The good and the bad of protein fibrillation

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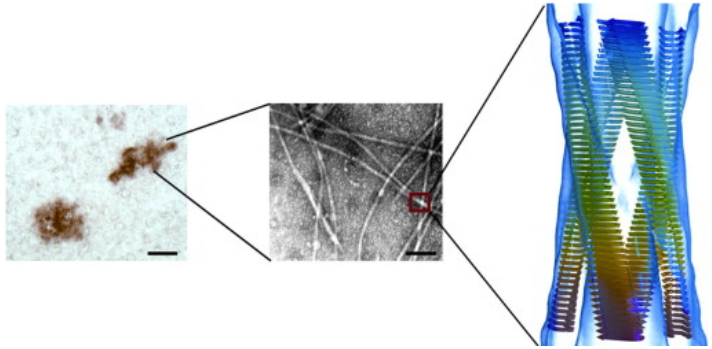
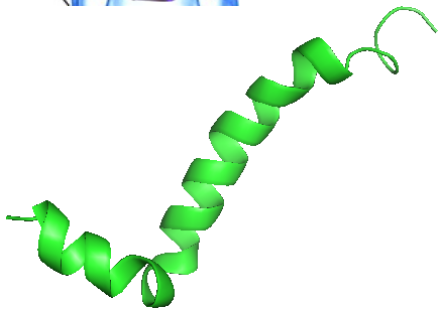
Outline

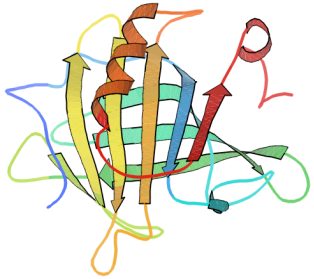
Folding, misfolding and amyloid structures



Amyloid-β (Aβ) peptide and β-sheet breaker peptides



β-lactoglobulin and epicatechin

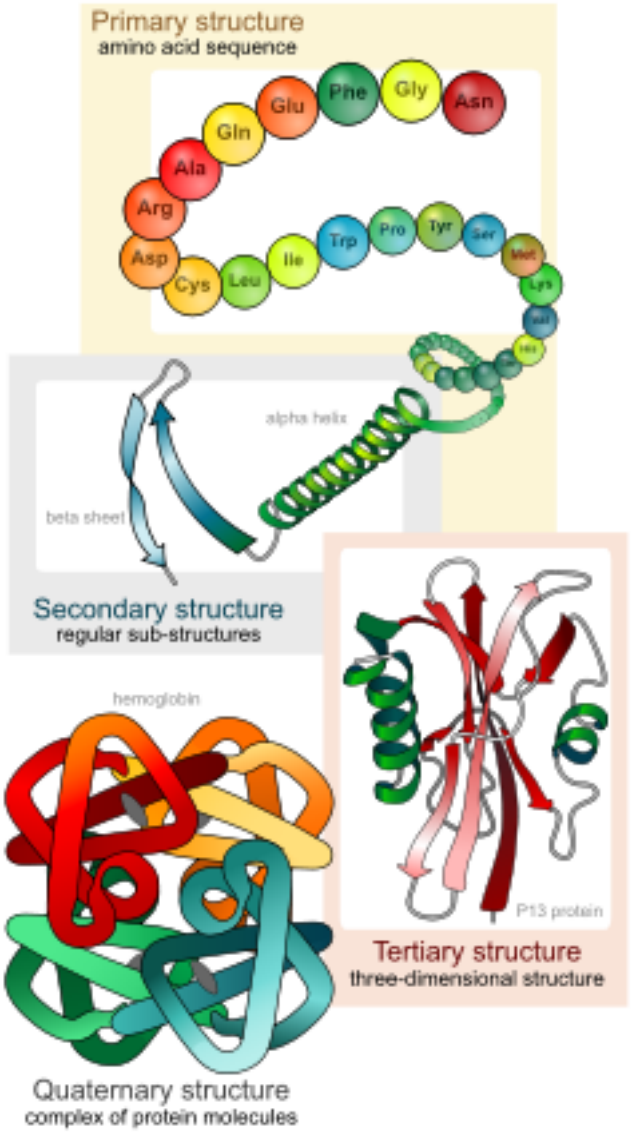


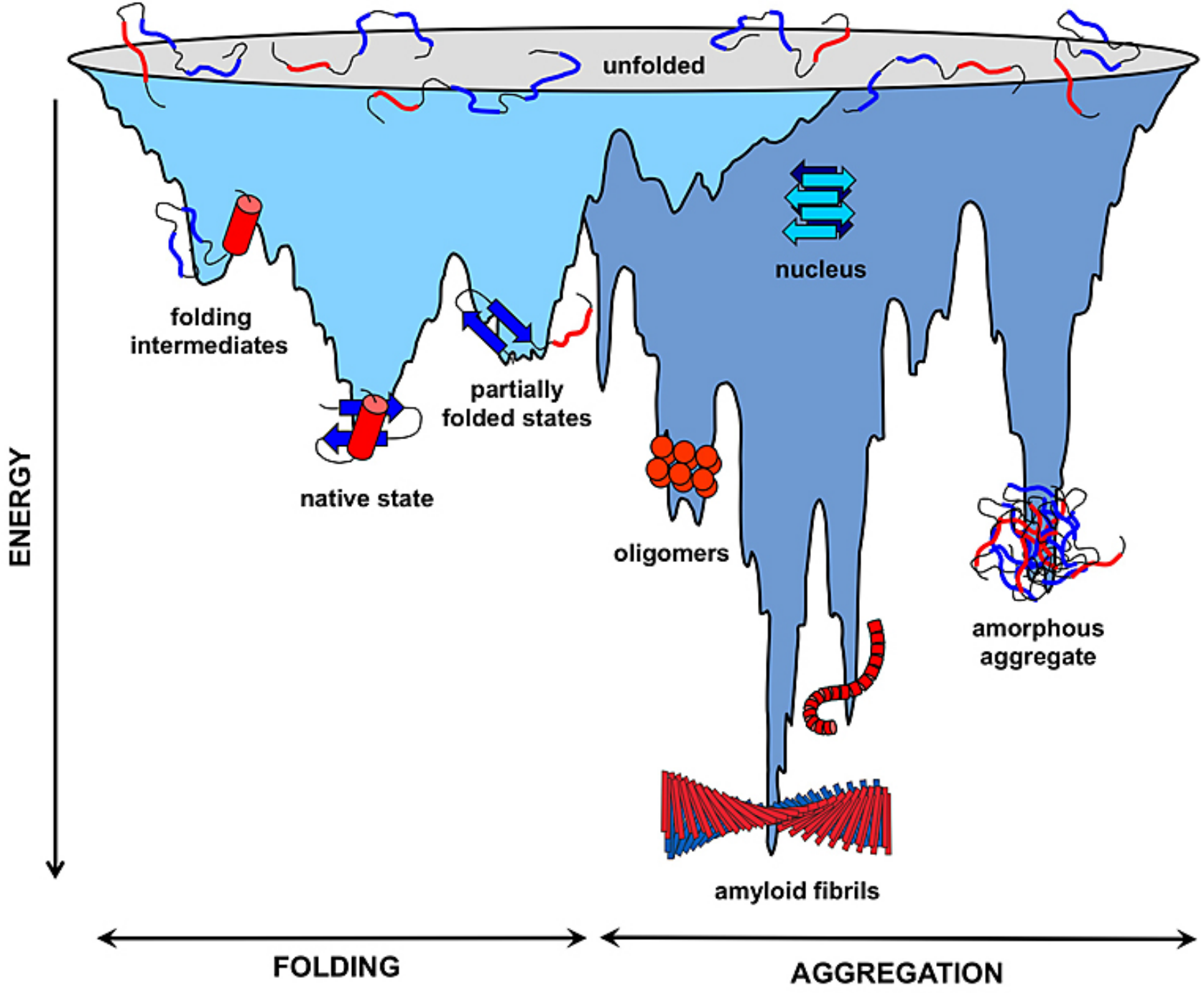
Conclusions

Protein folding and misfolding

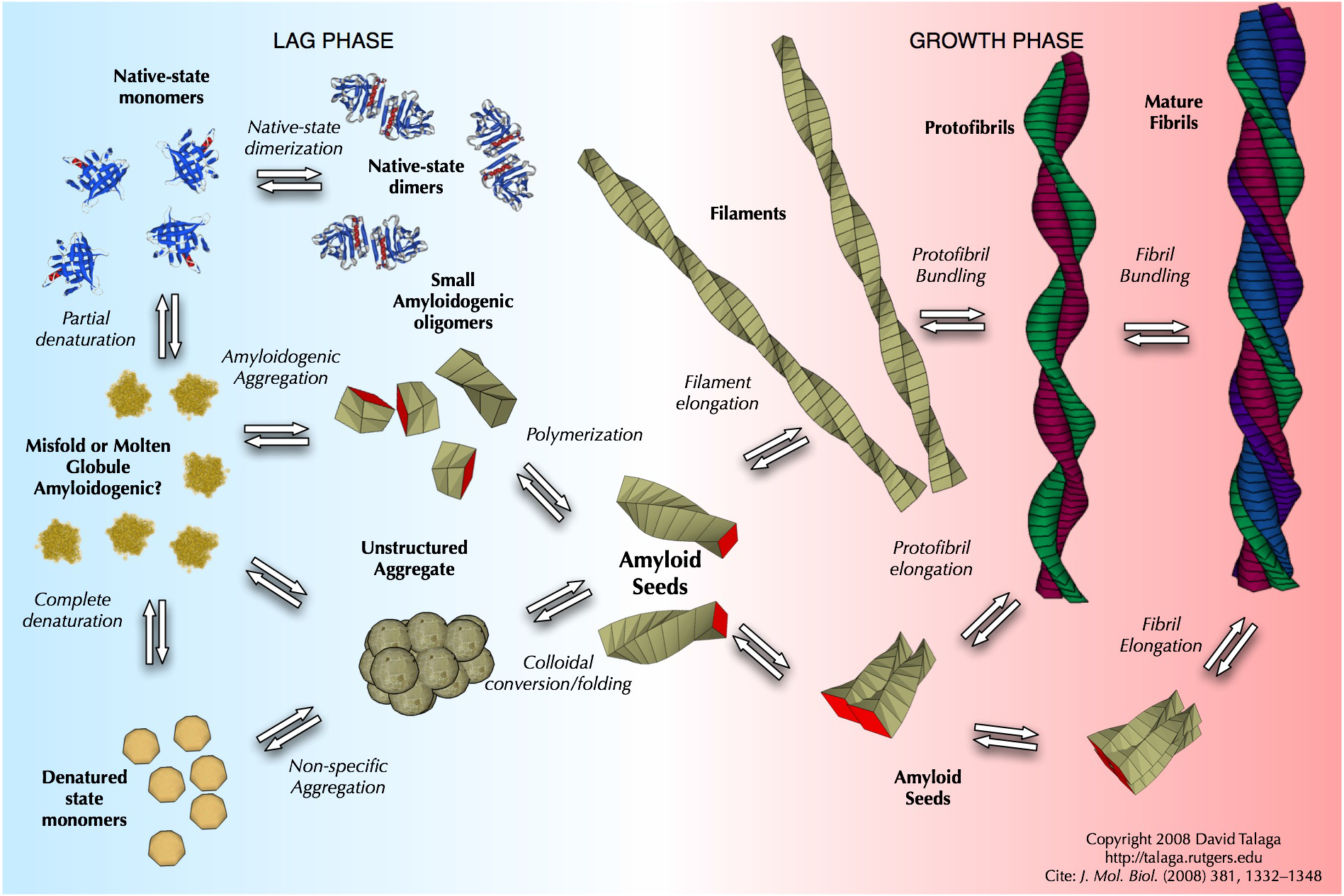
Folding: process by which a protein assumes its functional 3D structure

Misfolding: wrong folding of the (secondary and hence) tertiary protein structure. Misfolding is the basis of several diseases called protein misfolding diseases



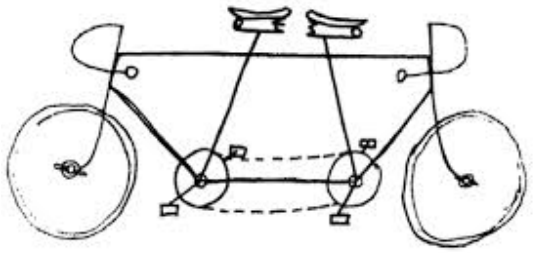

unfolded
folding intermediates
partially folded states
native state
nucleus
oligomers
amorphous aggregate
amyloid fibrils
ENERGY
FOLDING
AGGREGATION

Some of the states accessible to a polypeptide chain following its synthesis.



Amyloid structures

The formation of amyloid structures both *in vivo* and *in vitro* has become a major focus for research



by Bruno Touschek

aggregated (non-functioning) dimer

Amyloid structures are defined as the aggregation of misfolded or unfolded proteins or peptides

It is important to understand some of the basic principles that underlie aggregation and fibril formation

Amyloid structures

Folding depends largely on the primary amino acid sequence and is thermodynamically driven

correctly folded

misfolded

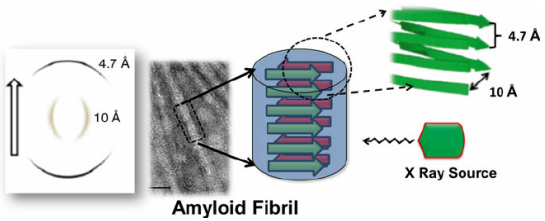
Unfolding may be reversible or irreversible and can occur for various reasons: change in temperature, pH, salt concentration, UV-light, protein concentration, pressure, …

Amyloid fibrils

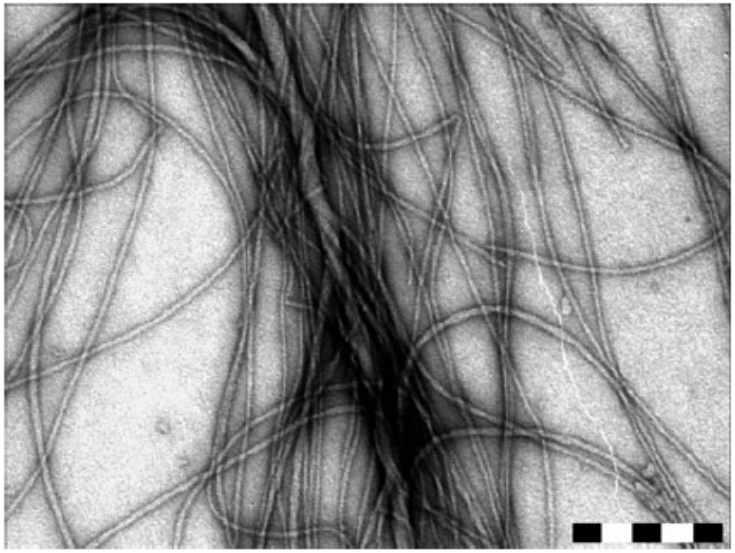
Characteristic dye binding capacities and optical properties → birefringence in the presence of Congo red

Fluorescence in the presence of Thioflavin T (ThT)

"cross-β" X-ray diffraction pattern



Relatively linear morphology and are between 60–300 Å in width

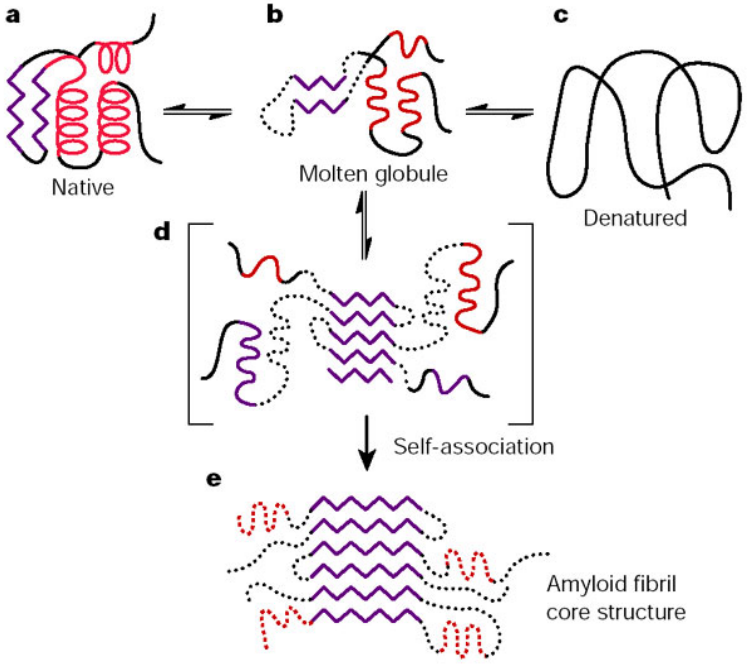


TEM micrograph of amyloid-like fibrils formed of crystallin proteins extracted from fish eye-lenses, 10 mg/ml, 80 °C, 24 h. 89,000 magnification, scale bar 0.2 µm.

Lassé et. al in J. R. Harris (ed.), Protein Aggregation and Fibrillogenesis in Cerebral and Systemic Amyloid Disease, Subcellular Biochemistry 65, © Springer Science+Business Media Dordrecht 2012

Neurodegenerative Amyloidosis (the bad)

Amyloidosis: a disorder in which insoluble protein fibrils are deposited in tissues and organs, impairing their function



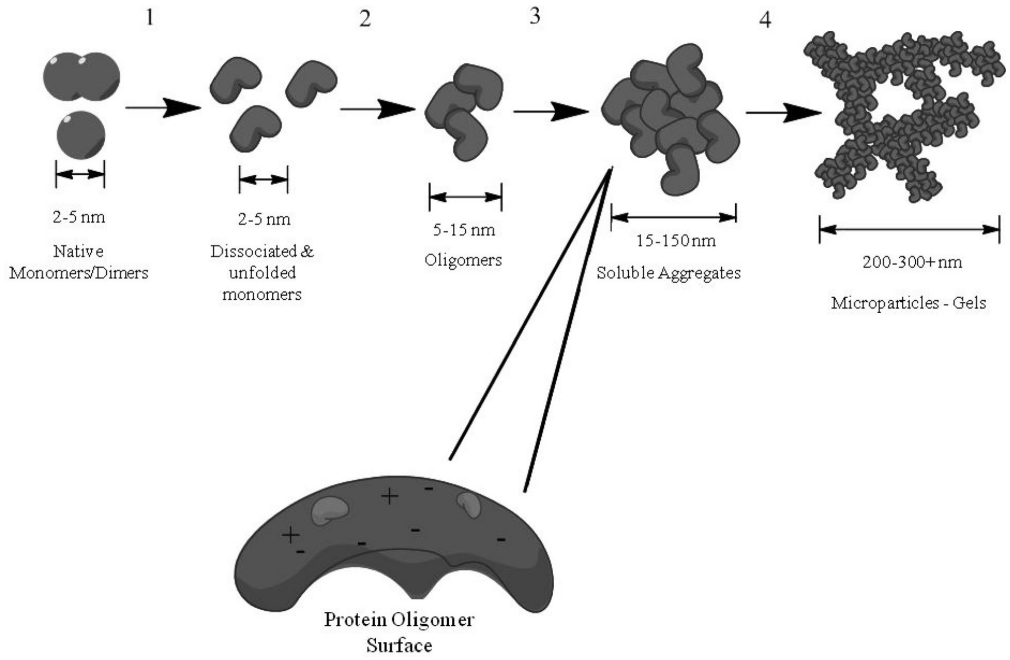
Protein aggregation characterises neuro-degenerative disorders

Protein Conformational Disorder	Fibril Subunit
Alzheimer's Disease	Aβ-peptide
Spongiform encephalopathies	Prion protein
Parkinson's disease	α-synuclein
Type II diabetes	Amylin
Thyroid carcinoma	Procalcitonin
Atrial amyloidosis	Atrial natriuretic factor
Amyotrophic lateral sclerosis	Superoxide dismutase
Huntington disease	Long Glutamine Stretches within proteins
Primary systemic amyloidosis	Ig light chains
Secondary systemic amyloidosis	Serum amyloid A
Senile systemic amyloidosis	Transthyretin (wild tipe)
Familial amyloidotic polyneuropathy I	Transthyretin (mutant)
Familial amyloidotic polyneuropathy II	Apolipoprotein A1
Familial Mediterranean fever	Serum amyloid A
Hemodialysis-related amyloidosis	b2-microglobulin
Finnish hereditary systemic amyloidosis	Gelsolin (mutant)
Lysozyme systemic amyloidosis	Lisozime
Insulin-related amyloidosis	Insulin
Fibrinogen systemic amyloidosis	Fibrinogen α chain

Food proteins (the good)

Important gelling agents and nutrients

Aggregates with amyloid character important for their rheological properties during the processes necessary for gel formation (transparent gels)



Amyloid fibrils have properties (elasticity, solubility, etc) favorable for food texturing or to produce special structures.

possible Inhibitors

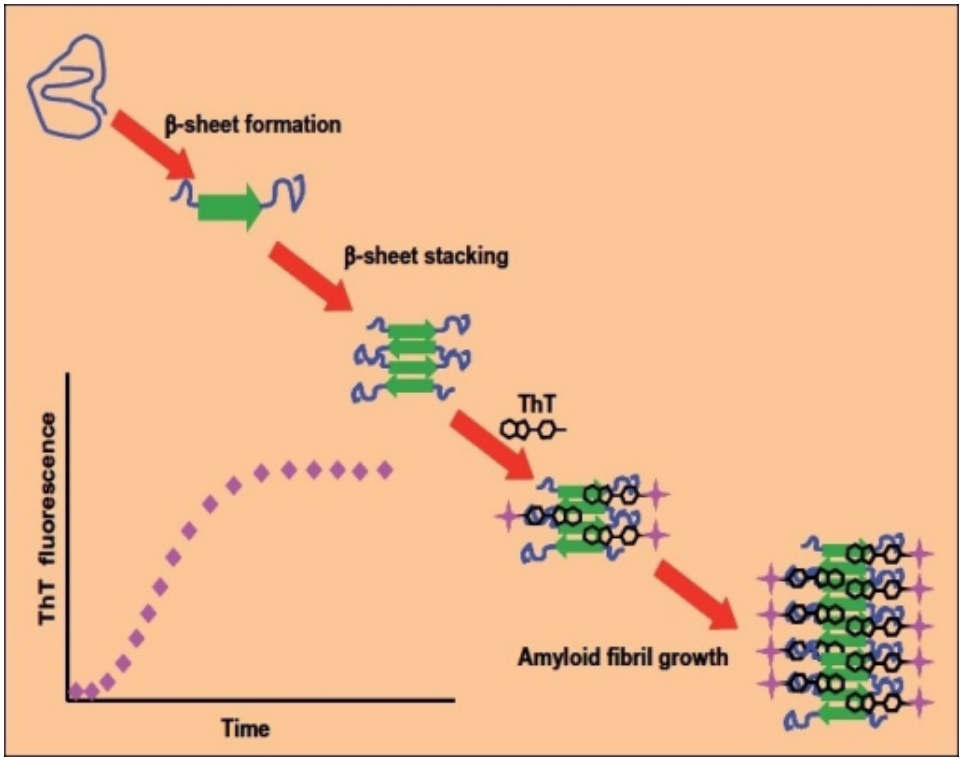
Flavonoids, organic compounds, synthetic compounds, …

Computational and Experimental Studies on β-Sheet Breakers Targeting $A\beta_{1-40}$ Fibrils

V. Minicozzi, R. Chiaraluce, V. Consalvi, C. Giordano, C. Narcisi,
P. Punzi, G.C. Rossi, S. Morante

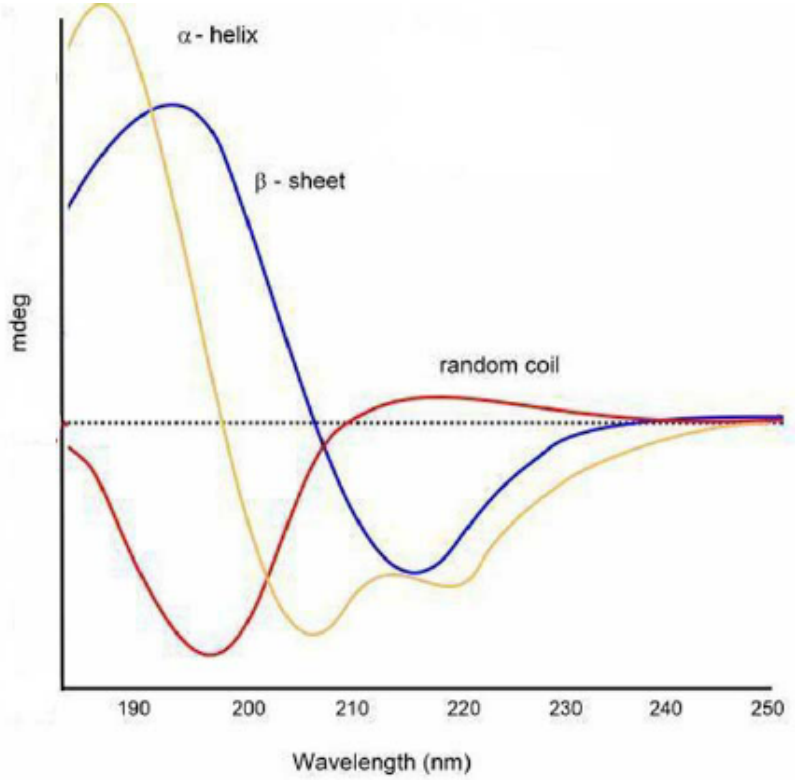
J. Biol. Chem. 2014, 289, 11242.

- **Molecular Dynamics (MD) simulations**
- **ThT fluorescence measurements**
- **Far-UV Circular Dichroism (CD) measurements**
- **Mass Spectrometry measurements**



ThT-fluorescence

far-UV CD



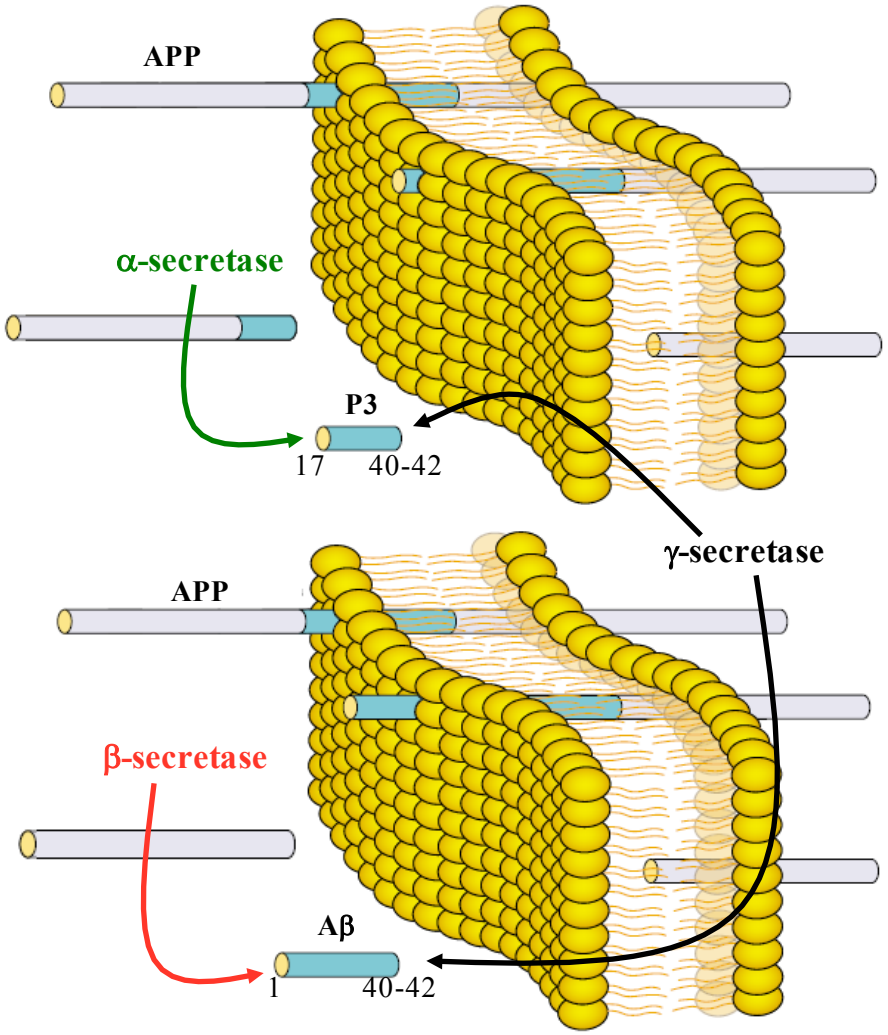
Amyloid β-peptide

•α- & γ-secretases cleavage ⇒ **non-pathological** peptide P3

• Aβ is derived from proteolitic cleavage of APP protein (Amyloid Precursor Protein).

•APP: 770 trans-membrane protein coded in chromosome 21

•β- & γ-secretases cleavage ⇒ **pathological** peptides Aβ1-40, Aβ1-42

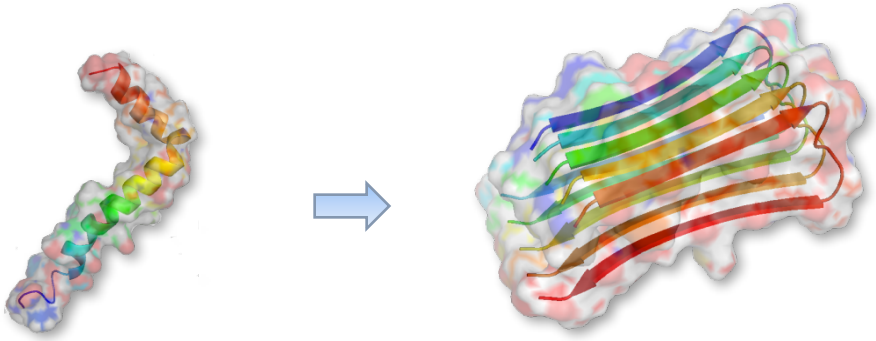


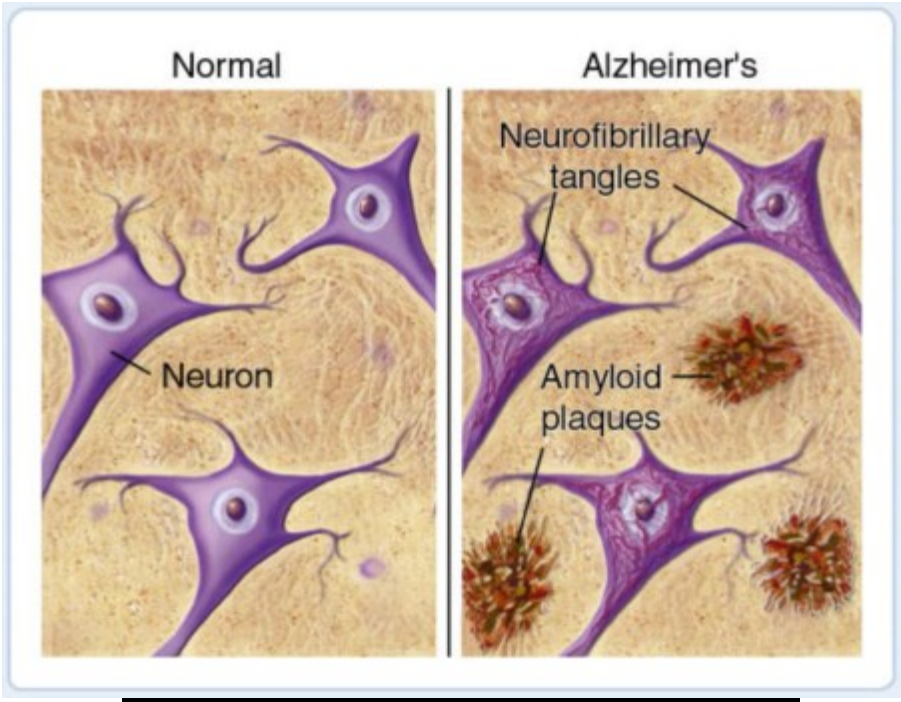
AD brains show two lesions

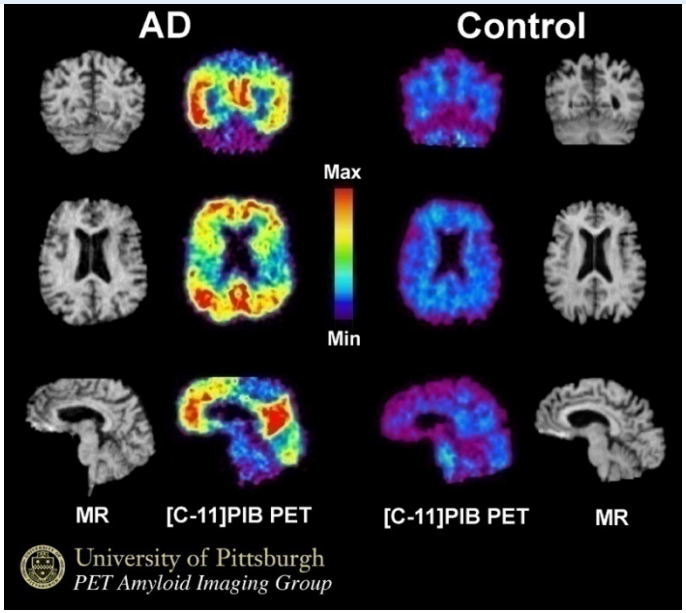
1- **Amyloid Plaques**:

Extracellular deposits of Amyloid β (Aβ) peptide

Almost spherical with a 10-100 mm diameter







2- **Neurofibrillar Tangles:**

Intracellular anormal elicoidal fibers mainly composed by *tau* protein

Development of a macromolecular strategy
to prevent aggregation and fibrils formation

β-sheet breakers

Small peptides, called β-sheet breaker peptides (BSBp's), are able to inhibit or delay the conformational transition of Aβ peptide from α-helix to β-sheet

Possible therapeutic strategy

17 21

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA

β-amyloid

Ac-LPFFD-NH_2

breaker

Classical Molecular Dynamics

MD simulations

- $A\beta_{1\text{-}40}$ = NH_3^+-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-COO^-

- Soto breaker = Ac-LPFFD-NH_2
- Soto breaker + taurine = Tau-LPFFD-NH_2

} Already studied experimentally

- Modified Soto D → N = Ac-LPFFN-NH_2 → New Peptide

Systems

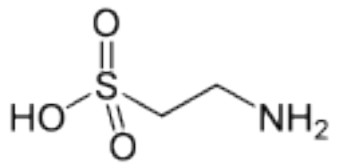
abeta = $A\beta_{1\text{-}40}$ + water + ions

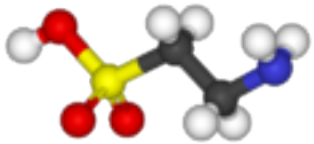
lpffd = $A\beta_{1\text{-}40}$ + 10 LPFFD + water + ions

taulpffd = $A\beta_{1\text{-}40}$ + 10 Tau-LPFFD + water + ions

lpffn = $A\beta_{1\text{-}40}$ + 10 LPFFN + water + ions

Taurine





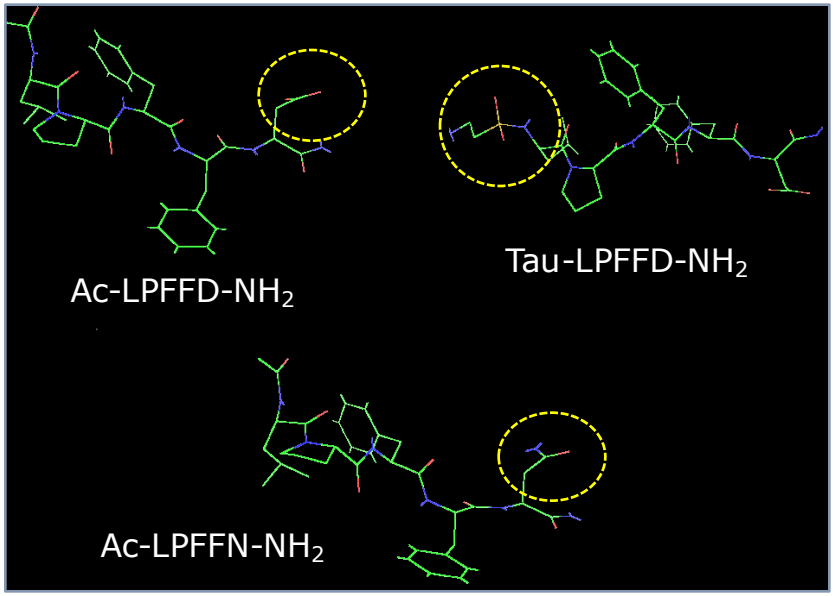
1 $A\beta_{1\text{-}40}$ + ~ 122,300 H_2O

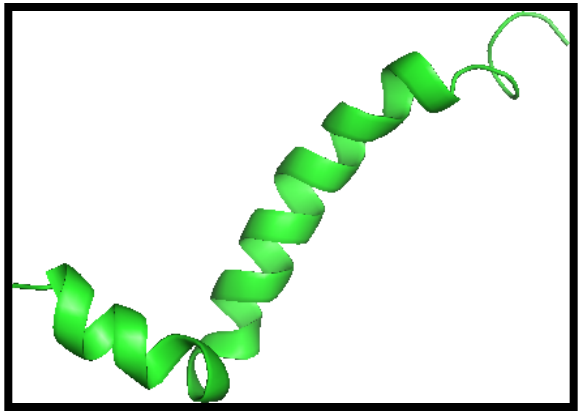
$[A\beta_{1\text{-}40}]$:[BSBp] = 1:10

$[A\beta_{1\text{-}40}]$ ~ 400μM

ions added to neutralize the systems

$A\beta_{1-40}$ and BSBp's structure





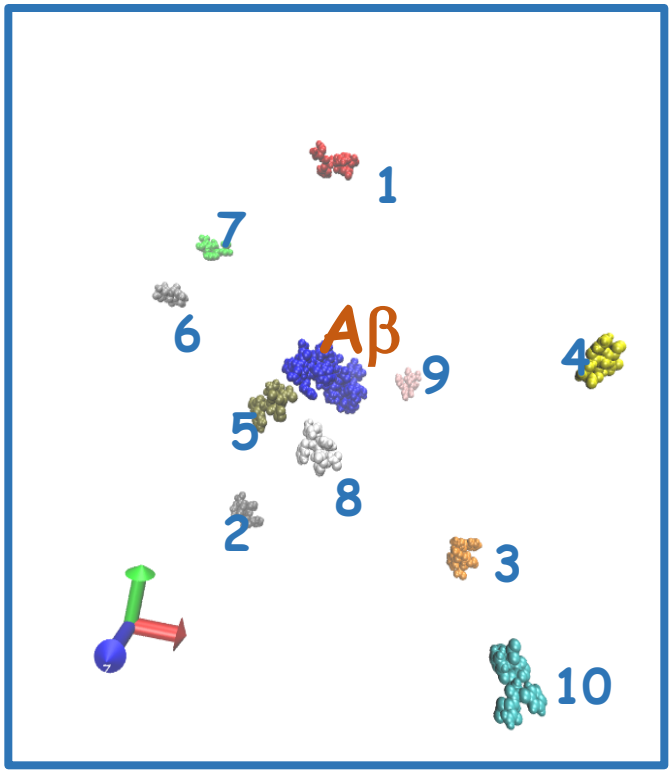
$A\beta_{1-40}$ starting structure from PDB-ID: 1IYT

MD simulation steps

- Steepest Descent minimization in vacuum
- Solvation: adding water molecules and counter-ions
- Solvent relaxation: 10 ps NVT MD at 200K
- Whole system relaxation 50 ps NVT MD at 300K
- **NpT MD at 300K for 80 ns is finally performed**

GROMACS package with GROMOS53A6 force field

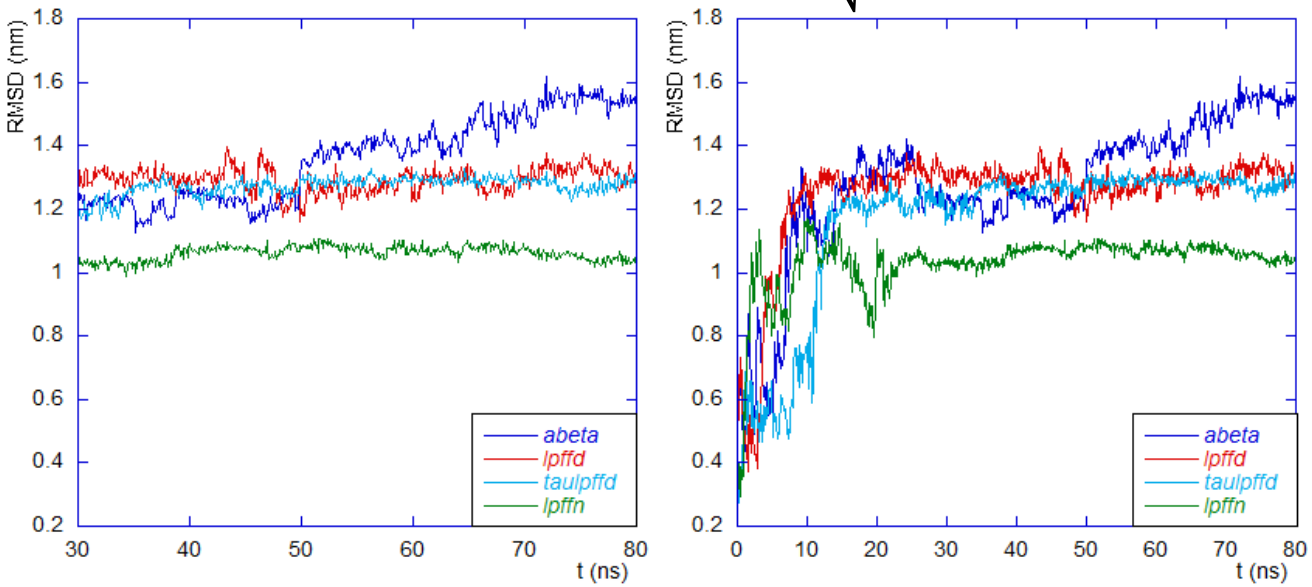
- SPC water model
- dt = 2 fs
- PME
- pair list (with a 1.4 nm cutoff) updated every ten steps



Starting positions of BSBps identical for all systems (water not shown)

RMSD

$$RMSD(t, t_0) = \sqrt{\frac{1}{M}\sum_{i=1}^{N} m_i \, \|r_i(t) - r_i(t_0)\|^2}$$

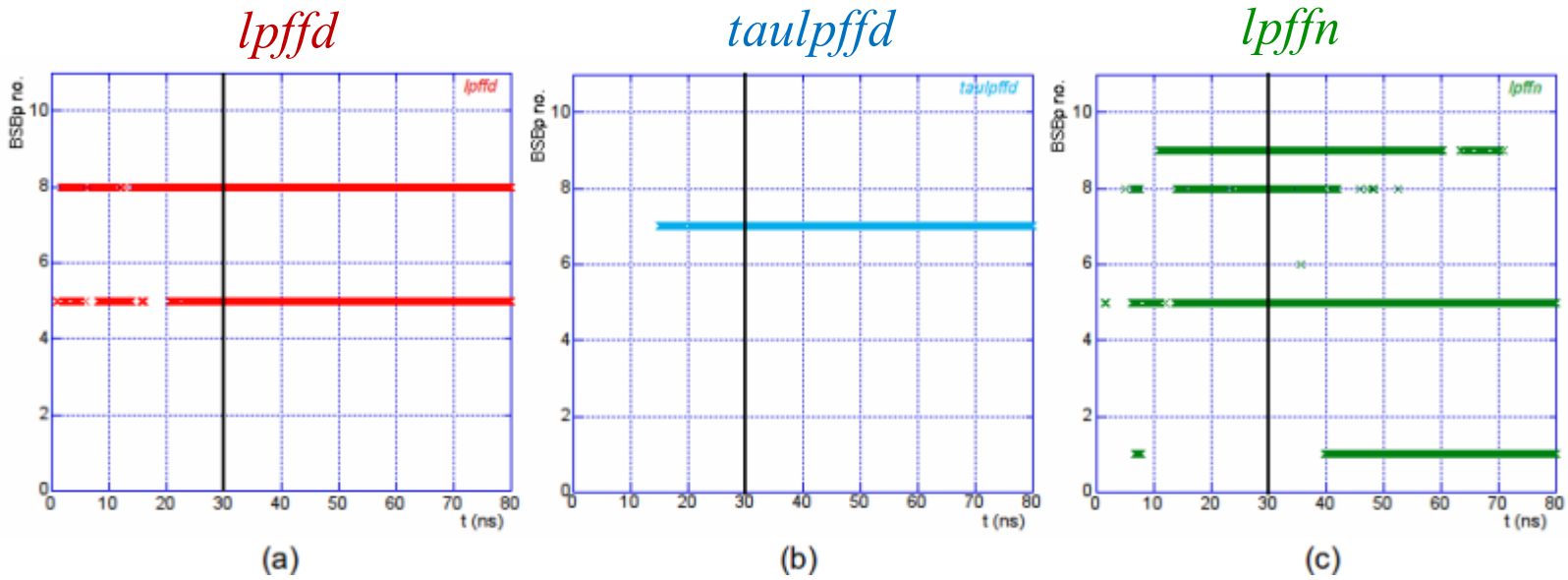


System	Average RMSD (nm) ± Std
abeta	1.4 ± 0.1
lpffd	1.29 ± 0.04
taulpffd	1.27 ± 0.03
lpffn	1.06 ± 0.02

$A\beta_{1\text{-}40}$ in interaction with **Ac-LPFFN-NH_2** changes very little from its initial configuration

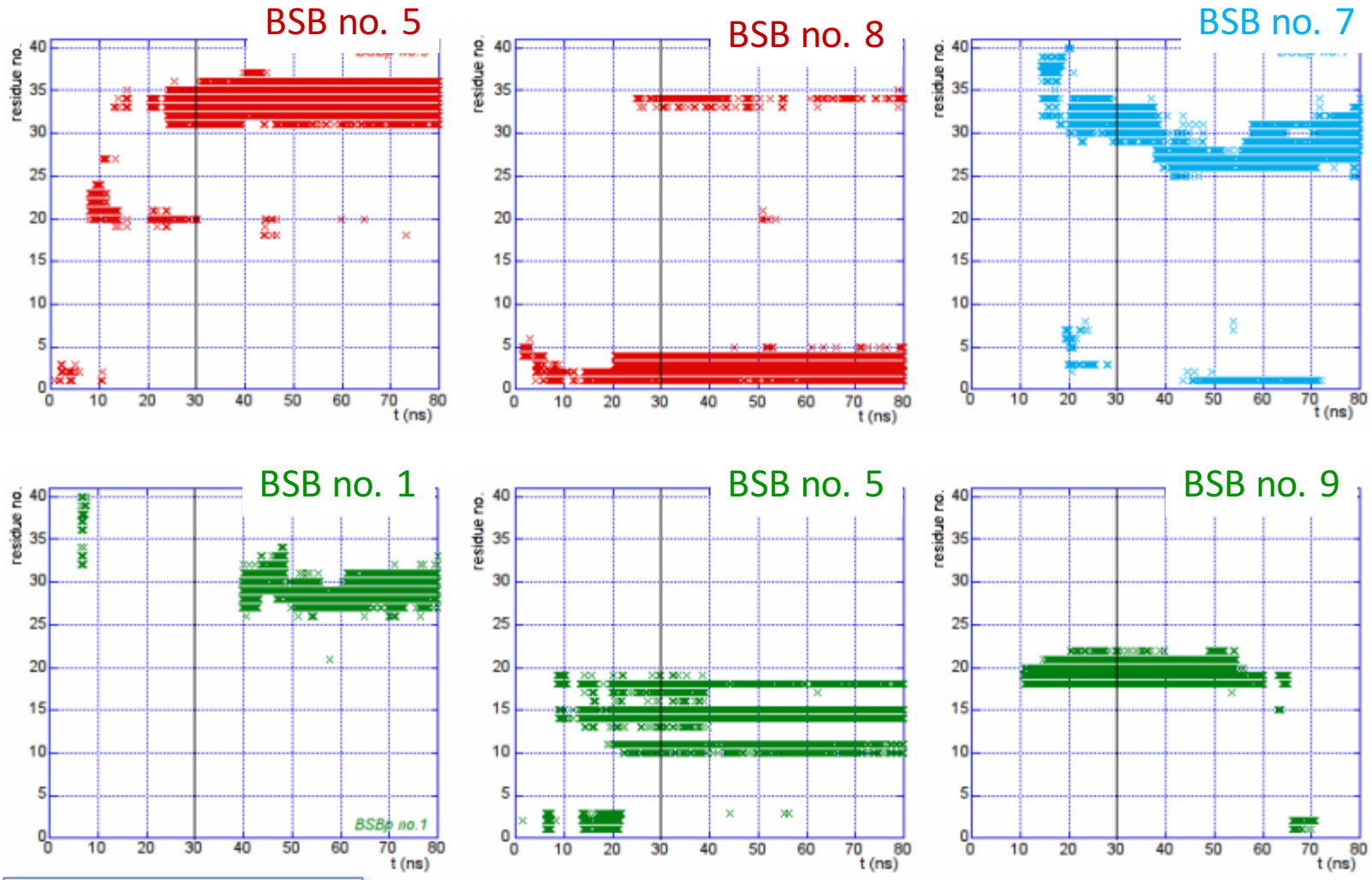
All the values are averaged over the last 50 ns simulation time

BSBp's within 8 Å from $A\beta_{1-40}$



lpffd	→ 2 BSBps approaches $A\beta_{1-40}$
taulpffd	→ only 1 BSB after about 15 ns
lpffn	→ more BSBps only two stably

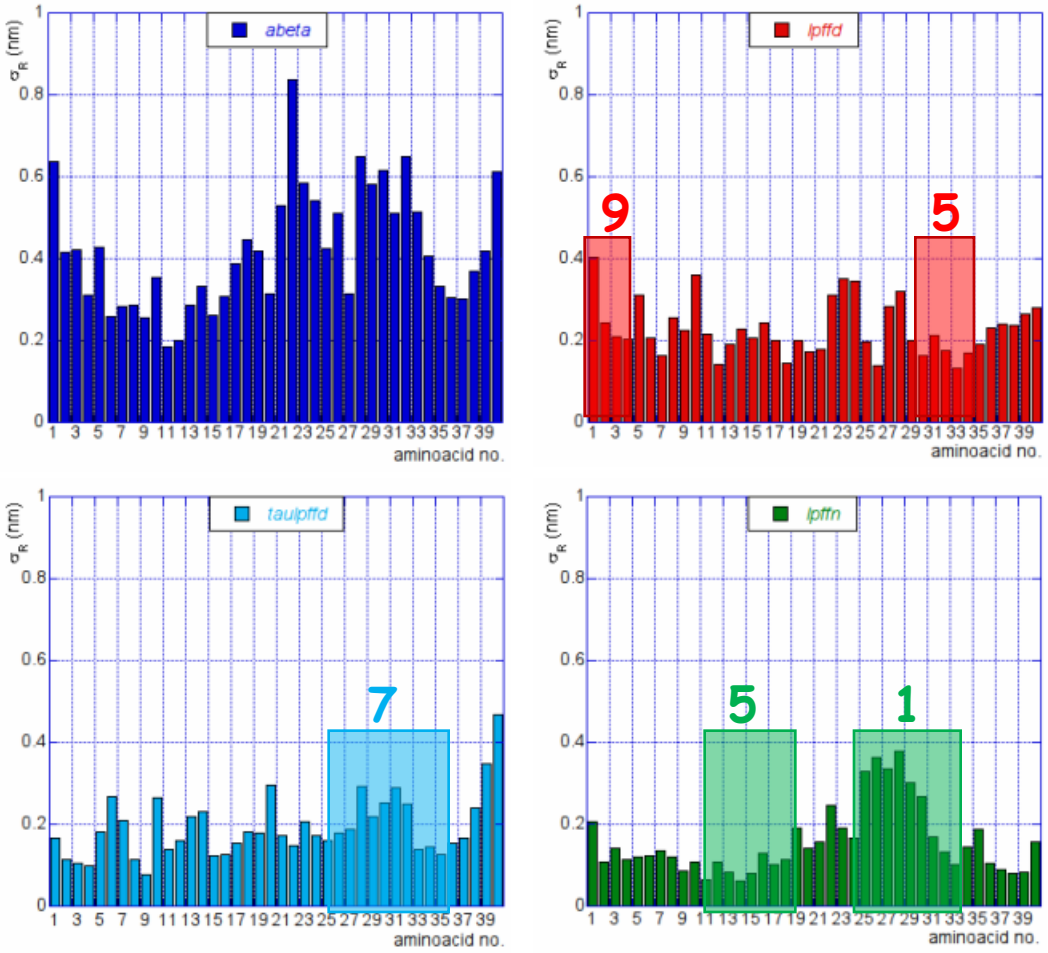
To which $A\beta_{1\text{-}40}$ aa is each BSBs nearer ?



red = lpffd
light blue = taulpffd
green= lpffn

Mobility

$$RMSF = \sqrt{\frac{1}{T}\sum_{t}\frac{1}{N_R}\sum_{j}\left|r_j(t) - \langle r_j \rangle\right|^2}$$



- $A\beta_{1\text{-}40}$ residue mobility is always reduced by the presence of any of the three BSBps

- The most effective BSBp seems to be Ac-LPFFN-NH_2

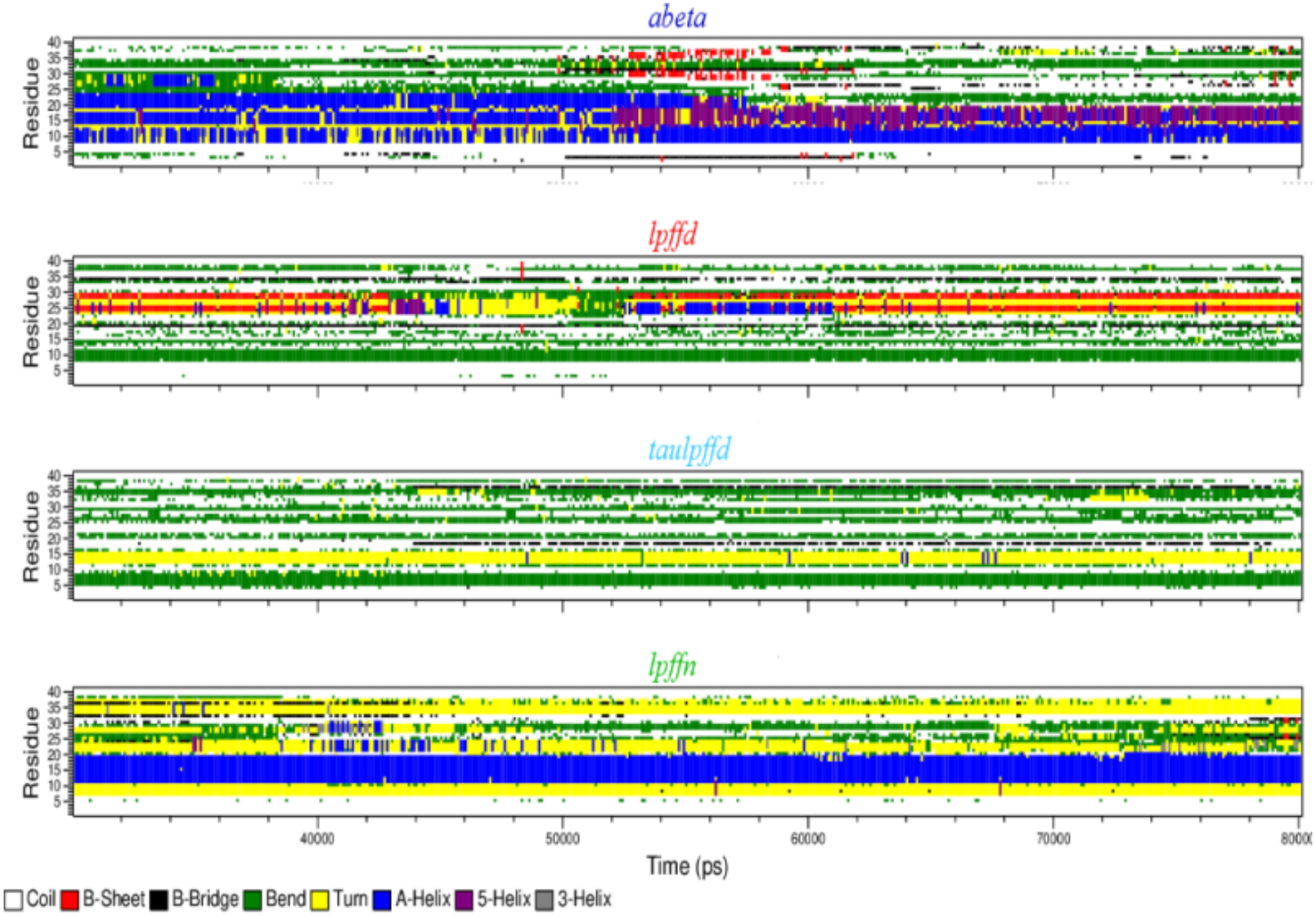
Secondary structure

abeta

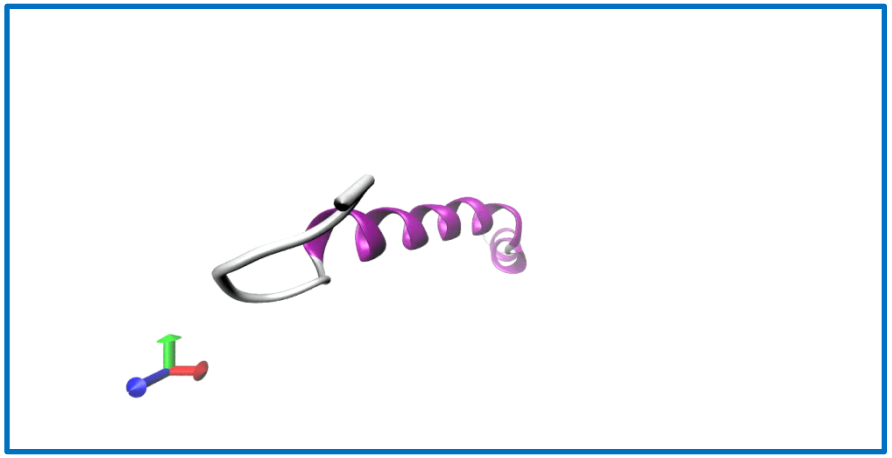
lpffd

taulpffd

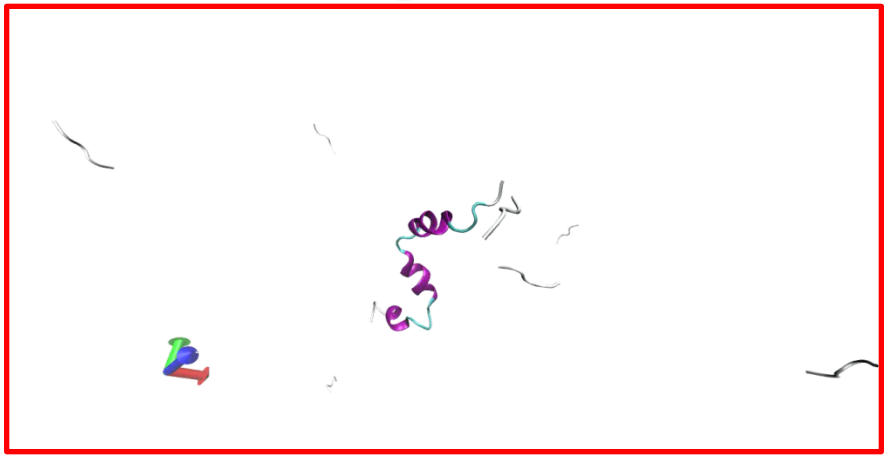
lpffn



abeta

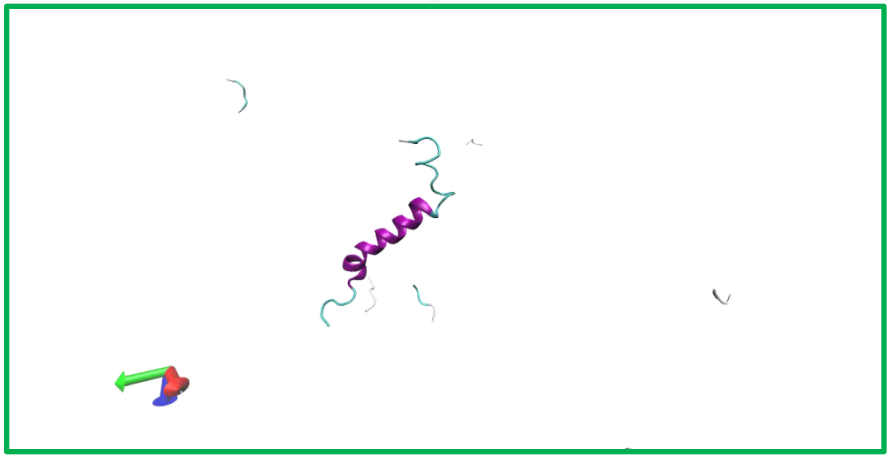


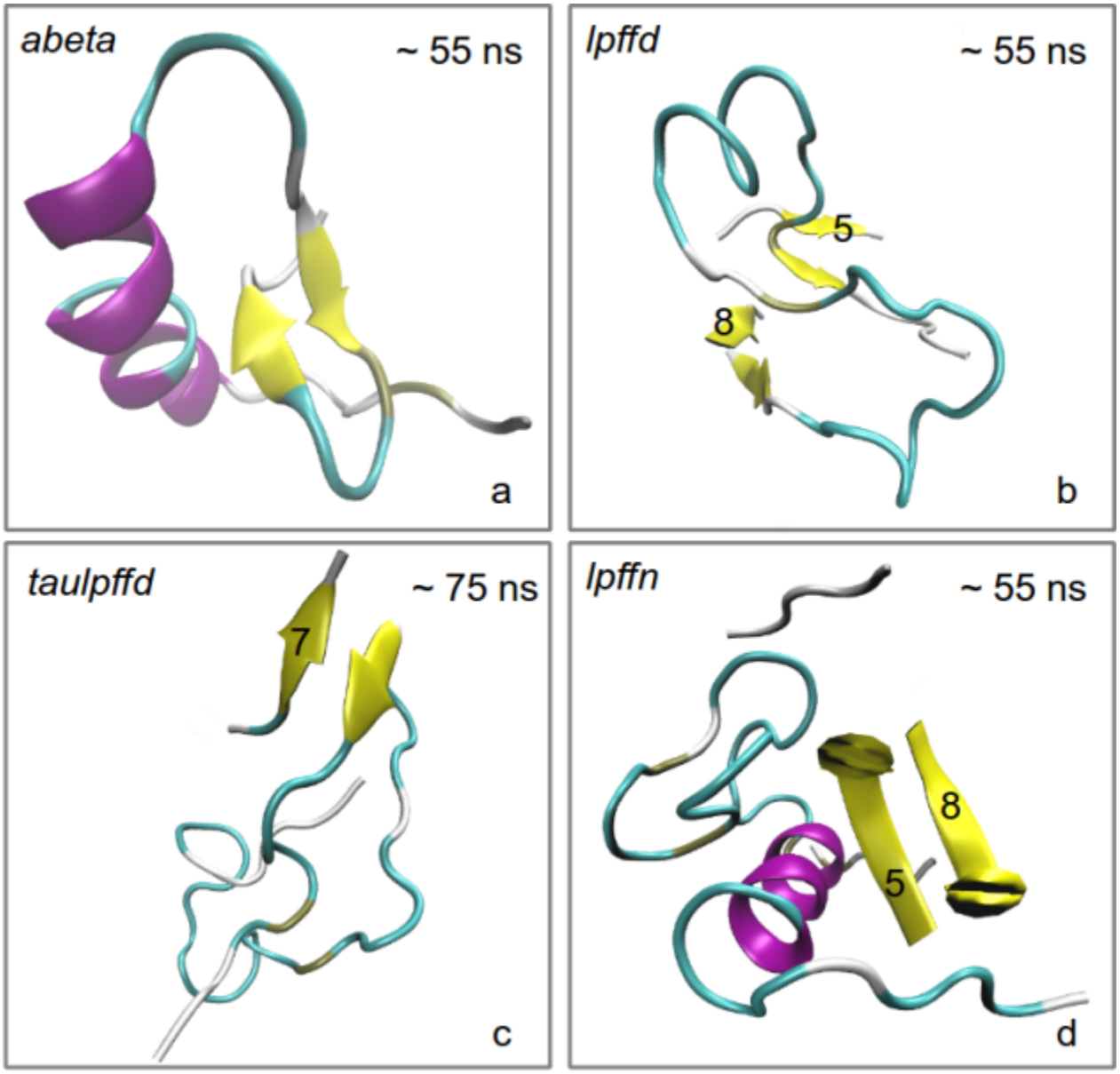
lpffd



taulpffd

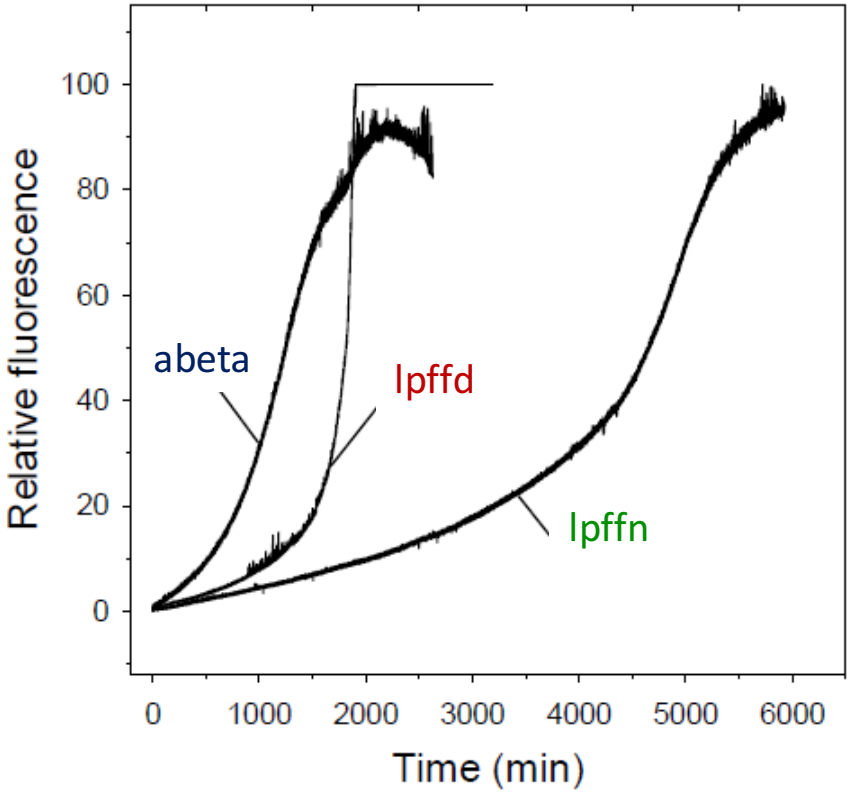
lpffn




abeta
~ 55 ns
a
lpffd
~ 55 ns
5
8
b
taulpffd
~ 75 ns
7
c
lpffn
~ 55 ns
8
5
d

ThT fluorescence

ThT fluorescence is useful to determine $A\beta_{1-40}$ fibril formation and aggregation



The longer the lag phase
the higher the inhibition of $A\beta_{1-40}$ aggregation

In the presence of BSBp, the lag phase is longer

$A\beta_{1-40}$ ~ 600 min

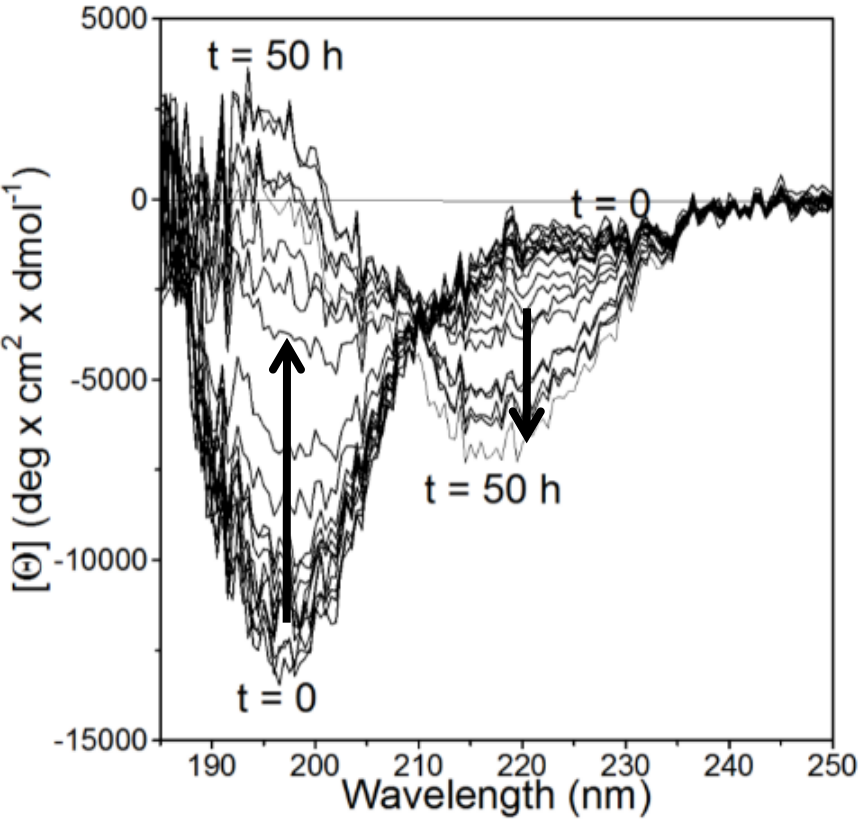
$A\beta_{1-40}$ +Ac-LPFFD-NH_2 ~ 1000 min

$A\beta_{1-40}$ +Ac-LPFFN-NH_2 ~ 3000 min

Ac-LPFFN-NH_2
the highest inhibition activity

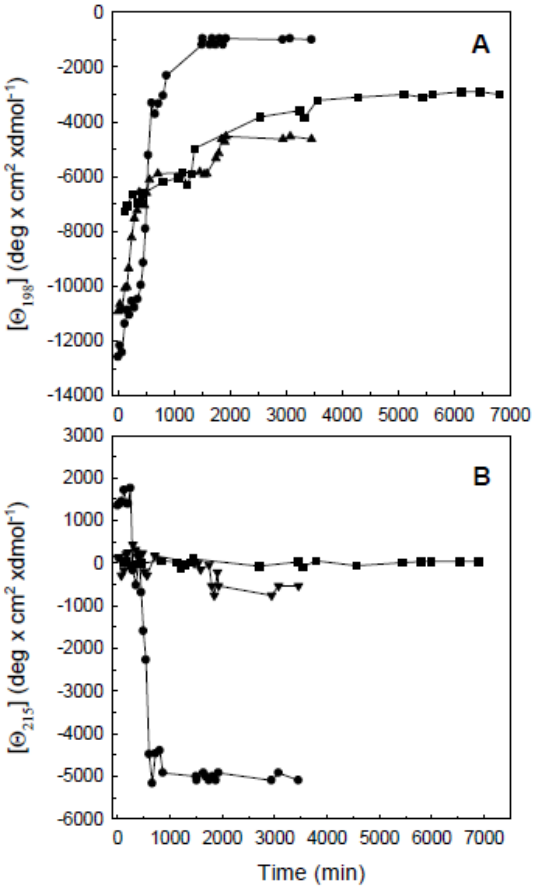
Far UV CD spectroscopy

CD spectroscopy gives information about changes in $A\beta_{1-40}$ secondary structure



$A\beta_{1-40}$ alone
turn structure → β-sheet structure

$A\beta_{1-40}$ alone (full dots) in the presence of Ac-LPFFD-NH_2 (full triangles) and in the presence of Ac-LPFFN-NH_2 (full squares)



The interaction between $A\beta_{1-40}$ and both BSBps reduces the peptide conformational transition propensity

Mass spectrometry

Experiments performed on samples containing fragments of $A\beta_{1-40}$ and the three different BSBp's

BSBp	% bond with $A\beta_{25-35}$	% bond with $A\beta_{17-21}$
Ac-LPFFD-NH_2	3.17	5.60
Tau-LPFFD-NH_2	2.47	7.18
Ac-LPFFN-NH_2	5.92	20.60

Ac-LPFFN-NH_2 is the BSBp which binds more strongly $A\beta_{1-40}$

It is the one with the highest affinity for $A\beta_{17-21}$ fragment

Consistent with MD simulations

Conclusions – Aβ+BSBs

From MD simulations we obtained that

→ All BSBp's are able to interact with $A\beta_{1-40}$ and to reduce its residues mobility (Ac-LPFFN-NH_2 being the most effective)

→ All BSBp's come close to 25-35 hydrophobic region but Ac-LPFFN-NH_2 interacts also with 17-21 region, which conserves its α-helix content

→ Ac-LPFFN-NH_2 works by stabilizing the starting α-helix secondary structure of $A\beta_{1-40}$ peptide

→ Ac-LPFFD-NH_2 and Tau-LPFFD-NH_2, act by somehow saturating (or hindering) the dangling bonds of a nascent β-sheet structure so that the latter is not anymore available for $A\beta_{1-40}$ aggregation

Conclusions – Aβ+BSBs

From *in vitro* experiments we obtained that:

- As shown from the longer lag phase in ThT Fluorescence spectra, in the presence of Ac-LPFFN-NH_2 fibrils formation is delayed;

- CD measurements have shown that Ac-LPFFN-NH_2 stabilizes $A\beta_{1-40}$ secondary structure thus reducing its propensity to form β-sheets;

- Mass Spectrometry confirms what emerges from MD simulations, that Ac-LPFFN-NH_2 is the BSBp which interacts more strongly with $A\beta_{1-40}$ and especially with 17-21 region.

Ac-LPFFN-NH_2 can thus be considered as a lead compound to prevent and/or destabilize (delay) $A\beta_{1-40}$ fibril formation and aggregation

V. M., R. Chiaraluce, V. Consalvi, C. Giordano, C. Narcisi, P. Punzi, G.C. Rossi and S. Morante, JBC (2014) **289**:11242-11252

Outlooks

$A\beta_{1\text{-}40}$ in the presence of Cu^{2+} and Zn^{2+} → add BSBp's
→ are there any difference in the presence of metal ions?

CD, ThT fluorescence, AFM, EXAFS measurements

Simulations → what kind of simulations in the presence of metal ions?

Role of dietary antioxidant (−)-epicatechin in the development of β-lactoglobulin fibrils

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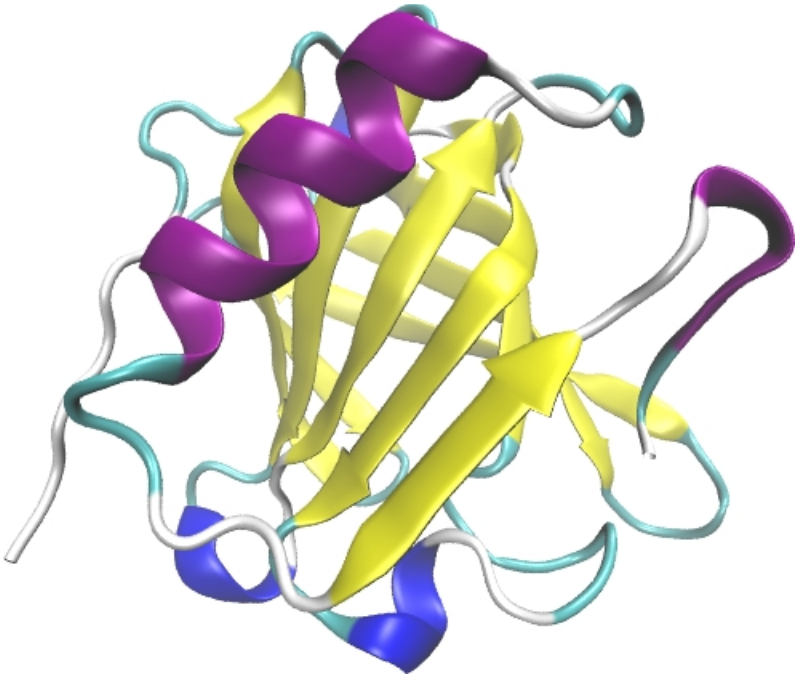
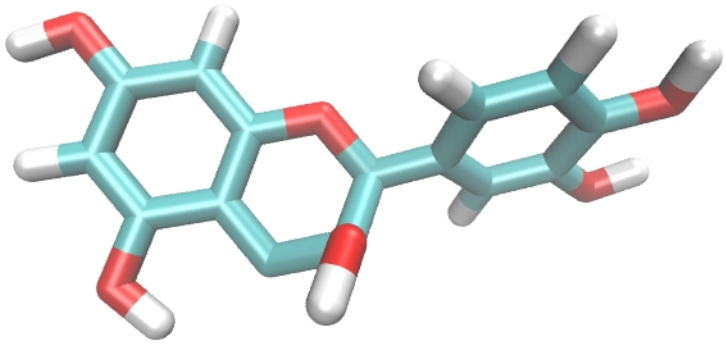
S. Morante, E. Nicolai, A. Nucara, E. Placidi, F. Stellato

BBA-Proteins and Proteomics 2016, 1864, 766.

- **AFM images**
- **ThT fluorescence measurements**
- **Fluorescence Correlation Spectroscopy (FCS) measurements**
- **Fourier Transform Infra Red (FTIR) measurements**
- **Molecular Dynamics (MD) simulations**

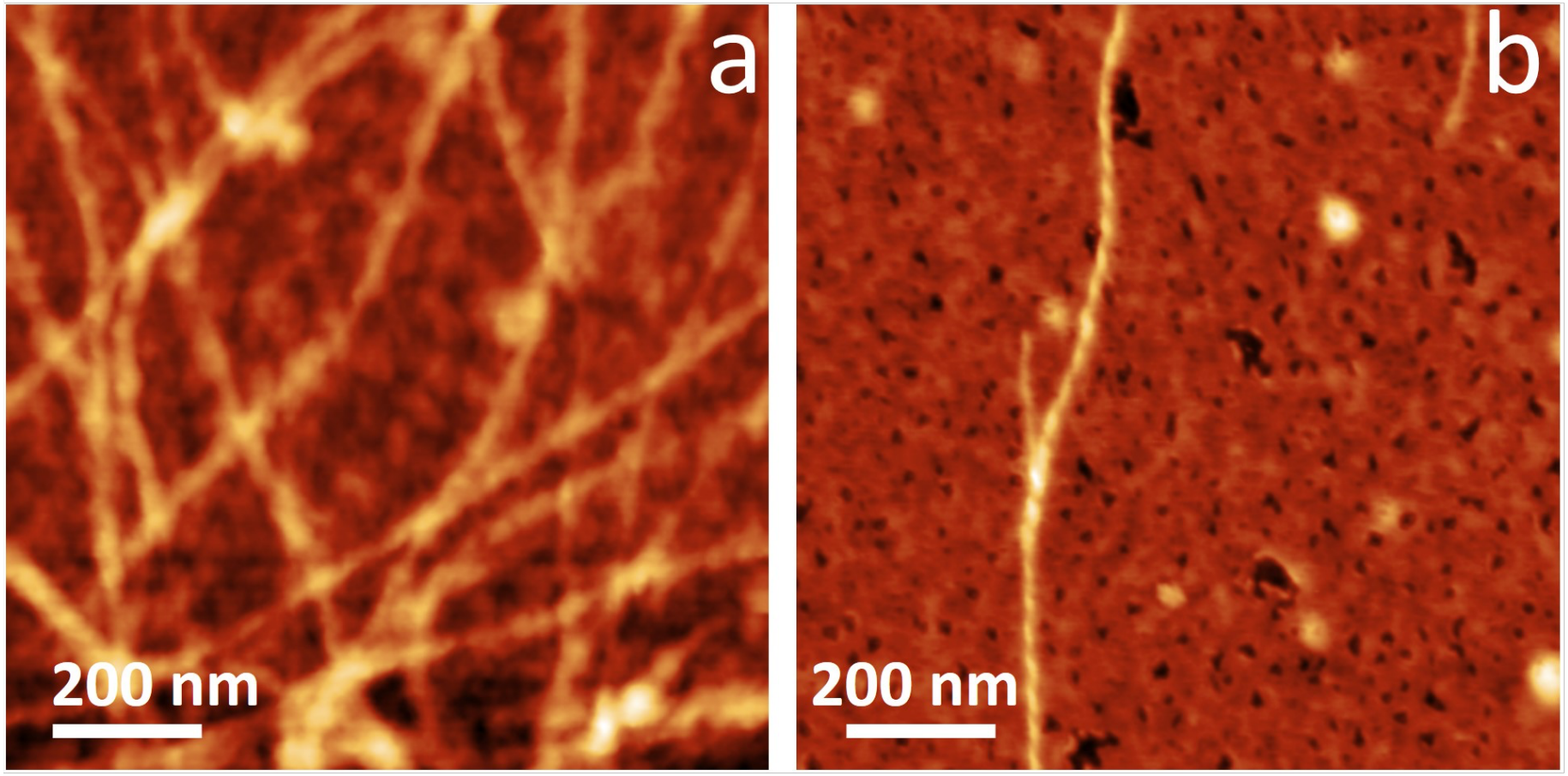
β-lactoglobulin and epicatechin





BLG fibrils are produced by heating the protein for 24 hours at 80 °C, pH 2.0 and low ionic strength

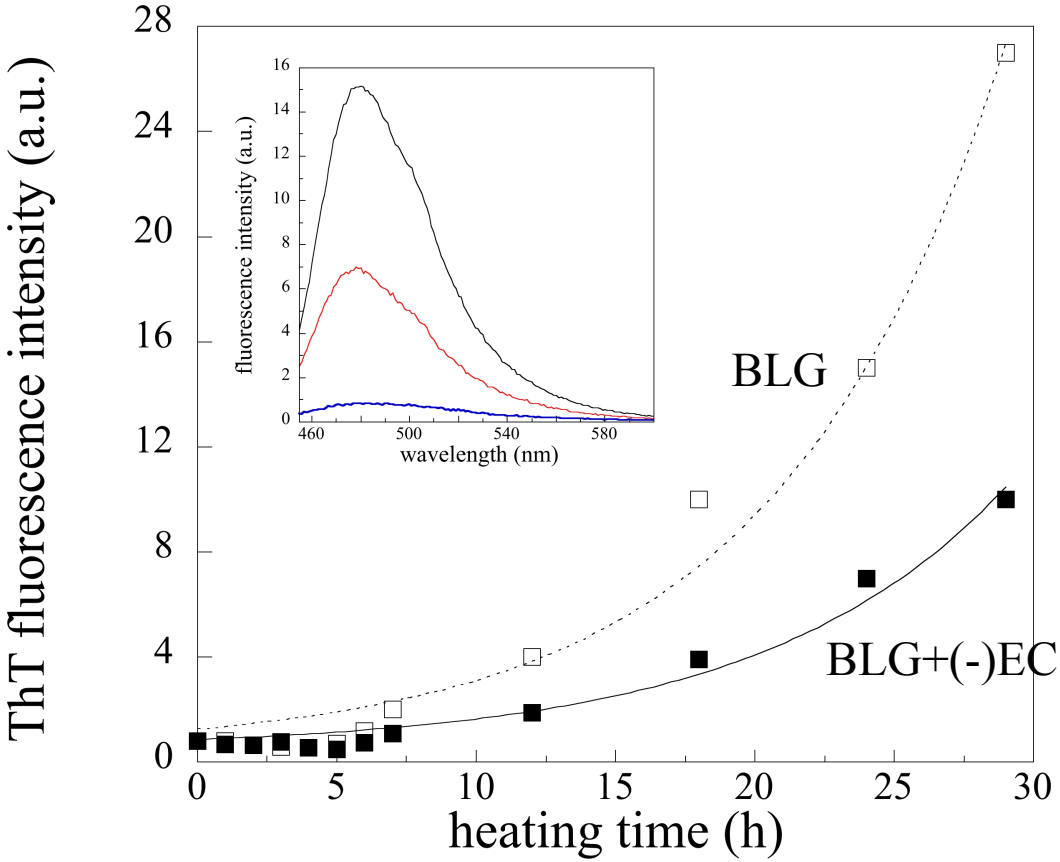
AFM images



(a)In the absence and (b) in the presence of (-)EC

Fibril periodicity = (38 ± 3) nm Fibril height = (4.7 ± 0.3) nm
not influenced by (-)EC presence

ThT-fluorescence measurements



Fluorescence intensity of ThT added to BLG in the absence (open squares) and in the presence (closed squares) of (-)EC

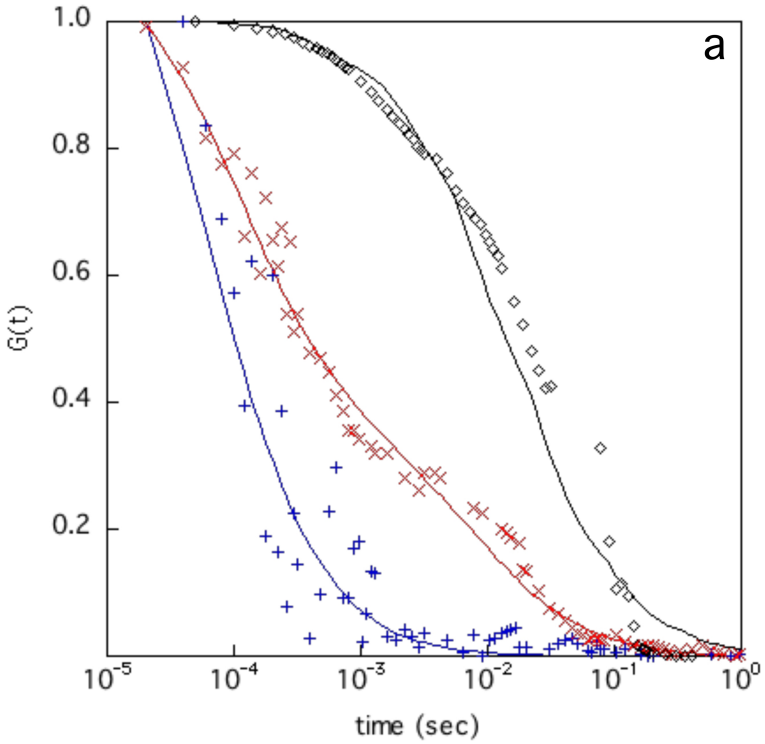
Exponential fit of the kind $I(t) = I_0 \exp(t/\tau)$ yields $\tau = 10.3$ hours for the BLG + (-)EC sample and $\tau = 8.7$ hours for BLG alone

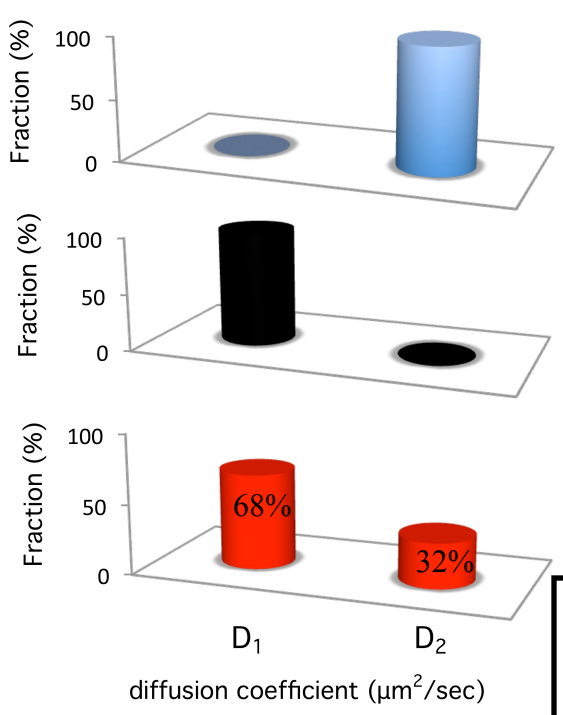
FCS measurements

t_{inc}=0 hours with and without (-)EC → blue
t_{inc}=24 hours without (-)EC → black
t_{inc}=24 hours with (-)EC → red

$$G(t) = \left(\frac{2\sqrt{2}}{\pi\sqrt{\pi}\omega_0^2 z_0\langle C\rangle}\right) \times \sum_{i=1}^{n} f_i \left(1+\frac{8D_i t}{\omega_0^2}\right)^{-1} \left(1+\frac{8D_i t}{z_0^2}\right)^{-1/2}$$

$$\sum_{i=1}^{n} f_i = 1,$$ **Temporal auto-correlation function**



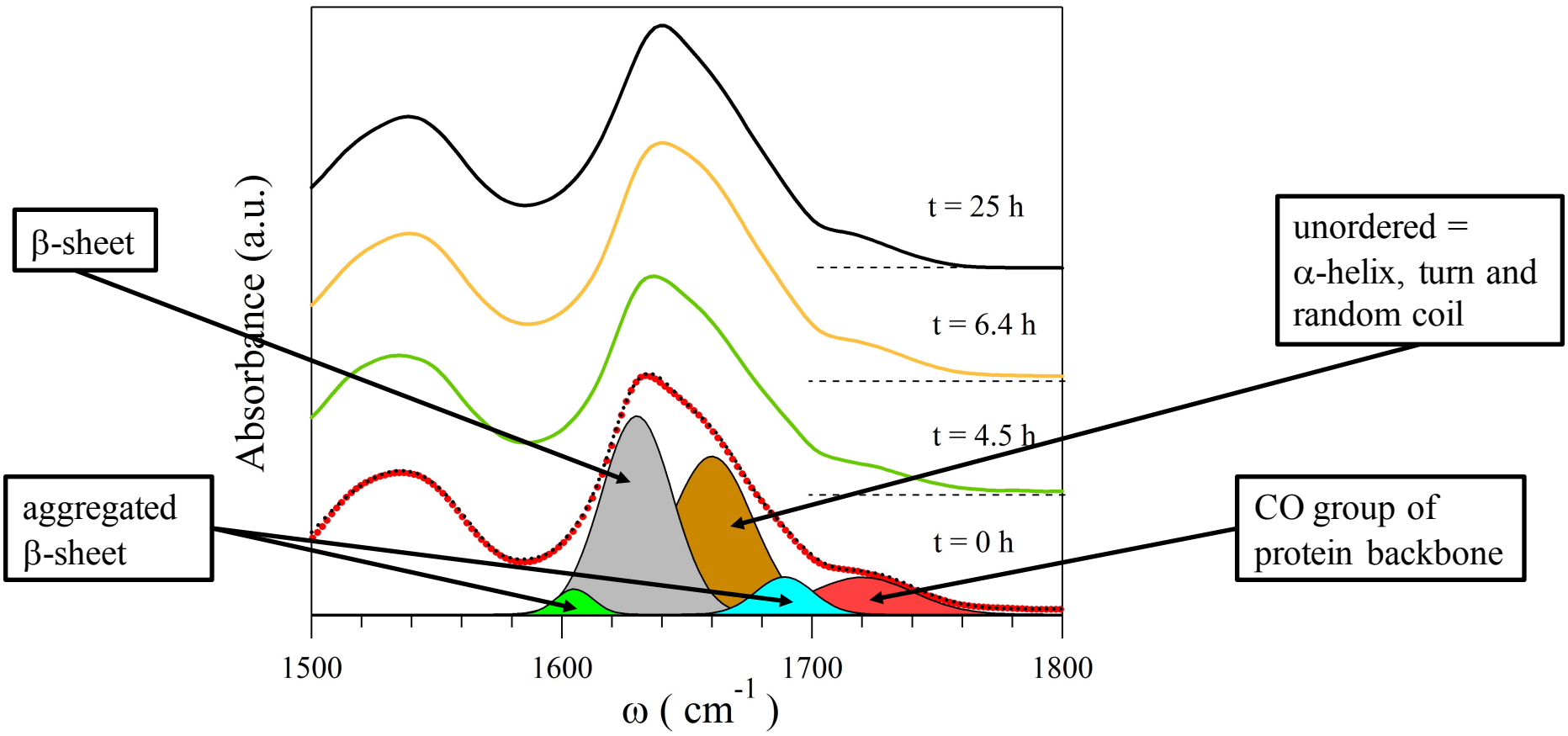


$D_1 = 10\ \mu m^2/s$

$D_2 = 250\ \mu m^2/s$

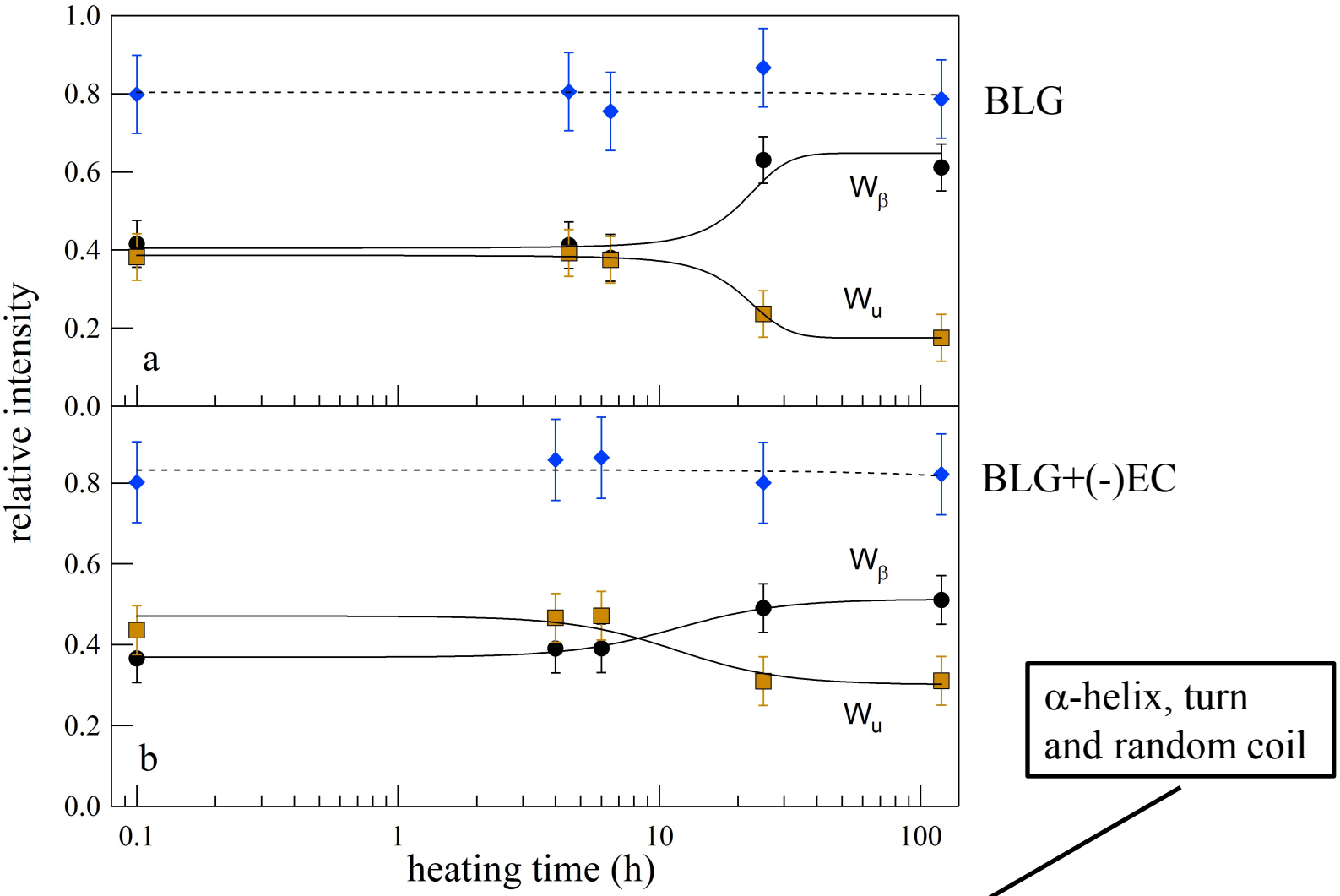
Panel a: FCS measurements of the BLG and BLG+(-)EC samples at pH 2.0.
Panel b: the percentage of each species is pictorially compared.

FTIR experiments



FTIR absorption spectra of pure BLG in the amide I and II regions at four selected incubation times

FTIR results



α-helix, turn and random coil

Relative intensity of β-sheet (W_β) and "unordered" structures (W_u)

MD simulations (@ pH 2)

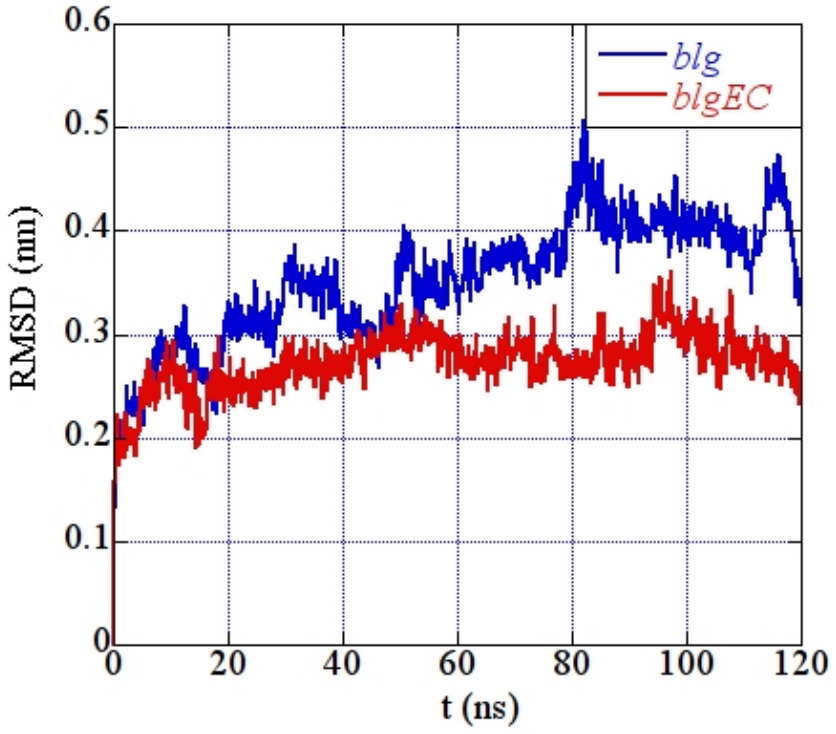
System name	Composition
blg	BLG monomer + 16545 H_2O + 48 Na^+ + 69 Cl^-
blgEC	BLG monomer + (-)EC + 16486 H_2O + 47 Na^+ + 68 Cl^-

- Steepest Descent minimization in vacuum
- Solvation: adding water molecules and counter-ions
- Solvent relaxation: 10 ps NVT MD at 200K
- Whole system relaxation 50 ps NVT MD at 300K
- **NpT MD at 353K for 120 ns is finally performed**

GROMACS package with GROMOS43A1 force field

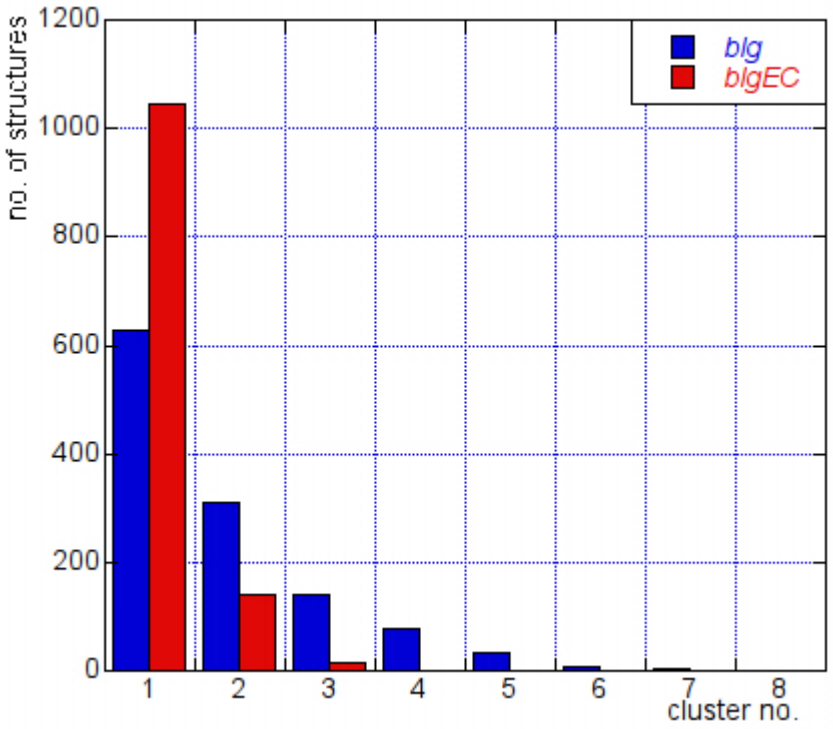
- SPC water model
- dt = 2 fs
- PME
- pair list (with a 1.4 nm cutoff) updated every ten steps

RMSD



Ligand binding stabilizes BLG structure

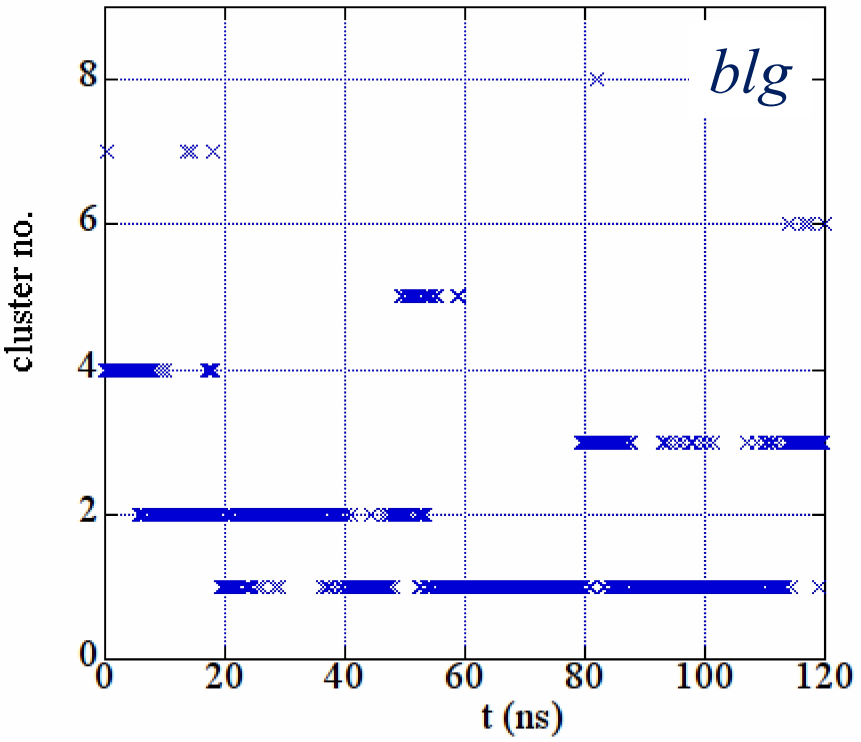
Clusterization

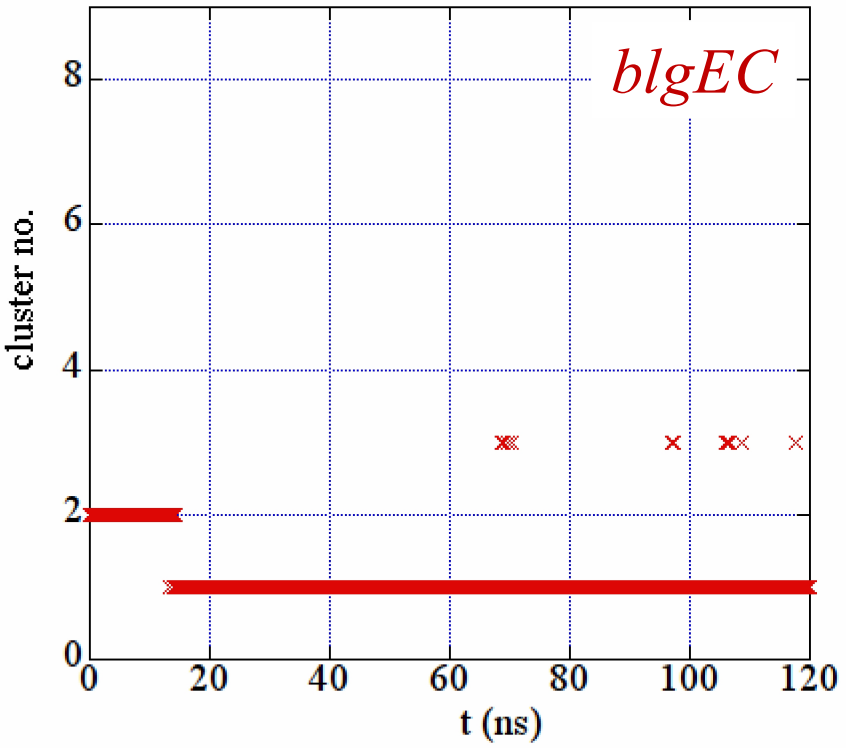


Structures whose RMSD differ less than 3 Å

In the presence of (-)EC only two clusters are appreciably populated

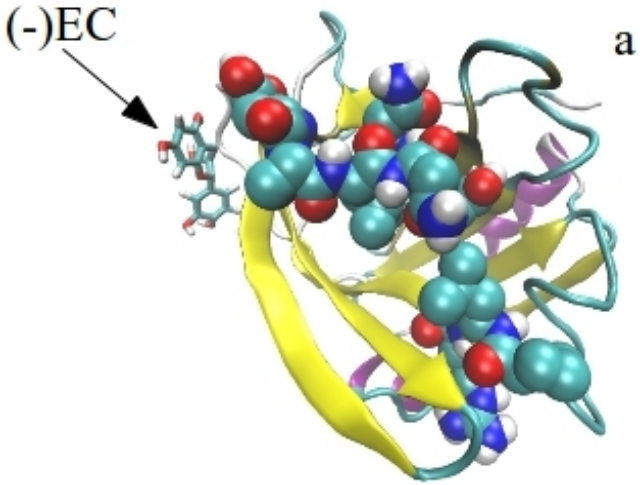
Clusters time evolution



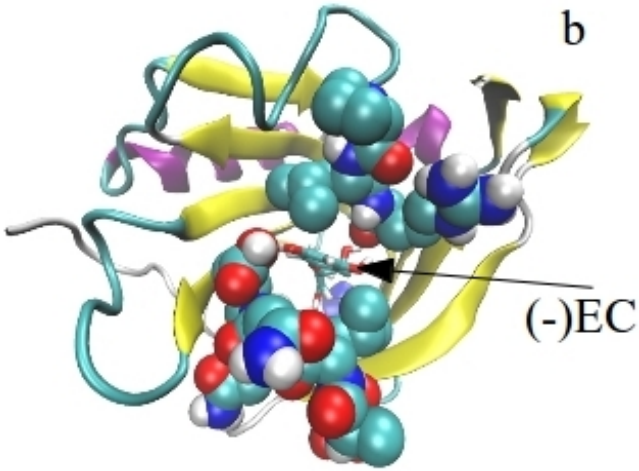


Cluster no. 2 is populated only in the first 15/18 ns, while cluster no. 1 for the remaining simulation time

Representative structures

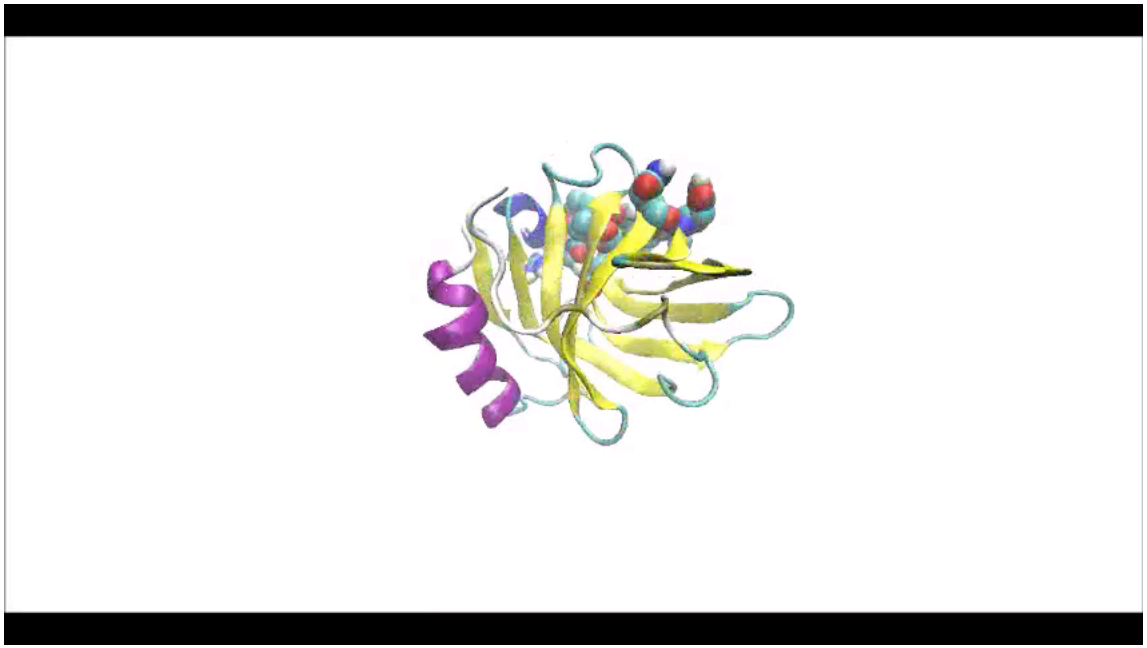


(-)EC outside the calyx
cluster no. 2

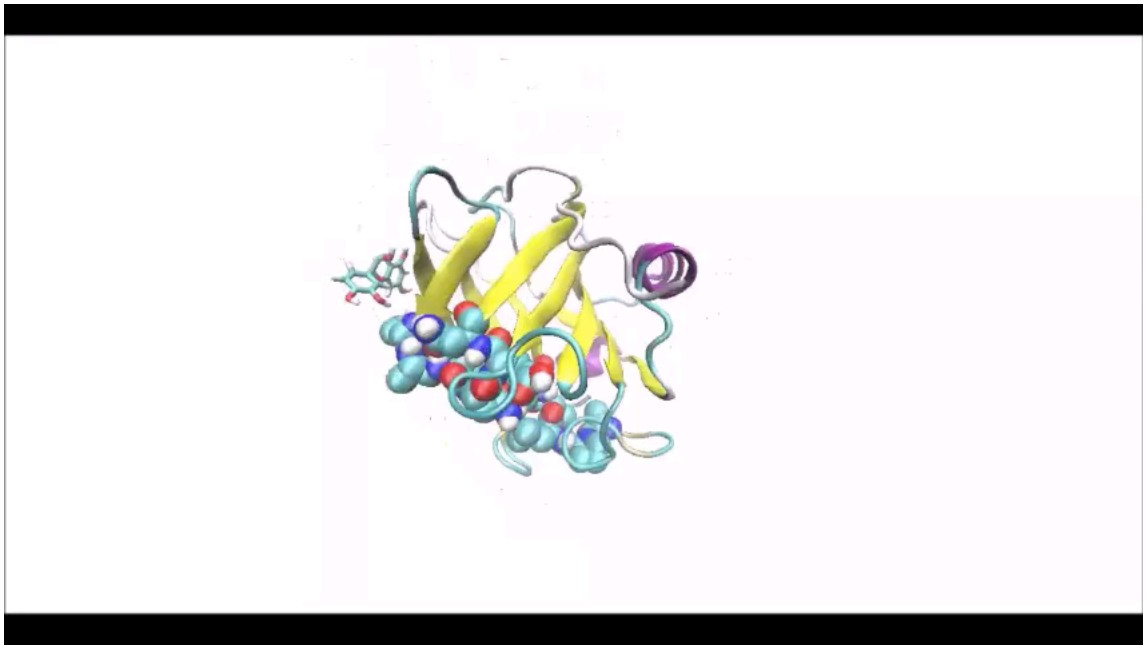


(-)EC inside the calyx
cluster no. 1

blg



blgEC



Conclusions + BLG+(-)EC

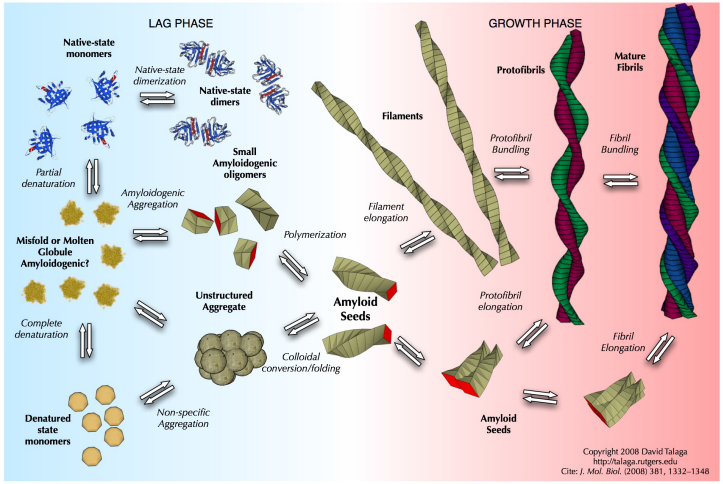
- ✓ (−)EC is able to slow down BLG aggregation process
- ✓ AFM and FCS show that (−)EC has the effect of reducing BLG fibrils
- ✓ ThT fluorescence and FTIR data reveal that aggregates formation starts concurrently with the onset of secondary structural changes of the molecule
- ✓ MD simulations unambiguously give evidence that (−)EC stabilizes the BLG secondary structure
- ✓ Moreover MD hypothesizes the mechanism by which (−)EC interacts with BLG

Summary– the good and the bad

Many techniques (MD, AFM, CD, FTIR, NMR, ThT-Fluorescence, Raman, EXAFS, SAXS,…)

Many systems (Aβ, PrP, Insulin, BLG, Amylin…) and different effectors (metals, flavonoids, peptides,...)

?



Collaborations

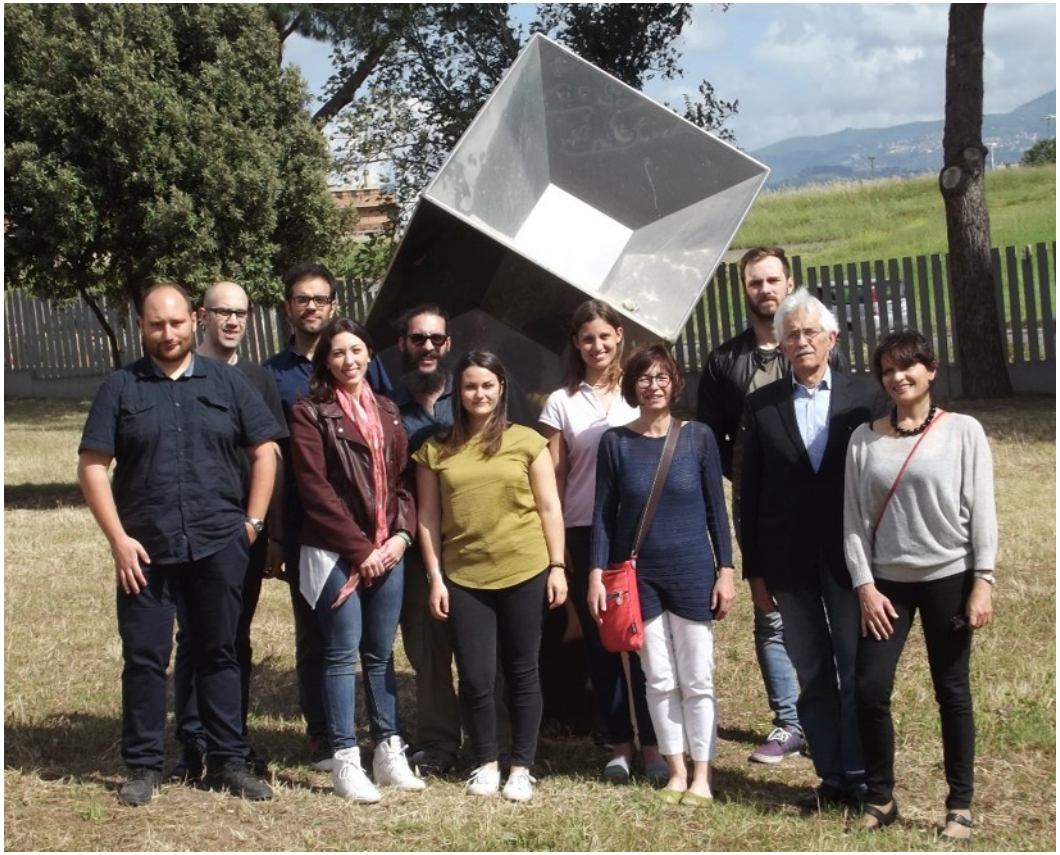
Roma → Biochimica - Sapienza, Fisica - Sapienza, Medicina - Tor Vergata, Fisica - Tor Vergata, Chimica - Tor Vergata

Italia → CNR – Firenze, Fisica – Udine, Fisica – Palermo, Chimica – Cosenza, LNF - Frascati

Resto del mondo → NIC – Zeuthen, ESRF – Grenoble, SLAC – Stanford, Chemistry – Santa Cruz, CFEL – Hamburg, EMBL – Hamburg, Chemistry - Pittsburg

The Biophysics Group in Tor Vergata

http://biophys.roma2.infn.it



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Antonio Vitale (graduate student)
Elisa Lenzi (graduate student)
Riccardo Marrocchio (graduate student)
Giulia Romoli (undergraduate student)

*in the quest for a position

Thank you for your attention!