Colloquio Interdisciplinare sulla Biologia

I metalli e le malattie neurodegenerative
Un rapporto sinergico tra esperimento e simulazioni numeriche

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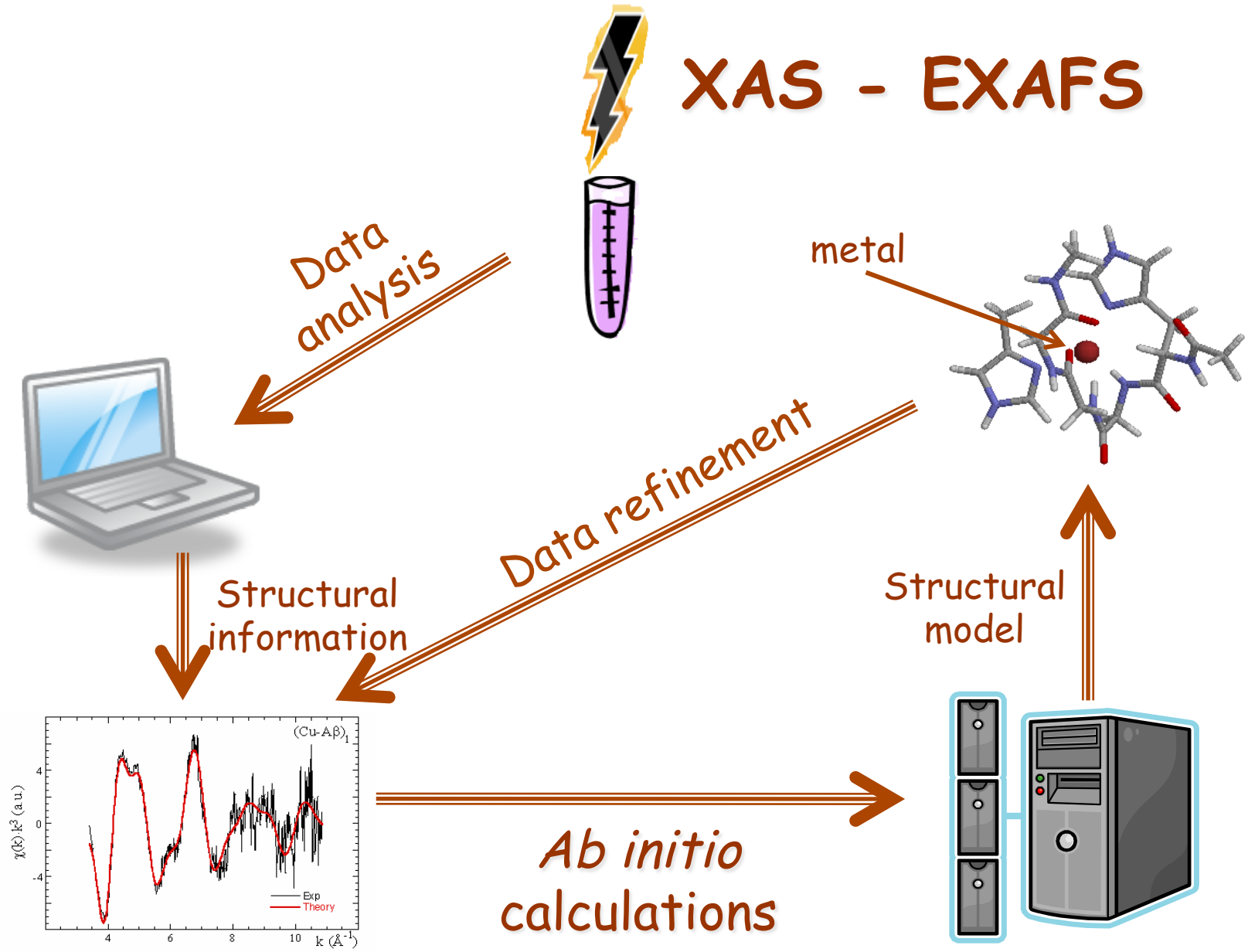
Dipartimento di Fisica

An example of a fruitful synergy



Outline

Biological challenge

understanding the molecular basis of
Protein Conformational Diseases (PCD's)
among which the Alzheimer disease (AD)

- misfolding and aggregation
- the Aβ-peptide
- metal ions and brain

Scientific instruments

focused experiments + numerical approaches

- XAS spectroscopy
- classical and *ab initio* molecular dynamics

Results

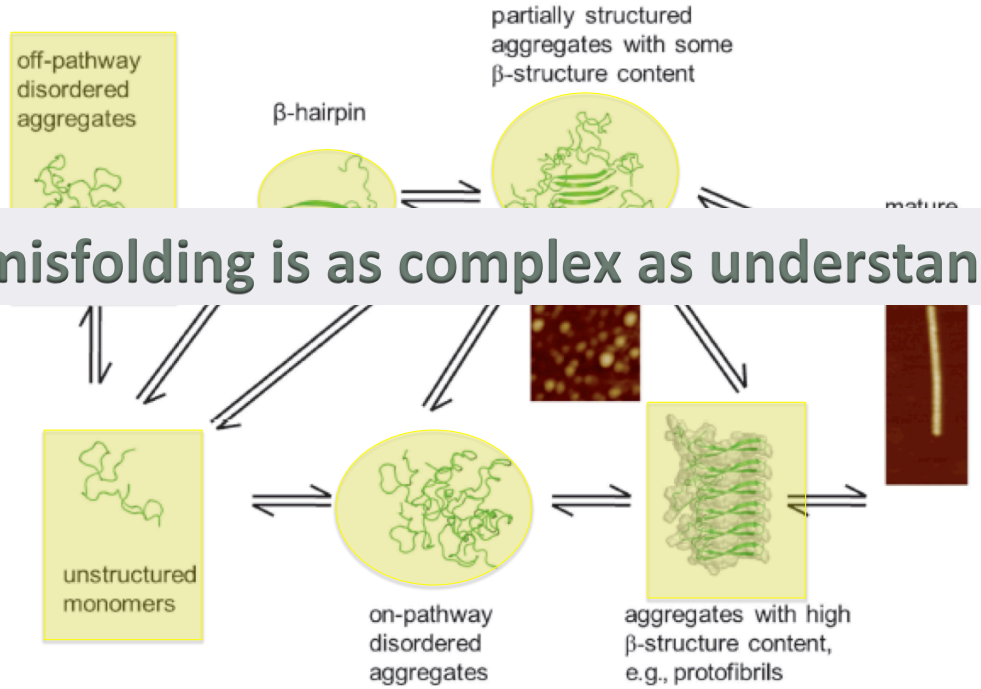
unveiling the (relevant) role of metal ions

Protein Conformational Diseases (PCD's)

Disease	Misfolded Protein
Alzheimer's Disease	Aβ-peptide
Transmissible Spongiform Encephalopathies (TSE)	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	Glutamine
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

Protein misfolding

From a *stable* functional structure
to a non-functional (or even infectious) *stable* structure
Misfolding is not limited to "special" proteins or peptides
Almost all proteins can undergo misfolding and aggregation



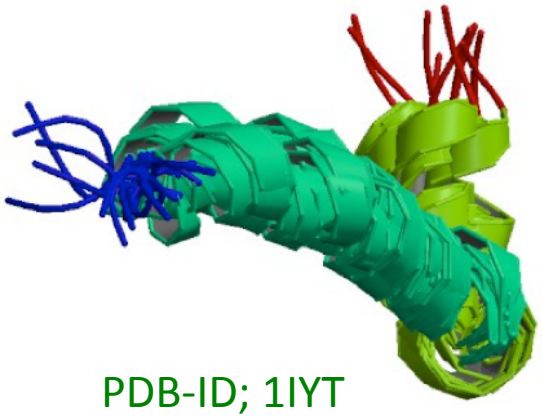
Understanding misfolding is as complex as understanding folding

Wärmländer et al., *ChemBioChem* (2013) 14: 1692 – 1704

Sequences that do not (easily) misfold and aggregate
have been *evolutionally selected to become proteins*

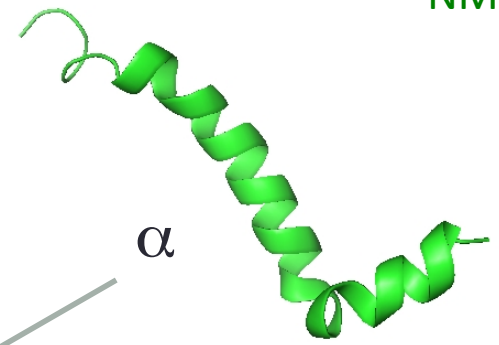
The Aβ-peptide aggregation

Crescenzi et al. (2002) Eur. J. Biochem. 269, 5642–5648



PDB-ID; 1IYT

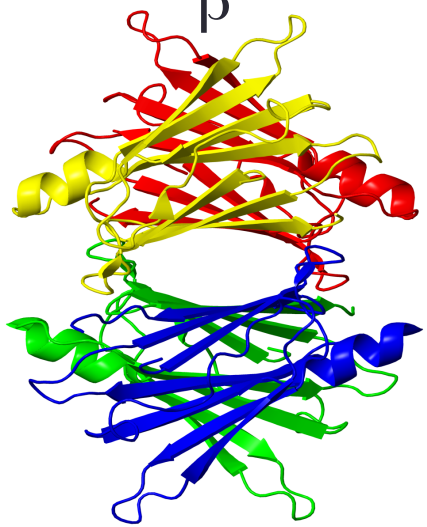
NMR



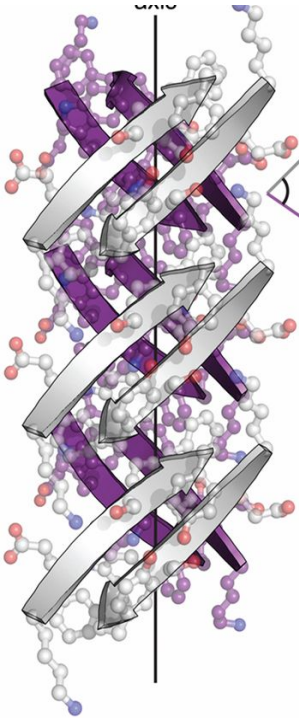
α

β

Oligomer

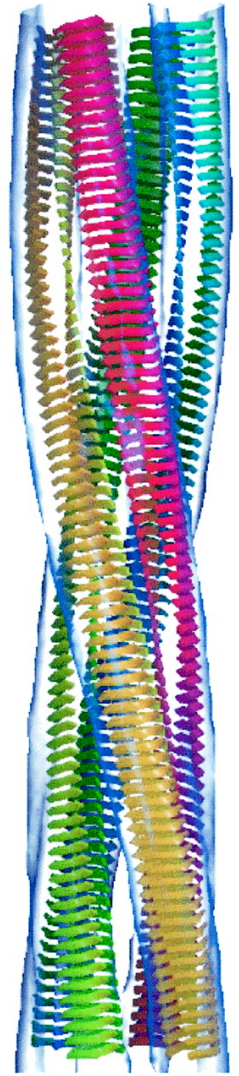


Proto-filament



Cross-beta fibril

⦸70-150 Å



Metal ions are essential cell components

They can easily and quickly form complexes with enzymes and other biological molecules

They are involved in a wide range of biological processes

≈ ¼ ÷ ⅓ of all proteins are estimated to require metals to function

Metalloproteins have many different functions in cells

- storage and transport
- enzymatic activity
- signalling and transduction
- …
- …

BUT METALS HIGH REACTIVITY CAN EASILY MAKE THEM HIGHLY TOXIC FOR THE CELL

Metals should cross the blood-brain barrier

Central Nervous System

- Metals are **essential** for neurochemical activity
- **Fe**, **Cu** and **Zn** are fairly abundant in **grey matter**
- during neuro-transmission processes, high concentrations of Zn (~300 μM) and Cu (~30 μM) are normally released
- **Fe**, **Cu** and **Zn** are continuously **trafficked** whithin the cell and also into and out of it
- Metals **deficiency** may cause **dysfunctionality and** metal **excess may** lead to **toxicity**
- **Failure of metal trafficking (dyshomeostasis)** is known to occur both in AD and in Parkinson diseases

In presence of dyshomeostasis, metal ions may

- interfere with normal nervous cells activities
- disrupt brain electrochemical balance
- •••
- **promote protein misfolding and aggregation (?)**

Protein Conformational Diseases (PCD's)

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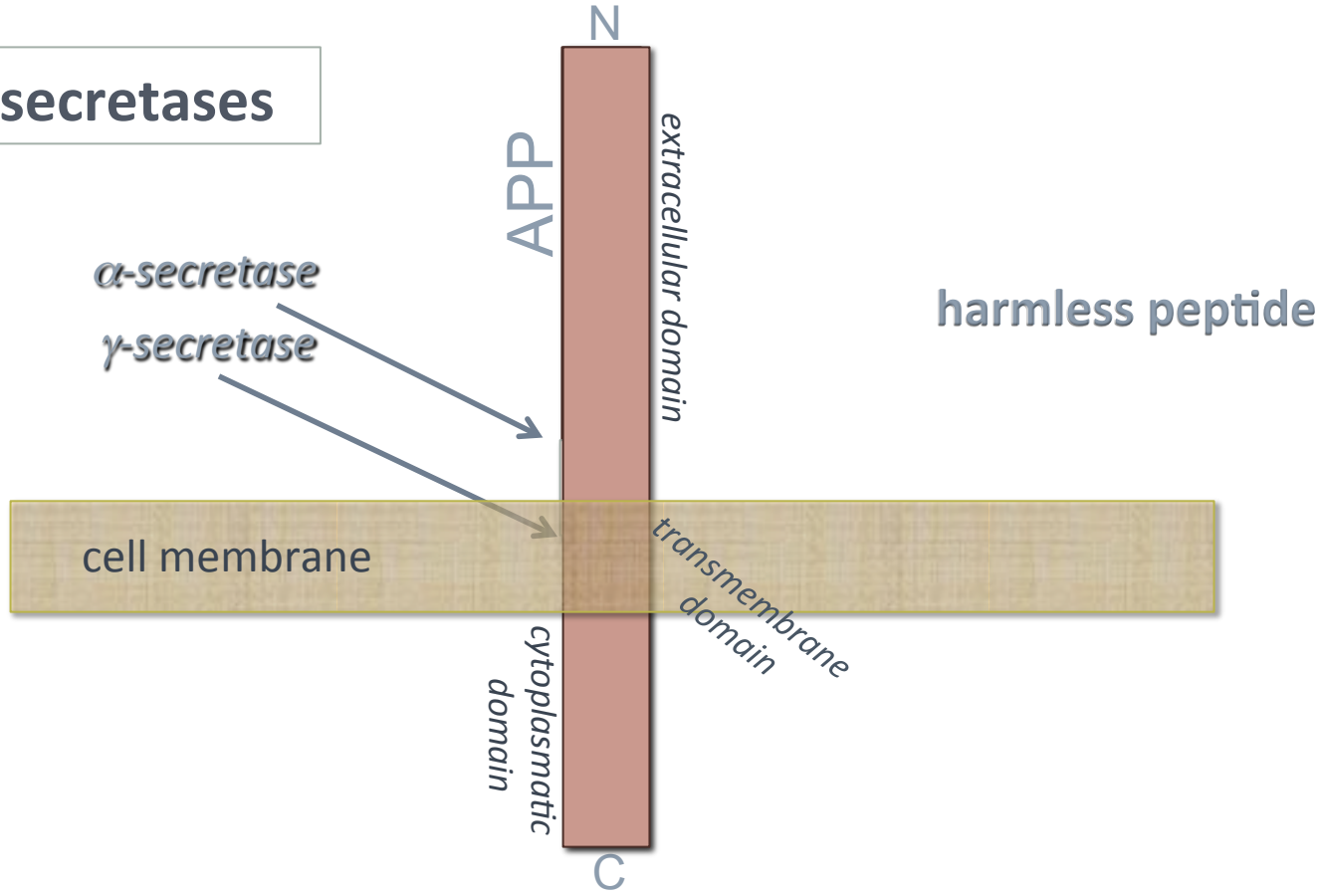
The amyloid β-peptide

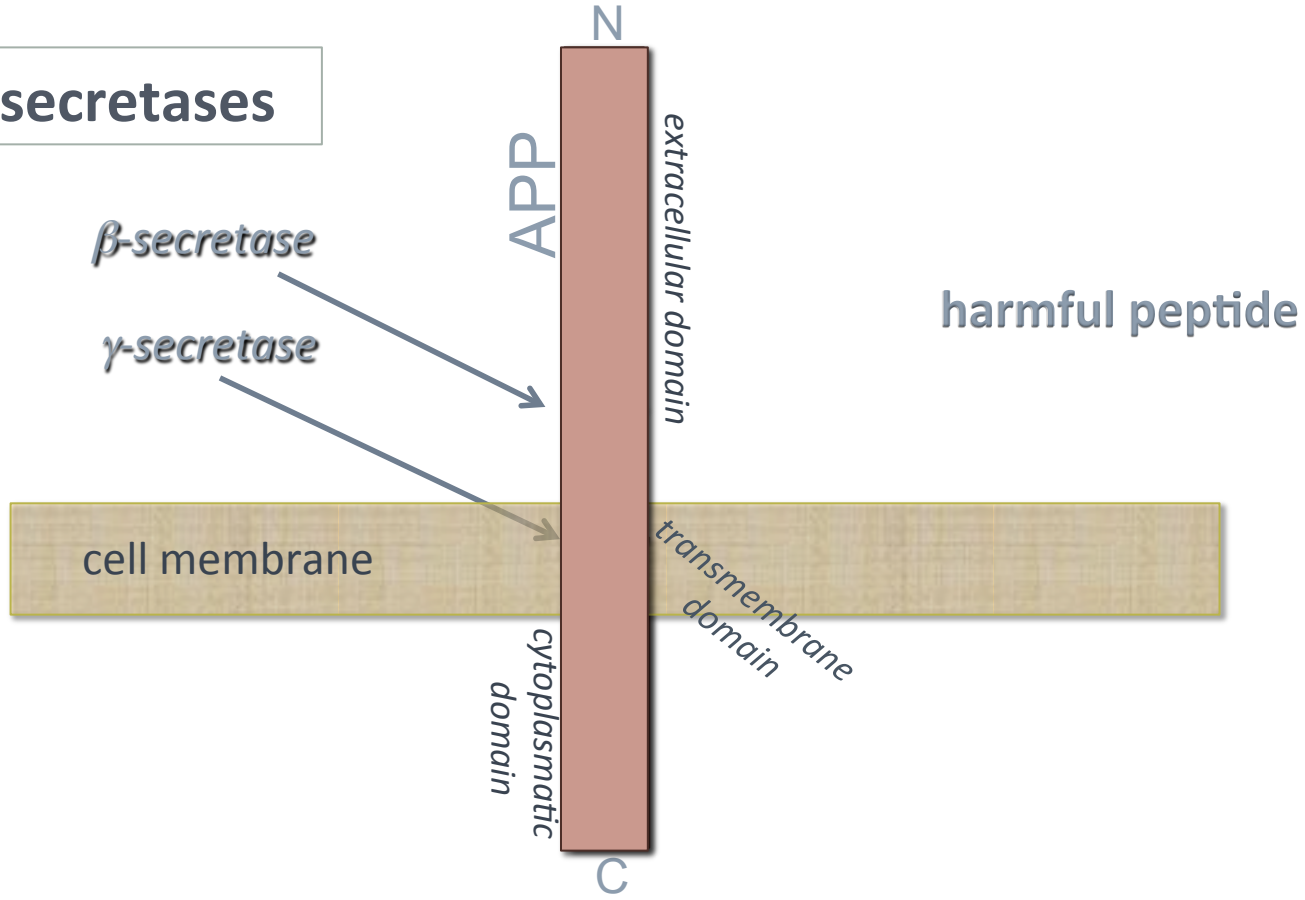
α-, β- and γ-secretases



APP (amyloid precursor protein): transmembrane 770 a.a.'s protein

The amyloid β-peptide

α-, β- and γ-secretases



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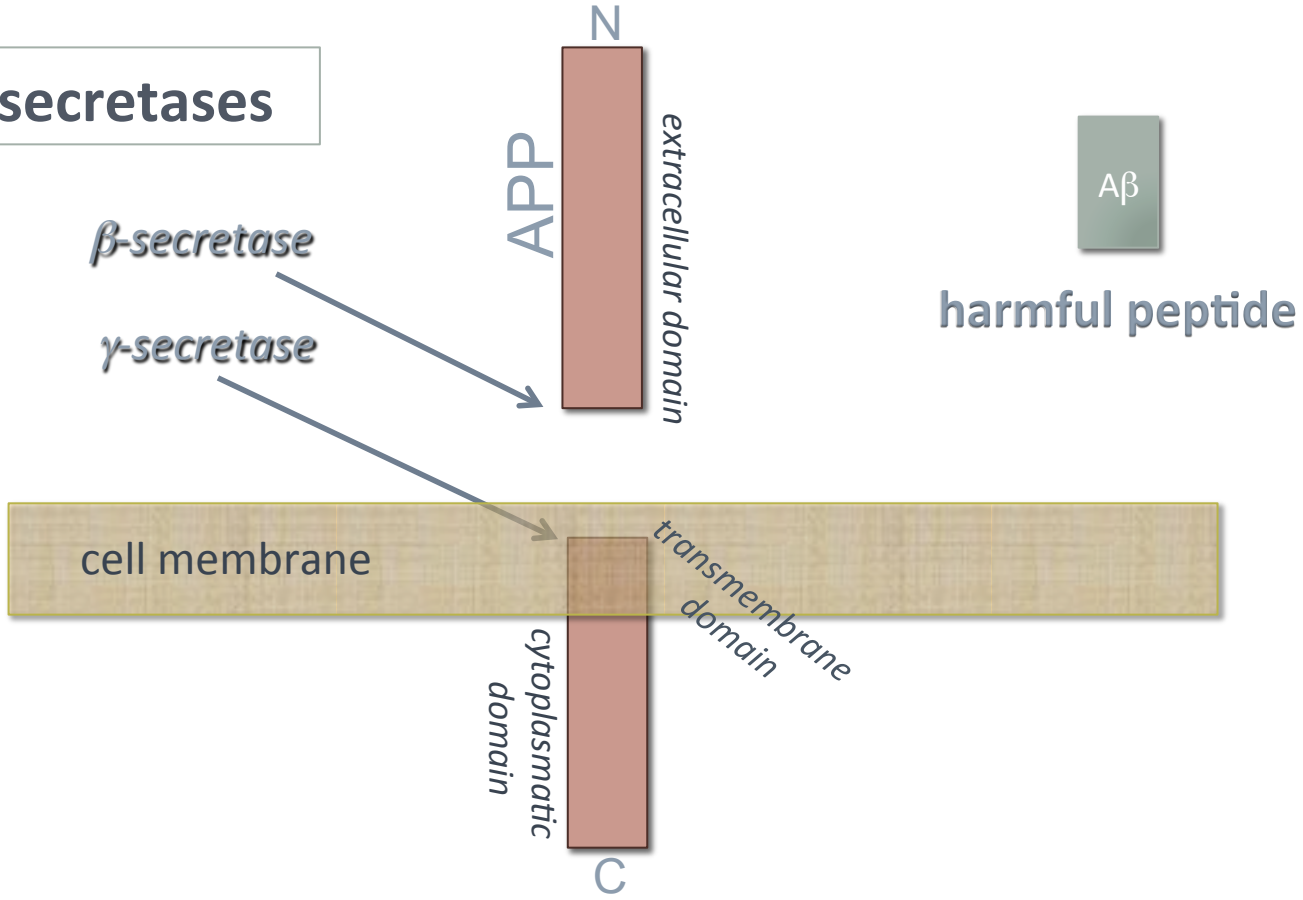
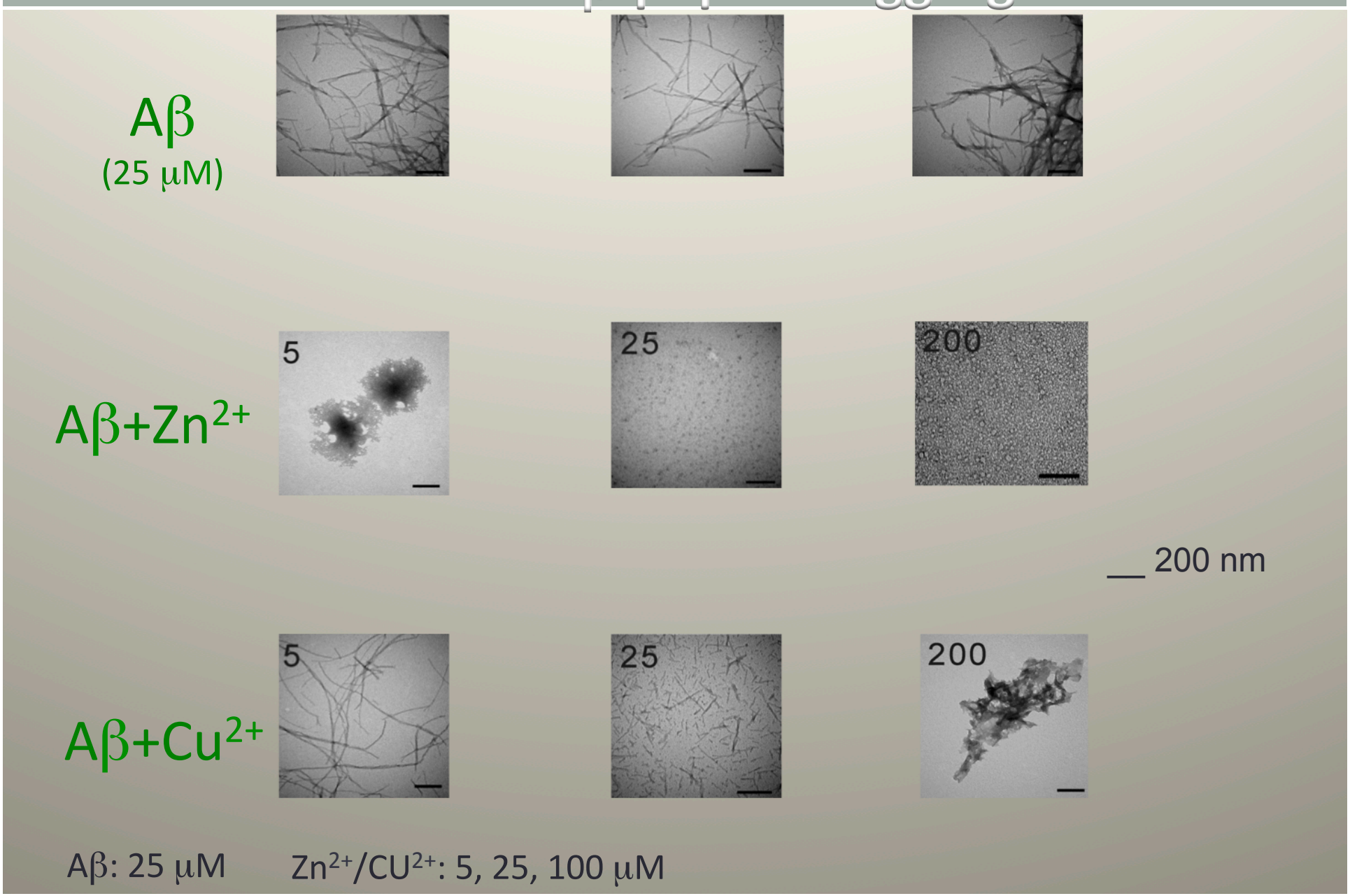
α-, β- and γ-secretases



672 DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV 711

$A\beta_{1\text{-}40}$

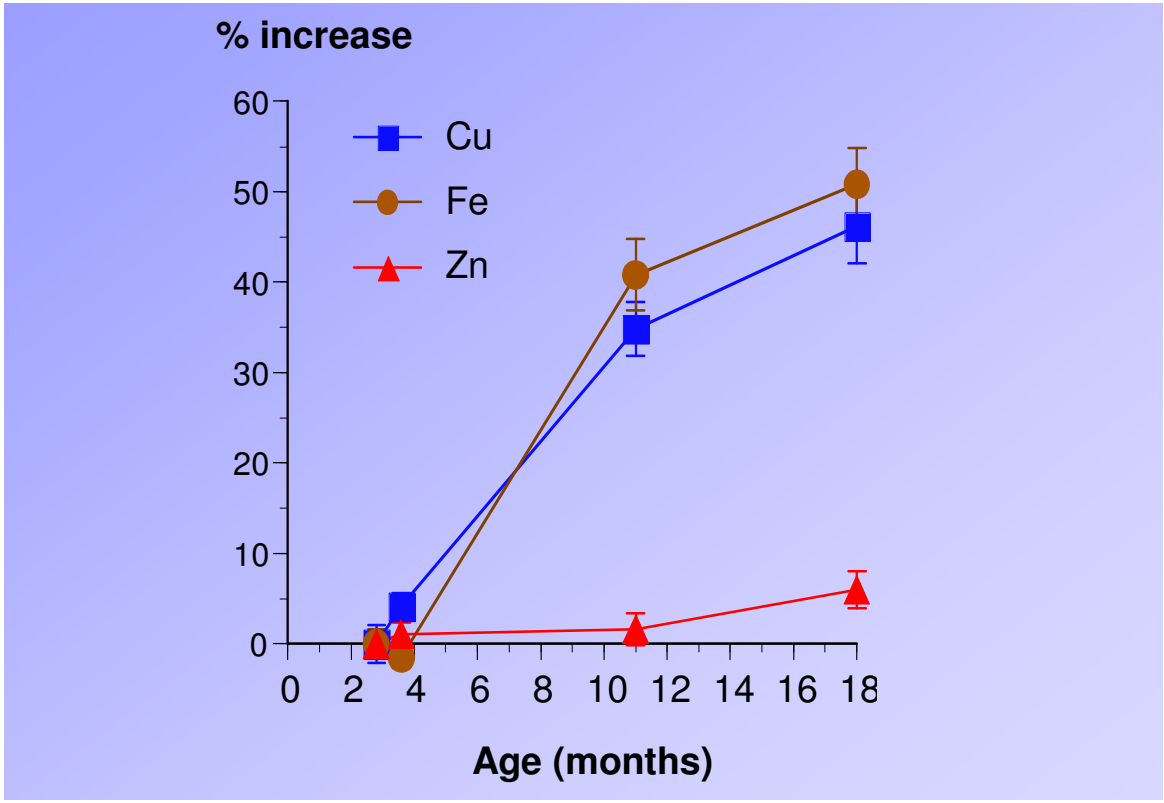
Metals affect Aβ-peptide aggregation



Chen et al. *J. Biol. Chem. 2011, 286:9646-9656.*

Normal aging and increased brain metal level

Maynard et al. (2002) *JBC*, 277: 44670



The triggering event is possibly metal dyshomeostasis

World Health Organization

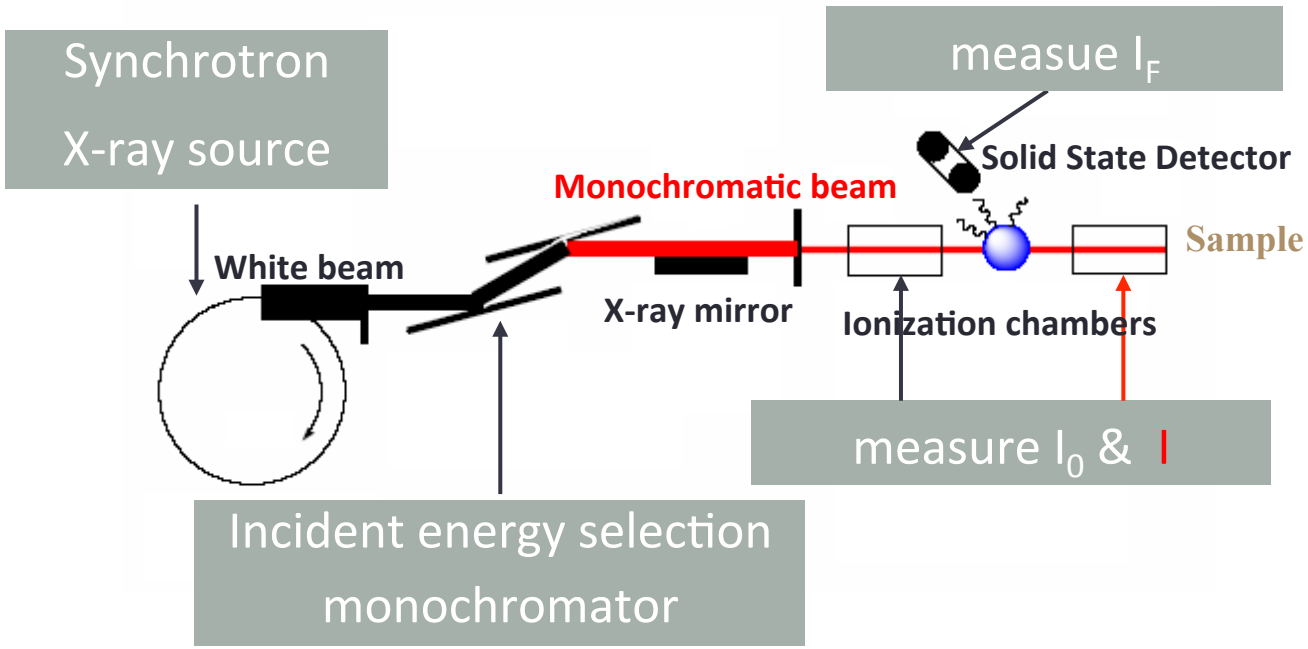
- The number of people aged 60 and over has doubled since 1980
- The number of people aged 80 years or older will be almost quadrupled to about 400 millions between 2000 and 2050
- The risk of dementia (like Alzheimer disease) rises sharply with age with an estimated 25-30% of people aged 85 or older having some degree of cognitive decline.

Horizon 2020: improve healthy ageing

The challenge is:
understand, control and (possibly) stop PCD's evolution

X-ray Absorption Spectroscopy - XAS

Photons are produced by synchrotrons



X-ray Absorption Spectroscopy - XAS

- It can be used to study samples in any aggregation state (not necessarily crystallized, frozen or transparent)

  proteins can be studied in physiological or controlled solution condition

- There are no selection rules for absorption

  a XAS spectrum can always be obtained

- Spectra registered in a relatively short time (few min's to few msec's)

  limited radiation damage

- High chemical selectivity -

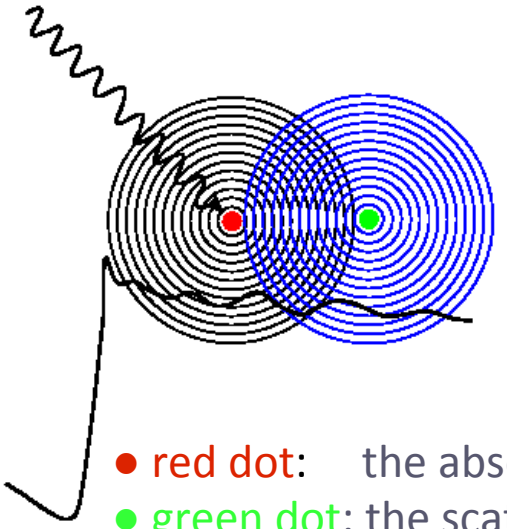
  local atomic environment around a selected absorber

  1s ionization energy (K-edge)

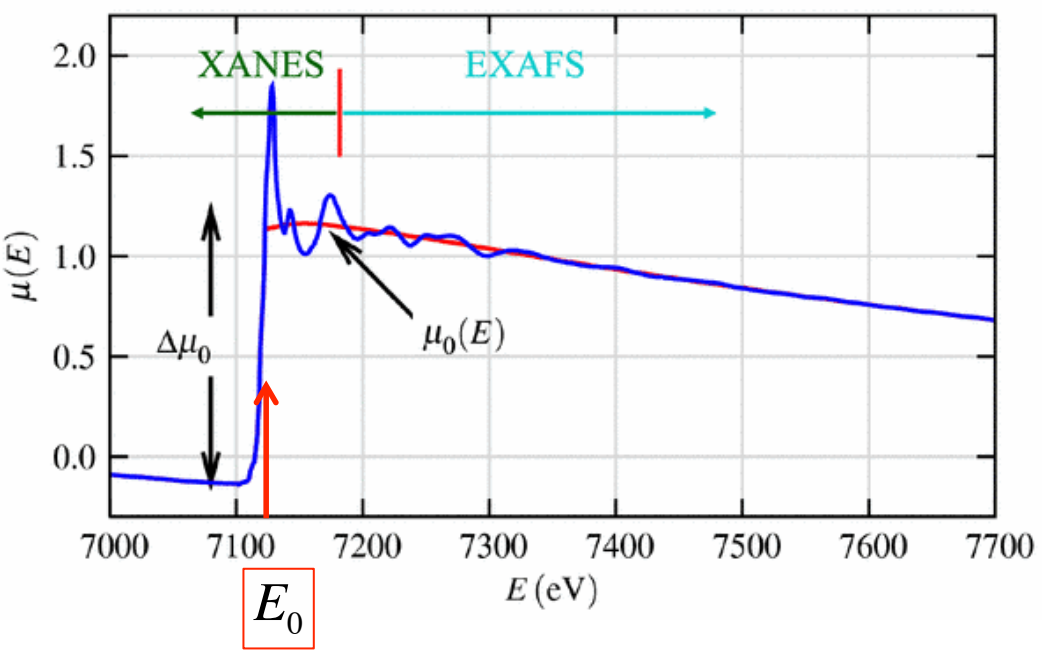
  $_{26}$Fe ≈ 7112 eV ; $_{29}$Cu ≈ 8993 eV; $_{30}$Zn ≈ 9673 eV

XAS of a multi atomic system

Absorption coefficient, μ, behaviour as a function of emitted photon energy



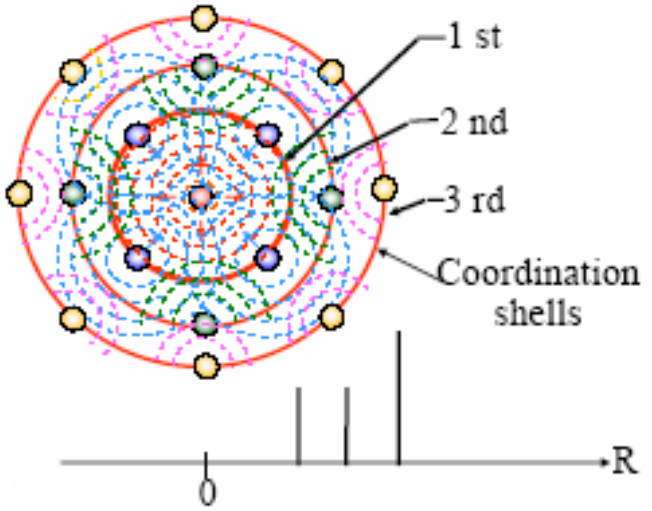
- red dot: the absorber
- green dot: the scatterer



$\hbar\omega >> E_0$ Single Scattering approximation

$$\chi(k) = \frac{\sigma_a - \sigma_0}{\sigma_0}$$

$$\chi(k) = S_0^2 \sum_i \frac{N_i}{kR_i^2} |f_i(k,\pi)| e^{-2k^2\sigma_i^2} e^{-2R_i/\lambda(k)} \sin\left[2kR_i + \Phi_i(k)\right]$$



Numerical simulations

CPMD (***Car Parrinello Molecular Dynamics***) a (smart) parallelized implementation of DFT (Density Functional Theory) that uses the Kohn and Sham reformulation of the Schroedinger equation for the calculation of the electron ground-state energy

Density Functional Theory -> the electronic structure (in the ground state)

The electronic energy is a functional of the electronic density, $n(\vec{r})$

$$E_{DFT} = \Gamma_{HK}\left[n(\vec{r})\right] + \int n(\vec{r}) V(\vec{r}) d\vec{r}$$

the potential seen by the atoms can be determined by E[n]
The value of *n(r)* at which E[n] is minimum is the ground-state electronic density.

The mimimum can be found by solving the Kohn-Sham equations

$$\left[-\frac{\hbar^2}{2m}\nabla^2 + V(\vec{r}) + V_H(\vec{r}) + V_{xc}(\vec{r})\right]\psi_i(\vec{r}) = \varepsilon_i \psi_i(\vec{r})$$

$$V_H(\vec{r}) = e^2 \int \frac{n(\vec{r}')}{|\vec{r} - \vec{r}'|} d\vec{r}' \quad \text{Hartree potential}$$

$$V_{xc}(\vec{r}) \quad \text{Exchange-correlation potential}$$

$$n(\vec{r}) = (2)\sum_i |\psi_i(\vec{r})|^2 \qquad \langle \psi_i | \psi_j \rangle = \delta_{ij}$$

The simulation strategy

Quantum-ESPRESSO package
www.quantum-espresso.org

1 "prepare" the initial state of the system (classical MD)
   type of atoms: number of electrons and bands → pseudopotentials
   atomic coordinates
   dimension of system cell
   cut-offs → plane wave expansion
   FFT grid spacing
2 electronic minimization (Steepest Descent, SD) with fixed ions → ground state
3 ionic positions minimization (SD or damped dynamics) → to relax the system
4 start the Car-Parrinello dynamics

NOTE: in order to reach the desired temperature
ions are coupled to Nosè-Hoover thermostat

QM key feature: bonds not imposed (like in MD)
but derived by solving the Schroedinger equation

The simulation strategy

GROMACS FAST. FLEXIBLE. FREE.

1 "prepare" the initial state of the system
   - atomic coordinates
   - atomic velocities
   - force field choice (effective potential)
   - dimension of system box
   - water
   - …
2 system equilibration: potential energy minimization (T=0 K)
3 system heating: rise temperature to the desired value
4 start MD: solve the Newton's equation of motion for the atoms

1. Zn-ion-induced aggregation pattern

XAS has allowed to identify the Zn^{2+}-binding site sequence in $A\beta_{1-40}$

^{1}DAEFR^{6}HDSGYEV^{13}H^{14}HQKLVFFAEDVGSNKGAIIGLMVGGVV^{40}

Besides the whole peptide, we measured the XAS spectra of the following samples

Zn^{2+}- $A\beta_{1-16}$ → minimal fragment containing His_6, His_{13}, and His_{14}, suggested to be involved in metal binding

Zn^{2+}- $A\beta_{17-40}$ → complementary to the previous sequence: no His is present

Zn^{2+}- $A\beta_{1-28}$ → besides the three His's, it contains a long hydrophobic region believed to be relevant in the aggregation process

V. Minicozzi … S.M. et al., *J Biol Chem* (2008) **283**: 10784
F. Stellato … S.M. et al., *Eur Biophys J* (2006) **35**: 340

1° Zn-ion-induced aggregation pattern

Synopsis of XAS results: comparing spectra

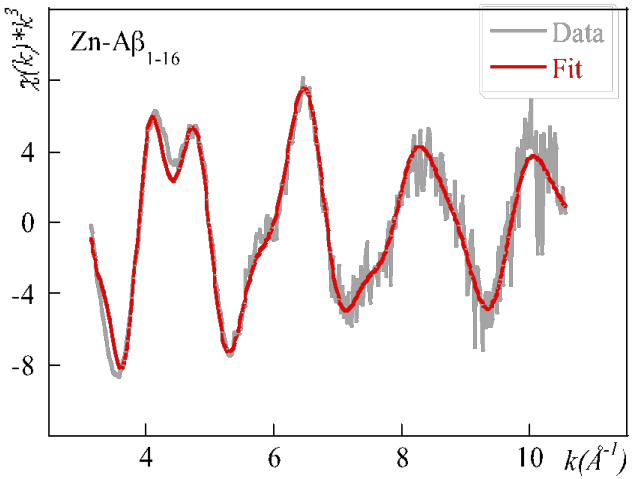
Zn^{2+}- $A\beta_{17-40}$ = Zn^{2+}- buffer → Zn^{2+} is unable to bind $A\beta_{17-40}$

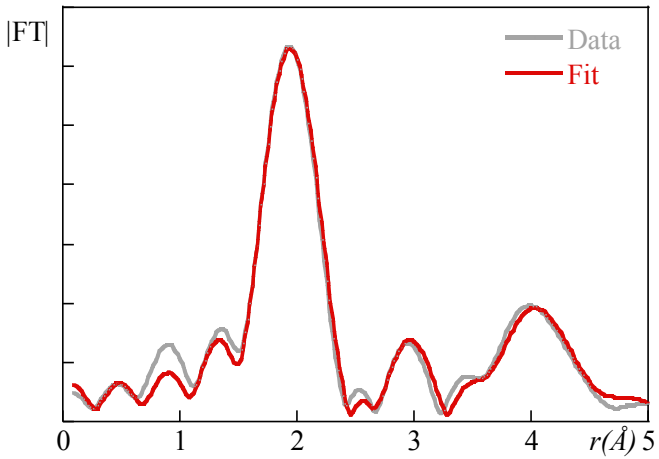
Zn^{2+}- $A\beta_{1-16}$ = Zn^{2+}- $A\beta_{1-28}$ = Zn^{2+}- $A\beta_{1-40}$ → Zn^{2+} always bind to the first 16 a.a. fragment

^{1}DAEFR^{6}HDSGYEV^{13}H^{14}HQKLVFFAEDVGSNKGAIIGLMVGGVV^{40}

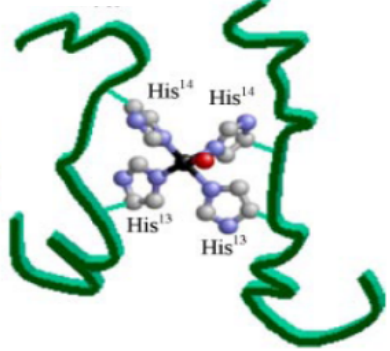
Zn^{2+} always binds with the same (*very crowded*) binding structure to the first 16 a.a.'s

Zn^{2+}-$A\beta_{1-16}$





Zn^{2+}-$A\beta_{1-16}$



- 4 histidines
- 1 water

1° Zn-ion-induced aggregation pattern

Many cases where Cu (not Zn) is coordinated to four His's side-chains belonging to the same monomer…

…but ALL of them concern proteins belonging to the wide class of Cu,Zn-SOD proteins.

(Actually a fully Zn-substituted SOD enzyme has been shown to be at least as stable as the wild-type heterodinuclear protein)

5854 PDB entries containing at least one Zn

in only one case Zn is coordinated to four His side-chains belonging to the same protein monomer

- PDB code 1PB0: Organism: E-Coli; Function: unknown

in two more instances the four His side-chains coordinated to Zn belong to different monomers:

- PDB code 1HWT: Organism: Saccharomyces Cerevisiae; Function: gene regulation/DNA and
- PDB code 1QP9: Organism: Saccharomyces Cerevisiae; Function: transcription/DNA

1° Zn-ions-induced aggregation pattern

Models and *ab-initio* calculations

Question:

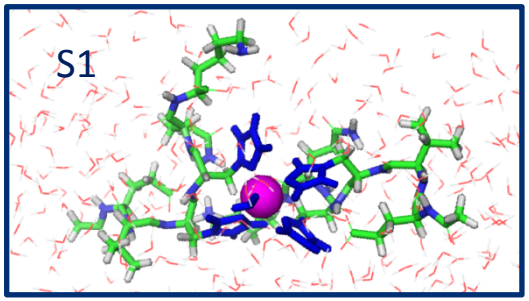
which structure stabilizes this (very crowded), 4 His's - Zn^{2+} coordination?

- XAS: structure information only within 5-6 Å from the absorber
- *ab-initio* simulations: structural information up to a longer distance without enforcing Zn coordination mode

Model building

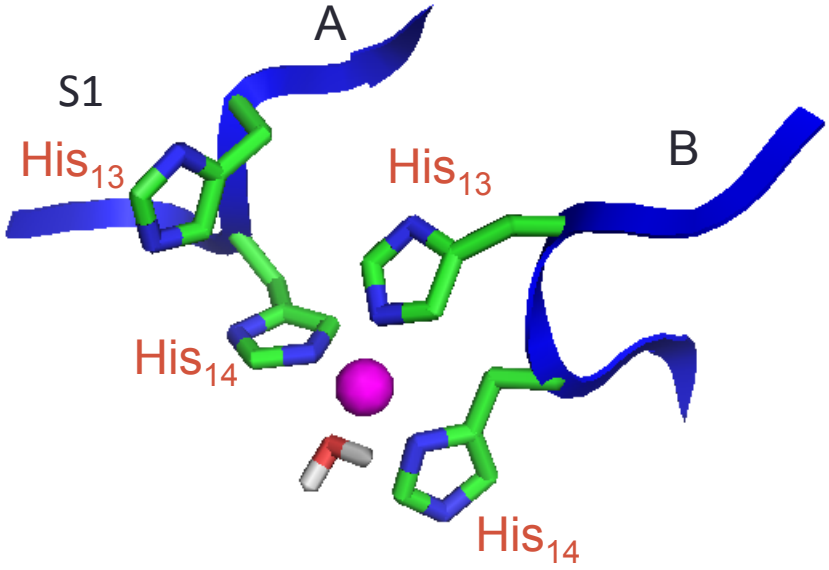
1. XAS → 4 His's coordination → two $A\beta_{1-16}$ – peptides (denoted A and B) with a single Zn atom bridging $N_\delta(His_{14}(A))$ and $N_\delta(His_{14}(B))$
2. minim + Monte Carlo Random Walk (MCRW) ("bad" contact discarded) → only configurations that fulfill structural constraints of point 1. are retained
3. $A\beta_{1-16}$ truncated to $A\beta_{11-16}$ and put in a cell with periodic conditions filled with TIP3P water molecules
4. Four trial binding geometries are chosen for *ab initio* computations

1° Zn-ion-induced aggregation pattern



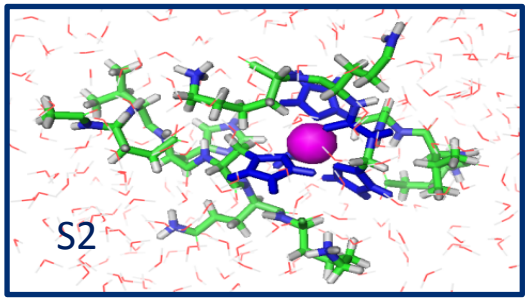
Besides the two His_{14}, also the two His_{13} happen to be within 3 Å from Zn

Name	Initial configuration
S1	Zn: $4N_{His} + 1O_W$



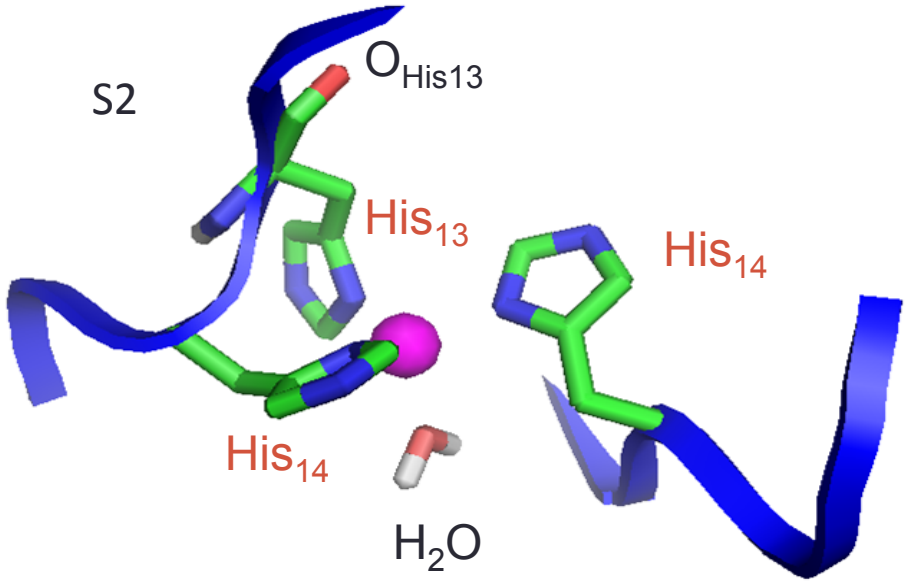
Name	Final configuration
S1	Zn: $3N_{His} + 1O_W$

1° Zn-ion-induced aggregation pattern



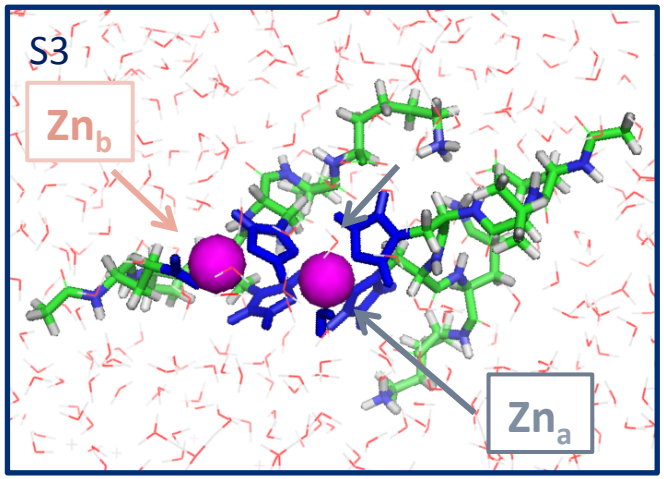
Only three His's. One of the His's is also bound by the main chain oxygen

Name	Initial configuration
S2	Zn: $3N_{His}$ +$1O_{His}$ + $1O_W$



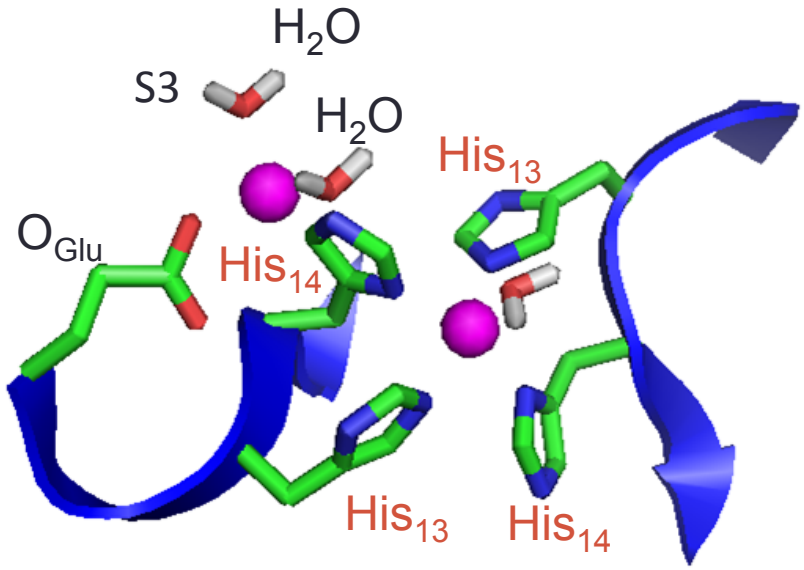
Name	Final configuration
S2	Zn: $3N_{His}$ + $1O_W$

1° Zn-ion-induced aggregation pattern



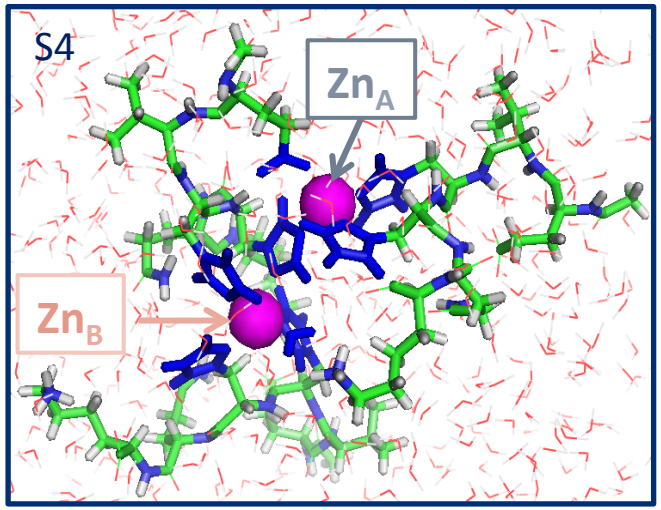
modified S1: the Hε of a His_{14} is substituted by a second Zn

Name	Initial configuration
S3	Zn_a: $4N_{His} + 1O_W$ Zn_b: $1N_{His} + 2O_{Glu}$



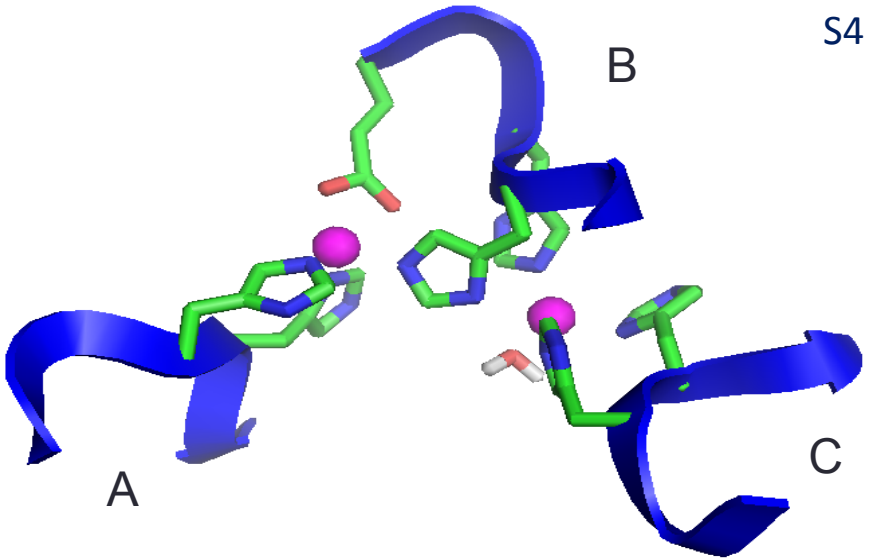
Name	Final configuration
S3	Zn_a: $4N_{His} + 1O_W$ Zn_b: $1N_{His} + 1O_{Glu} + 2O_W$

1° Zn-ion-induced aggregation pattern



modified S3: a third peptide is bound to the second Zn

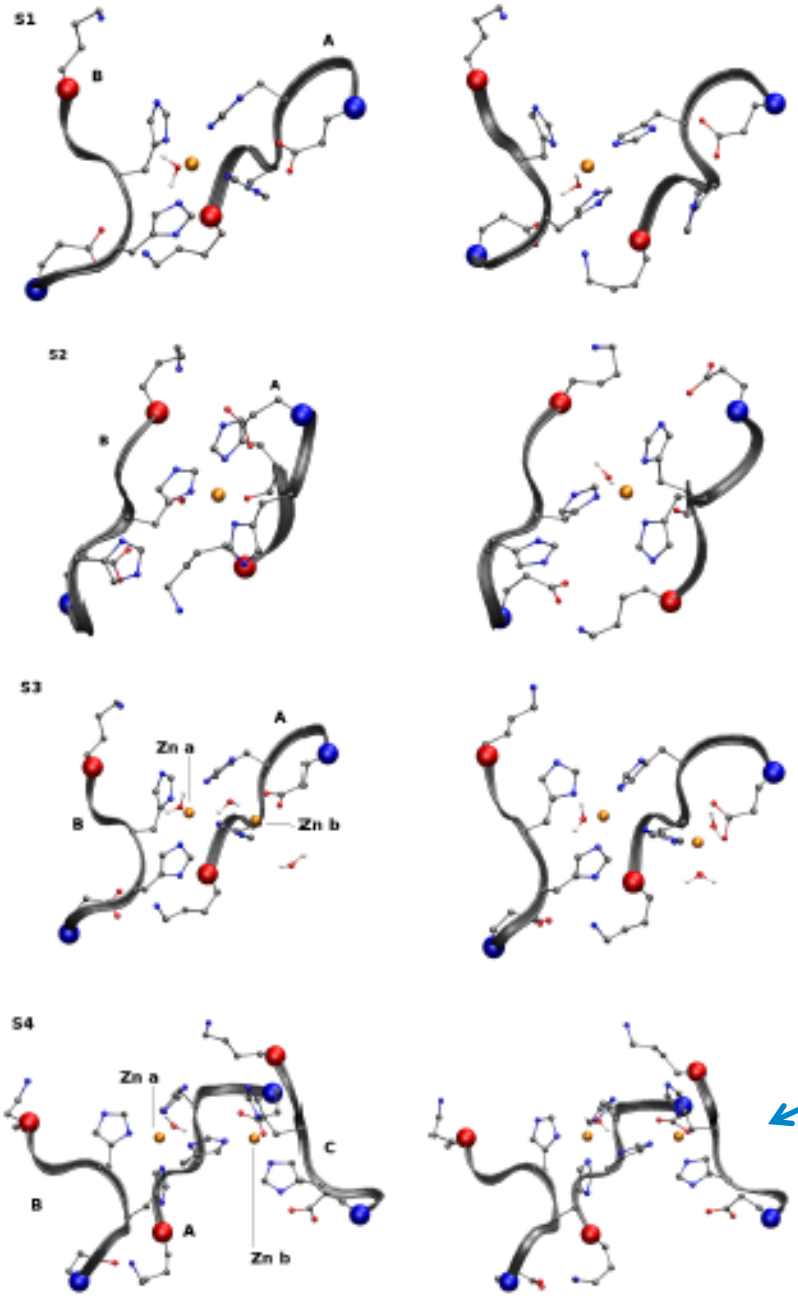
Name	Initial configuration
S4	Zn_a: $4N_{His} + 1O_W$ Zn_b: $3N_{His} + 1O_{Glu}$



Name	Final configuration
S4	Zn_a: $4N_{His} + 1O_W$ Zn_b: $3N_{His} + 1O_{Glu}$

1° Zn-ion-induced aggregation pattern

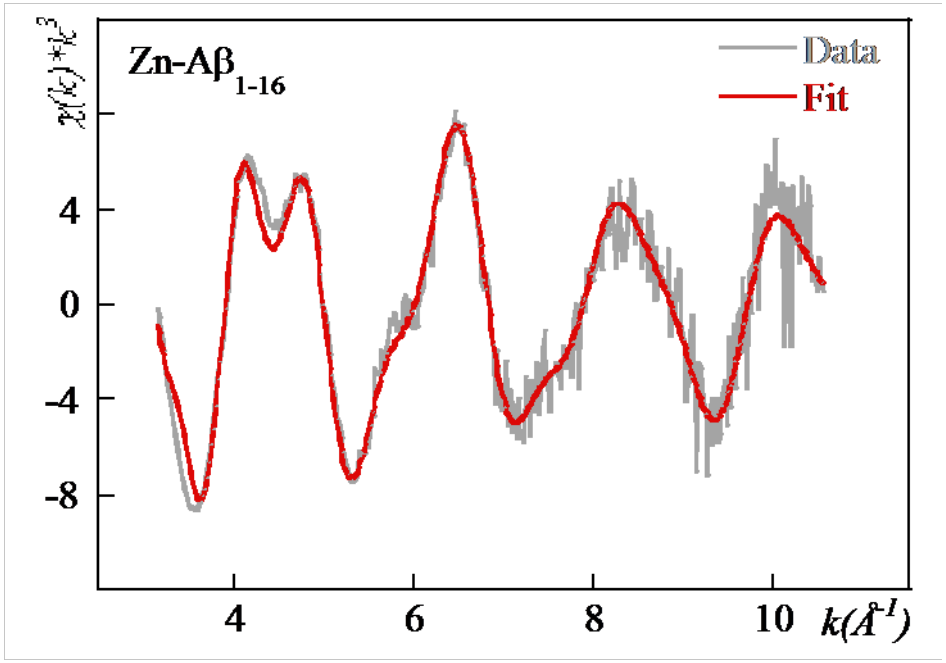
P. Giannozzi, K. Jansen, G. La Penna, V.Minicazzi, S. M., G.C. Rossi, F. Stellato. *Metallomics* (2012)



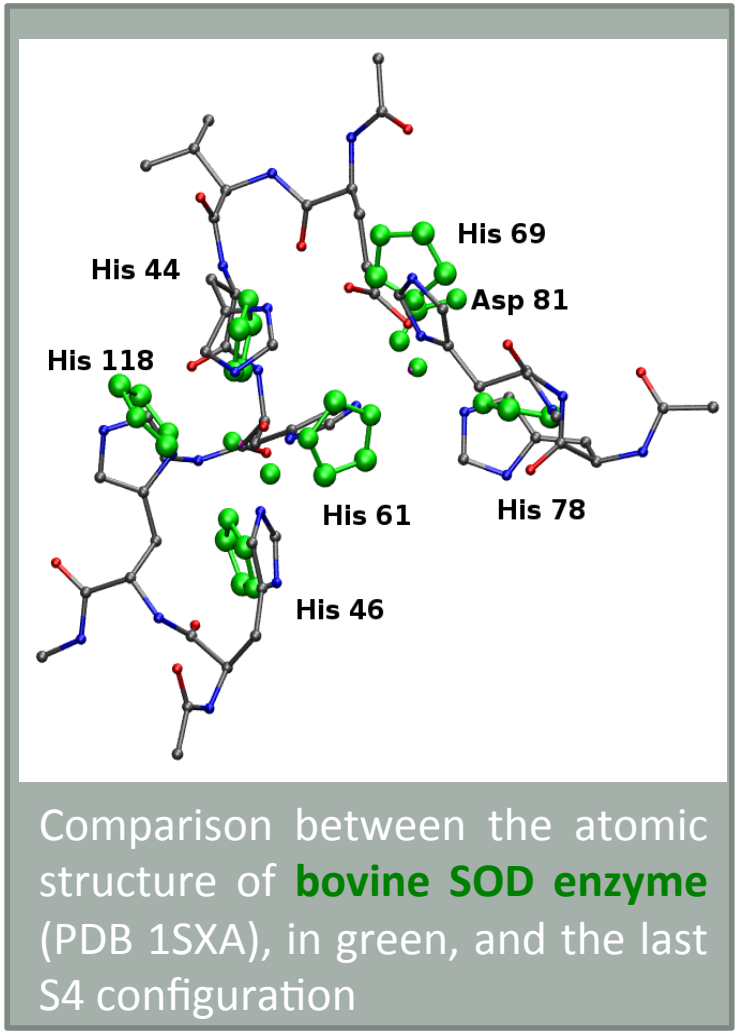
Zn site structures in the four models

Our favorite model

1° Zn-ion-induced aggregation pattern



Comparing the experimental XAS spectrum with the theoretical one of the S4 model



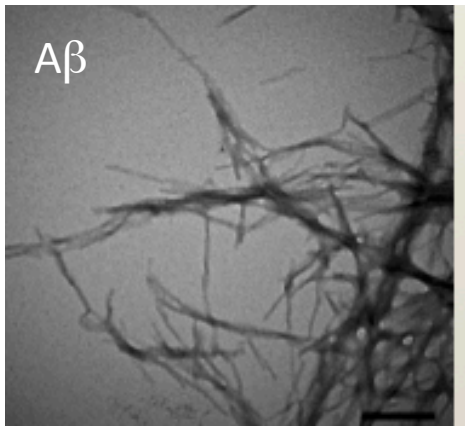
Comparison between the atomic structure of **bovine SOD enzyme** (PDB 1SXA), in green, and the last S4 configuration

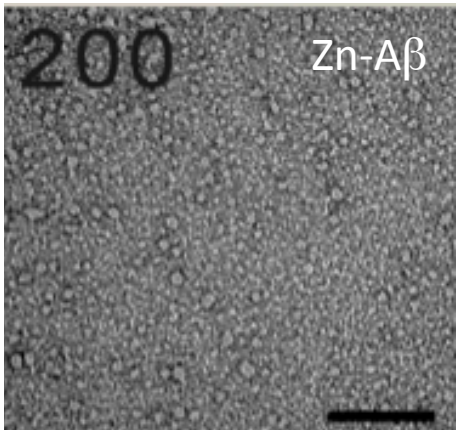
His's "crowding" in SOD enzymes is helped by the presence of two metal ions bridged by the His imidazole (in the unusual form of an imidazolate anion)

1° Zn-ion-induced aggregation pattern

Conclusions

- Zn promotes oligopeptides formation





- XAS cannot discriminate among different structural models, but
- empirical + first-principle simulations → stable Zn-His-Zn structure (≈ SOD)
- Zn binding stability largely depends on the formation of a Zn–His–Zn bridge assisted by an unusual deprotonated imidazole ring (imidazolate) (≈ SOD)

2° Cu^{2+}-Zn^{2+} cross modulation in Aβ

XAS measurements: Cu and Zn K-edge

Sample name	$A\beta_{1-16}$:Cu:Zn	K-edge energy		Metal ions addition order
		Cu	Zn	
S1	1:1:1	✓	✓	Simultaneously
S2	1:1:4	✓	✓	Simultaneously
S3	1:1:1	✓	✓	Cu first
S4	1:1:1	✓	✓	Zn first
S5	1:1:4	✓	✓	Cu first
S6	1:1:4	✓	✓	Zn first
bCu	Cu in buffer	✓		Only Cu
bZn	Zn in buffer		✓	Only Zn

the second metal is added after 15 hours of incubation of the first one

2° Cu^{2+}-Zn^{2+} cross modulation in Aβ

Conclusions …

The order in which metals are added matters

- When Zn^{2+} is added first, it prevents Cu^{2+}-Aβ complex formation
- When Cu^{2+} is added first, it does not completely prevent Zn^{2+}-Aβ complex formation
- Cu^{2+} and Zn^{2+} bind to the peptide through different numbers of histidines (2/1 and 3)

… but

it would be of the utmost importance to know what happens,
to the whole peptide structure as a result of the metal binding competition, …

… we are going toinvestigate this problem via MD and CPMD simulations

E. De Santis, V. Minicozzi, , S. Morante, O. Proux, G. C. Rossi, S. Saxena, K. I. Silva, F. Stellato
"Cu(II)-Zn(II) cross-modulation in Ab peptide binding: an X-ray Absorption Spectroscopy study"
(2015) *J. Phys. Chem. B.* **119(52)**: 15813-20

3° Beta sheet breakers: targeting Aβ fibrils

MD simulations

- $A\beta_{1-40}$
- Soto breaker = Ac-LPFFD-NH_2
- Soto breaker + taurine = Tau-LPFFD-NH_2

} Already studied in the literature

- Soto breaker D → N = Ac-LPFFN-NH_2 ← Newly tested

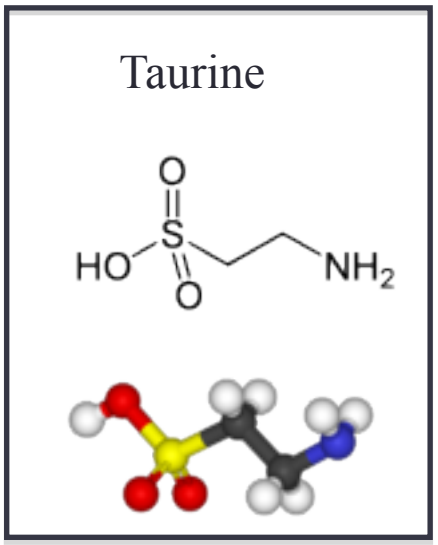
Simulated systems

abeta = $A\beta_{1-40}$ + 3 Na^+ + water

lpffd = $A\beta_{1-40}$ + 10 LPFFD + 13 Na^+ + water

taulpffd = $A\beta_{1-40}$ + 10 Tau-LPFFD + 13 Na^+ + water

lpffn = $A\beta_{1-40}$ + 10 LPFFN + 3 Na^+ + water



immersed in ~ 122,300 H_2O

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

[$A\beta_{1-40}$] ~ 400mM

Na^+ added to neutralize the systems

Note: D: Aspartic acid (hydrophilic); N: Asparagin (hydrophobic)

3° Beta sheet breakers: targeting Aβ fibrils

MD Simulations

The simulation strategy

GROMACS FAST. FLEXIBLE. FREE.

1 "prepare" the initial state of the system
   - atomic coordinates
   - atomic velocities
   - force field choice
   - dimension of system box
   - water
   - …

2 system equilibration: potential energy minimization (T=0 K)

3 system heating: rise temperature to the desired value

4 start MD: solve the Newton's equation of motion for the atoms

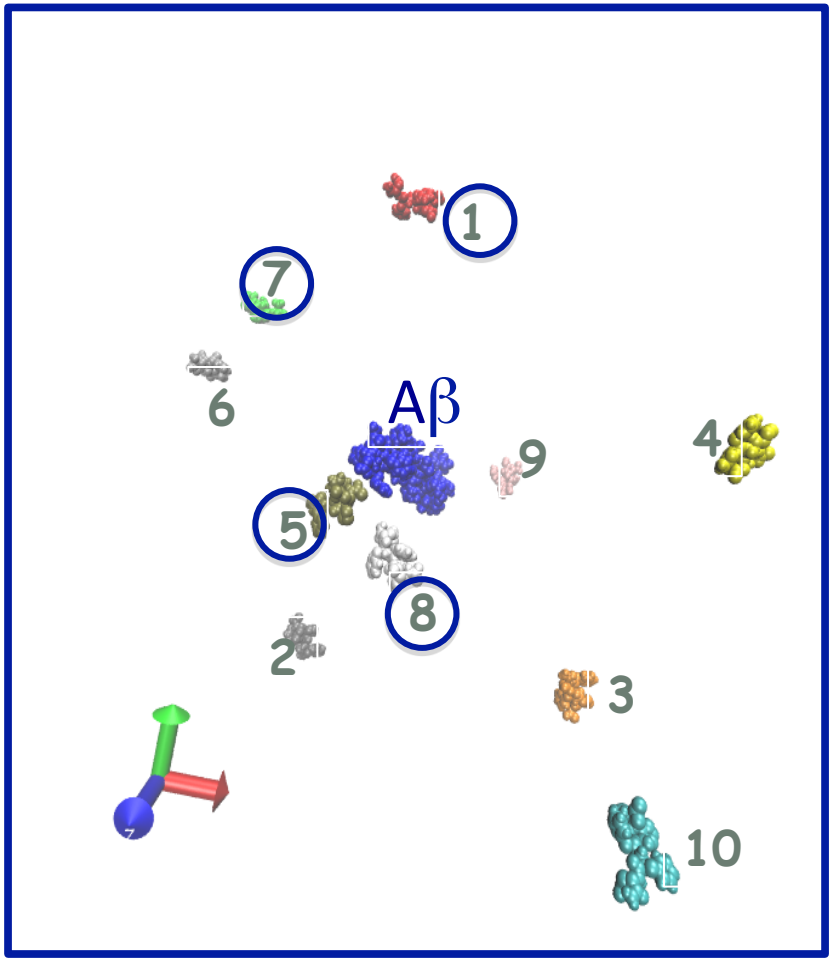
3° Beta sheet breakers: targeting Aβ fibrils

Step 1: prepare the initial state

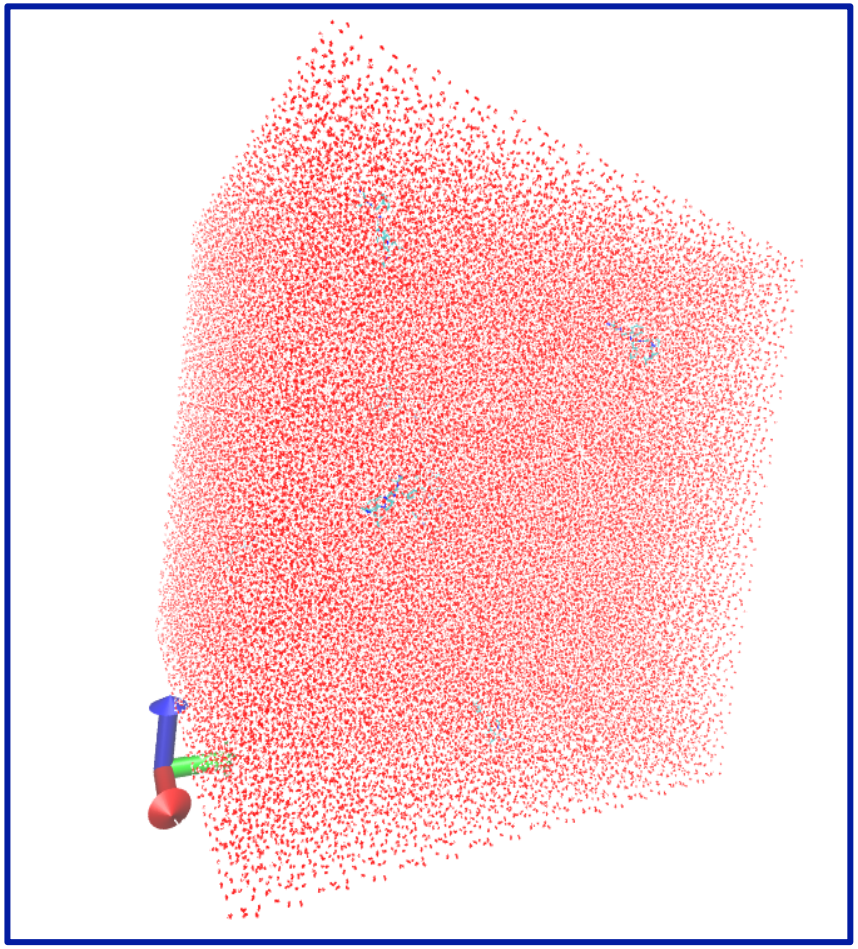
BSB's are located at the same (randomly chosen) starting positions

inside the simulation box

BSB's initial location



Solvated system

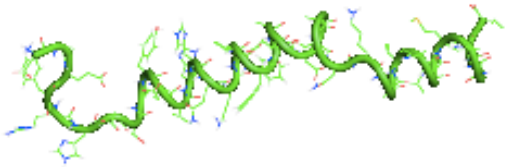


3° Beta sheet breakers: targeting Aβ fibrils

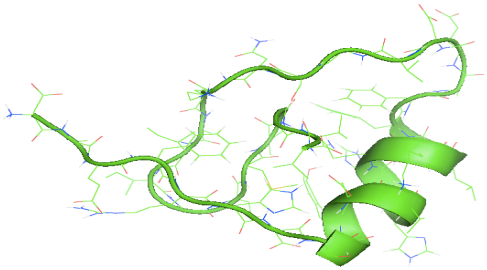
Time evolution of the $A\beta_{1-40}$ structure

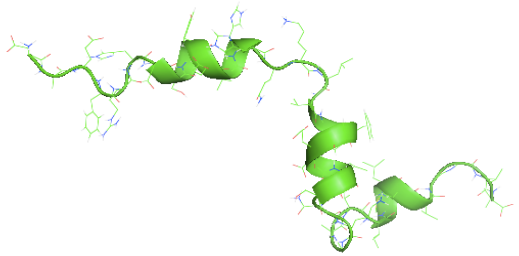
After 2-3 equilibration steps

After step 4: 80 ns NpT MD @300 K

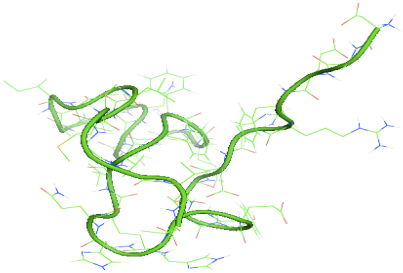


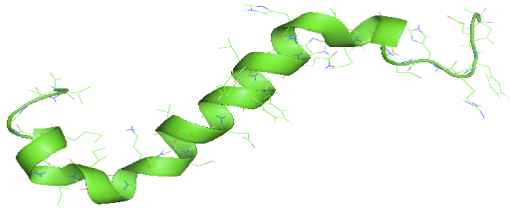
abeta



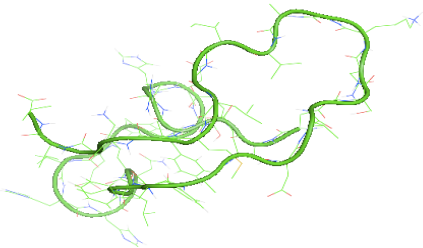


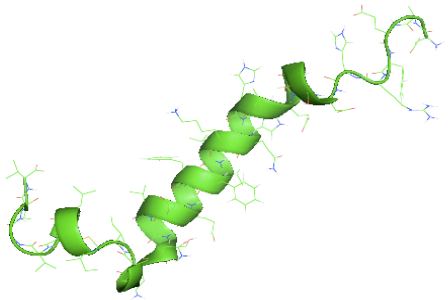
lpffd



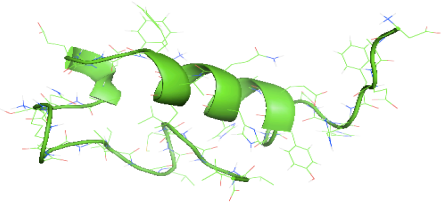


tau-lpffd



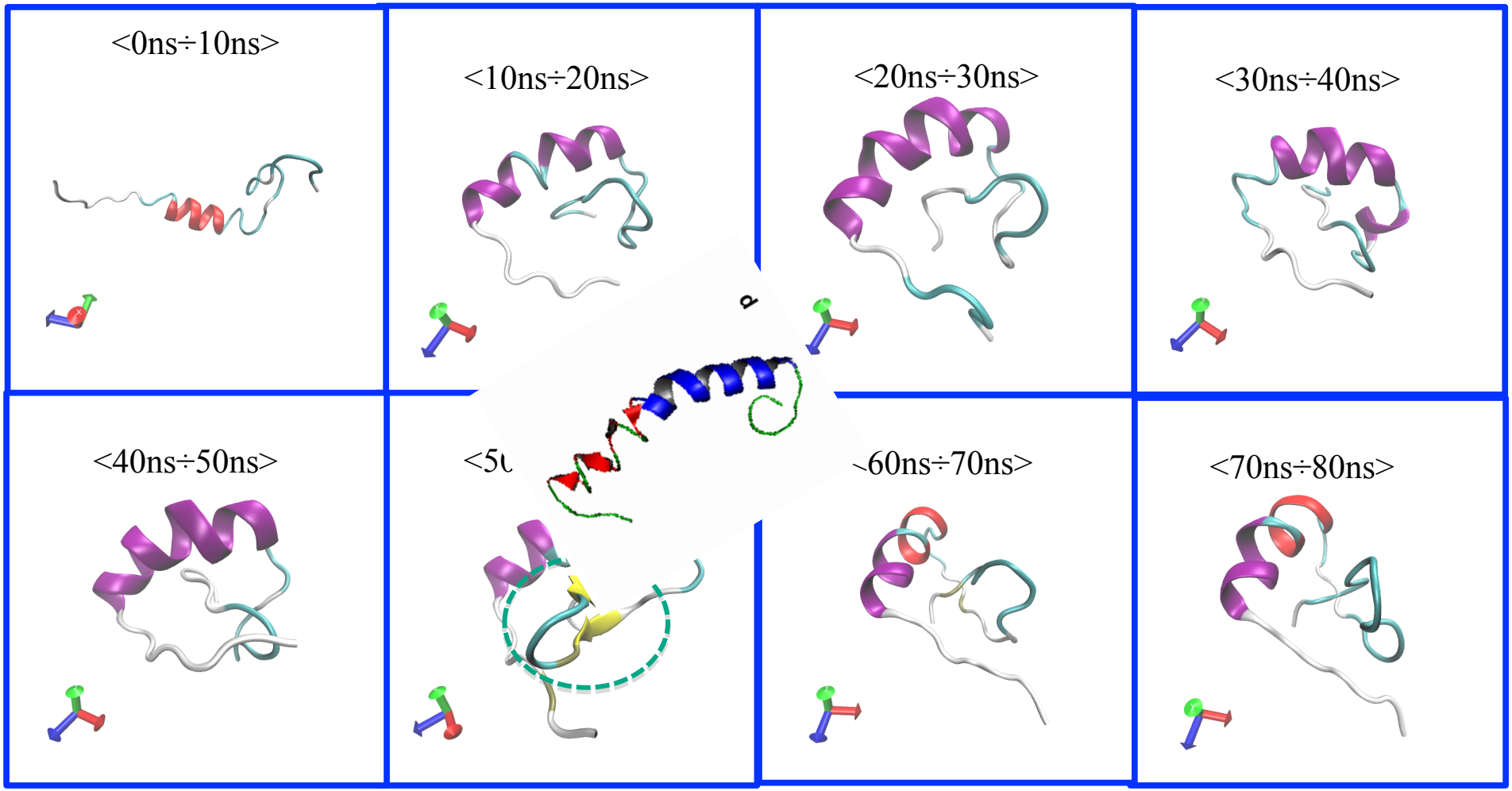


lpffn



3° Beta sheet breakers: targeting Aβ fibrils

abeta

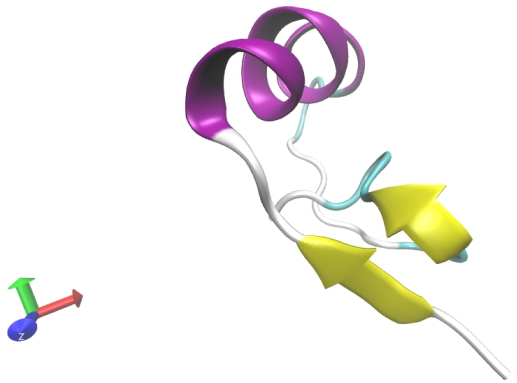


Colour code

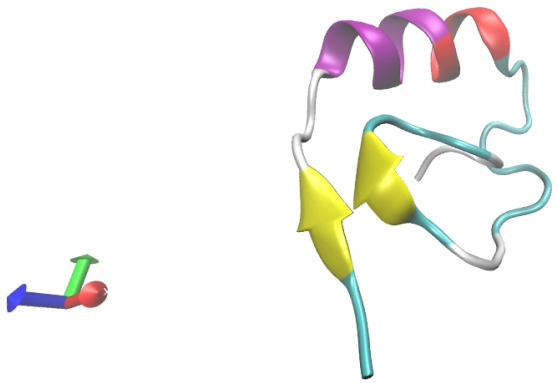
purple: a-helix; steel blue: turn; light yellow: b-helix;

orange: isolated bridge; red: p-helix; white: coil

In agreement with Ito et al. (PLoSONE, 2012, 7/1:e30510; PLoSONE, 2011, 6/3:e17587)



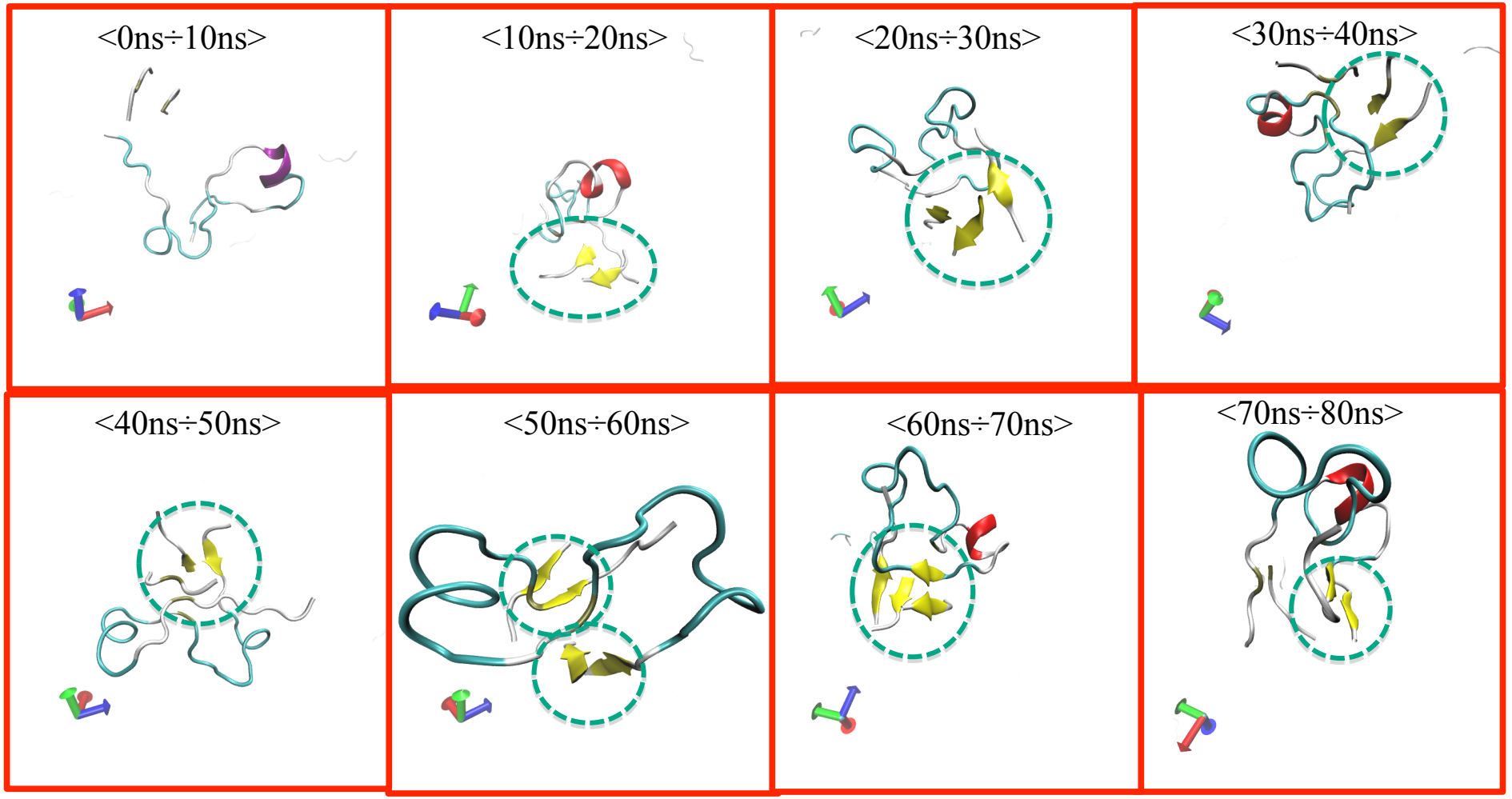
(80÷90) ns



(90÷100) ns

3° Beta sheet breakers: targeting Aβ fibrils

lpffd



Colour code

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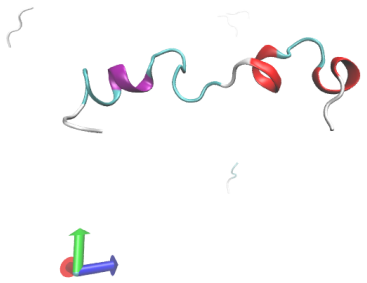
3° Beta sheet breakers: targeting Aβ fibrils

tau-lpffd

<0ns÷10ns>



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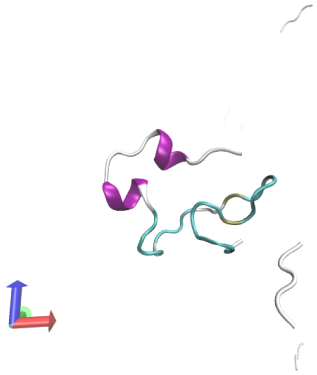

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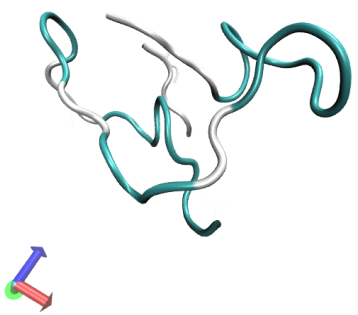

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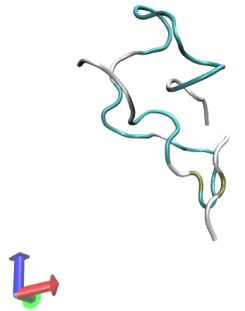

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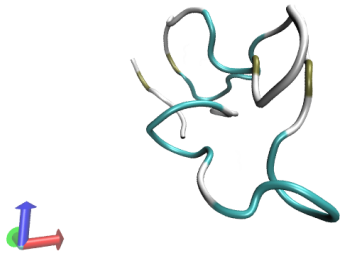

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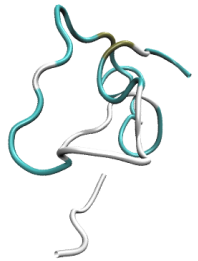

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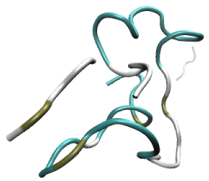

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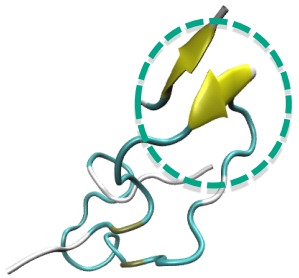

Colour code

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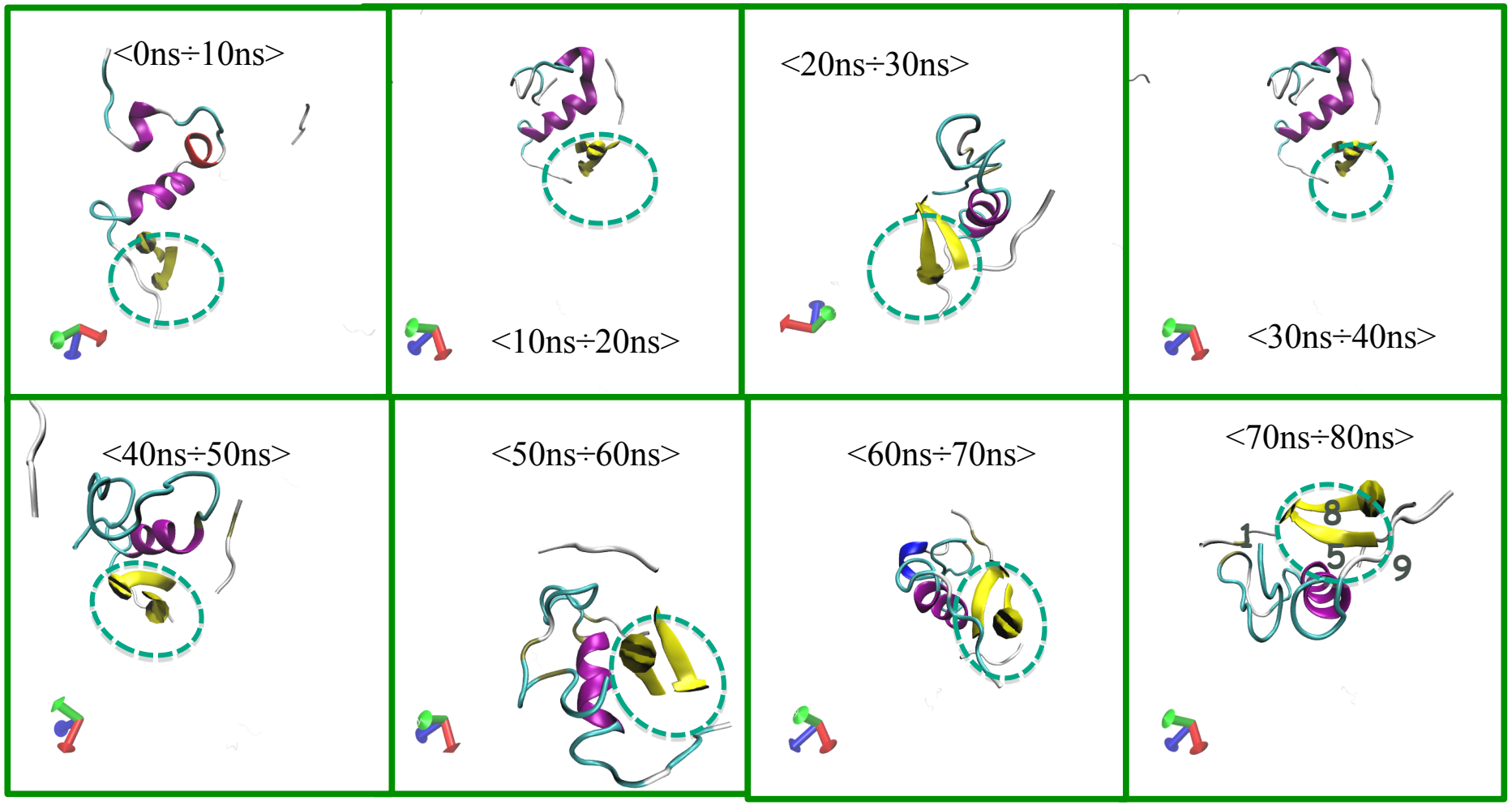
orange: isolated bridge; red: p-helix; white: coil

3° Beta sheet breakers: targeting Aβ fibrils

lpffn



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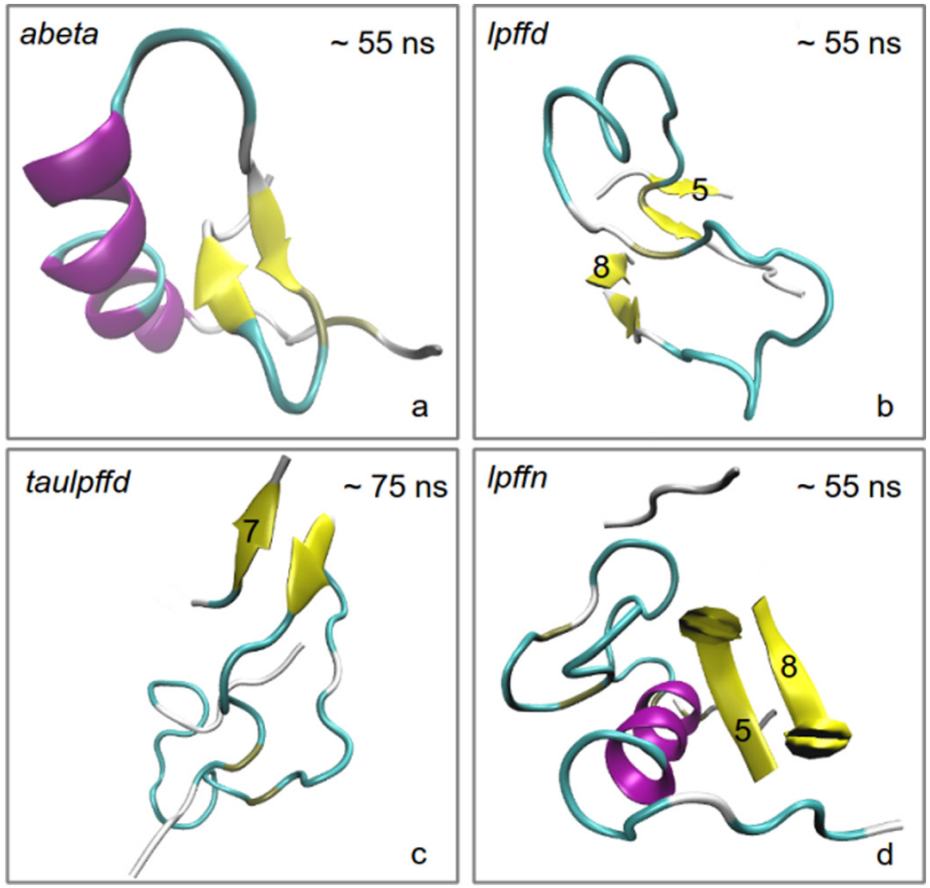
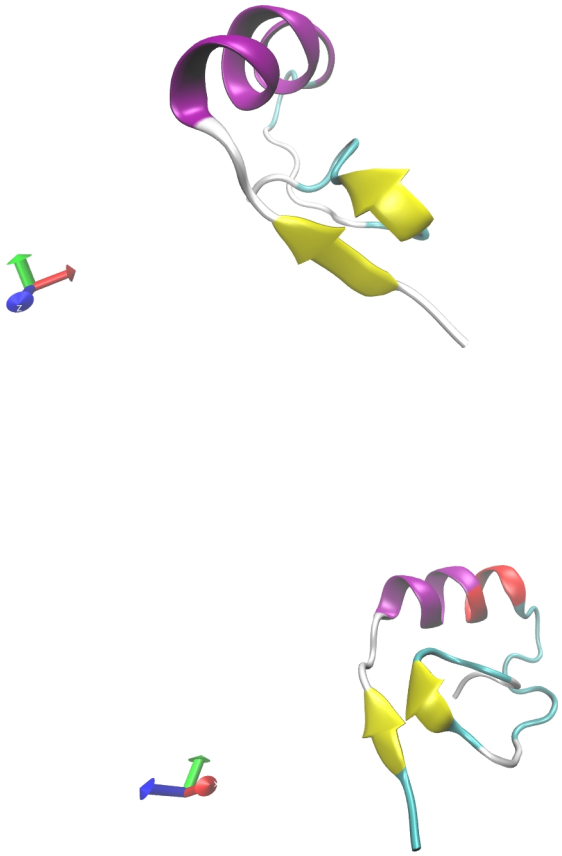
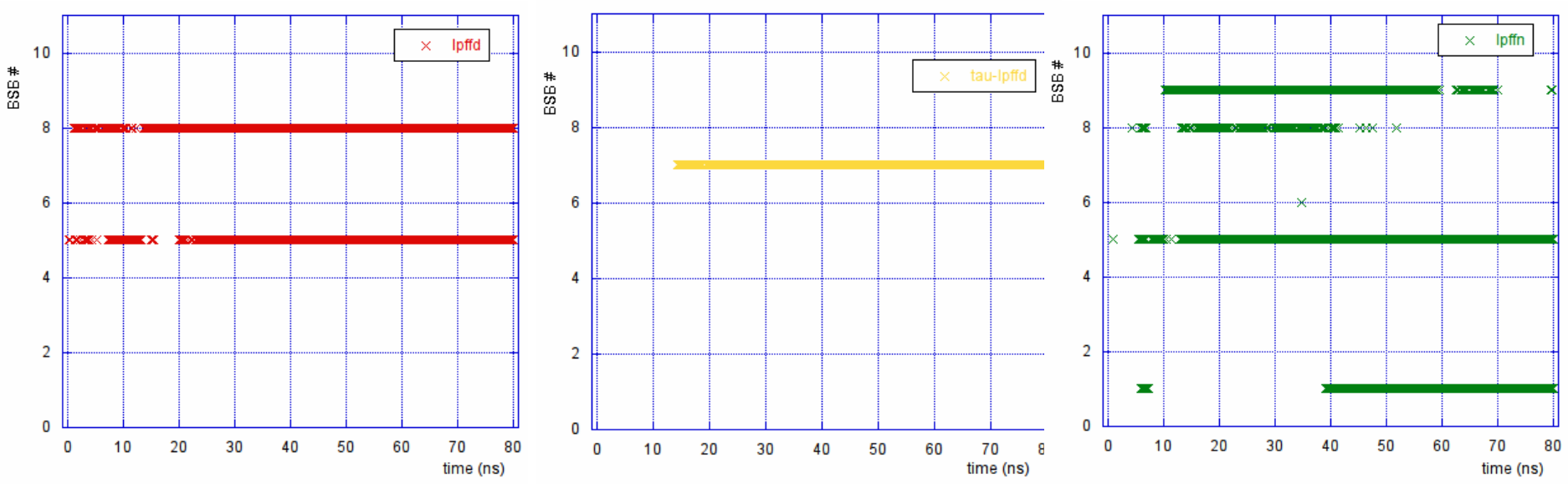
3° Beta sheet breakers: targeting Aβ fibrils





3° Beta sheet breakers: targeting Aβ fibrils

BSB's within (backbone-backbone) 8 Å from $A\beta_{1\text{-}40}$



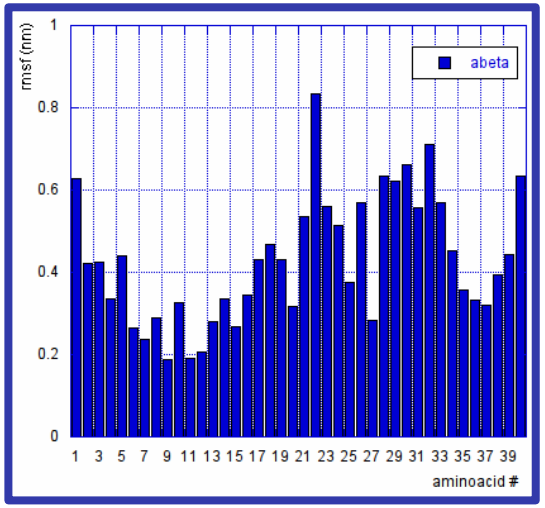
5 - 8 7 1 - 5

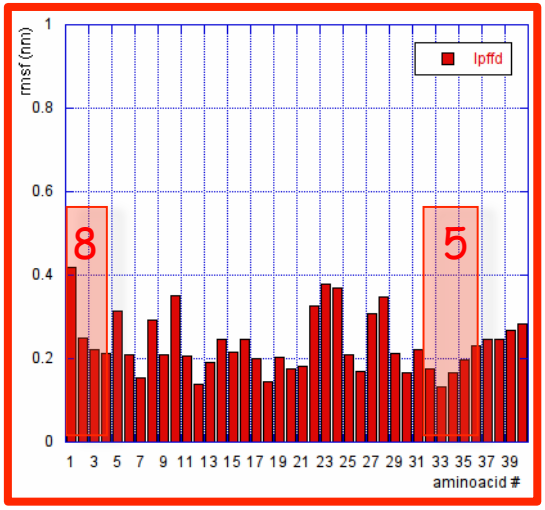
3° Beta sheet breakers: targeting Aβ fibrils

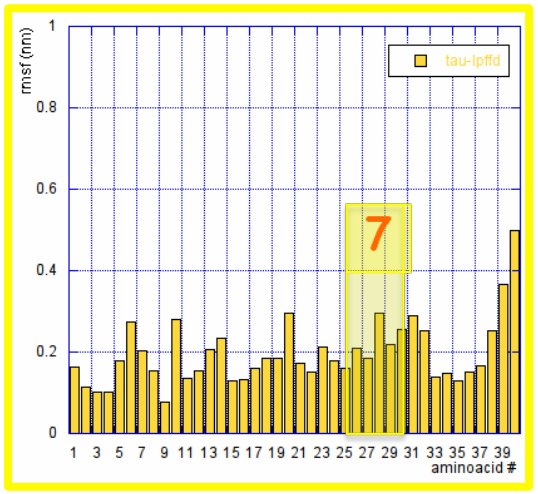
Residue Mobility

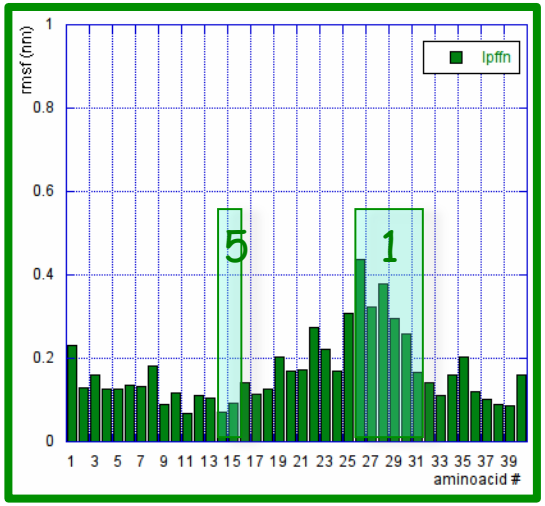
$$\sigma_R^2 = \frac{1}{T}\sum_k \frac{1}{N_R}\sum_{i=1}^{N_R} |\vec{r}_i(t_k) - \langle \vec{r}(t_k)\rangle|^2$$

T: trajectory length (30÷80 ns)
R: amino acidic residue
N_R: number of atoms belonging to R residue side chain
$\{r_i(t_k); i: 1, \ldots, N_R\}$: position of the R residue n^{th} atom
$\langle r(t_k)\rangle$: mean residue position at time t_k





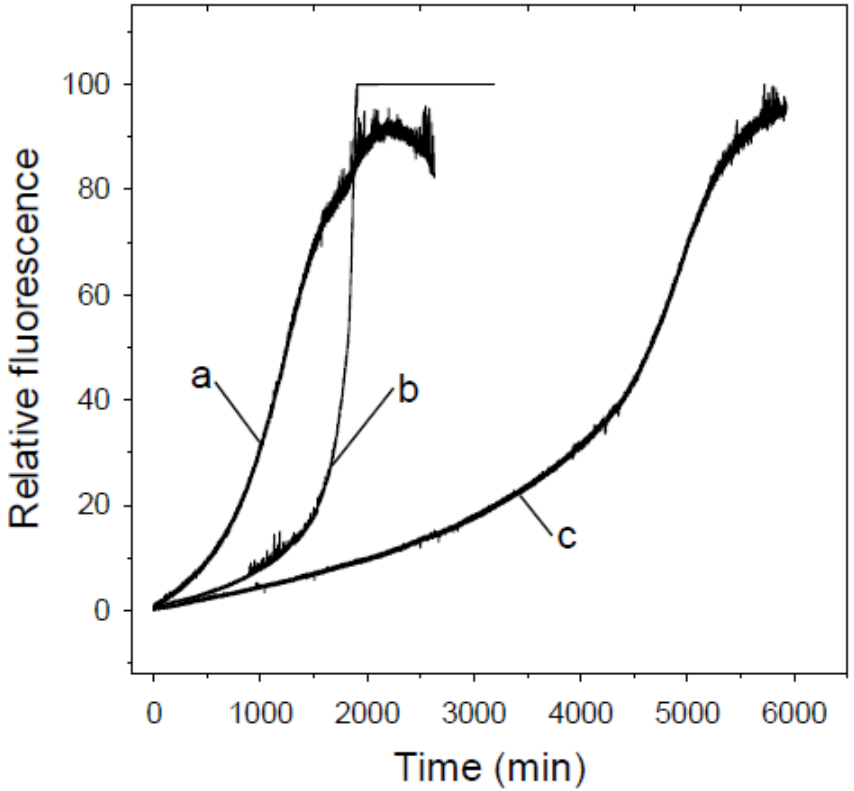




3° Beta sheet breakers: targeting Aβ fibrils

ThT fluorescence

ThT fluorescence intensity increases proportionally to $A\beta_{1-40}$ fibril formation



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

all BSBp's lengthen the lag phase duration

a: $A\beta_{1-40}$ alone : 600 min
b: $A\beta_{1-40}$ +Ac-LPFFD-NH_2 : 1000 min
c: $A\beta_{1-40}$ +Ac-LPFFN-NH_2 : 3000 min

Ac-LPFFN-NH_2
The most effective one

3° Beta sheet breakers: targeting Aβ fibrils

Conclusions …

LPFFN is the most "efficient" BSB

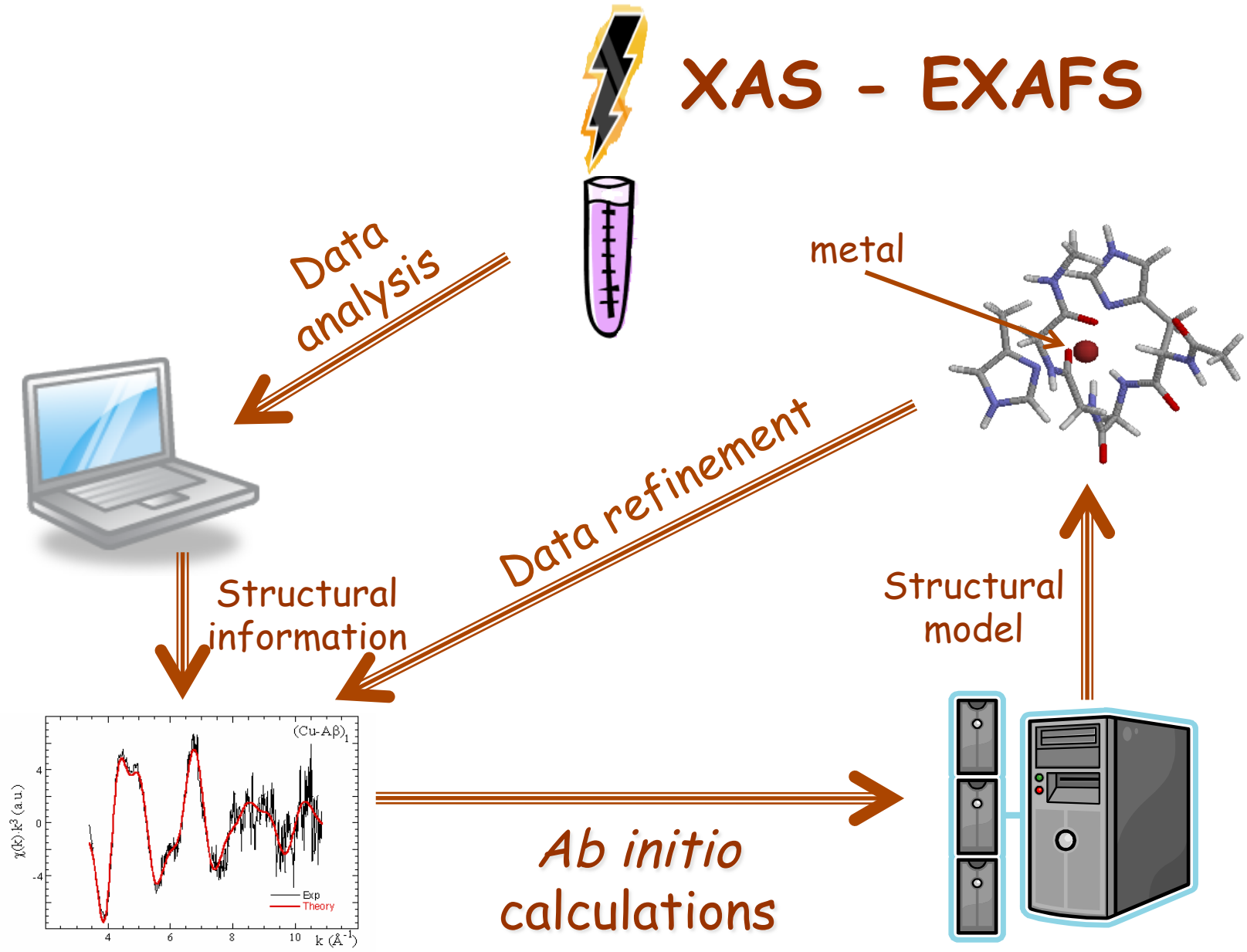
- Highest number of BSB's stay near Aβ
- Highest α-helix content stably conserved in Aβ
- Strongest effect on Aβ mobility
- Smallest RMSD → smallest Aβ structural modifications

… but

It would be of great interest to study how BSB's affect metals-Aβ interaction

… we are on the way of performing experimental studies
of the same systems in the presence of Cu or/and Zn

General conclusions



Outlook…already started

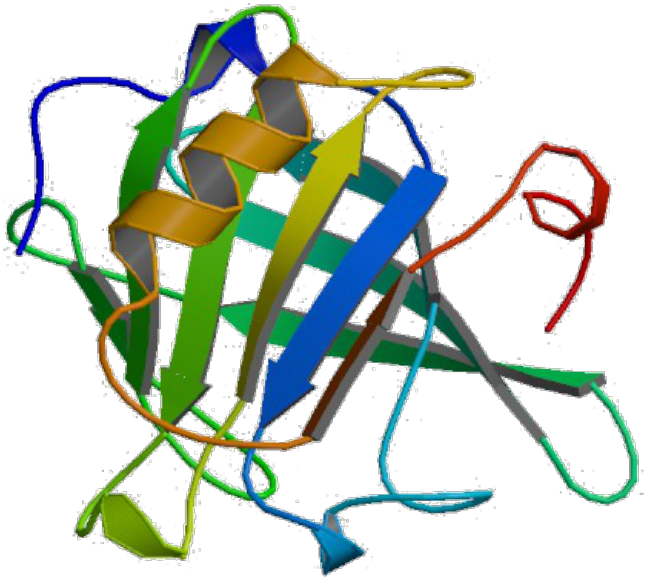
Aggregation & Misfolding: industrial applications

Amyloid-like aggregates are now widely studied also in **non-medical** contexts: food science, pharmacology, nanomaterials …

- Gel and foam production (drugs, food integrators, …)
- Bio-material and bio-electronic device developments (nanowires, bio-sensors, …)
- Food preservatives and taste enhancers, …
- …

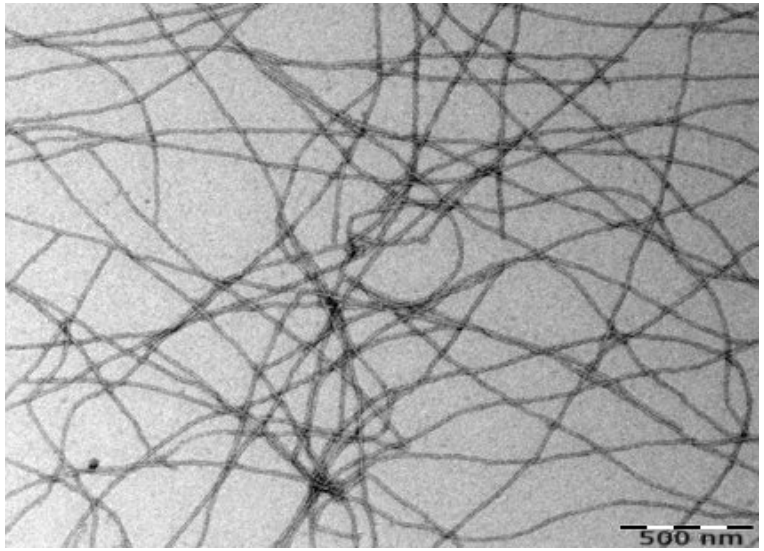
Outlook…already started

An important example: β-lactoglobulin



It is the most abundant protein in the milk whey (mainly as a dimer). 38% is in a β-sheet secondary structure.
It is considered a potential new food ingredient both as an enhancer of gel propensity and as a nutritional ingredient.

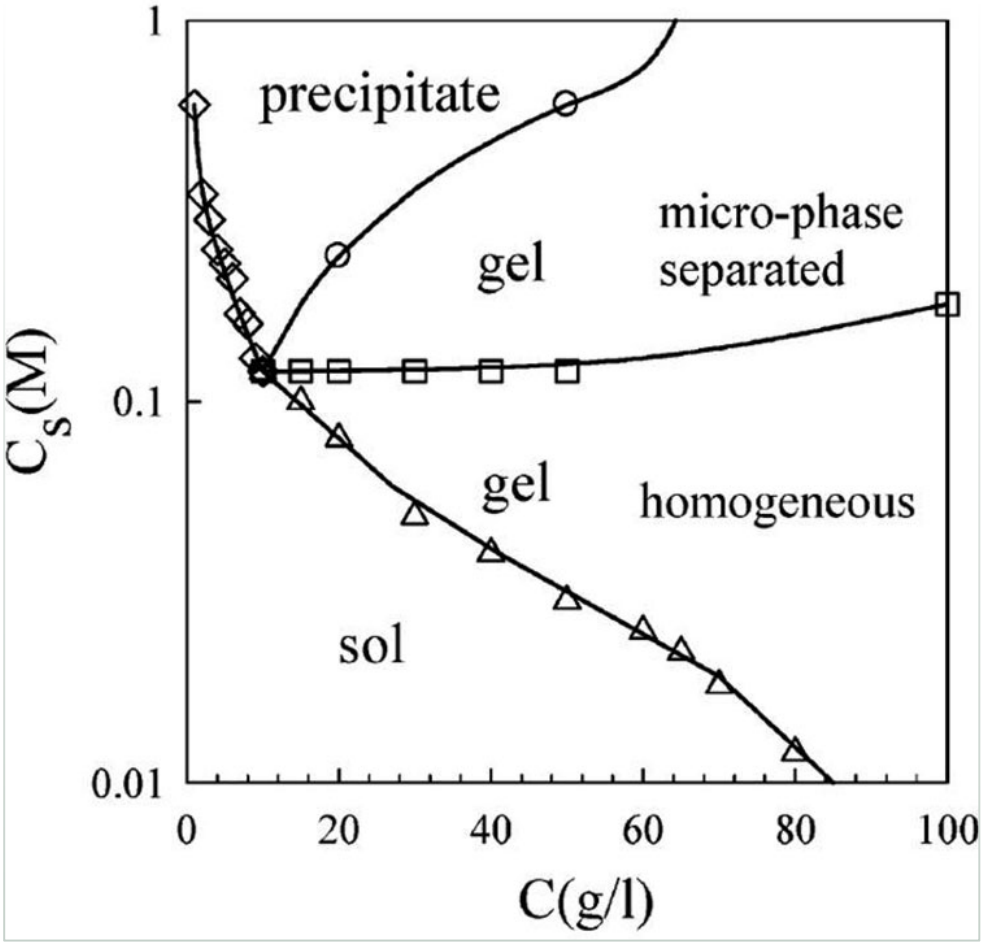
In extreme conditions (@ pH=2 & 80-110 °C for > 20 h) it is able to form amyloid fibrils



M. Carbonaro, A. DiVenere, A. Filabozzi, P. Maselli, V. Minicozzi, S. Morante, E. Nicolai, A. Nucara, E. Placidi, F. Stellato "Role of dietary antioxidant (-)-epicatechin in the development of b-lactoglobulin fibrils" (2016) *BBA proteins and proteomics*, submitted

Outlook…already started

Phase diagram



C_S(M): molar protein concentration

C(g/l): NaCl concentration

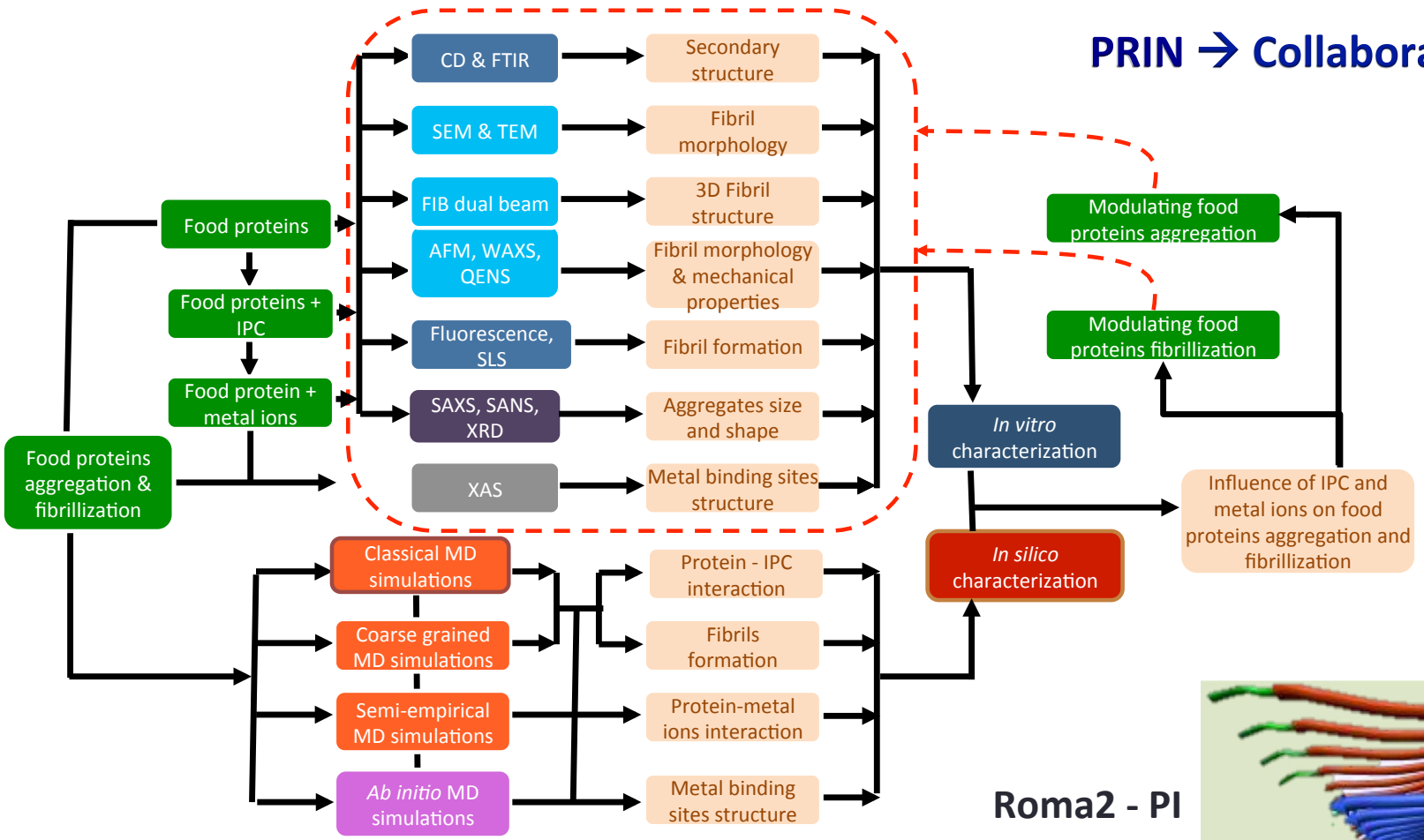
(pH=7)

Ako K, Nicolai T, Durand D, Brotons G. (2009) "Micro-phase separation explains the abrupt structural change of denatured globular protein gels on varying the ionic strength or the pH" *Soft Matter* **5(20)**:4033–41.

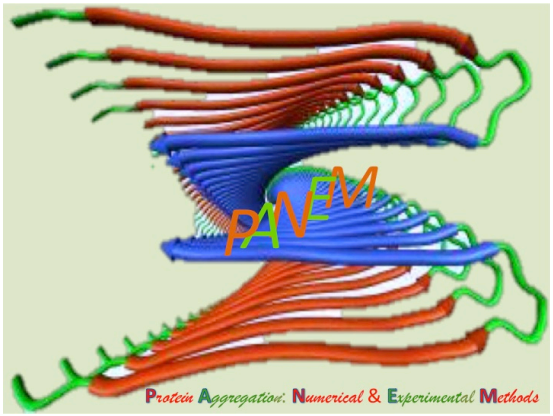
Outlook…already started

A diffuse cutting-edge experimental and numerical laboratory for the study of protein misfolding and aggregation. An application to food science.

PRIN → Collaboration



Roma2 - PI
Roma1
Firenze
Ancona



The Biophysics Group

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Velia Minicozzi

Francesco Stellato Post Doc

Emiliano De Santis PhD

Emilia Capozzi*

Simone Aureli*

Zelio Fusco*

* in the quest for a position

(Permanent) Collaborators

Giovanni La Penna (CNR); Olivier Proux (ESRF)

Glenn Millhauser (UCSC); Sunil Saxena (U. Pittsburgh)

Ann Spevacek (UCSC); Valerio Consalvi (RM1); …

Thank you