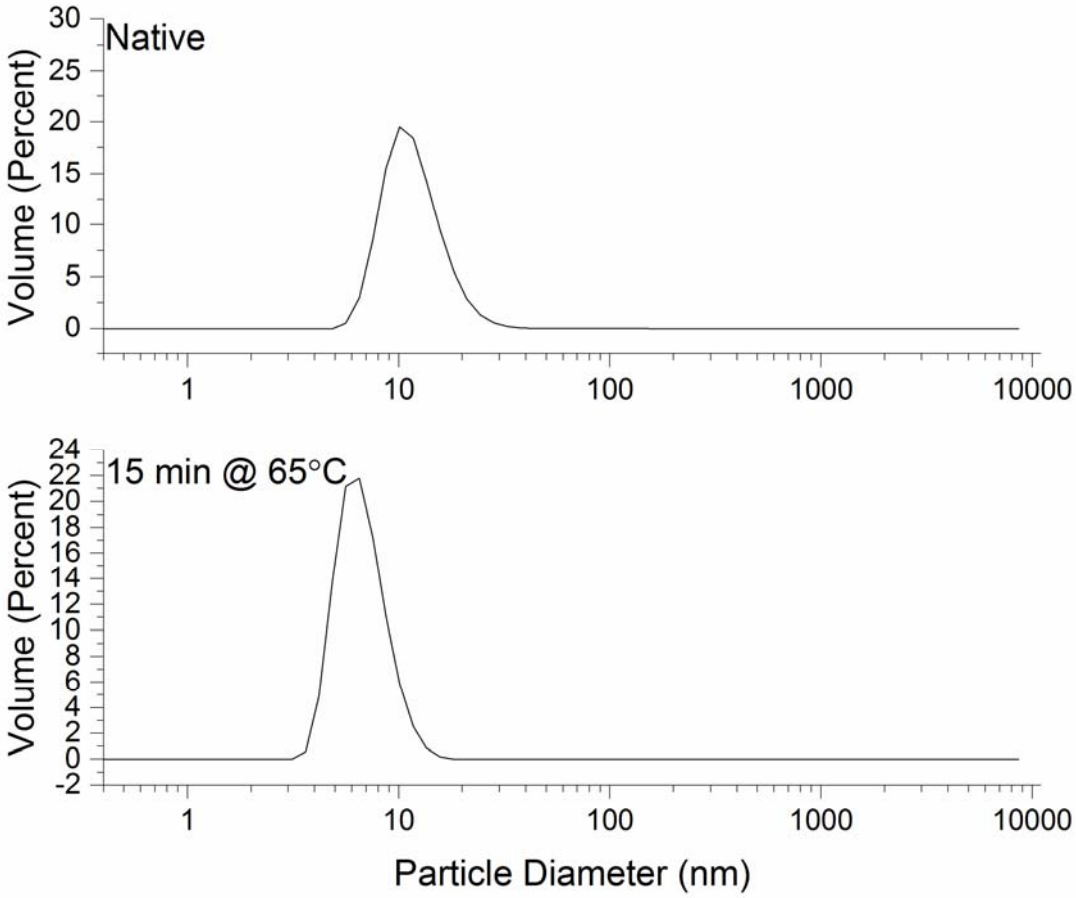


Figure S10: Dynamic light scattering data for HGH before (top) and after heating at 65 ^{o}C for 24 h (bottom). These data demonstrate that HGH undergoes some degree of compaction upon heating.