Technische Universität Braunschweig

Wätzig et al.
Institut für Medizinische und Pharmazeutische Chemie

Protein quality and binding affinities by Affinity CE and 2-dimensional separations

Hermann Wätzig, Christin Scheller, Finja Krebs, Mais Olabi, Ratih Ratih, Rebecca Wiesner, Robert Minkner, Holger Zagst, Matthias Stein, Imke Oltmann-Norden
Institute of Medicinal and Pharmaceutical Chemistry, University of Braunschweig, Germany

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Protein quality and binding affinities by Affinity CE and 2-dimensional separations

Importance of protein classification

2-dimensional separations

Case study: isoelectric points of SARS-CoV-2 and ACE2 proteins

Case study: collagen

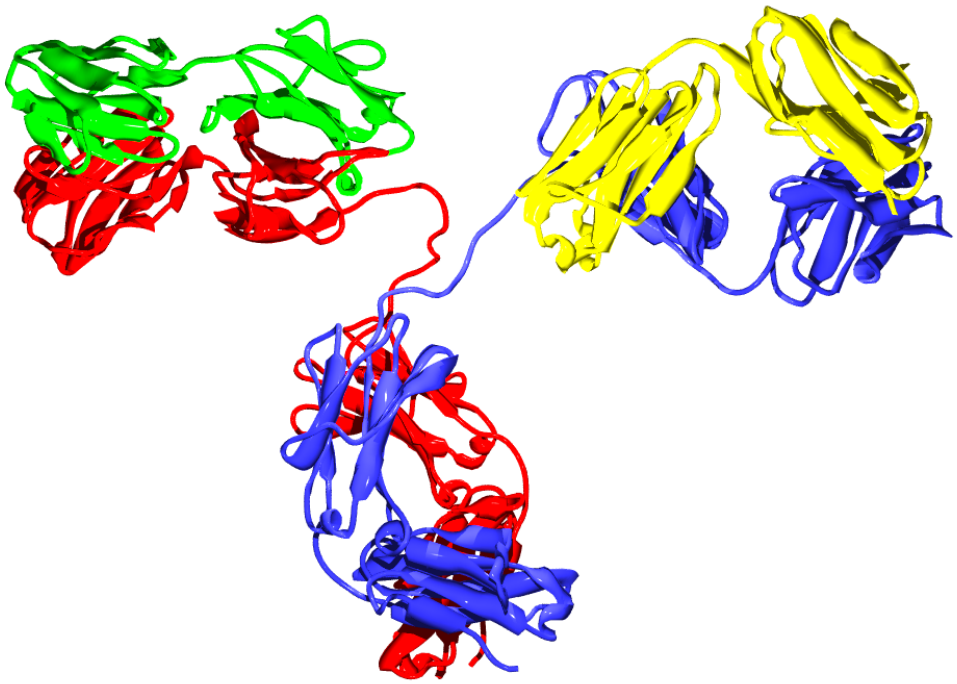
Affinity CE (ACE)

Uncertainty of Ligand Binding Assays

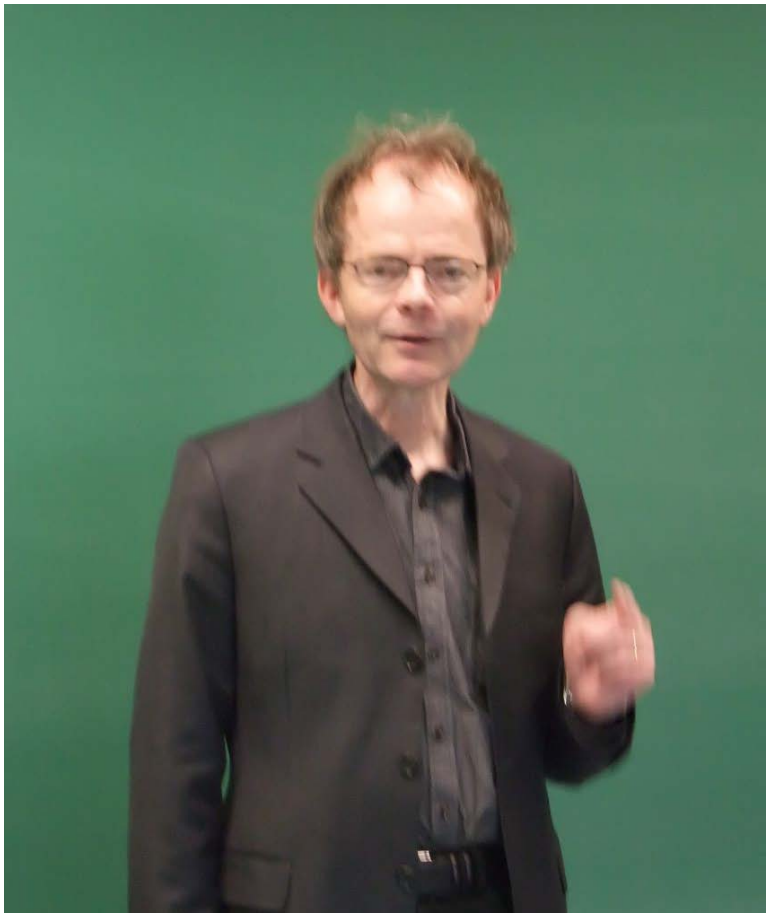
Conclusions and outlook

Physicochemical properties of the SARS-CoV-2 for drug targeting, virus inactivation and attenuation, vaccine formulation and quality control
Christin Scheller, Finja Krebs, Robert Minkner, Isabel Astner, Maria Gil-Moles, Hermann Wätzig
Electrophoresis 2020, 41, 1137-1151; http://dx.doi.org/10.1002/elps.202000121

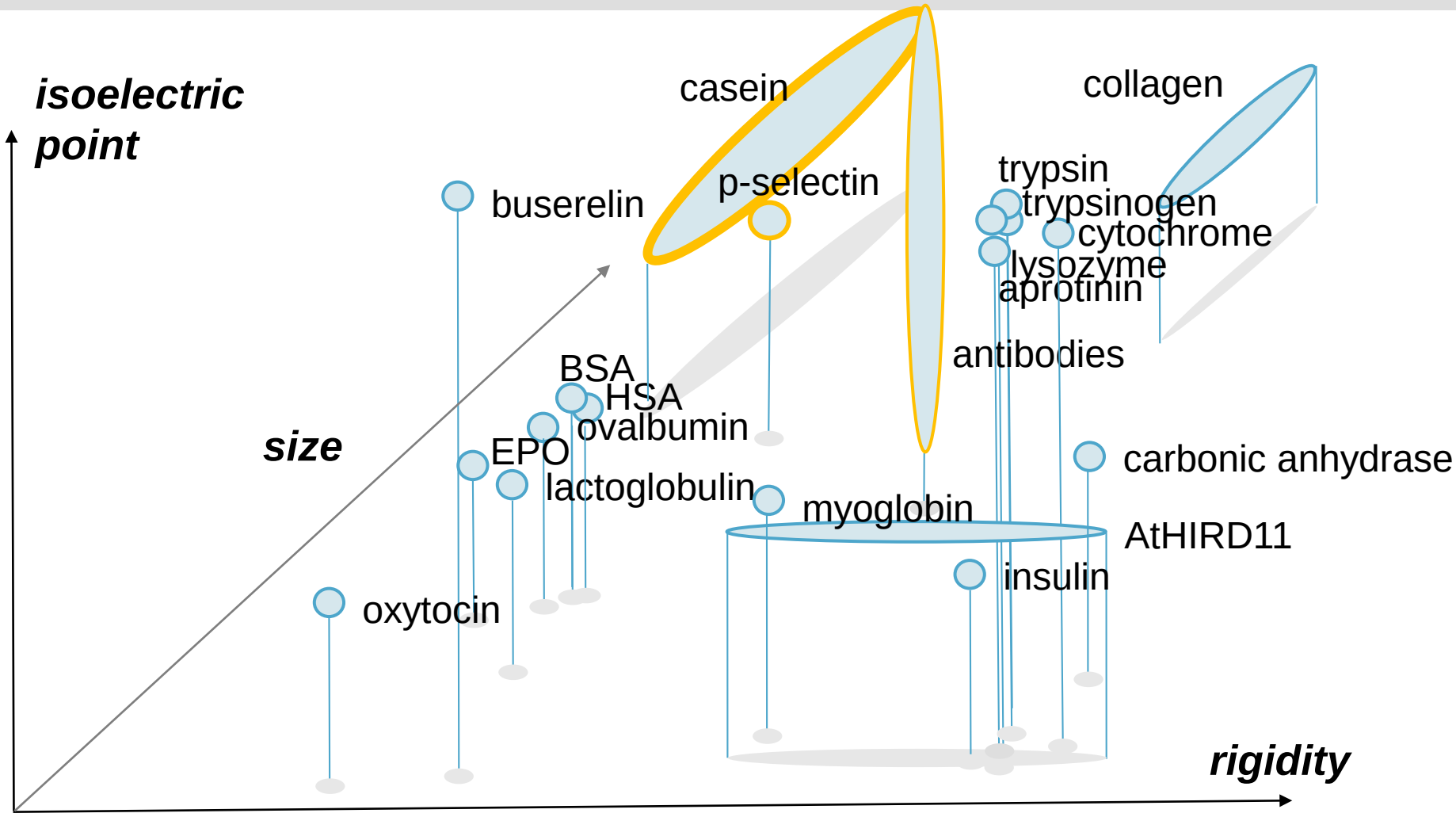
A very beautiful therapeutic protein



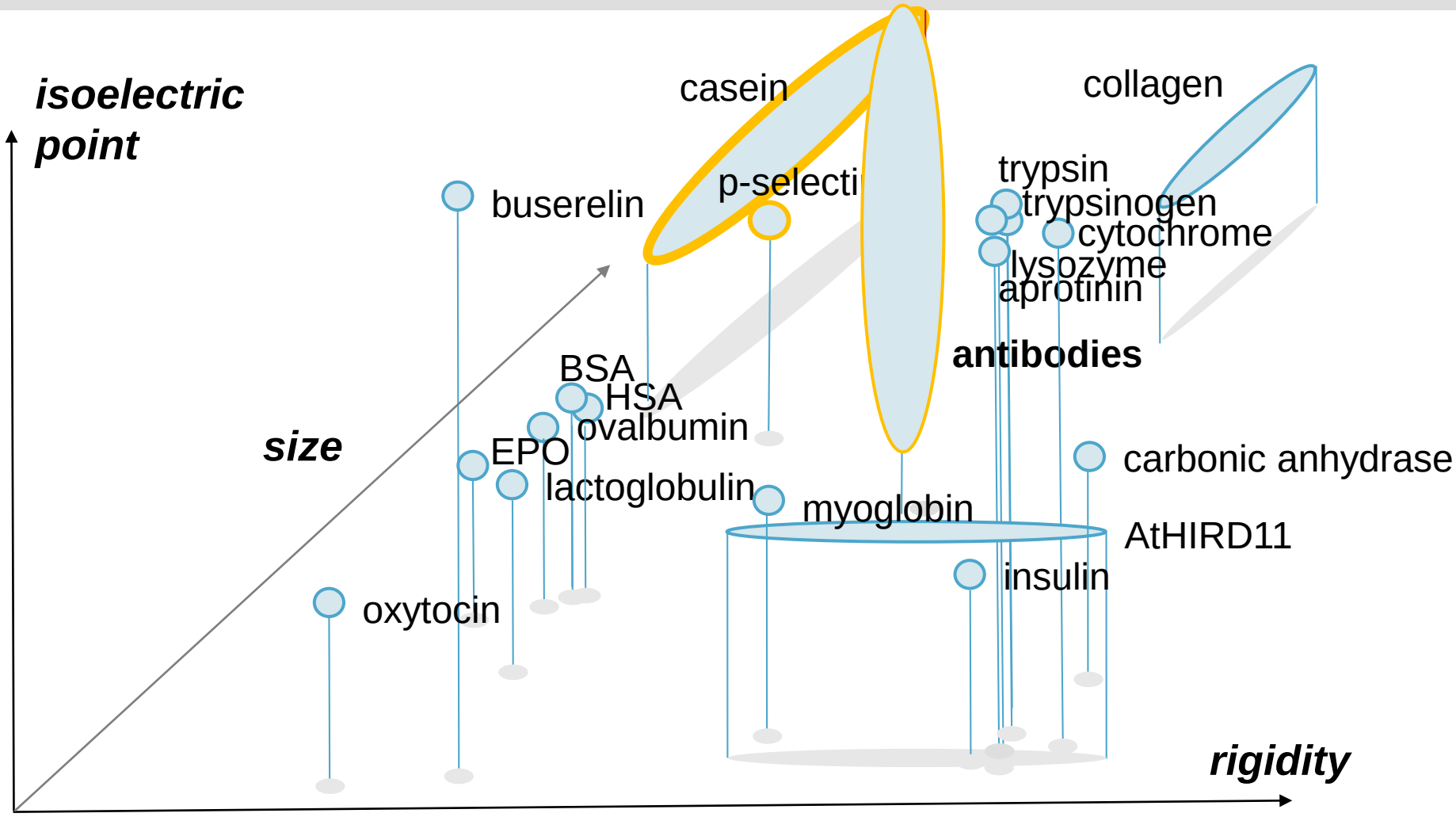
TimVickers at English Wikipedia / Public domain



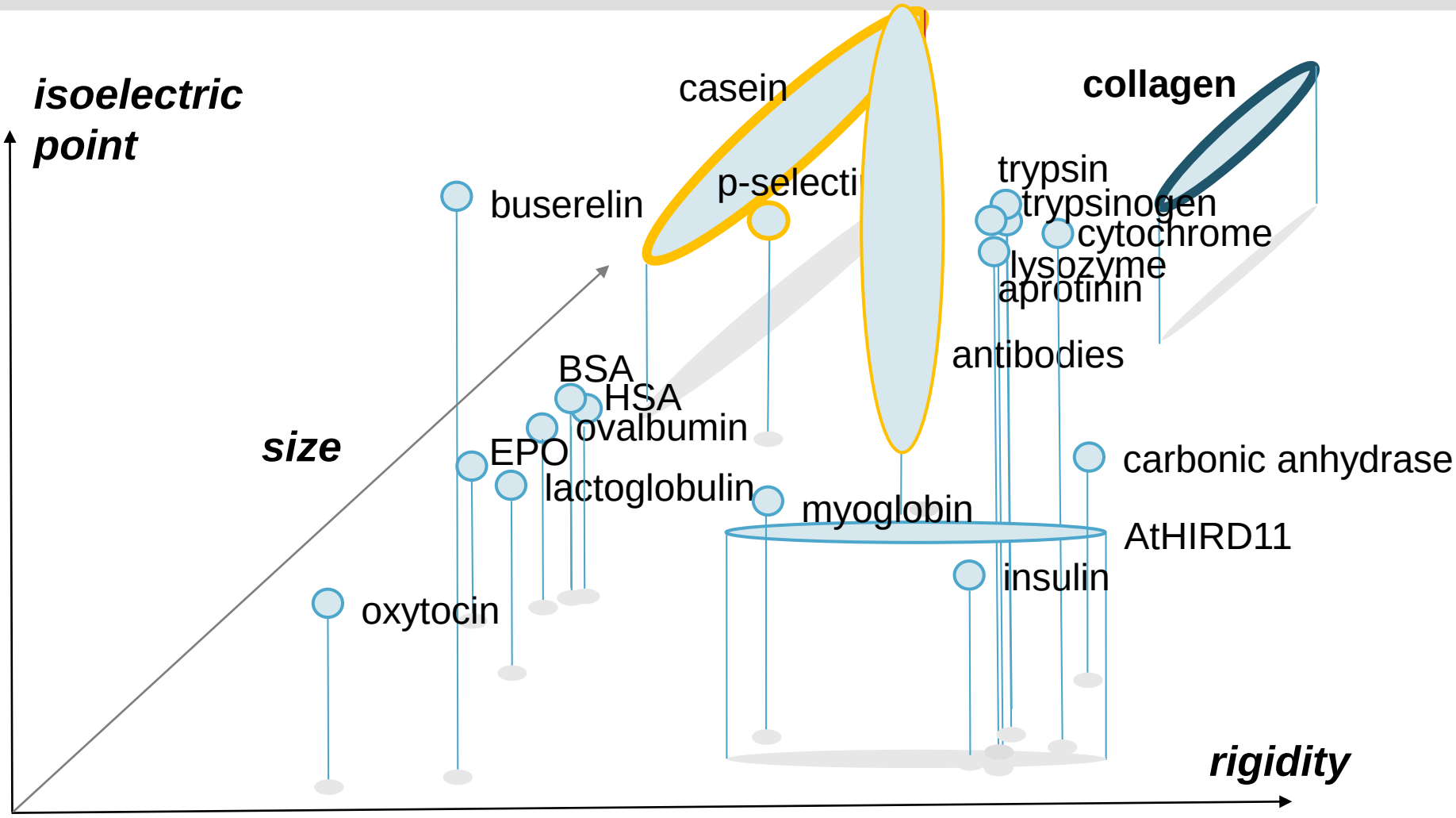
Physicochemical Properties of Proteins

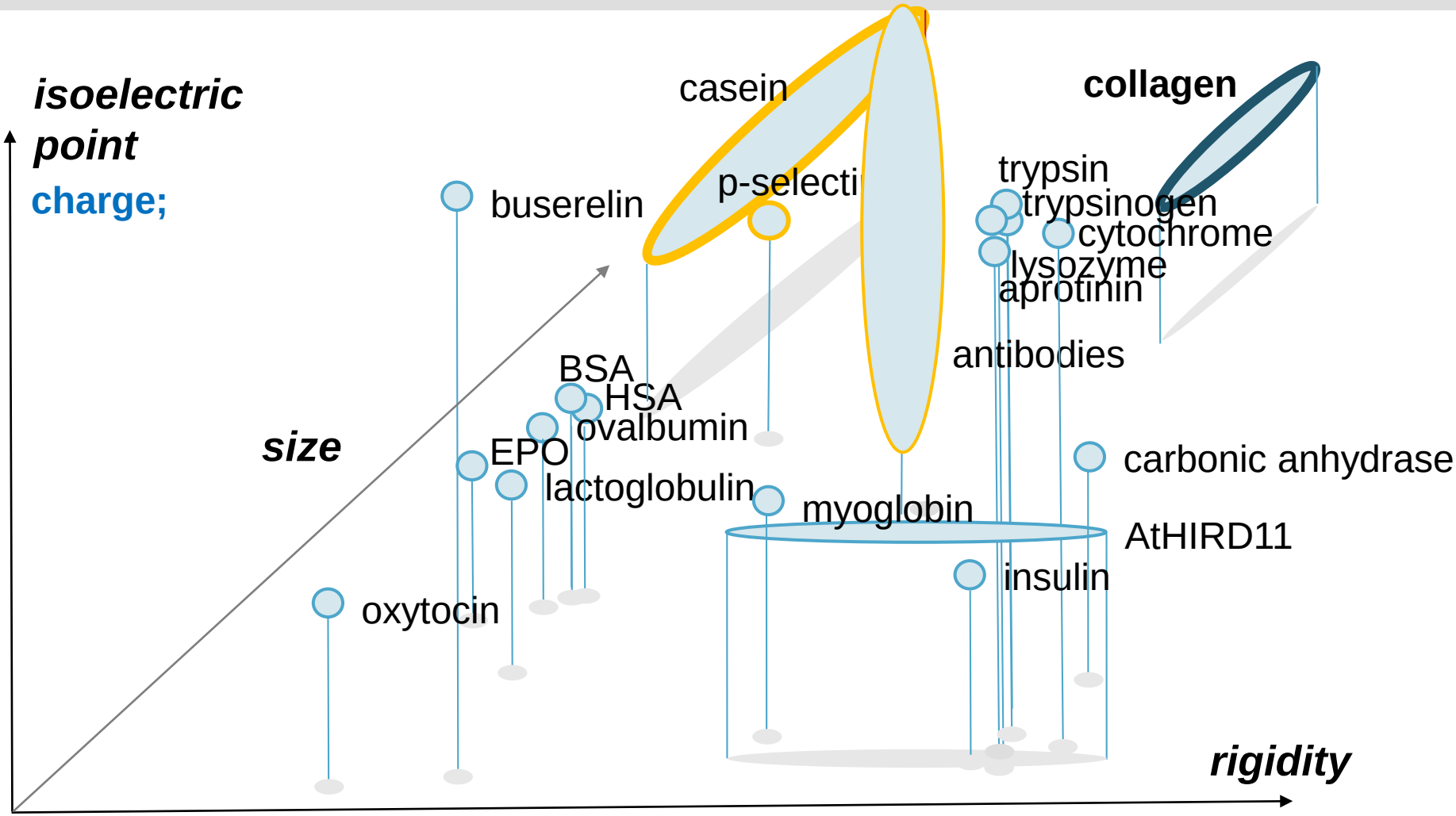


Physicochemical Properties of Proteins

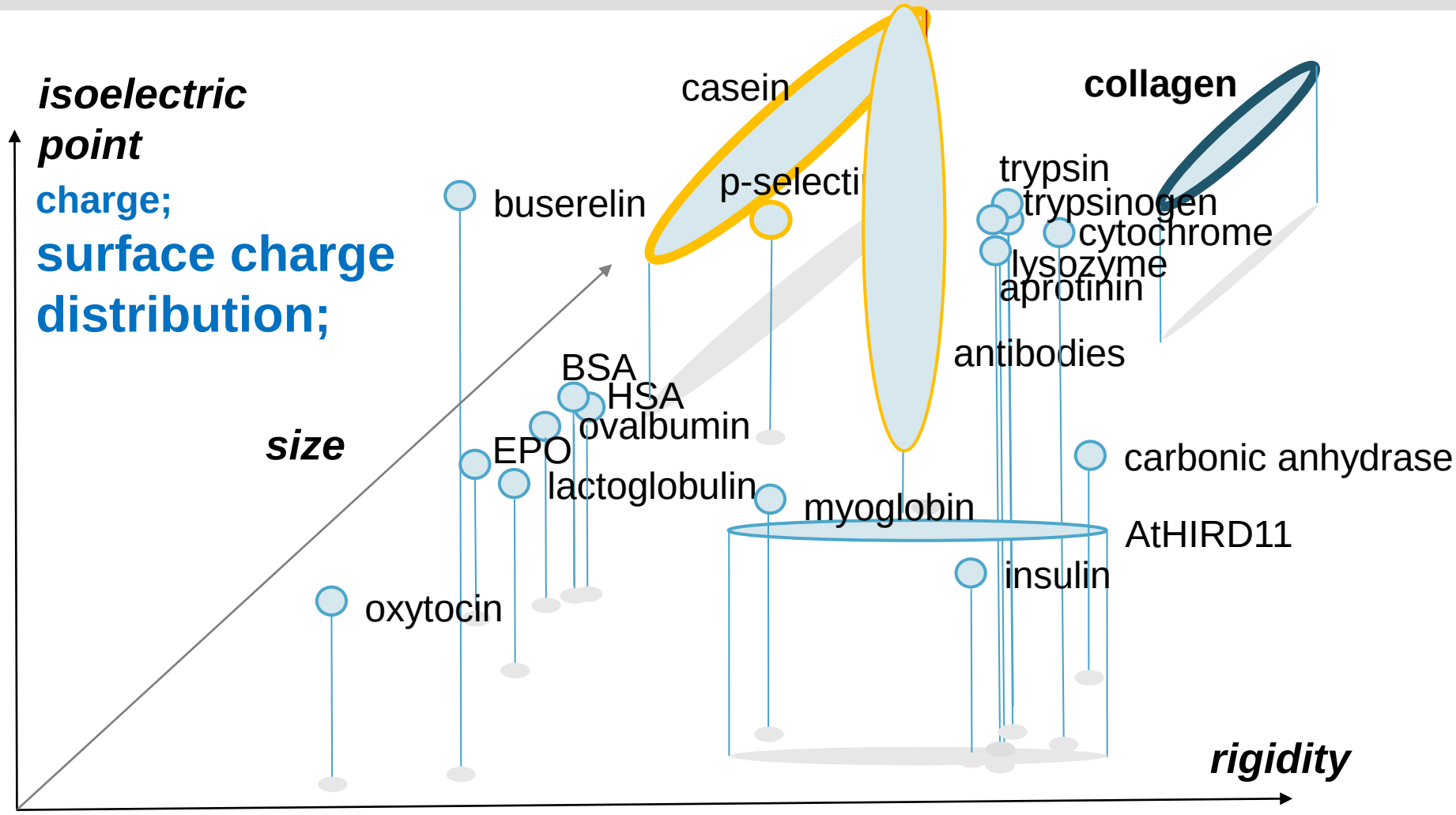


Physicochemical Properties of Proteins

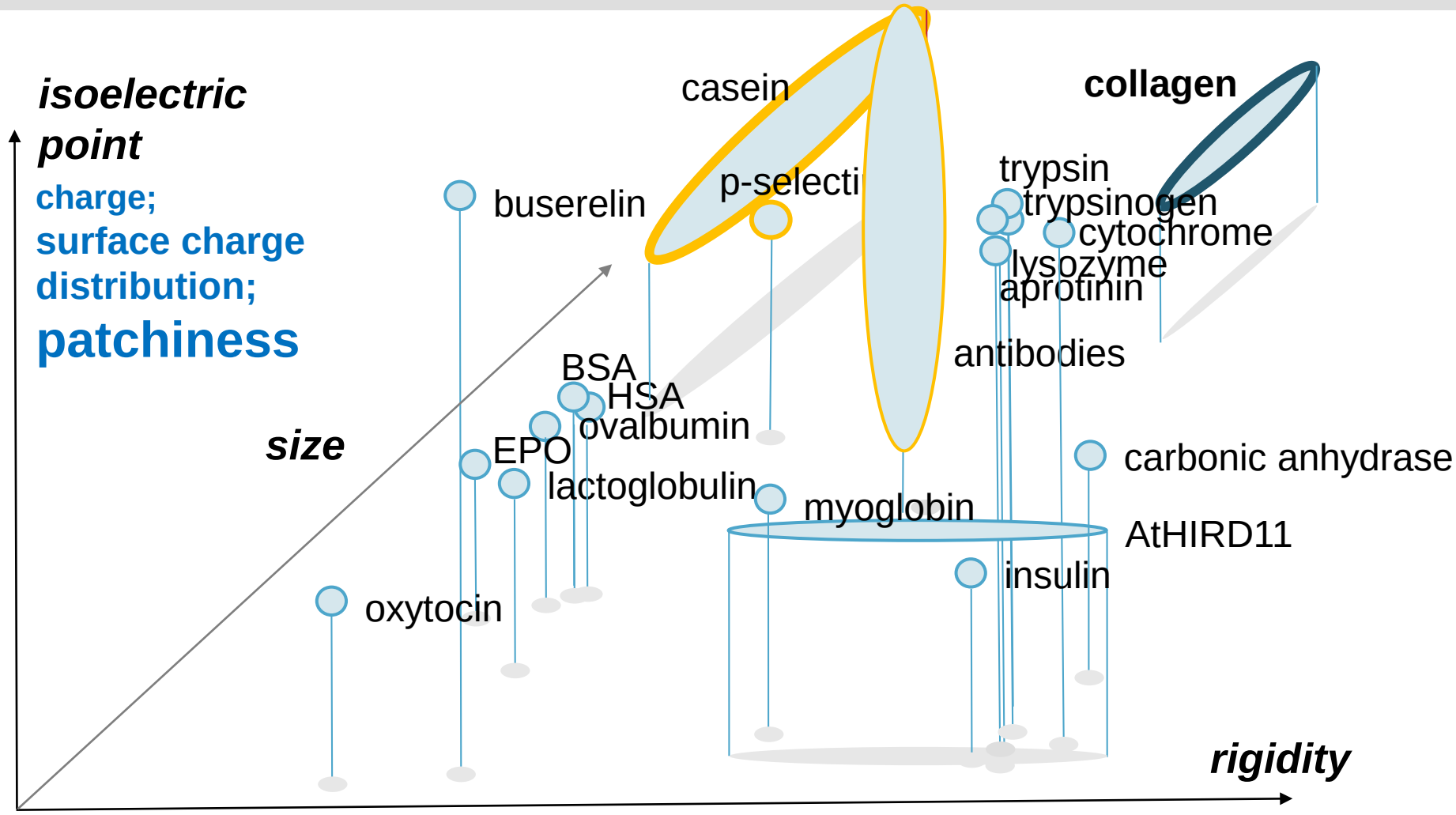



isoelectric point
charge;
size
rigidity
casein
collagen
p-selecti
buserelin
trypsin
trypsinogen
cytochrome
lysozyme
aprotinin
antibodies
BSA
HSA
ovalbumin
EPO
lactoglobulin
myoglobin
carbonic anhydrase
AtHIRD11
insulin
oxytocin

Surface Charge Distribution



Surface Charge Distribution



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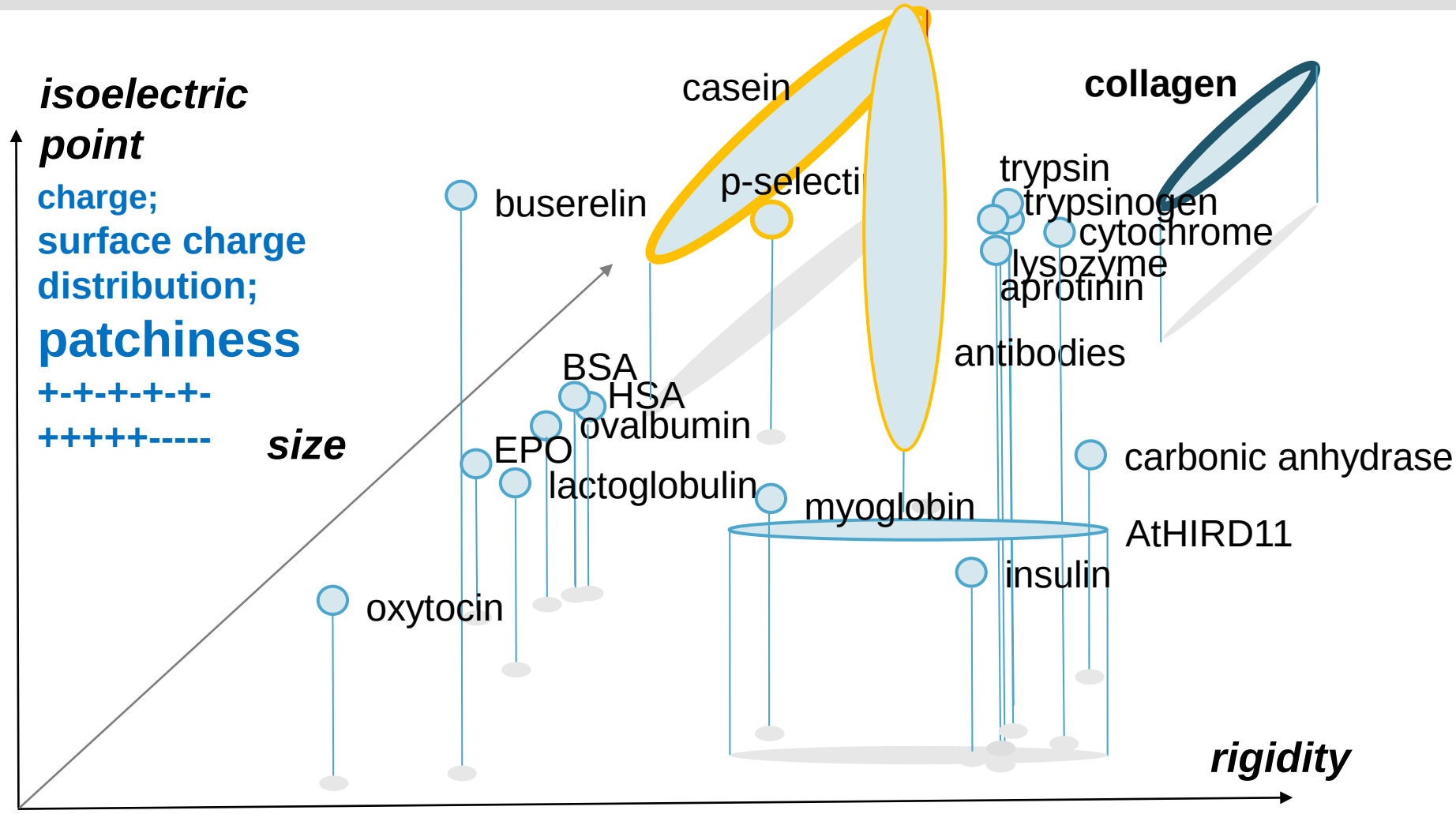

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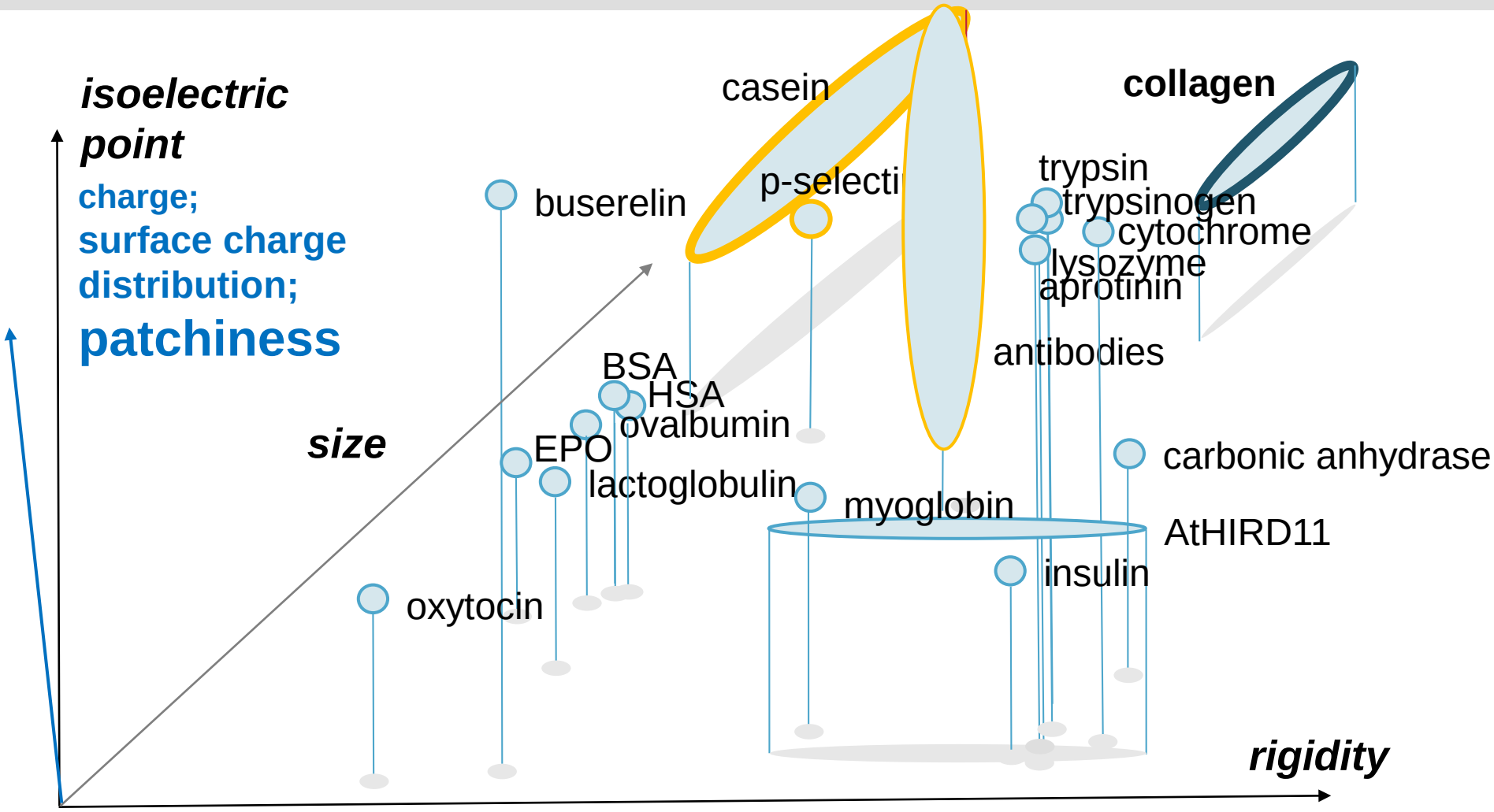
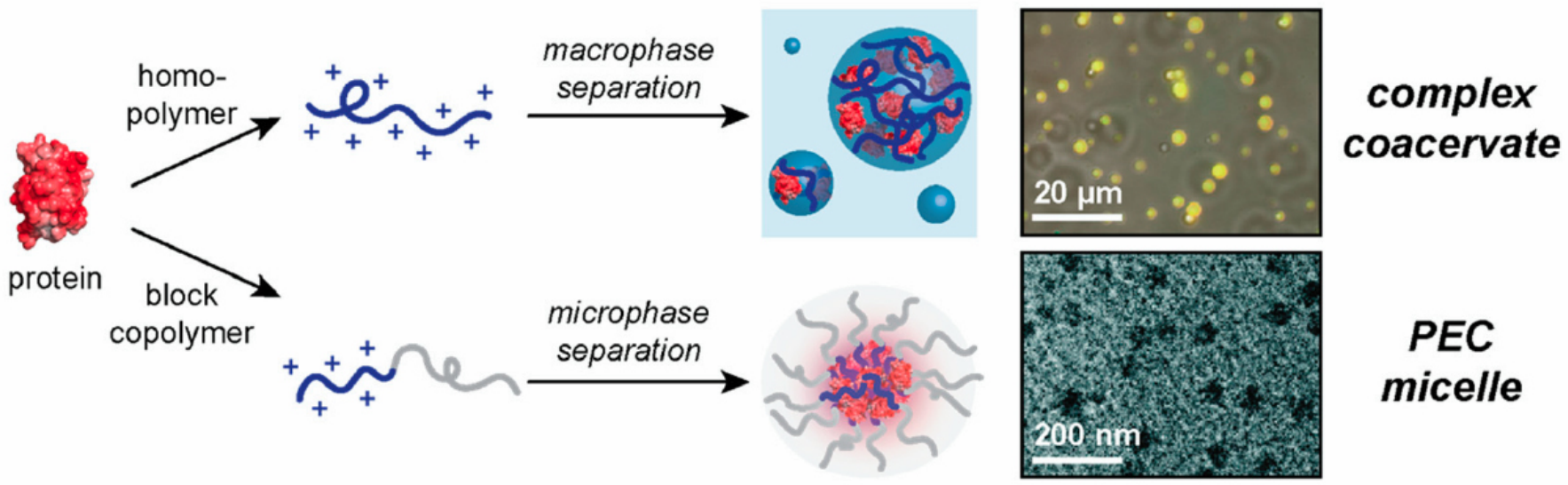


Surface Charge Distribution

Horn, Kapelner, Obermeyer, Polymers **2019**, 11, 578;
doi:10.3390/polym11040578 and [34, 35] cited therein



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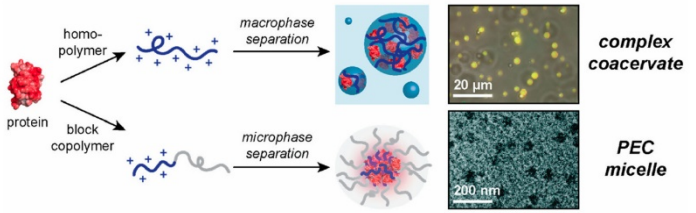
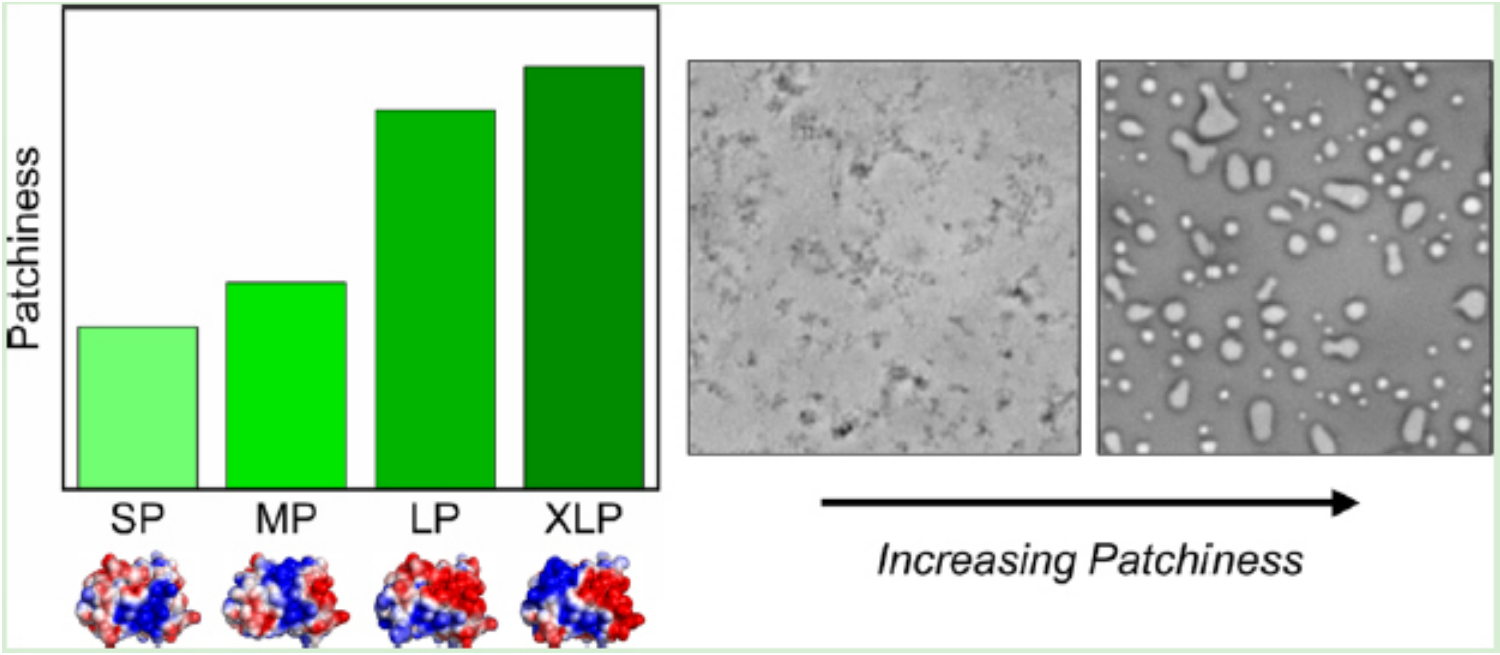
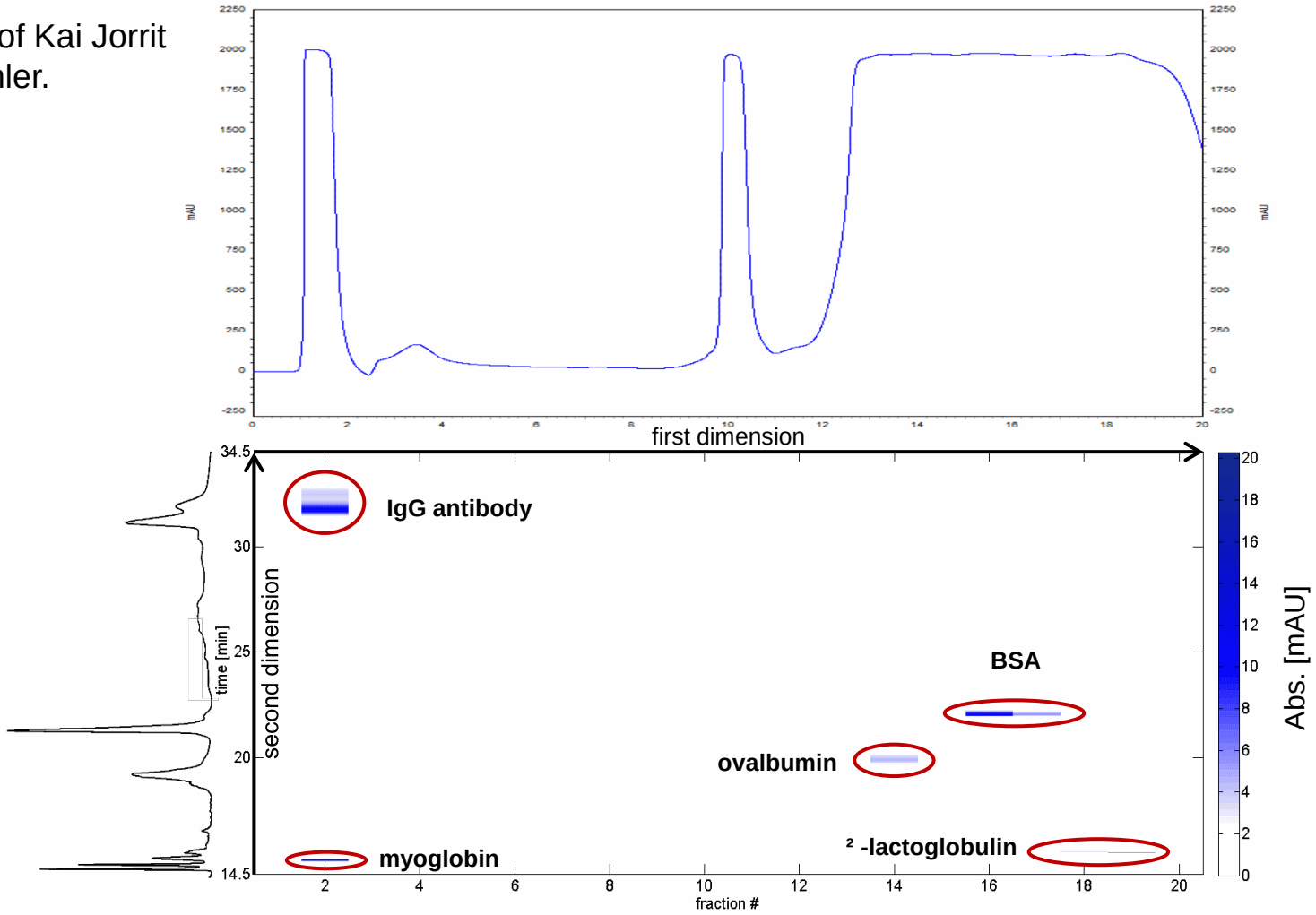


Kim, Sureka, Kayitmazer, Wang, Swan, Olsen, Biomacromolecules 2020, 21, 3026−3037

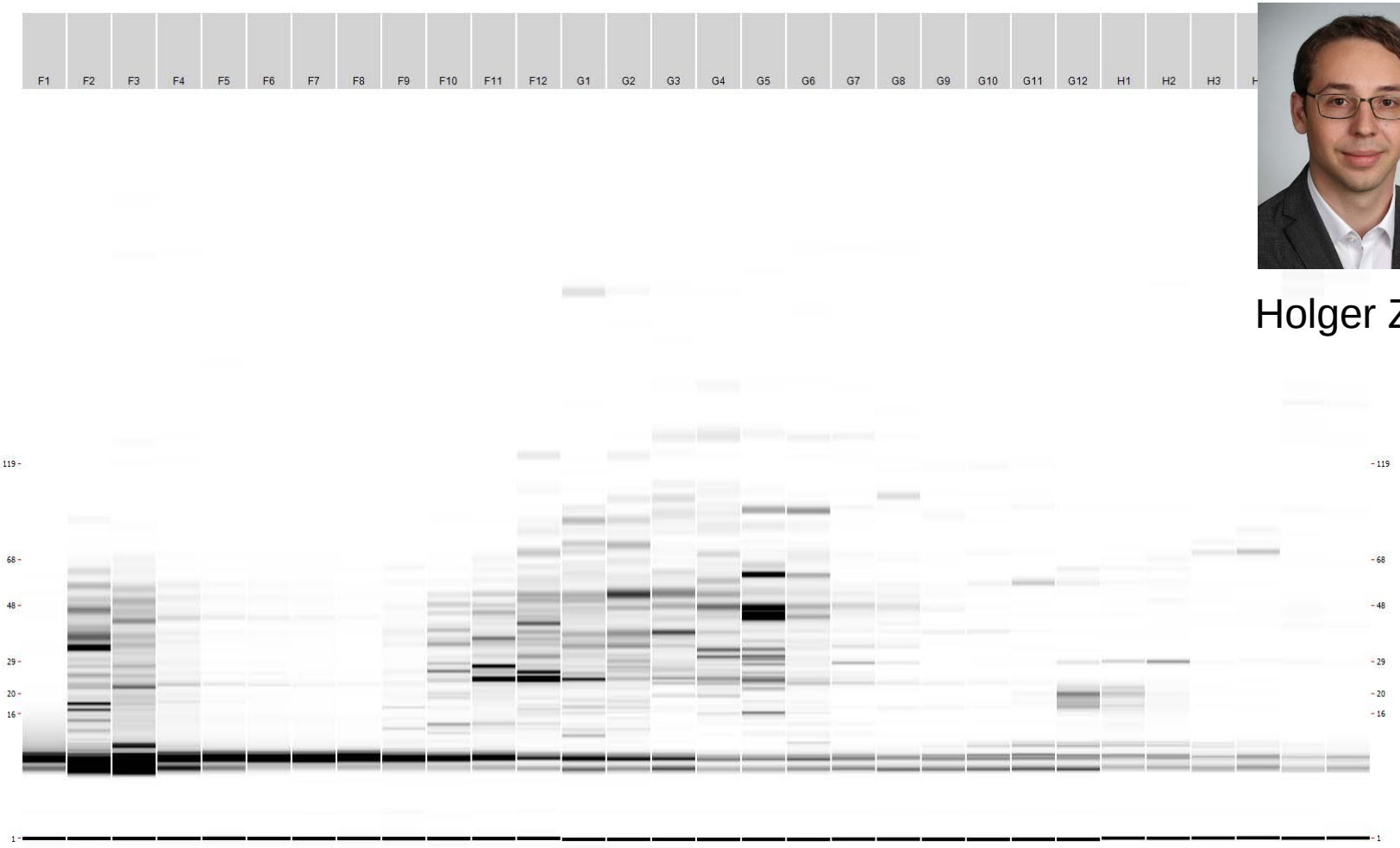


Mixture of Model Proteins, 2nd Dimension CE-SDS Patent WO 2018/127515 A1

courtesy of Kai Jorrit Maul-Köhler.



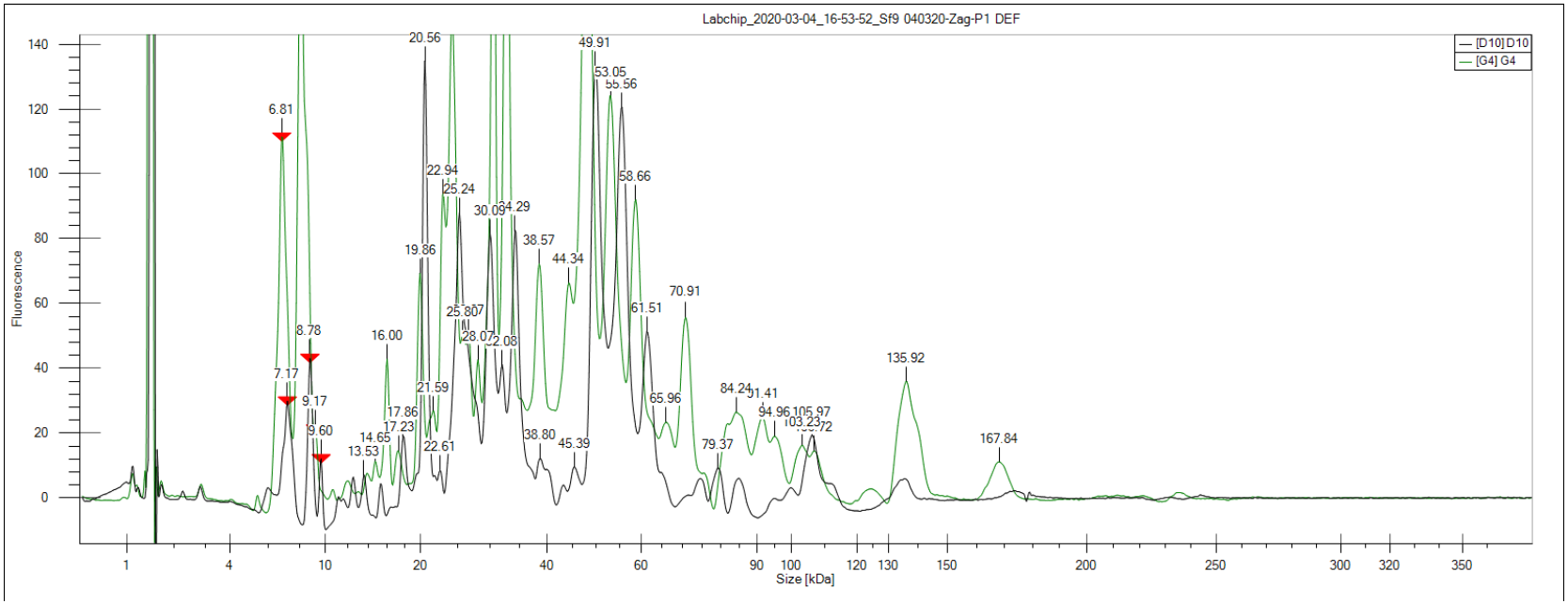
Sf9-Cytosol (transfected)



Holger Zagst

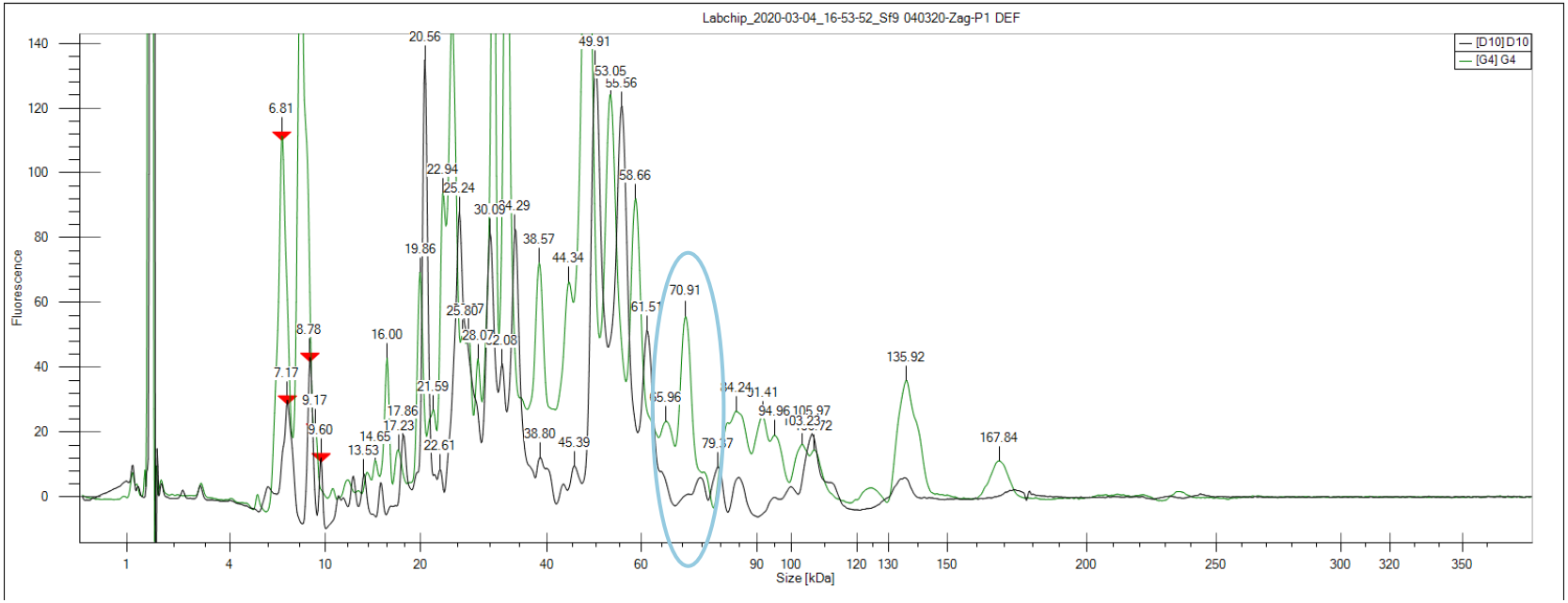
Sf9-Cytosol (transfected)

Overlay of two electropherograms from different samples.

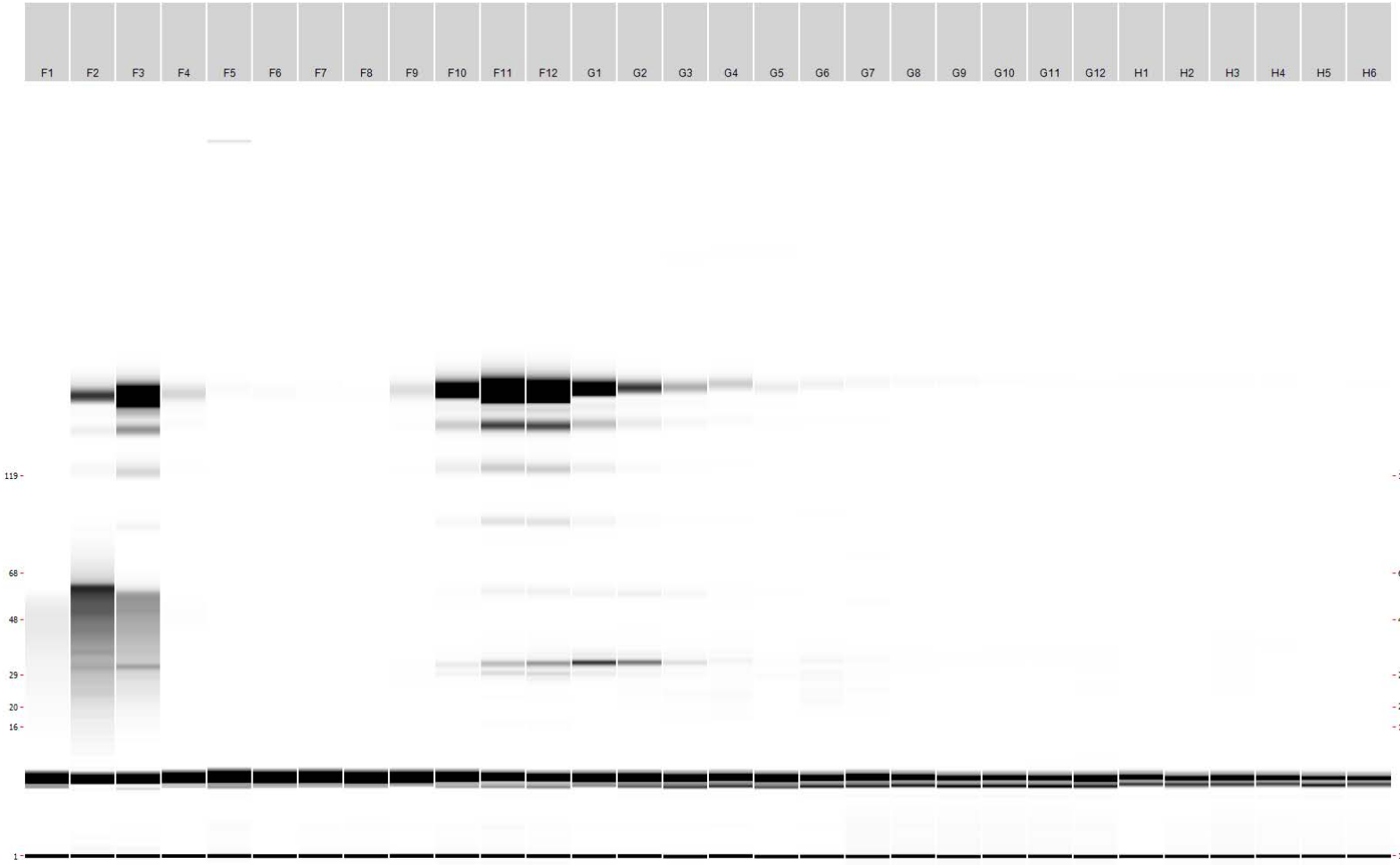


Sf9-Cytosol (transfected)

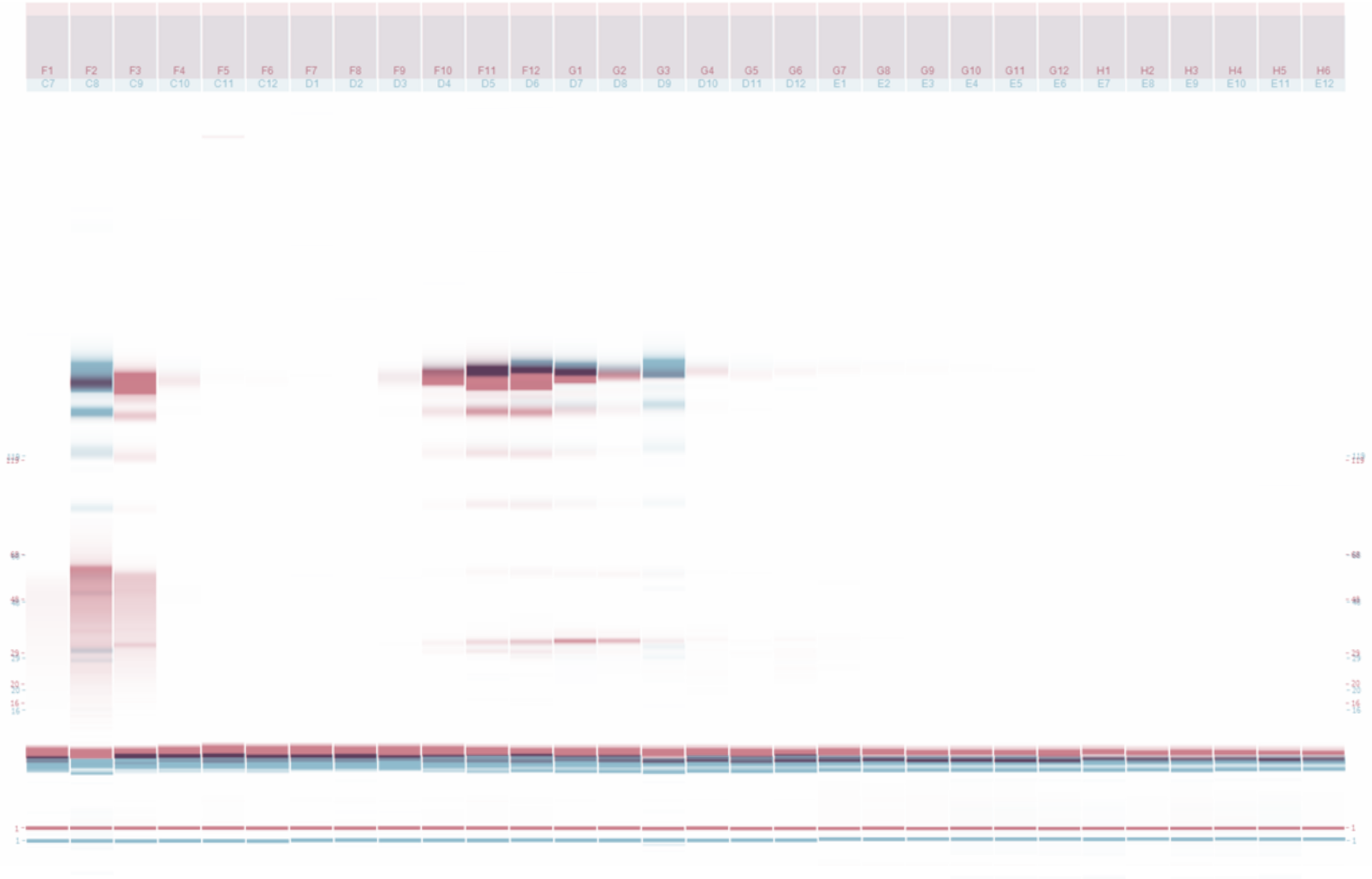
Overlay of two electropherograms from different samples.
Blue circle marks protein of interest



Example II – Sample from Biotechnological Process



Example II – Samples from Biotechnical Process: Both Separations Superimposed for Comparision



Outlook

- Exploration of further possible applications, e.g. diagnostics, process analytics, impurity analysis, etc.

- Determination of relevant method parameters
  - Linearity & LOD
  - Reproducibility
  - Accuracy

- Possibilities for method adjustments

Electrophoresis 2020, *41*, 1137–1151

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Review

Physicochemical properties for drug targeting, virus in and attenuation, vaccine f and quality control

The material properties of the severe acute respira CoV-2) and its proteins are discussed. We review the ity, isoelectric point, buoyant density and centrifuga temperature, UV light, gamma radiation, and susc including solvents and detergents. Possible inactiv conditions are given including suitable buffers an methods. This information supports vaccine develop authorities during vaccine approval and is certainly r hygienics. Several instructive tables are given, inclu dropathicity (GRAVY) of SARS-CoV-1 and -2 prote SARS-CoV-2 are similar in many regards, so inform unusually stable, but sensitive at their lipophilic m small differences can have strong effects, for examp

Table 1: Molecular Weight (MW), isoelectric Point (pI) and grand average of hydropathicity (GRAVY) of the SARS-CoV-1 and SARS-CoV-2 proteins predicted by ProtParam [54], including the corresponding sequence identifier [48]

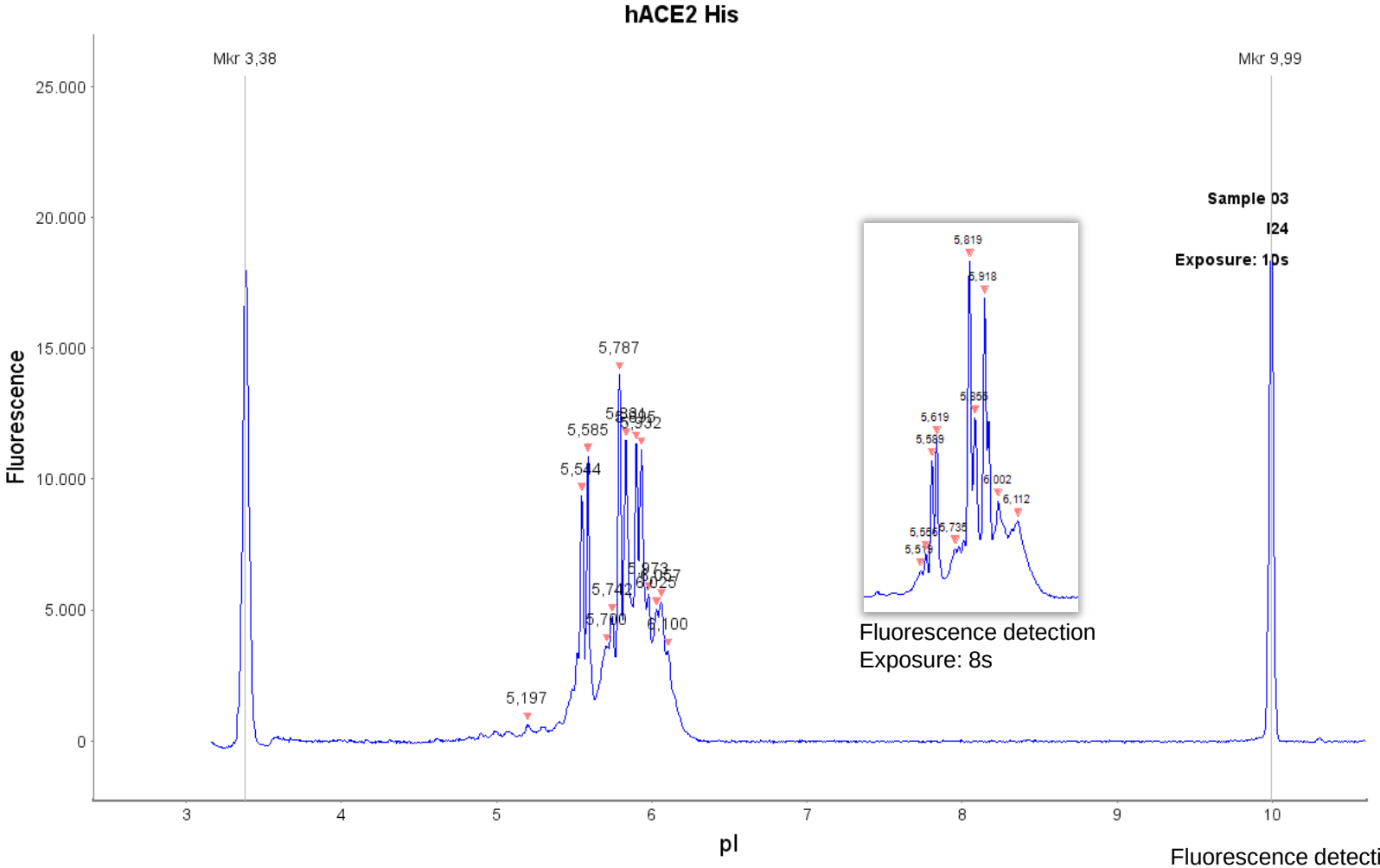
Virus	Protein	UniProt-ID	MW [kDa]	pI	GRAVY
SARS-CoV-1	Replicase polyprotein 1a	P0C6U8 · R1A_CVHSA	486.373	5.91	-0.020
	Replicase polyprotein 1ab	P0C6X7 · R1AB_CVHSA	790.248	6.19	-0.071
	Spike glycoprotein	P59594 · SPIKE_CVHSA	139.125	5.56	-0.045
	Nucleoprotein	P59595 · NCAP_CVHSA	46.025	10.11	-1.027
	Protein 3a	P59632 · AP3A_CVHSA	30.903	5.75	0.239
	Protein 7a	P59635 · NS7A_CVHSA	13.941	8.24	0.218
	Envelope small membrane protein	P59637 · VEMP_CVHSA	8.361	6.01	1.141
	Membrane protein	P59596 · VME1_CVHSA	25.061	9.63	0.417
	Nonstructural protein 3b	P59633 · NS3B_CVHSA	17.750	10.82	0.099
	Nonstructural protein 6	P59634 · NS6_CVHSA	7.527	4.64	0.297
	Protein 9b	P59636 · ORF9B_CVHSA	10.802	4.90	-0.122
	Protein nonstructural 7b	Q7TFA1 · NS7B_CVHSA	5.302	3.77	1.414
	Nonstructural protein 8b	Q80H93 · NS8B_CVHSA	9.560	9.45	-0.029
	Nonstructural protein 8a	Q7TFA0 · NS8A_CVHSA	4.327	8.30	0.644
	Uncharacterized protein 14	Q7TLC7 · Y14_CVHSA	7.852	6.25	0.310
SARS-CoV-2	Replicase polyprotein 1a	P0DTC1 · R1A_SARS2	489.989	6.04	-0.023
	Spike glycoprotein	P0DTC2 · SPIKE_SARS2	141.178	6.24	-0.079
	Replicase polyprotein 1ab	P0DTD1 · R1AB_SARS2	794.058	6.32	-0.070
	Protein 3a	P0DTC3 · AP3A_SARS2	31.123	5.55	0.275
	Membrane protein	P0DTC5 · VME1_SARS2	25.147	9.51	0.446
	Protein 7a	P0DTC7 · NS7A_SARS2	13.744	8.23	0.318
	Nucleoprotein	P0DTC9 · NCAP_SARS2	45.626	10.07	-0.971
	Envelope small membrane protein	P0DTC4 · VEMP_SARS2	8.365	8.57	1.128
	Nonstructural protein 6	P0DTC6 · NS6_SARS2	7.273	4.60	0.233
	Protein 9b	P0DTD2 · ORF9B_SARS2	10.797	6.56	-0.085
	Nonstructural protein 8	P0DTC8 · NS8_SARS2	13.831	5.42	0.219
	Uncharacterized protein 14	P0DTD3 · Y14_SARS2	8.050	5.79	0.603
	Protein nonstructural 7b	P0DTD8 · NS7B_SARS2	5.180	4.17	1.449
		A0A663DJA2 · A0A663DJA2_SARS2	4.449	7.93	0.637

cIEF with SARS-CoV-2 proteins – RBD Fc (pI 8.55)

Finja Krebs

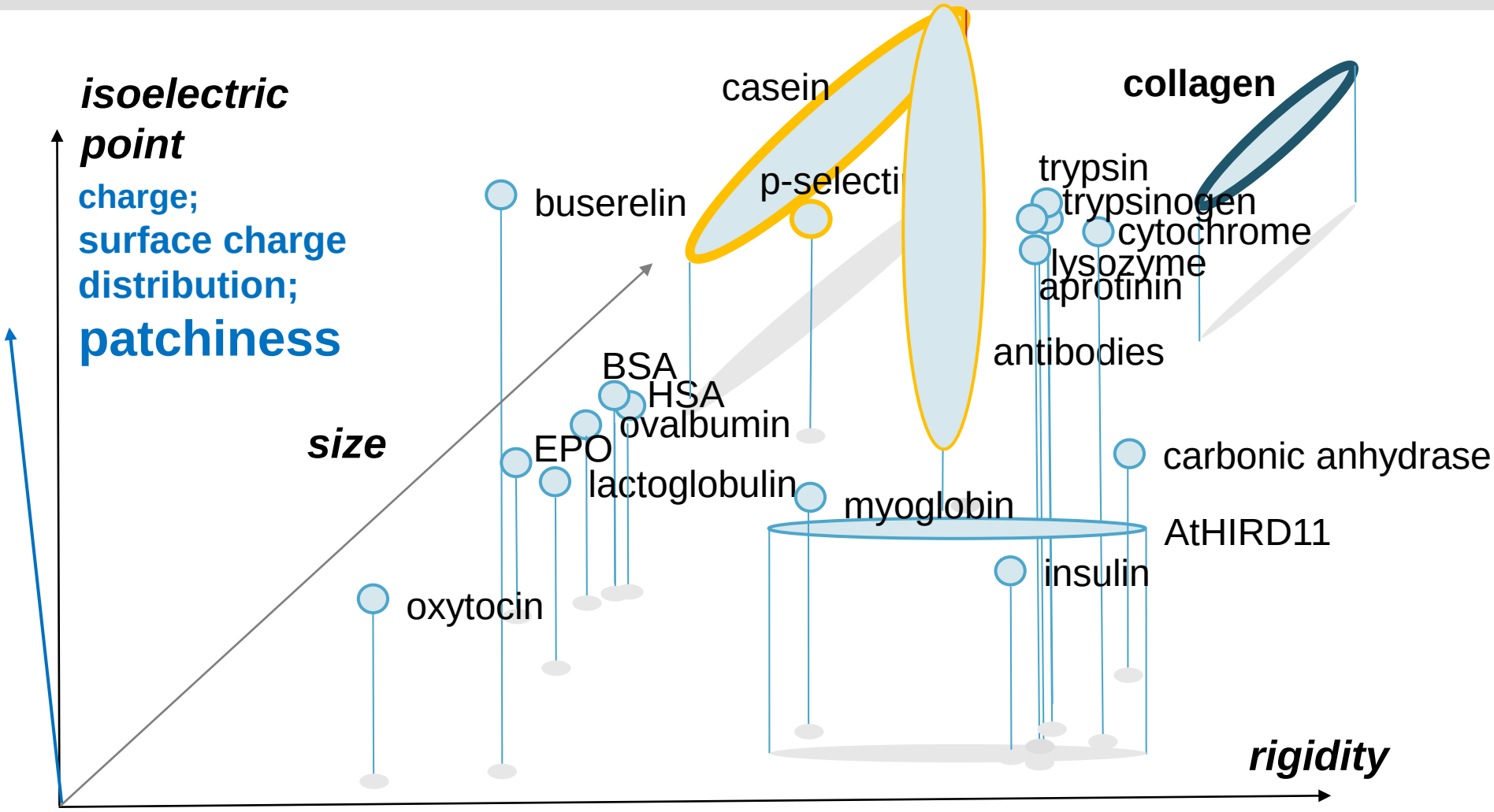
Fluorescence detection
Exposure: 5s

cIEF with SARS-CoV-2 proteins – hACE2 His (pI 5.6)

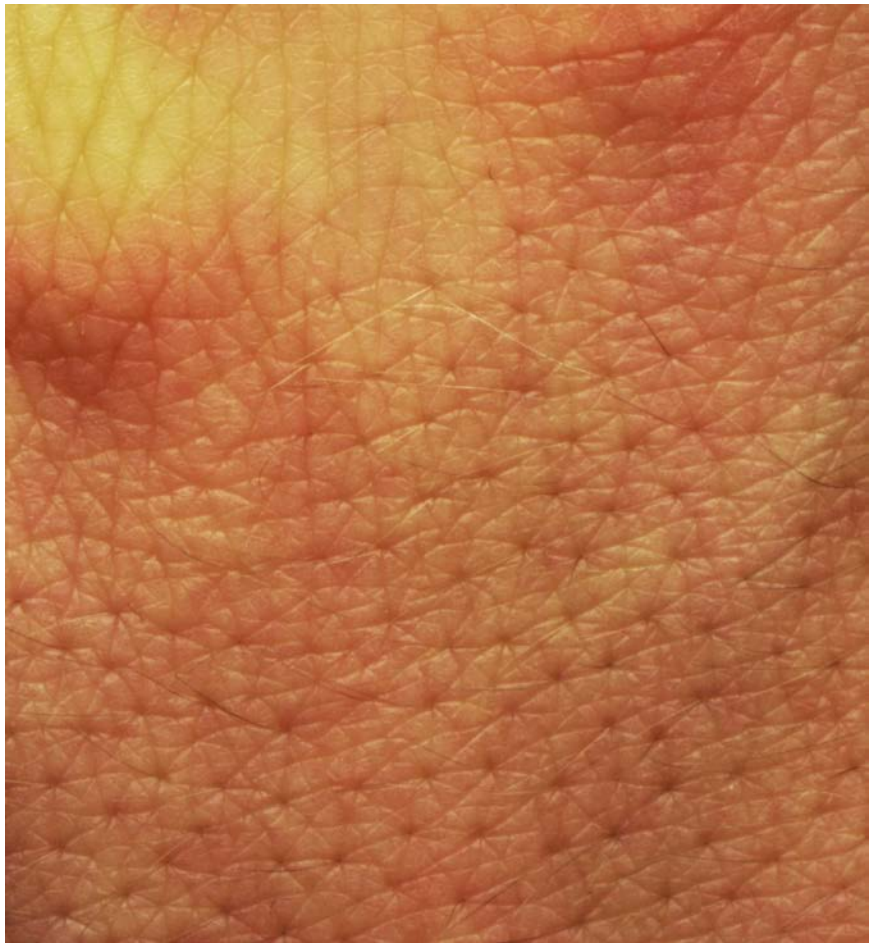


Fluorescence detection
Exposure: 10s

Surface Charge Distribution



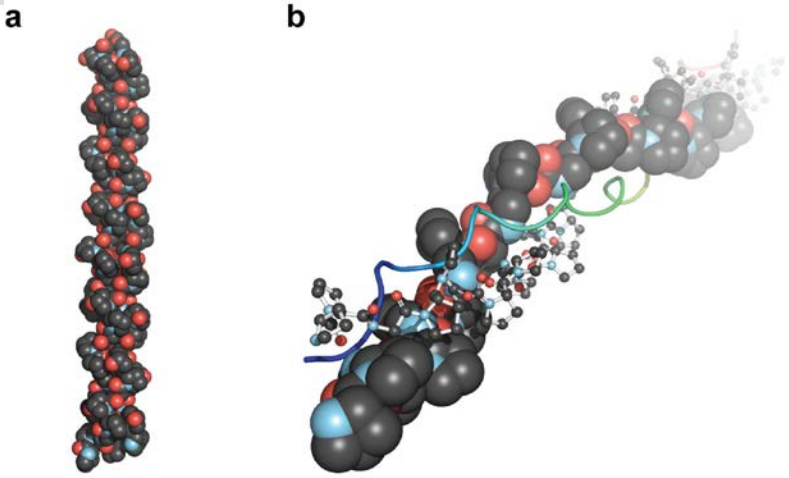
Collagen: main constituent of connective tissues



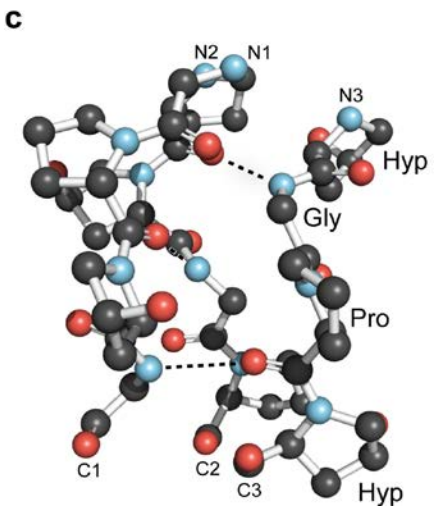
- skin
- bones
- tendons
- etc.

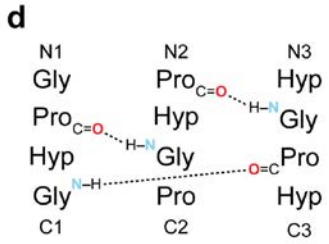
Human skin, from Klafubra, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=5409304

Collagen-Structure: Triple-Helix

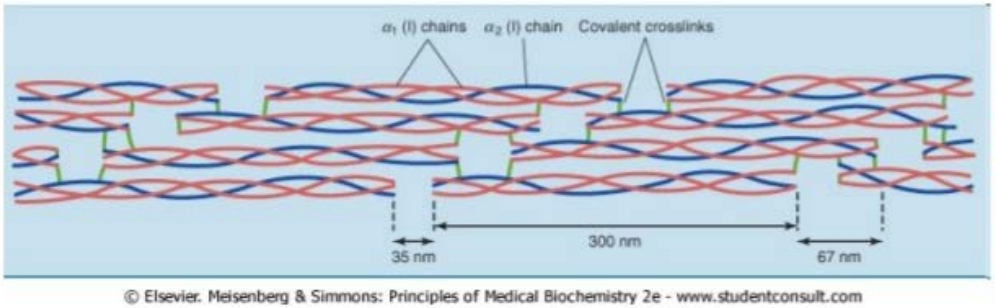


Dr. Imke Oltmann-Norden





Source: M.D. Shoulders and R.T. Raines „Collagen Structure and Stability“ Annu. Rev. Biochem. 2009; 78:929-958



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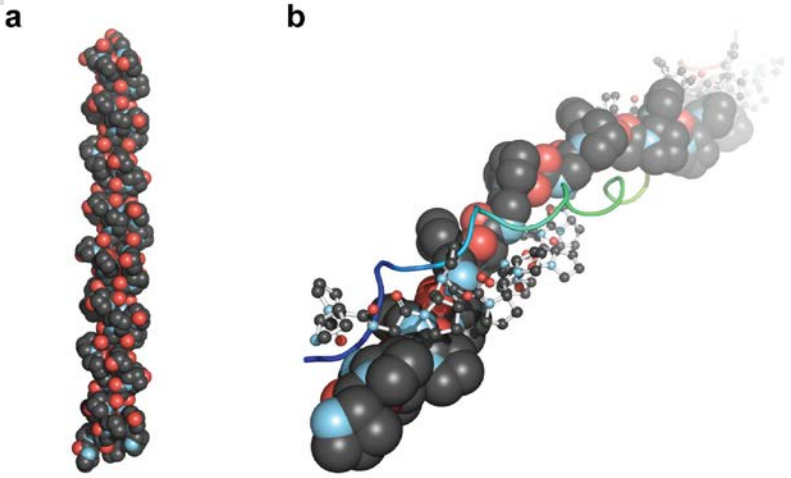
Collagen-Structure: Triple-Helix



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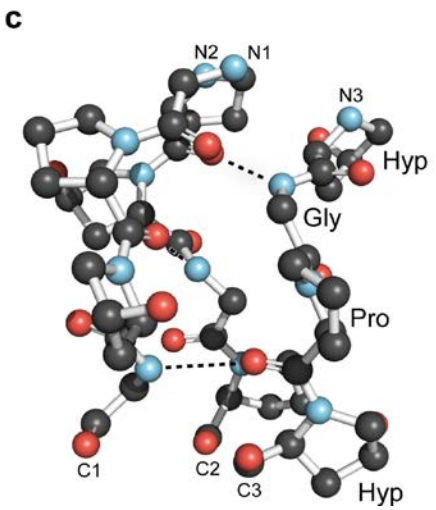

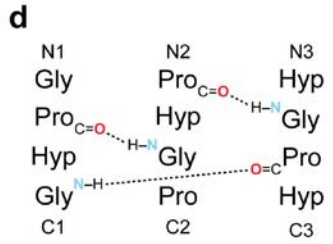

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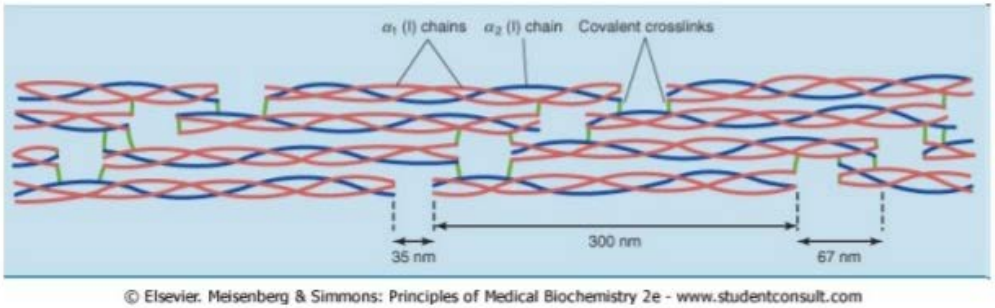

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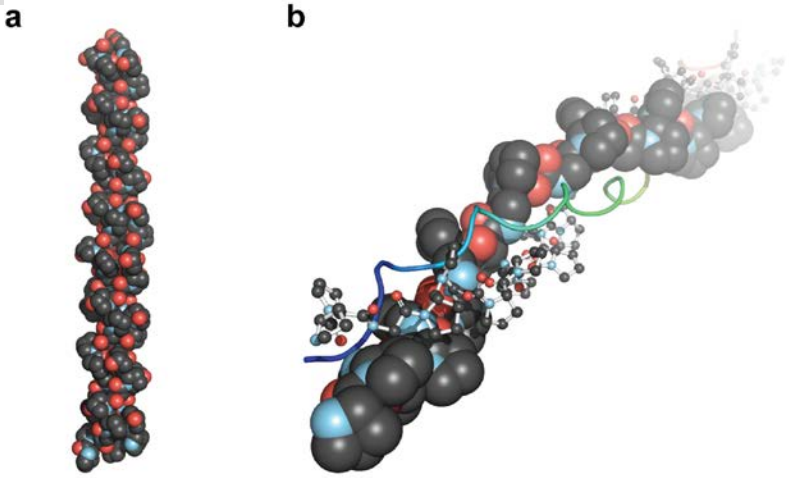

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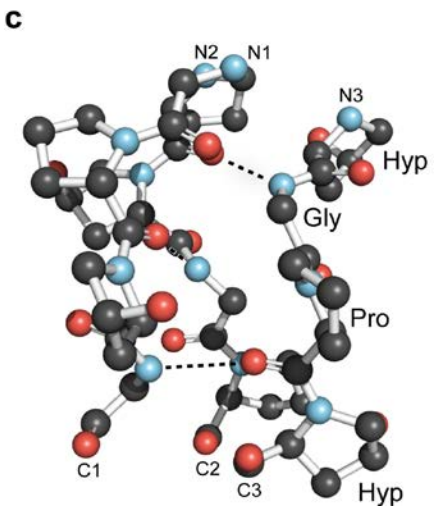

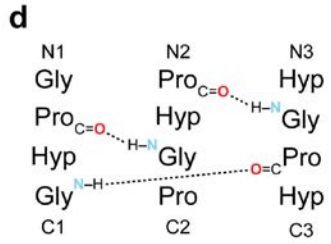

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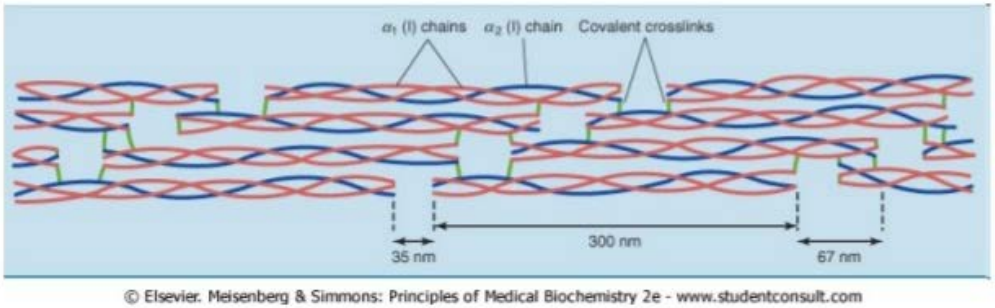

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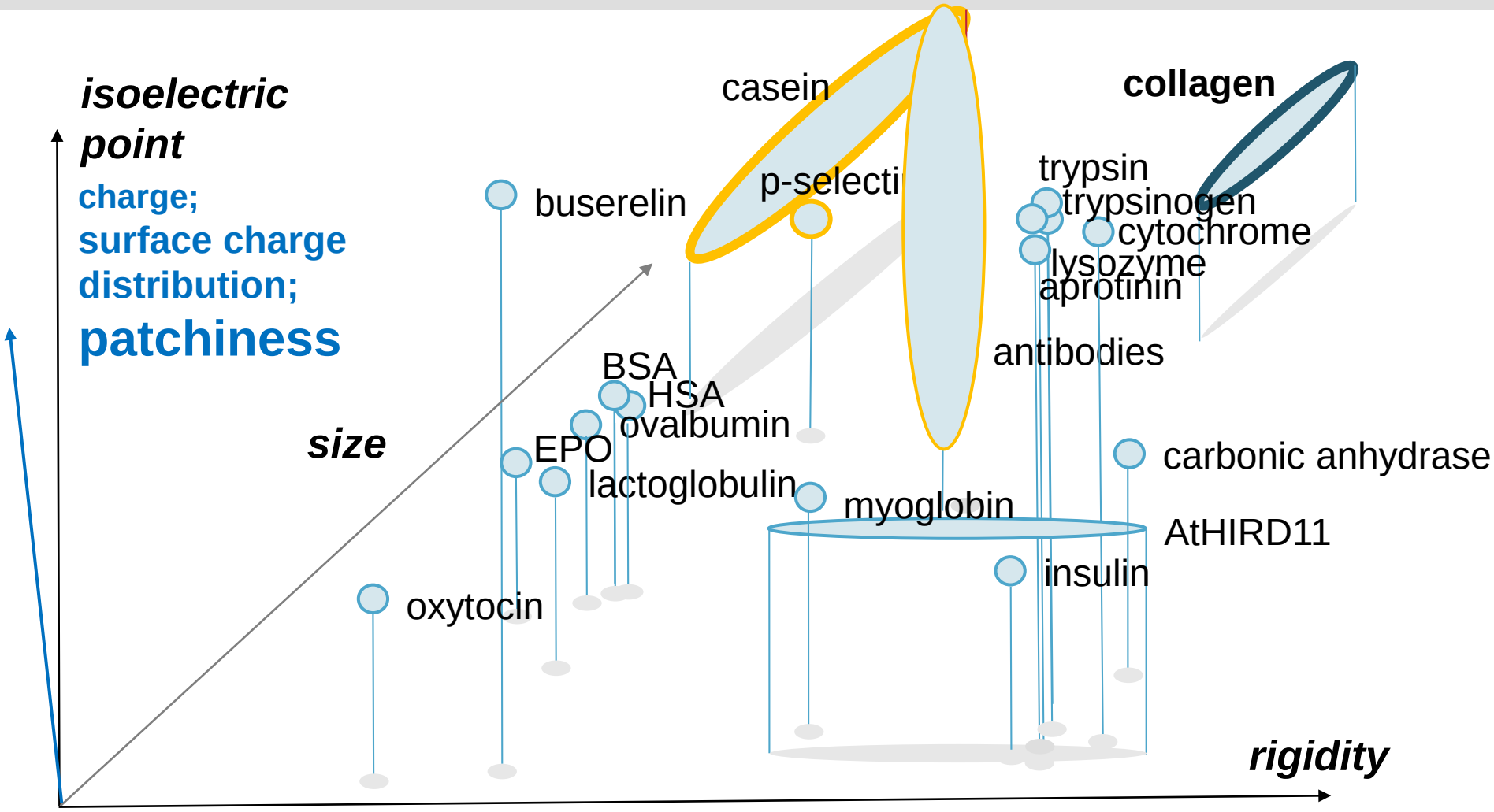
Surface Charge Distribution



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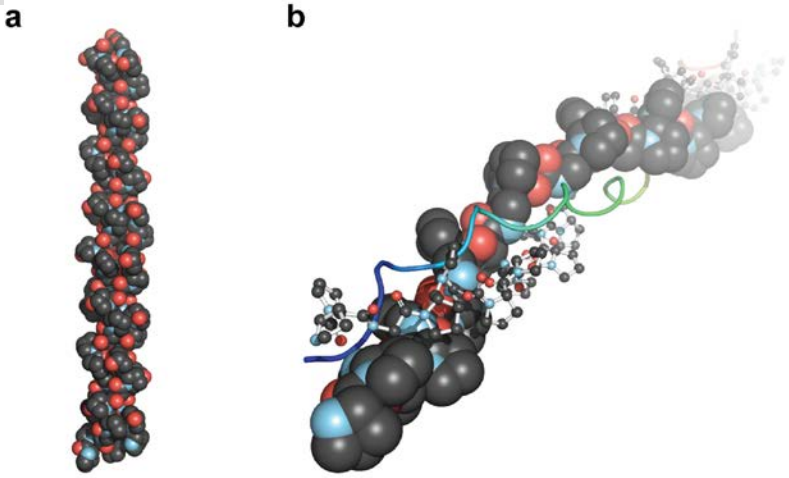
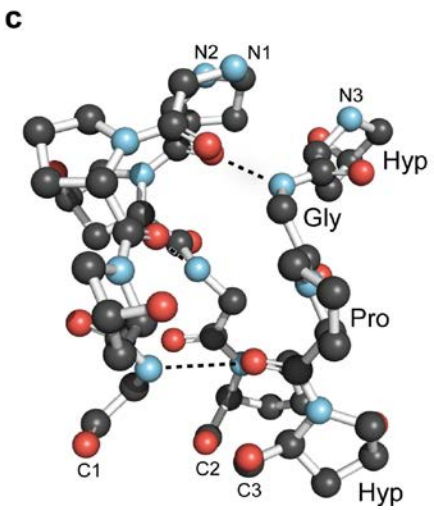
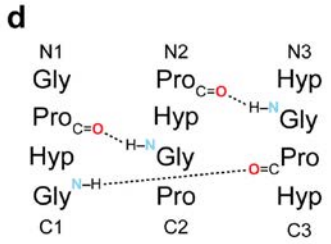


Dr. Imke Oltmann-Norden

Christin Scheller





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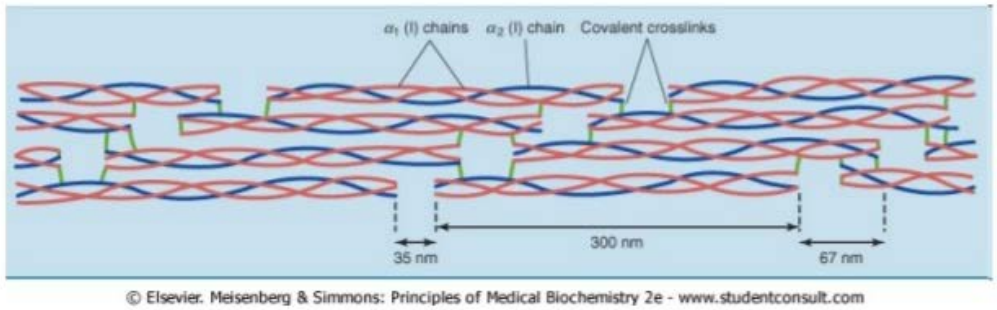
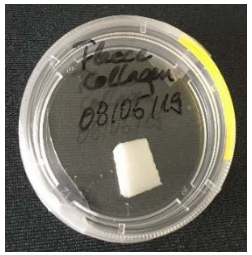


How to prepare a suspension of collagen



Grinding

+ H_2O =

Suspension

Filtration
5µm

Suspension, dp d 5µm

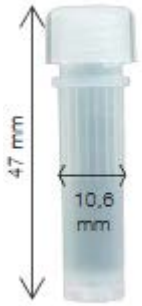
Freeze Drying

membrane fleece

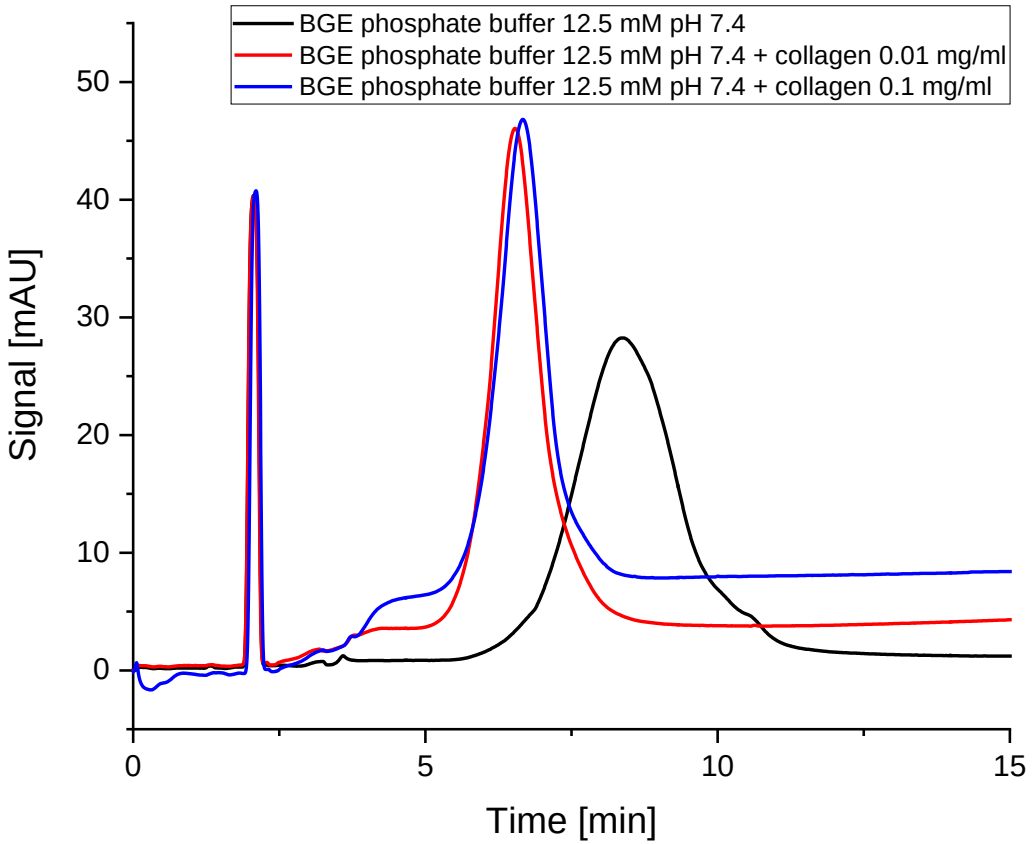
Solid material to prepare suspension of known concentration and dp d 5µm

Grinding using the dual centrifuge Zentrimix 380 R (Hettich)

- Grinding body: ZY-P Mahlkörper SiLibeads® (zirkonium oxide, stabilized by yttrium), 0,8-1,0 mm
- Weight: 30% grinding body ≙ 2,2g
- Temperature centrifuge: cooling to -20°C, real temperature during grinding process: between -9°C and -14°C
- Grinding: 1000 rpm for 1min, then freezing (N_2(l)), then repetitive grinding (four times altogether)



ACE in suspension

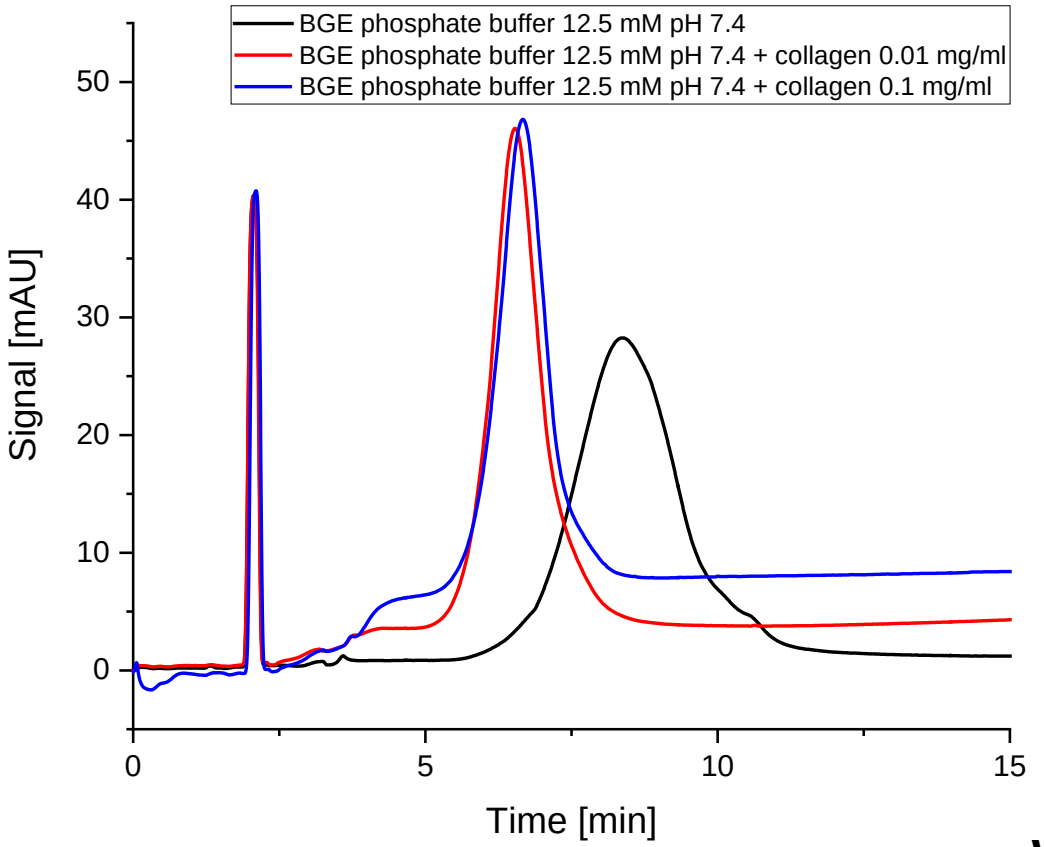


Christin Scheller

LPA ID 75 µm (Polymicro Technologies), total length: 32.5 cm; effective: 24.0 cm
Phosphate buffer 12.5 mM pH 7.4 (+ various amounts of collagen particles)
HSA 0.2 mg/ml + potassium hydrogen phthalate 40 µg/ml as mobility marker

0.6 W (limits: -18 kV; 50 µA)

ACE in suspension



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Working at constant power P = U * I

Ligand Binding Studies: Measurement Uncertainty

Experimental design and measurement uncertainty in ligand binding studies by affinity capillary electrophoresis

Matthias Stein, Rob Haselberg, Mona Mozafari-Torshizi, Hermann Wätzig

Electrophoresis 2019 Apr;40(7):1041-1054. doi: 10.1002/elps.201800450

Uncertainty in Ligand Binding Assay Data

Christian Kramer et al. dx.doi.org/10.1021/jm300131x
J. Med. Chem. 2012, 55, 5165−5173

Journal of Medicinal Chemistry
Article
pubs.acs.org/jmc

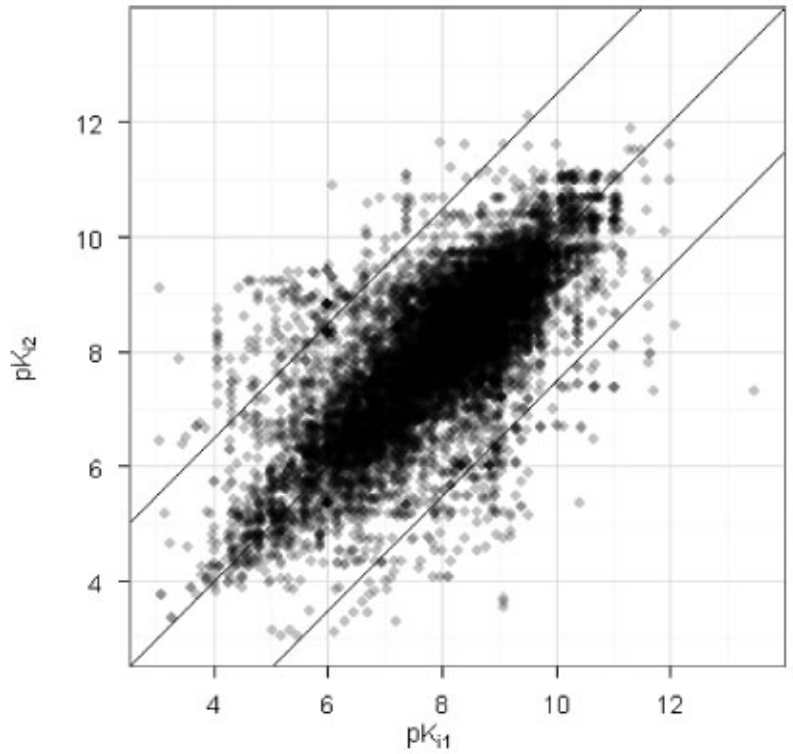

Figure 5. Plot of all pairs of measured values (curated dataset 1). Lines indicate the 2.5 log unit border for removing unreliable pairs of measurement (leaving curated dataset 2).

Quite often more than 2.5 log units difference in pK data

This means: one binding constant is by more than a factor of 300 greater than the other!

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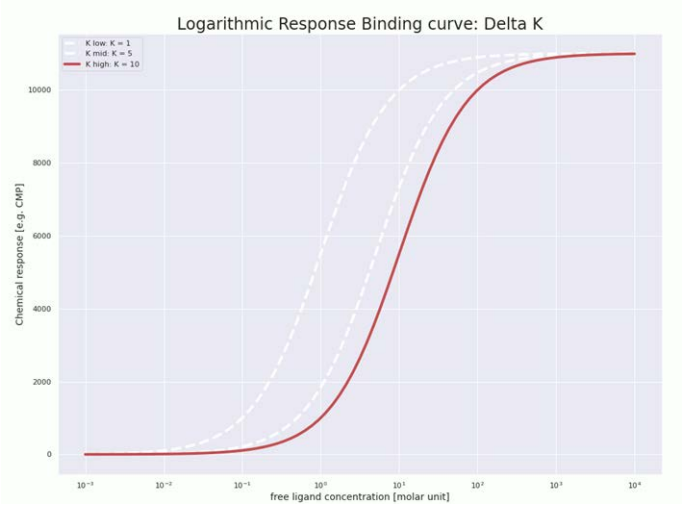
Generalization LBA - The Model

The five-parameter logistic model [3,4] and its Parameters in ˜



$$f(c,t|\theta) = B\,t\left(\frac{c^h}{c^h + K^h}\right)^g + b\,c + a \qquad \theta = [K,h,g,B,b,a]^\top$$

[3,4]

Total binding (*T*) = specific binding (*S*) + non-specific binding (*N*)

[3] Paul G. Gottschalk, John R. Dunn, The five-parameter logistic: A characterization and comparison with the four-parameter logistic, Analytical Biochemistry, Volume 343, Issue 1, 2005, Pages 54-65
[4] https://www.graphpad.com/support/faq/asymmetrical-five-parameter-logistic-dose-responsecurves/

Calculation of the best estimate for Theta $\hat{\theta}$ in RBA

Example LBA: Radio ligand Binding Assay

T: Total Binding Experiment [5]

Incubation of the target with ***n*** ligand concentrations

N: Non-specific Binding Experiment [6]

Incubation of the target with ***m*** ligand concentrations in the **presence** of a surplus of **competitor**.

$$y = f(c,t|\theta) + e \qquad f(c,t|\vartheta) = B\,t\left(\frac{c^h}{c^h + K^h}\right)^g + b\,c + a$$

$$\hat{\boldsymbol{\theta}} = \arg\min_{\theta \in D} \sum_{i=1}^{n} \frac{1}{w_i} [y_i - f(c_i, t_i|\theta)]^2$$

e: Measurement Uncertainty of y; w: weighting factor

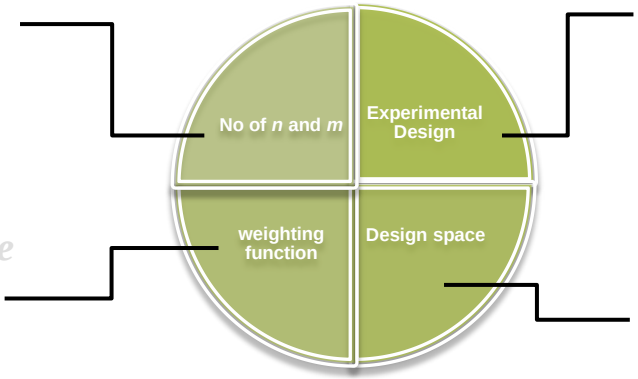
t: dummy variable to distinguish T and N, $t \in \{1,0\}$

c: total ligand concentration, $c \in C \subseteq \mathbb{R}^0 | \{C \leq c_{max}\}$

- *n: 24*
- *m: {all or short}*

- *no*
- *specific variance function u*
- *Response y*

Optimization of the process



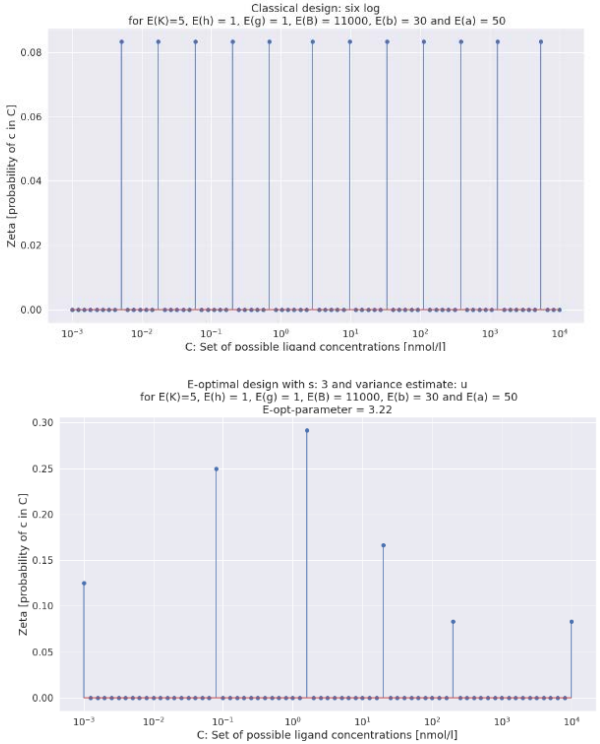
- ***classical***
  - ***6-log, 4-log, quadratic, inverse linear***
- ***Optimal***
  - ***D, E, A***

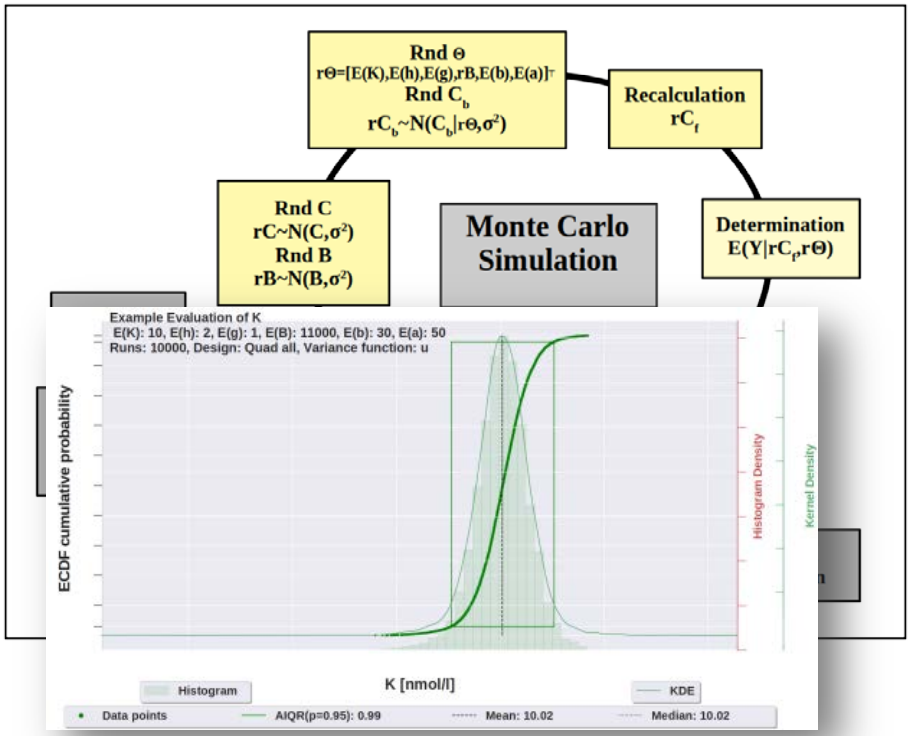
- *Knowledge about the influences*

[5] Radioligand binding methods: practical guide and tips, **D. B. Bylund and M. L. Toews, American Journal of Physiology-Lung Cellular and Molecular Physiology 1993 265:5, L421-L429**

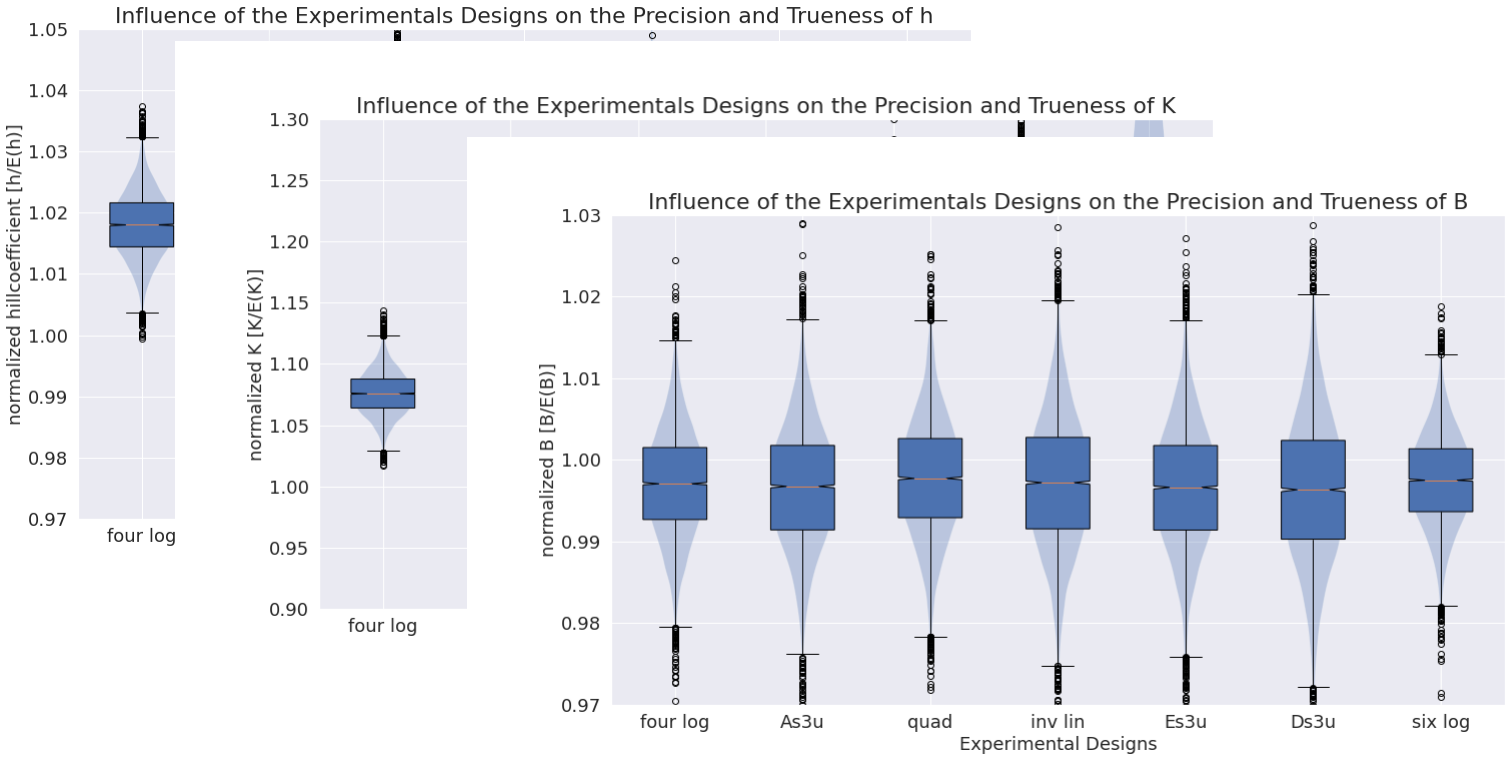
[6] https://www.originlab.com/doc/Origin-Help/Nonlinear-Curve-Fit

MC Simulation



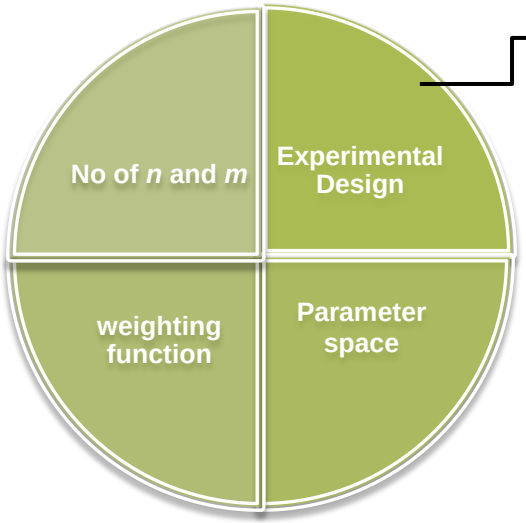


[7] GUM sup1 https://www.bipm.org/utils/common/documents/jcgm/JCGM_101_2008_E.pdf



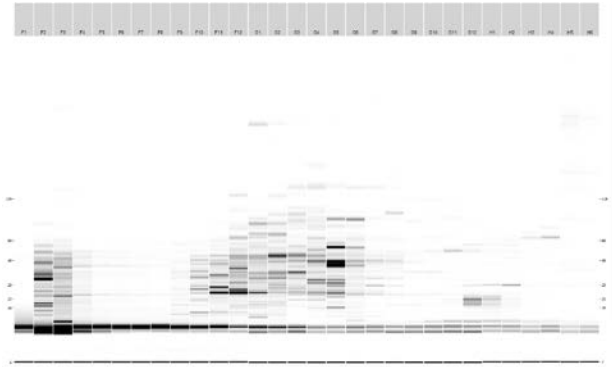
[7] GUM sup1 https://www.bipm.org/utils/common/documents/jcgm/JCGM_101_2008_E.pdf

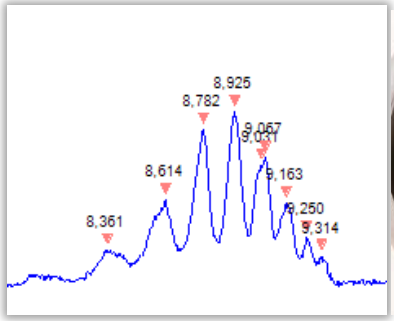
Preliminary Conclusion

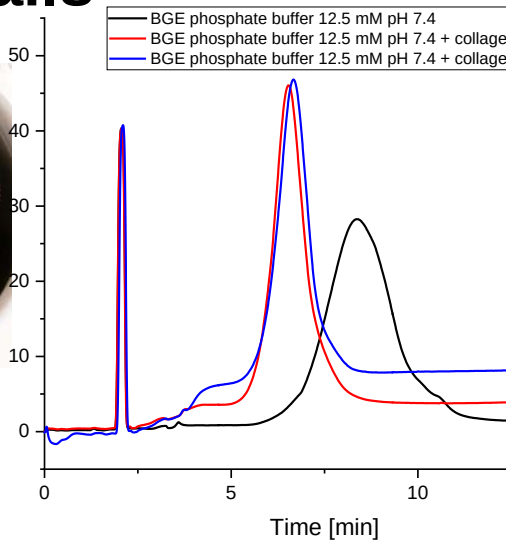


- **Both the precision and the trueness are depending strongly on the Measurement Design**
- **In this case the strongest effects could be observed for K**
- **The impact differs between the BPs**
  - **Ds3 was best for h and worst for K**

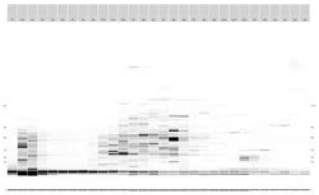
Overall conclusions and outlook: details

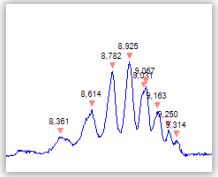


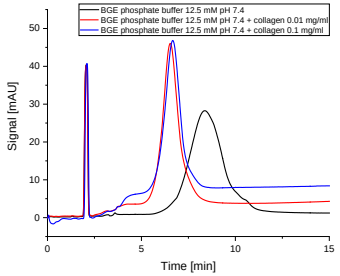




Overall conclusions and outlook: details and general concepts







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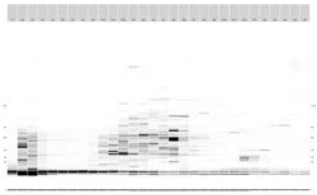

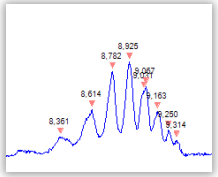

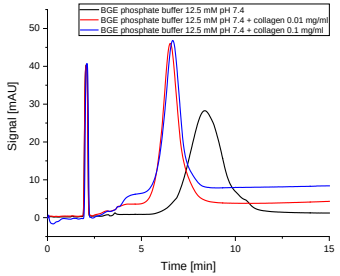

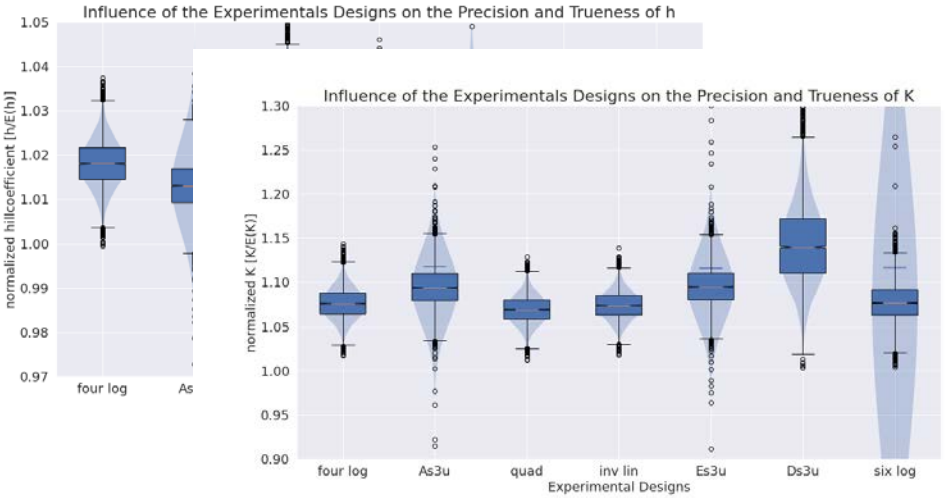

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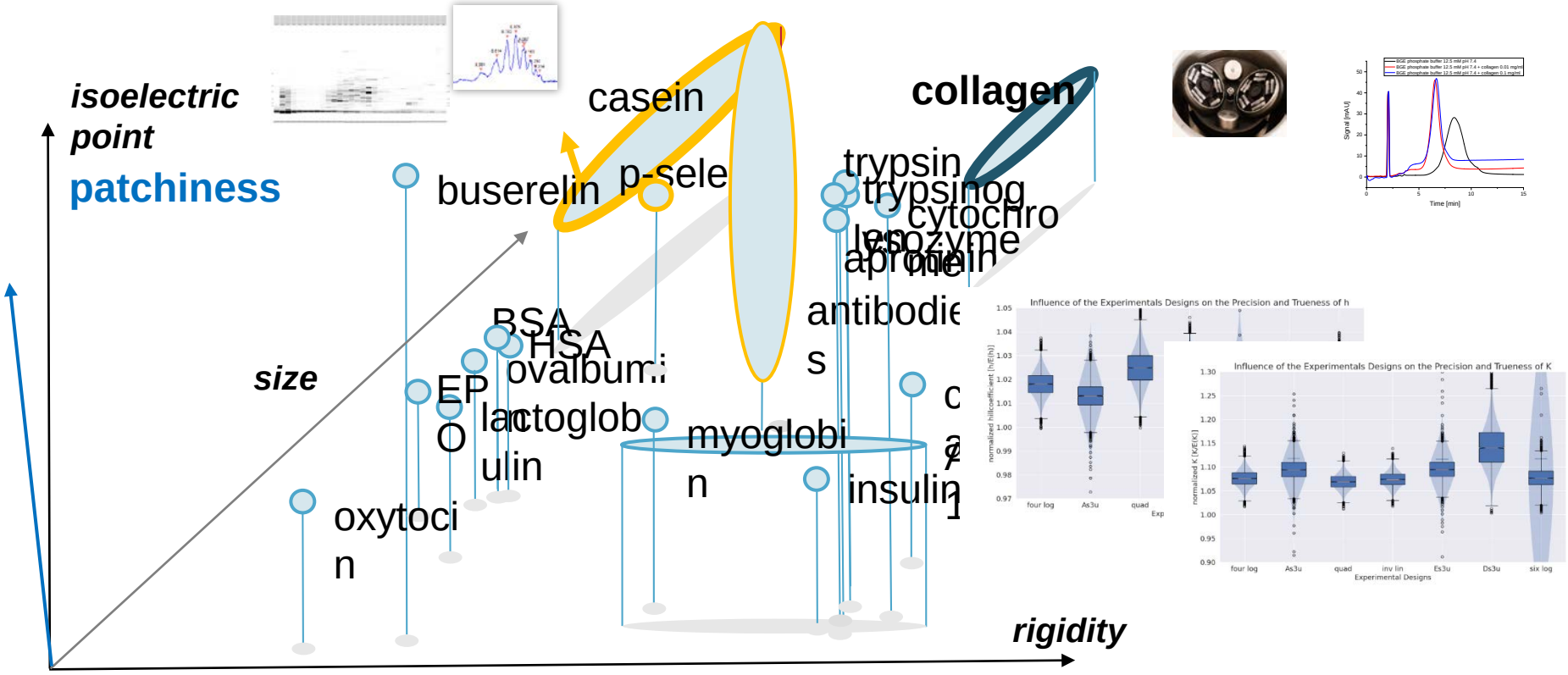

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Thank you very much!

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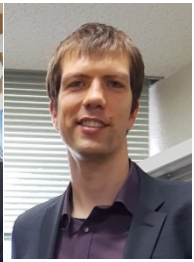

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