Understanding immobilised enzymes and immobilised substrates

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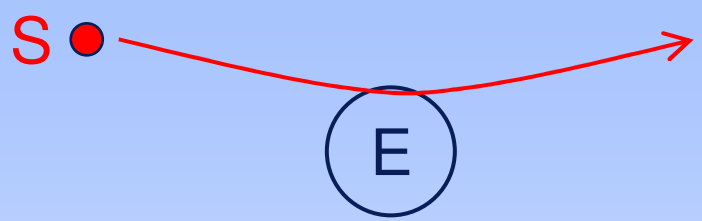
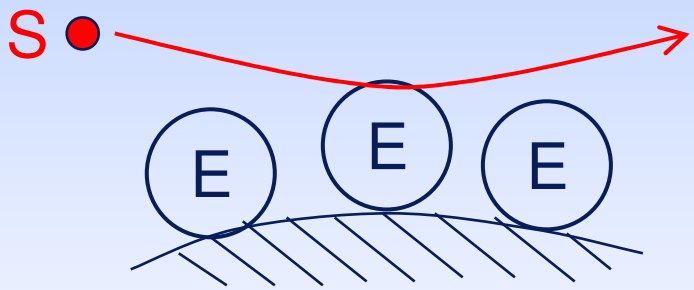
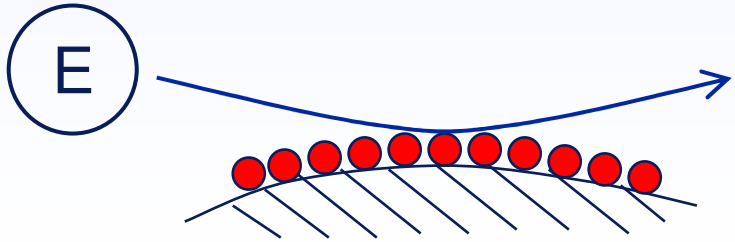
- Normal enzymology



- Immobilised enzymes



- Solid-phase (immobilised) substrates



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Why use enzymes with their substrates attached to a solid particle?

- Applications in solid phase chemical synthesis
- Assay or screening of enzymes on substrate arrays
- Fundamental relevance to *in vivo* state

NB extensive literature on enzymes evolutionally adapted to attack solid substrates, e.g. cellulases

How will enzyme behaviour change when substrates are attached to a solid support?

- Studies for solid-phase synthesis applications often show disappointing rates and/or yields
- But little fundamental study of reasons why
- Understanding these should help improve applications

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Equilibrium positions will not be affected, will they?

- Well, yes they will

e.g. thermolysin-catalysed condensation with saturated aqueous Fmoc-Gly, pH 7.4

		Equilibrium conversion to peptide
H_3N^+–Phe–O–●	⇌ Fmoc–Gly–Phe–O–●	99%
H_3N^+–Phe–OMe	⇌ Fmoc–Gly–Phe–OMe	2.3%

Solid phase is $PEGA_{1900}$,
Rein Ulijn et al, 2003.

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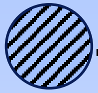
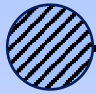
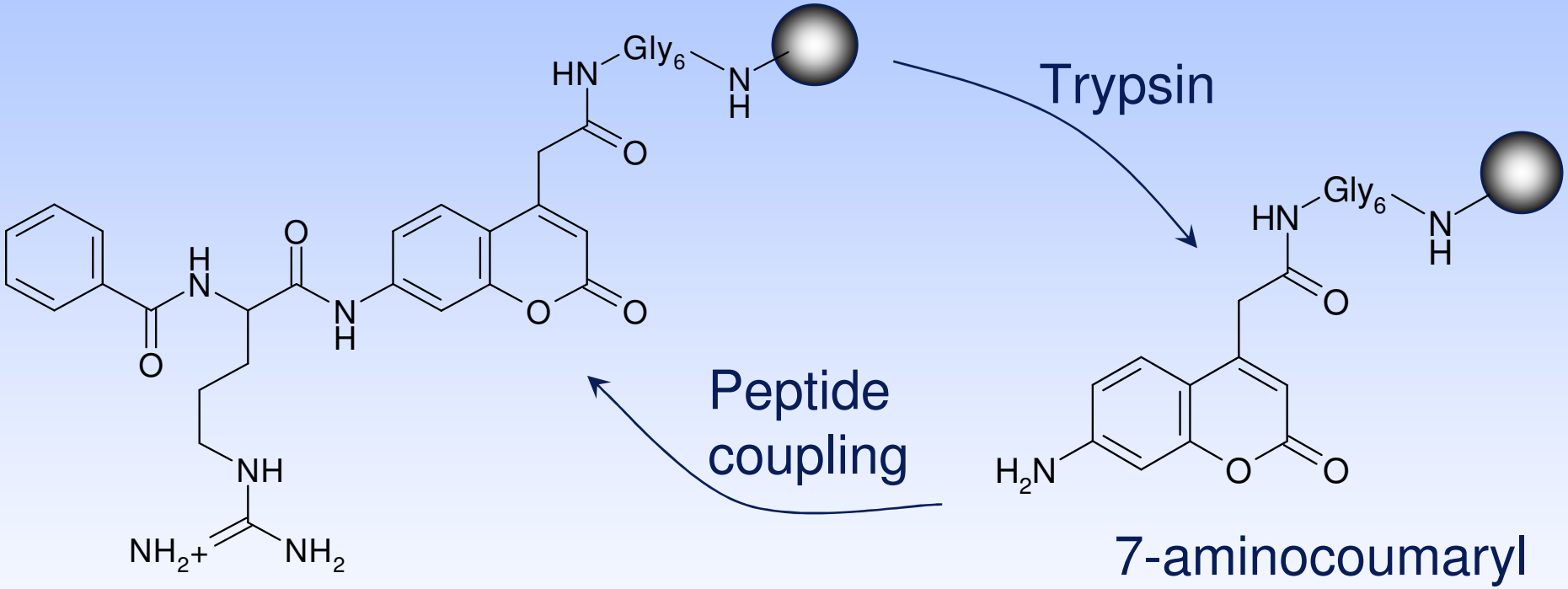
Why should the positions of these two equilibria differ?

 $-NH_3^+ \quad + \quad RCOO^- \rightleftharpoons$  $-NHCOR$

$R'NH_3^+ \quad + \quad RCOO^- \rightleftharpoons R'NHCOR$

- ... hydrophobic surface favours amide synthesis (is near surface and/or bead interior, where hydrophobic hydration is less unfavourable)
  - practically useful
  - theoretically trivial, allowed for in comparison just shown
  - ... free amino group important in making amide hydrolysis favourable in aqueous solution, so suppression shifts equilibrium
  - so not the only factor

Real-time spatially resolved enzyme kinetics on solid-phase substrates



Bz-Arg-coumarin
Non-fluorescent

7-aminocoumaryl
acetamide
Highly fluorescent

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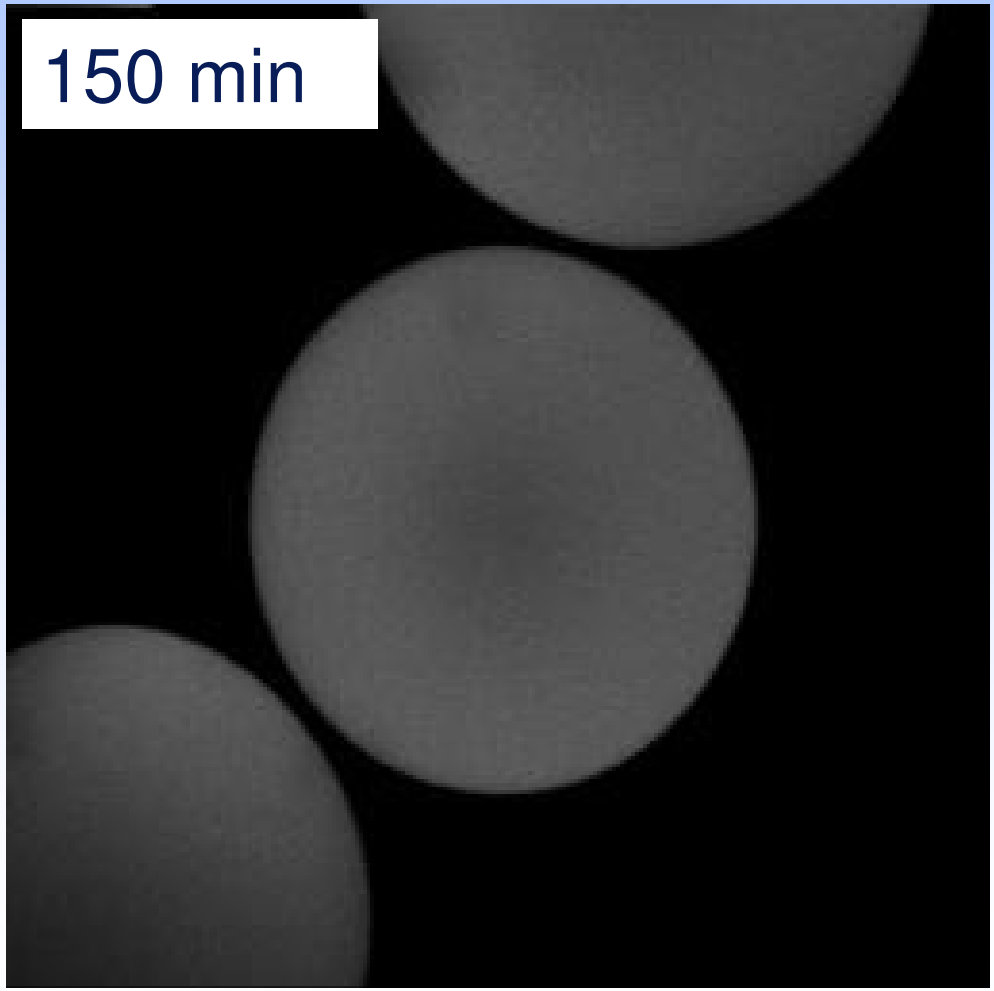
Real time images of trypsin action in PEGA bead



Optical section by two-photon microscopy. PEGA beads with attached Bz-Arg-coumarin-$(Gly)_6$-

Joe Deere et al, 2007

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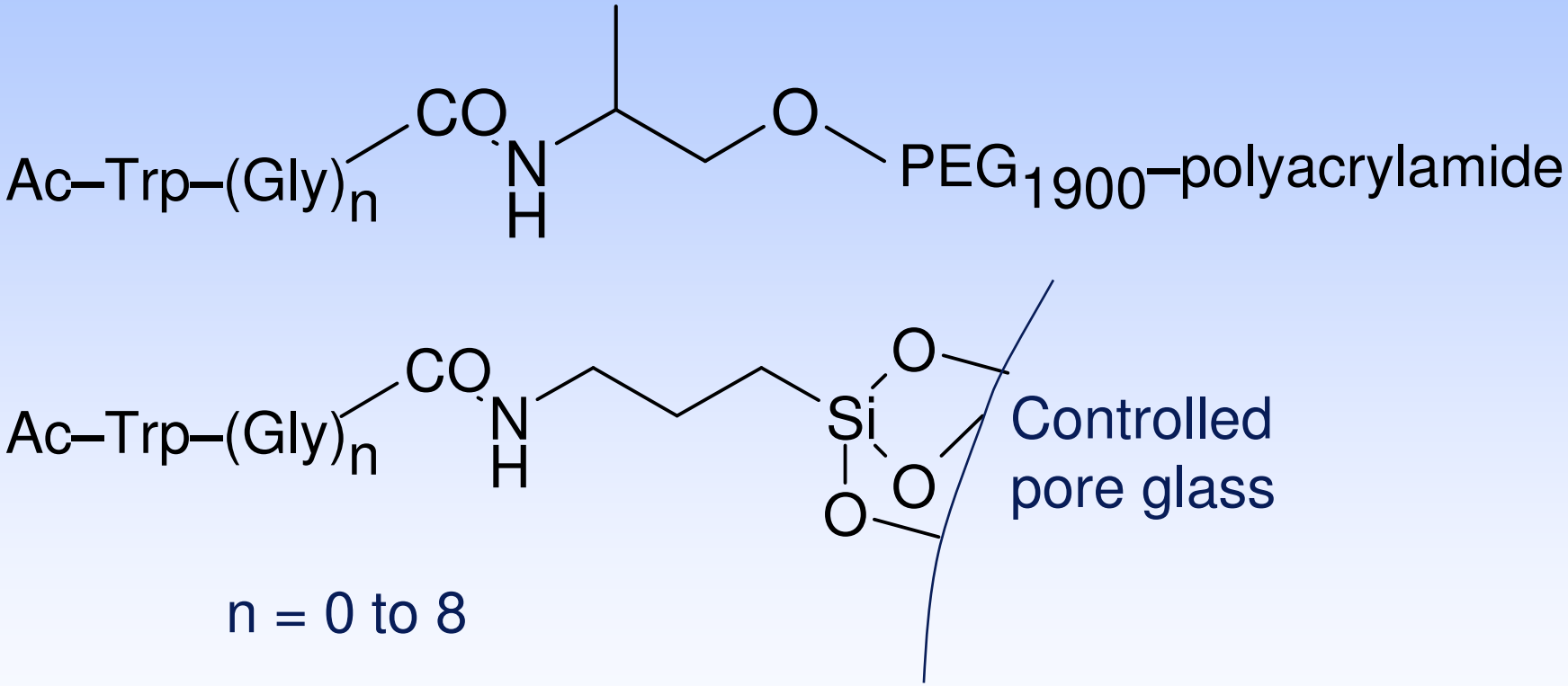
How should substrate moiety be attached to surface?

- Logical to use extended linker (“spacer”) to avoid interference by surface
- Analogy with affinity adsorbents and immobilised enzymes
- Commonly used, but no systematic study

Effect of spacer length: substrates used



Chymotrypsin releases Ac-Trp for analysis

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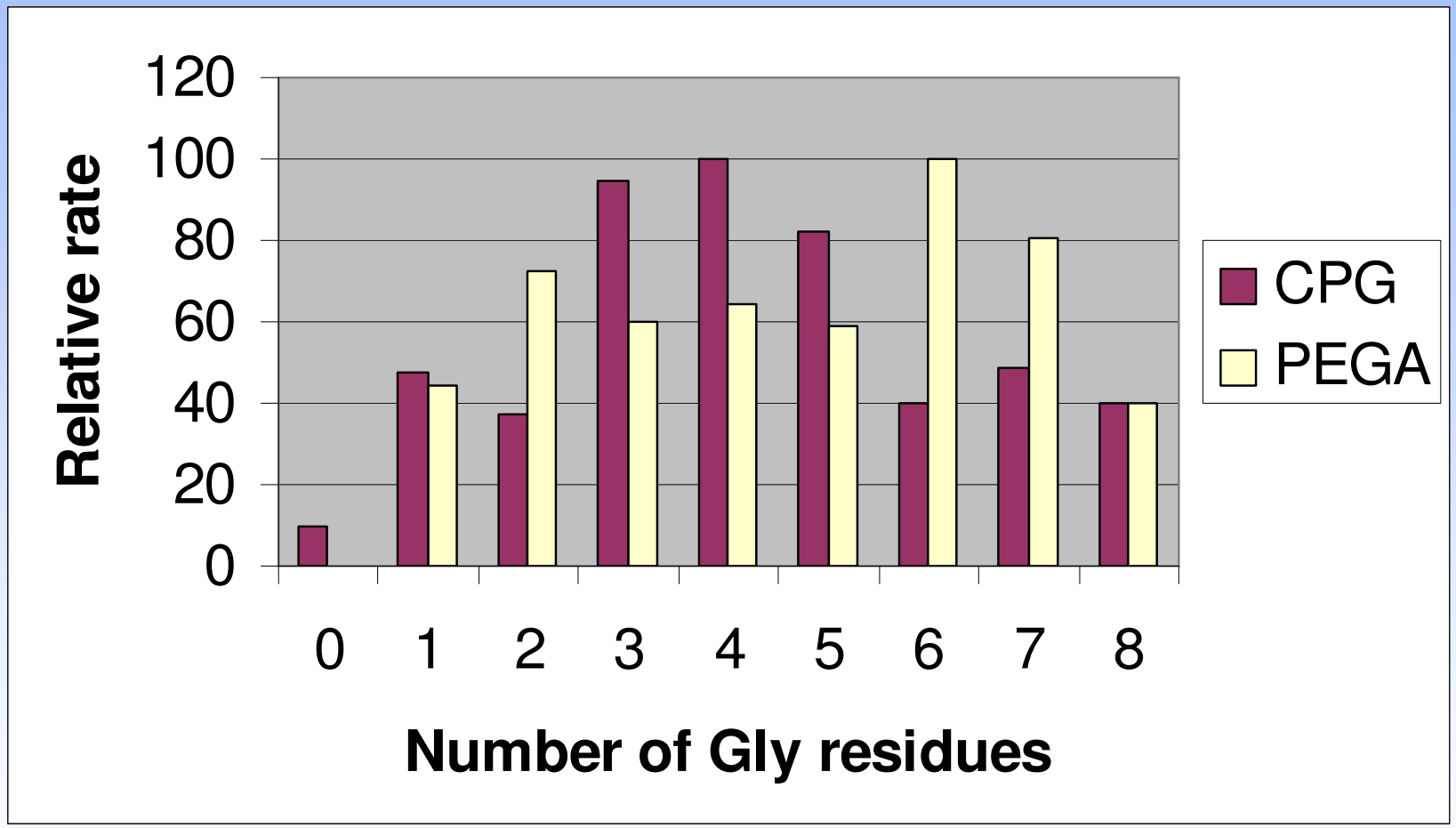
Effect of oligo-Gly spacer length on rate



Joe Deere, Antonia Lalaouni,
Laura Solares et al, 2008

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Circular dichroism on biocatalyst particles

- Far UV spectrum can give secondary structure composition
- Changes in near UV are sensitive indication of 3D structure changes

- Achiral scattering just means need more intense source – state-of-the-art bench instruments suitable
- Rotating cell to keep particles in uniform suspension
- Cell close to detector to minimise differential scattering

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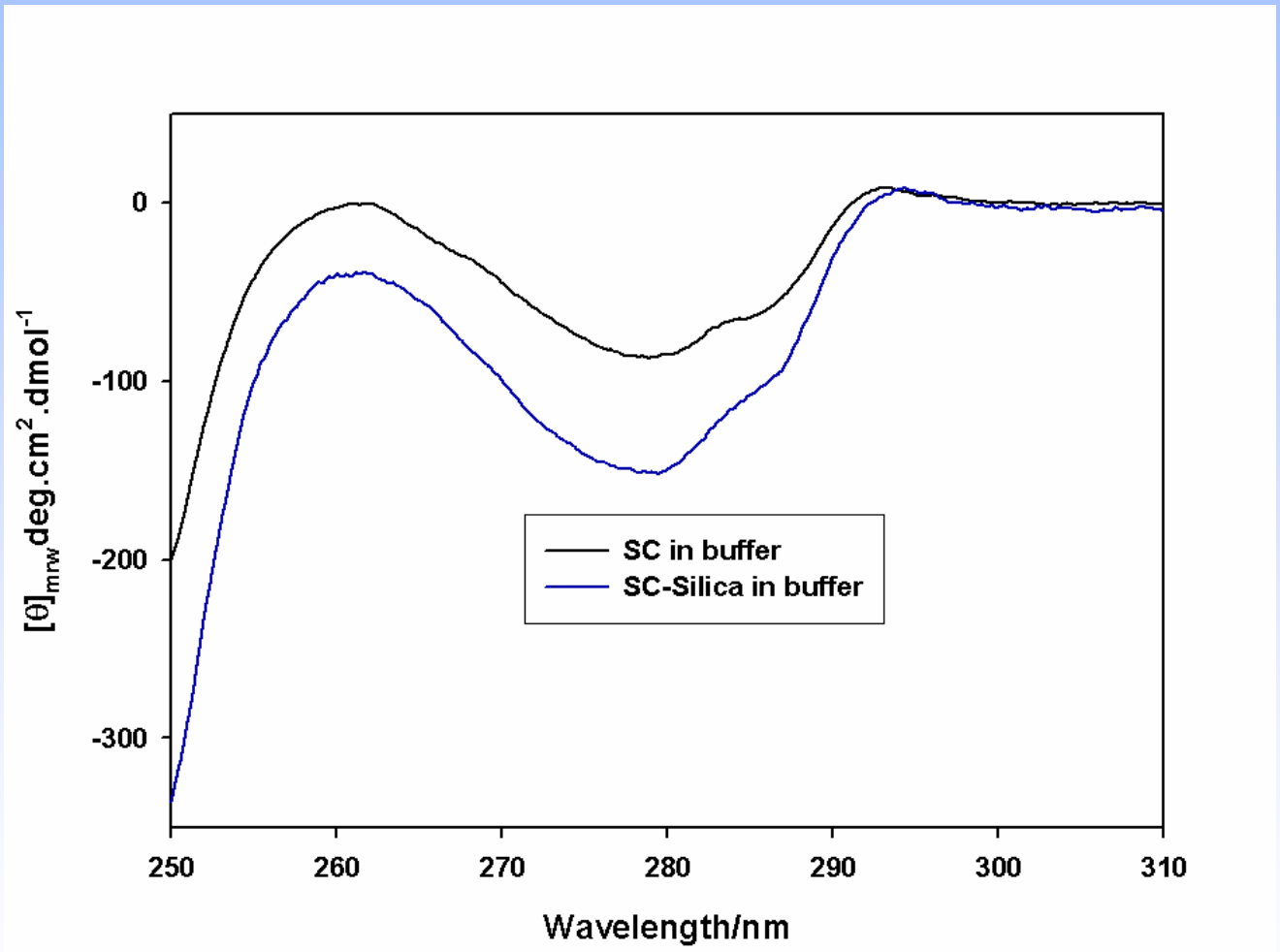
Effect of subtilisin immobilisation on near UV CD



Both in aqueous buffer

Ashok Ganesan et al, 2006

Increased intensity, same shape - probably means same structure but more rigid

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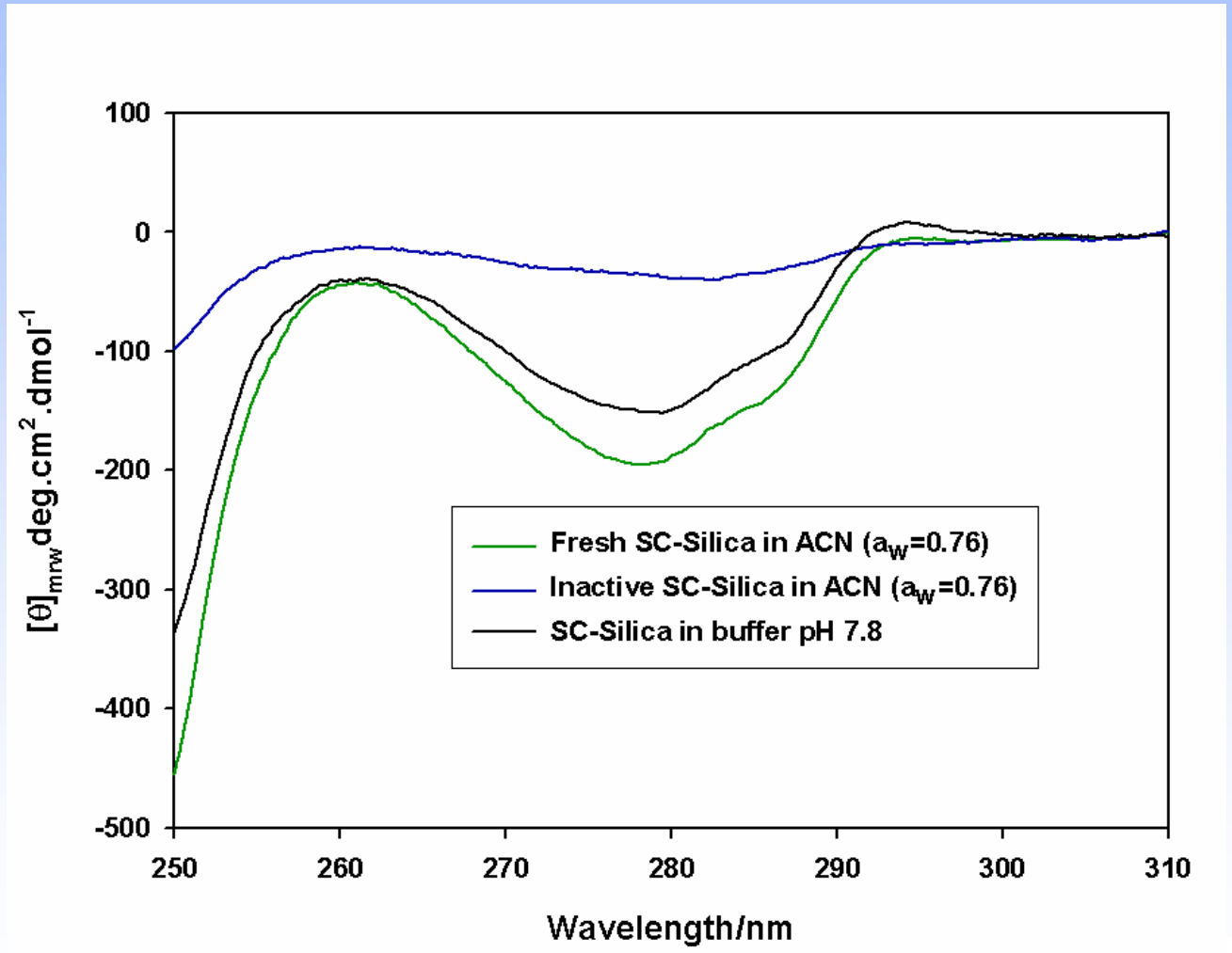
Near UV CD shows loss of tertiary structure after immobilised subtilisin is inactivated in acetonitrile



Ashok Ganesan et al, 2006

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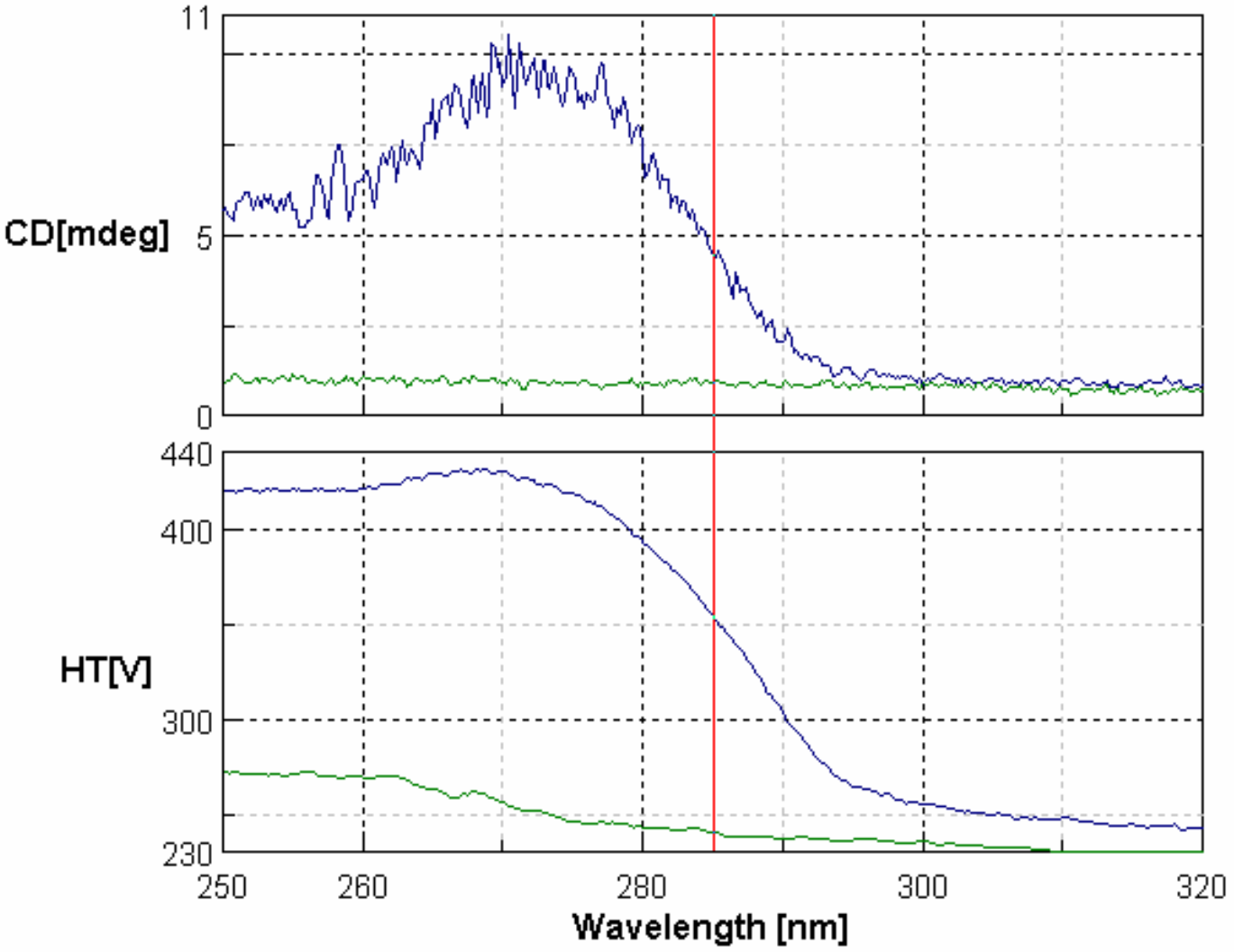
Near UV CD is possible with polymer supports [1P]



Green: Lewatit VPOC 1600
Blue: Novozyme 435
Both in acetonitrile (1% water).

Ashok Ganesan at al, 2009.

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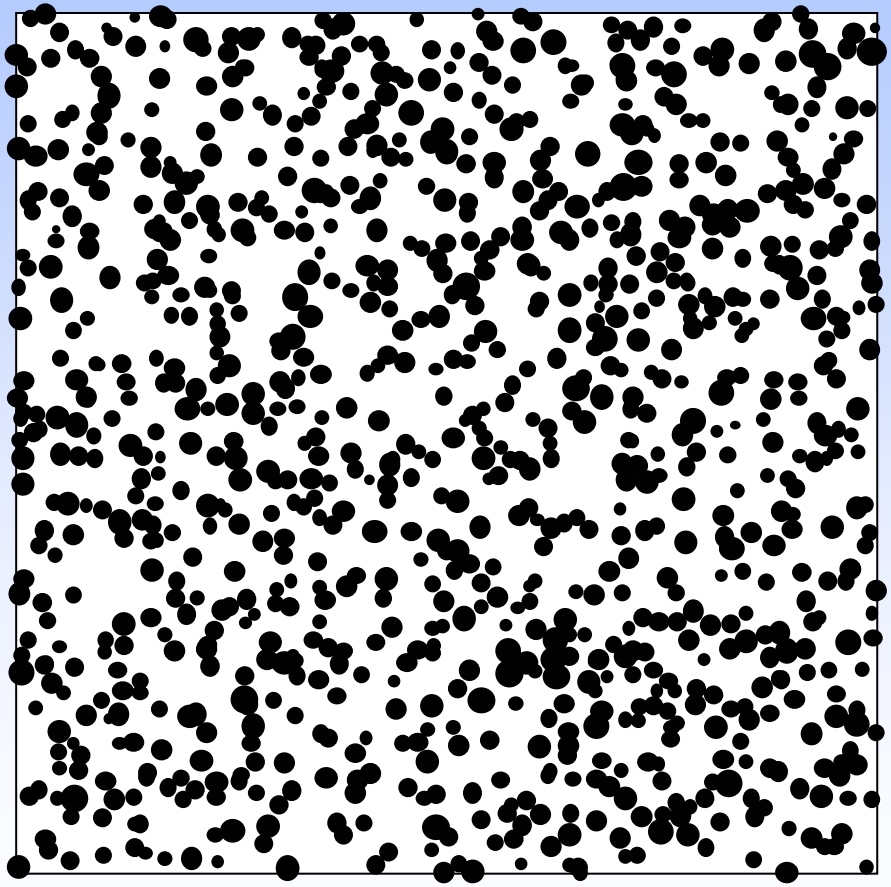
Absorption flattening

- CD signal (and absorbance) of a suspension is lower than if the same amount of chromophore was uniformly distributed
- Reduced by largest factor at wavelengths where absorbance greatest
- So distorted spectrum must be corrected before analysis

Cause of flattening best seen from appearance of suspension viewed along light path



Volume fraction = 0.1, pathlength = 2 X mean particle size.

Absorbance cannot exceed 0.15, however high the chromophore concentration in the particles

Calculation of flattening effects by simulation

- Place particles randomly within a simulated volume, to give required volume fraction
- For array of light paths through the simulated volume, calculate transmission along each, based on extent to which it meets particles
- Hence calculate average transmissions, and thus absorbance and CD signals

- Accurate answers in a few seconds on desktop computer

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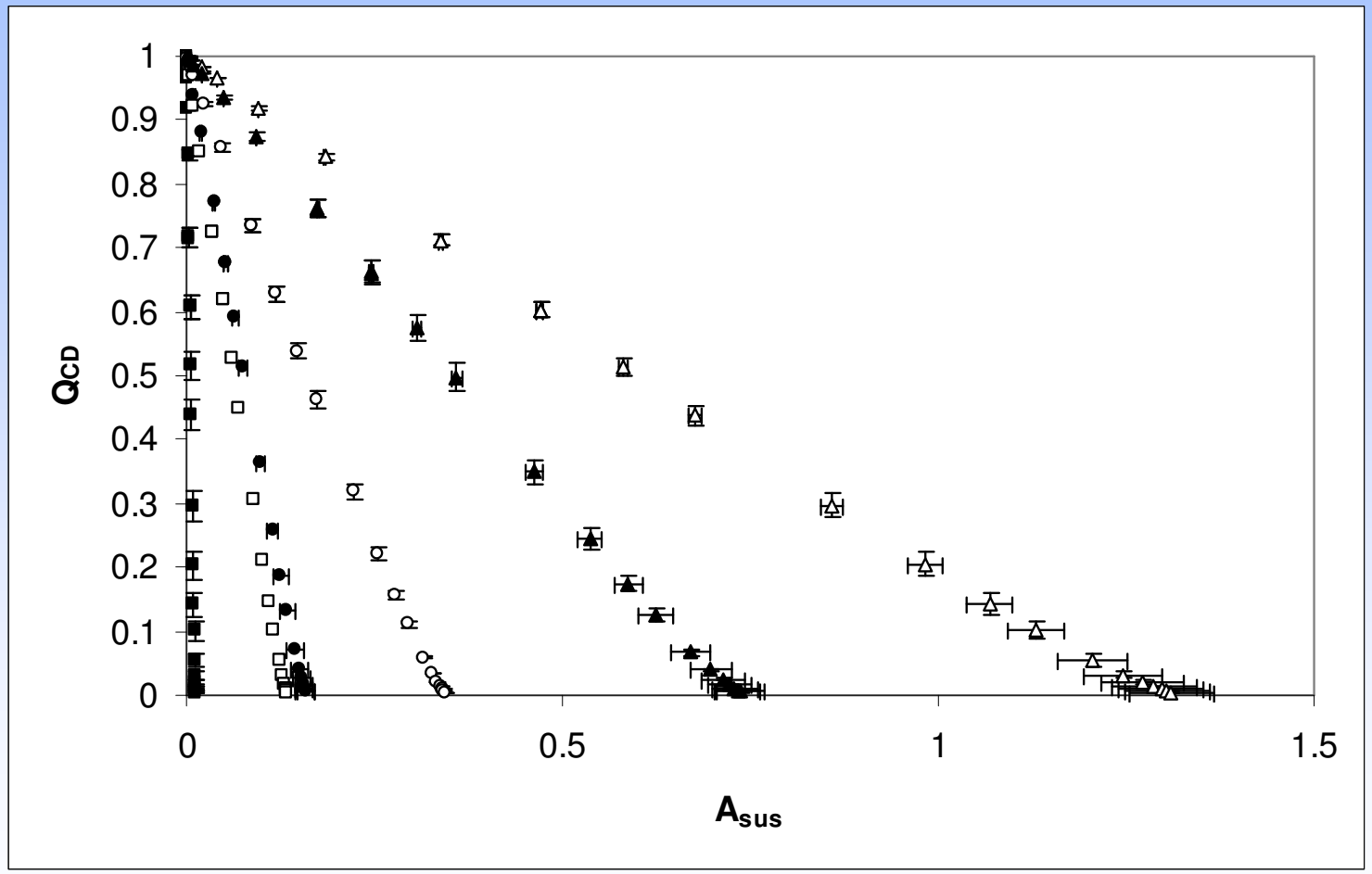
How to correct experimental data: relationship of Q_{CD} to measurable absorbance of suspension (A_{sus})



Each series shows effect of increasing particle absorbance for set values of particle size and volume fraction. Halling (2009).

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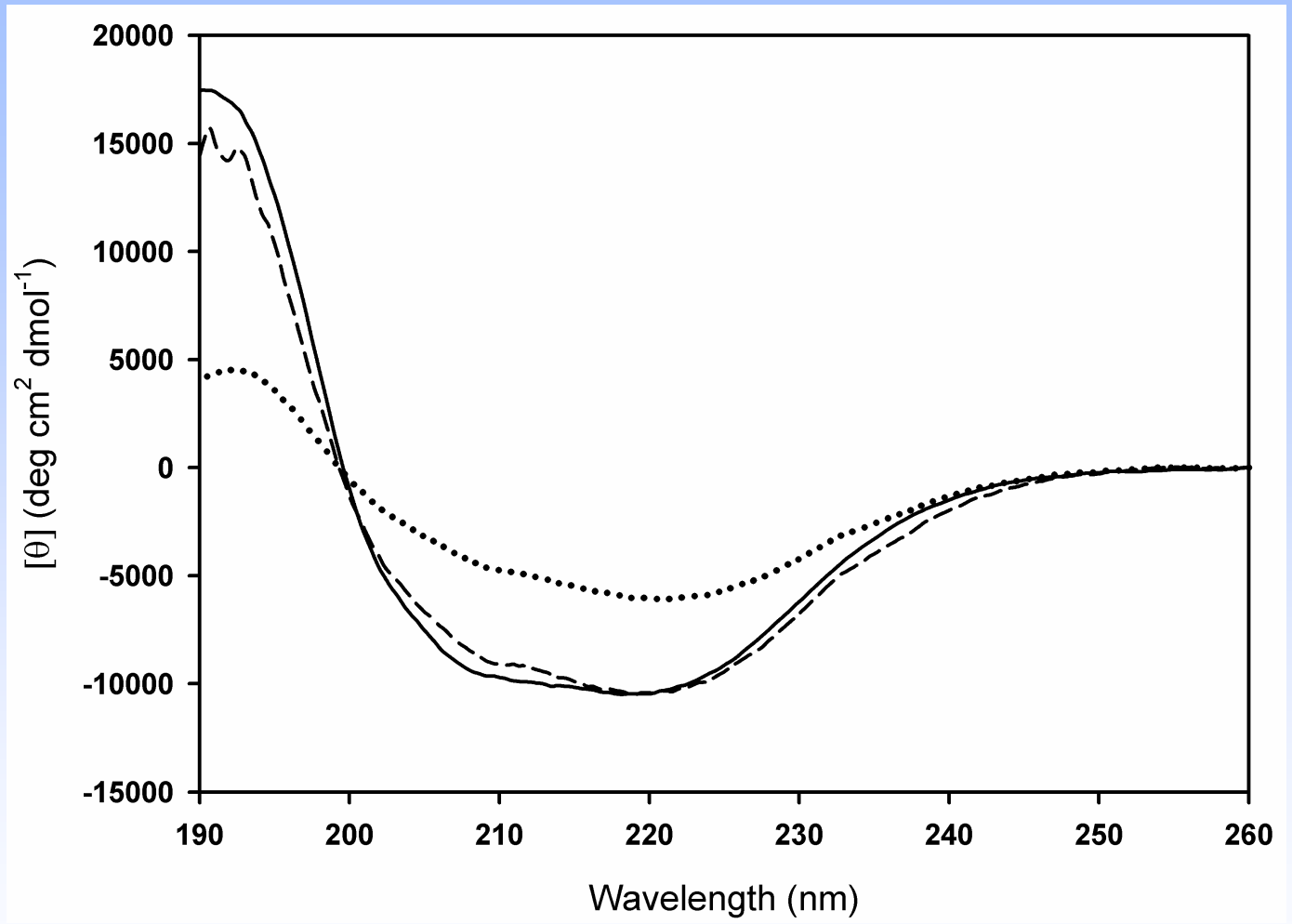
Correction of far UV CD for absorption flattening [1P]



Aqueous buffer with dissolved free subtilisin (——) or suspended silica-immobilised enzyme (·····), and after correction (- - - -).

Ashok Ganesan et al, 2006

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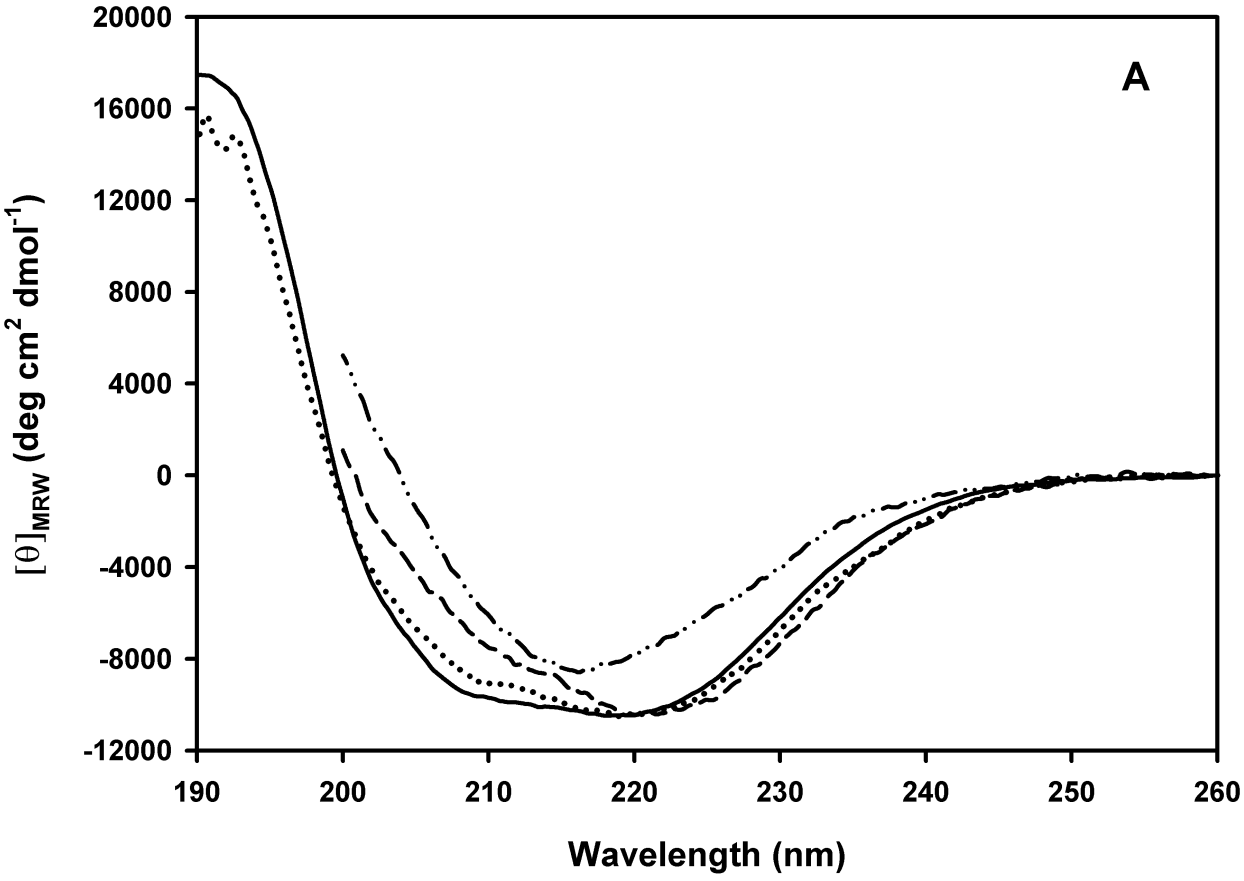
Far UV CD is significantly changed on inactivation



Subtilisin in aqueous solution (——).

Silica-immobilised enzyme: in aqueous (·····), in ACN (- - - -), inactivated ((-·-·-·-).

Corrected for absorption flattening.

Ashok Ganesan et al, 2006.

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Inactivation is accompanied by increase in beta structure

Sample	Helix	Sheet	Other	NRMSD
Aqueous solution	0.26	0.14	0.60	0.062
Immobilised in aqueous	0.29	0.11	0.59	0.095
Immobilised in ACN	0.31	0.11	0.58	0.183
Inactivated in ACN	0.30	0.22	0.49	0.218

Secondary structure contributions calculated using K2D algorithm.
Ashok Ganesan et al, 2006.

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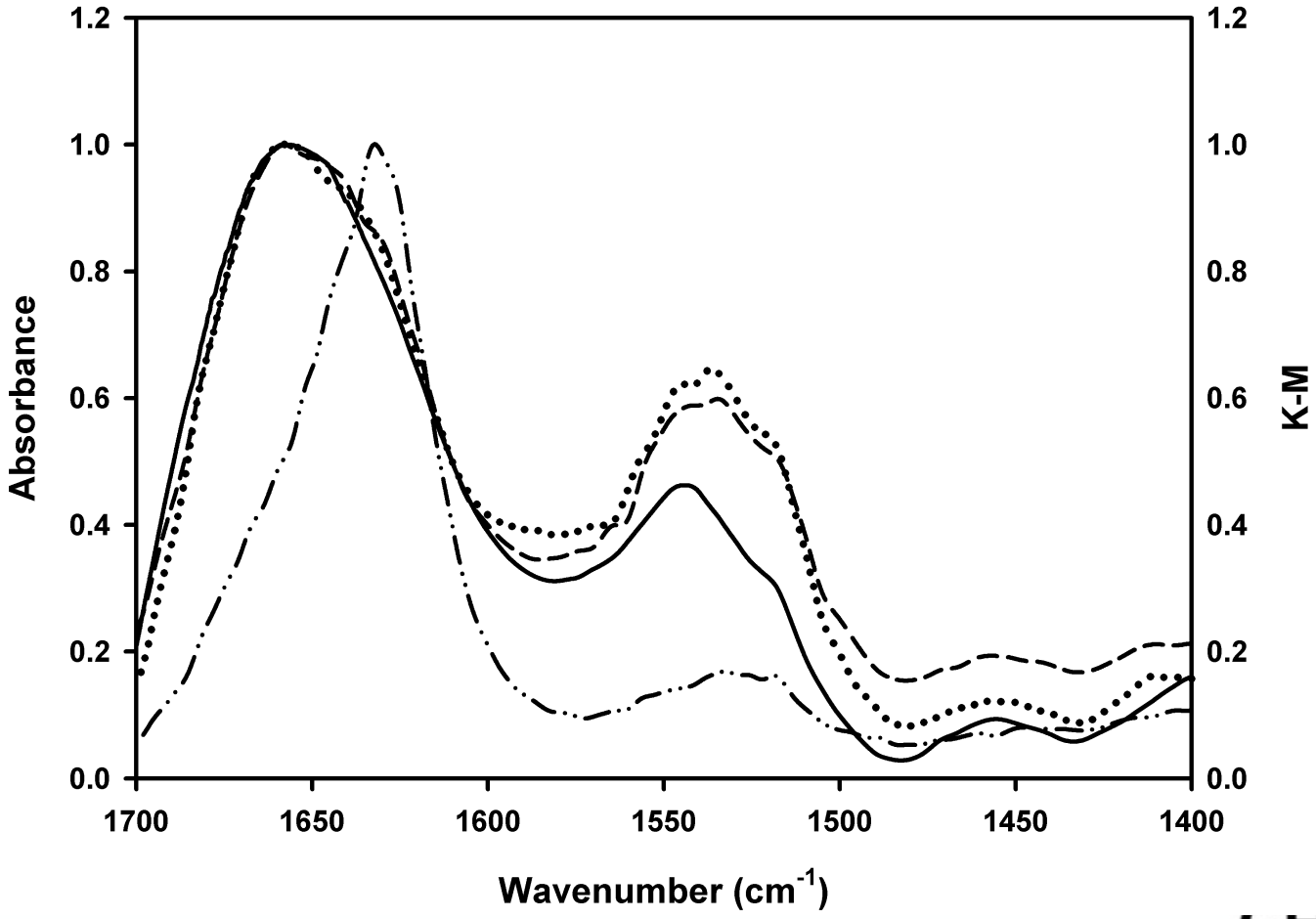
IR spectrum little effected by immobilisation or medium, but suggests shift to beta structure on inactivation



Aqueous solution (—), silica-subtilisin (- - -), after brief ACN exposure (·····), and inactivated (-·-·-·-).

Ashok Ganesan et al, 2009.

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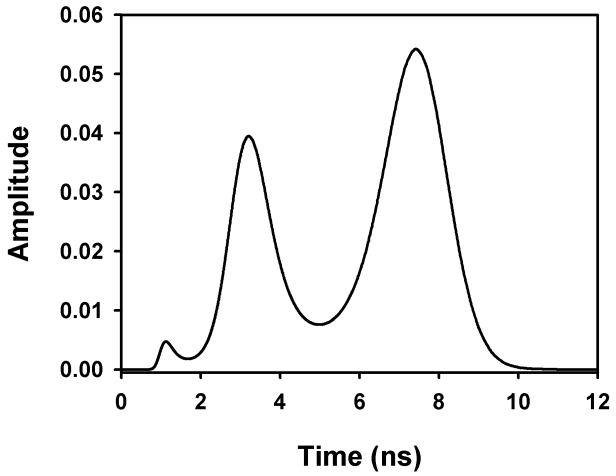
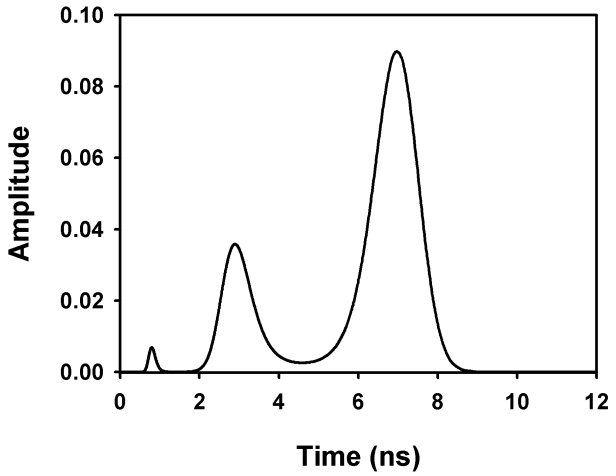
Fluorescence lifetime distribution

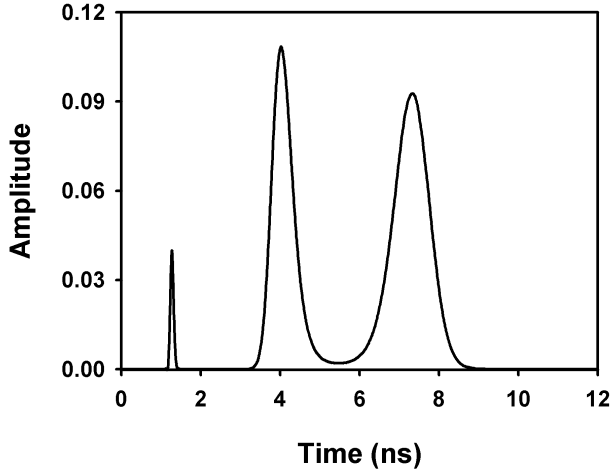
Aqueous solution



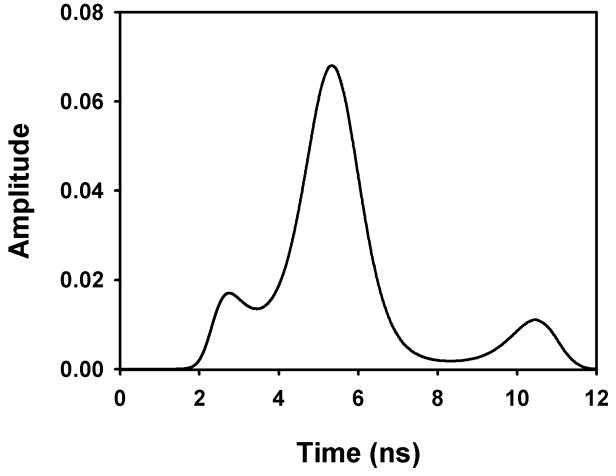
Immobilised in aqueous



Immobilised in ACN



Inactivated in ACN

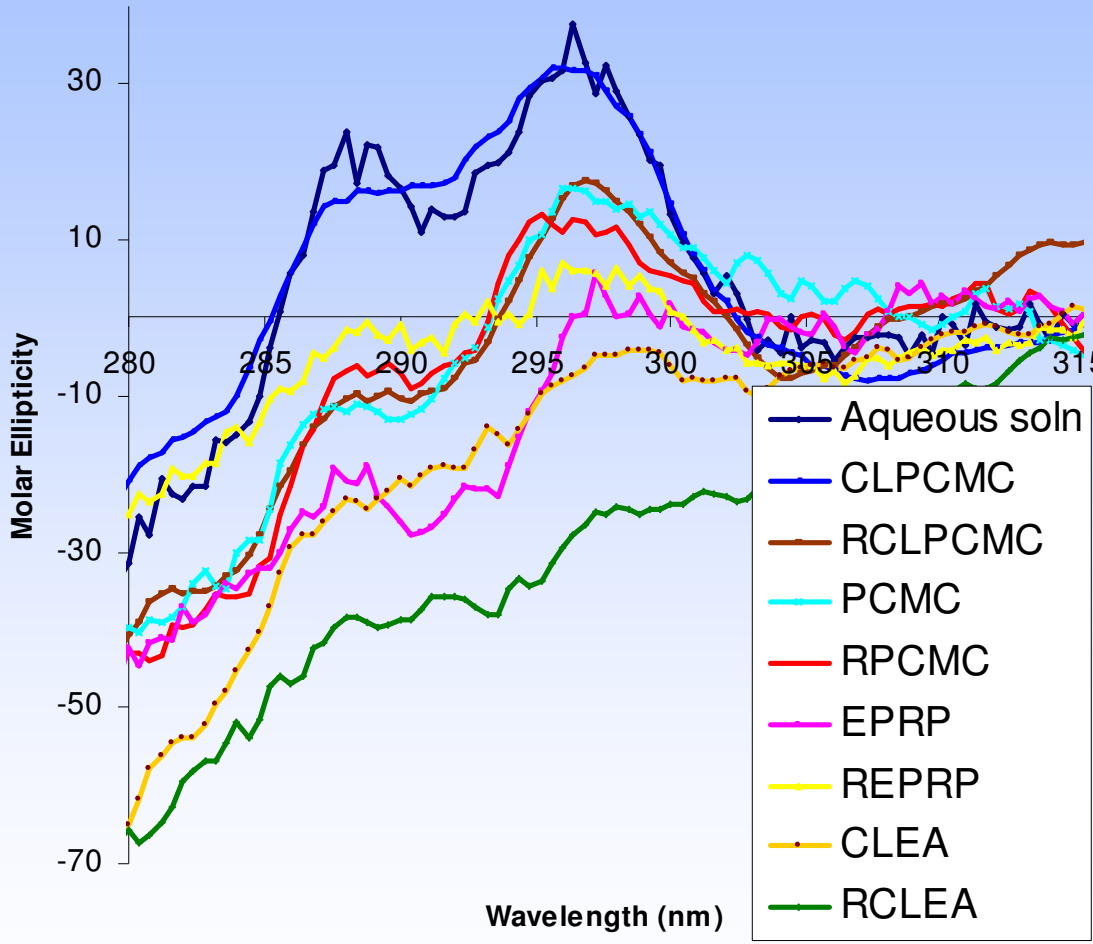


Time-resolved decay curves analysed by maximum entropy method

Ashok Ganesan et al, 2009

University of Strathclyde Glasgow

Prominence of 297 nm CD band correlates well with catalytic activity in octane



Catalytic activity (nmol $min^{-1}mg^{-1}$)
124
54
94
68
40
20
21
13

Various chymotrypsin preparations

Kusum Solanki, Munishwar Gupta, Peter Halling, unpublished

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CONCLUSIONS

- Improving understanding of effects on equilibrium and kinetics during enzyme action on immobilised substrates
- Better understanding of the state of enzyme molecules in immobilised preparations using spectroscopic techniques, especially circular dichroism

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ACKNOWLEDGEMENTS

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- Gail McConnell, John Girkin (Strathclyde Biophotonics)
- Sabine Flitsch (Manchester)
- Rein Ulijn (now back at Strathclyde)
- Nick Price, Sharon Kelly (CD facility, Glasgow Univ)
- Kusum Solanki, Munishwar Gupta (Chemistry, IIT Delhi)

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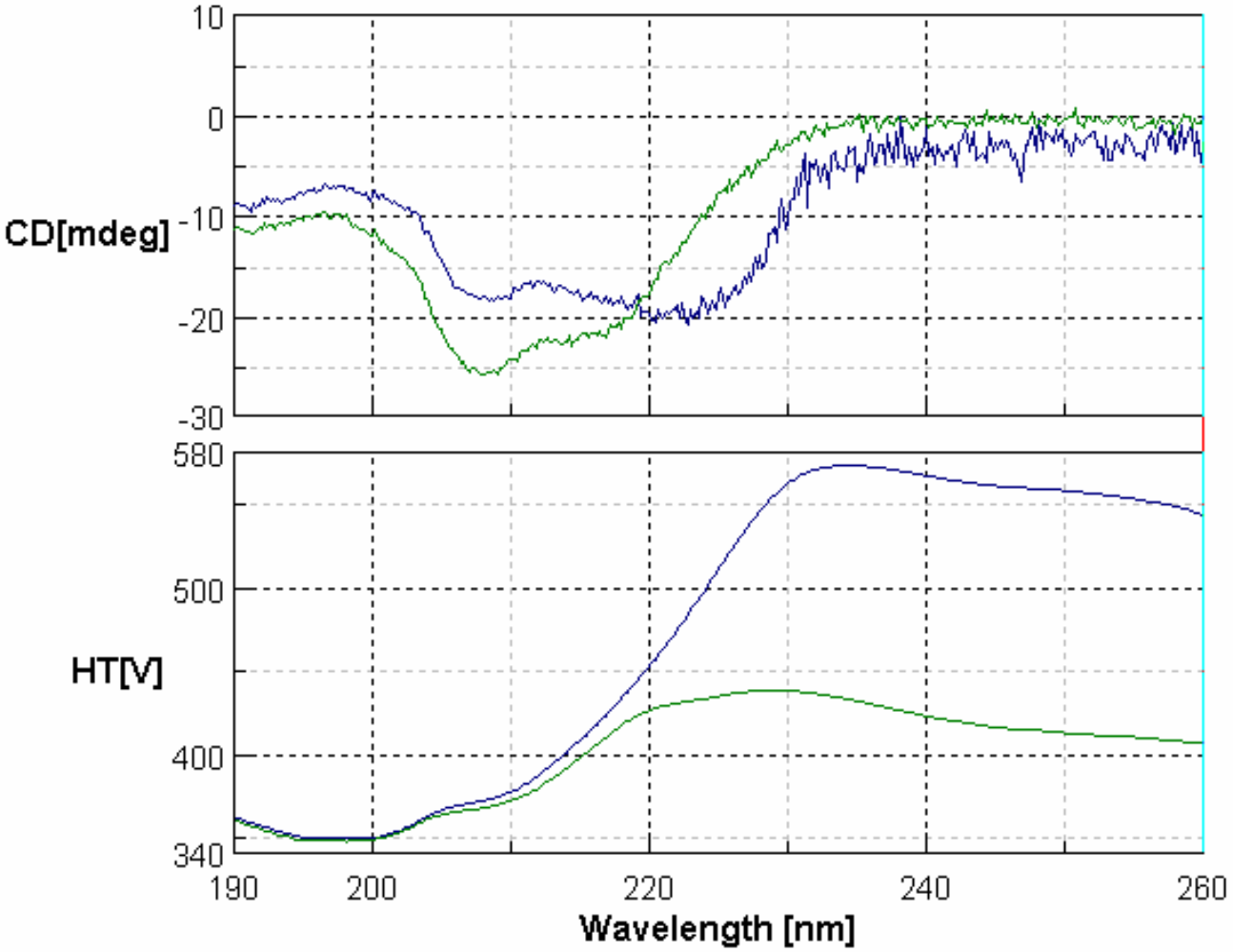
Far UV CD is masked by signal from support



Green: Lewatit VPOC 1600
Blue: Novozyme 435
In ACN 1% water.

Ashok Ganesan et al, unpublished

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Strong CD signal from support is totally unexpected and theoretically almost impossible!

- Shows that this synthetic polymer has significant chirality
- Reproducible, even different batch numbers
- Polymer has plenty of chiral centres, but generation from achiral monomers (methyl and butyl methacrylates, divinylbenzene) should generate equal amounts of R and S
- Presumably not the Nobel Prize for discovering parity violation – perhaps some stray chiral impurities during synthesis
- Found also in Duolite and an anion exchange Lewatit

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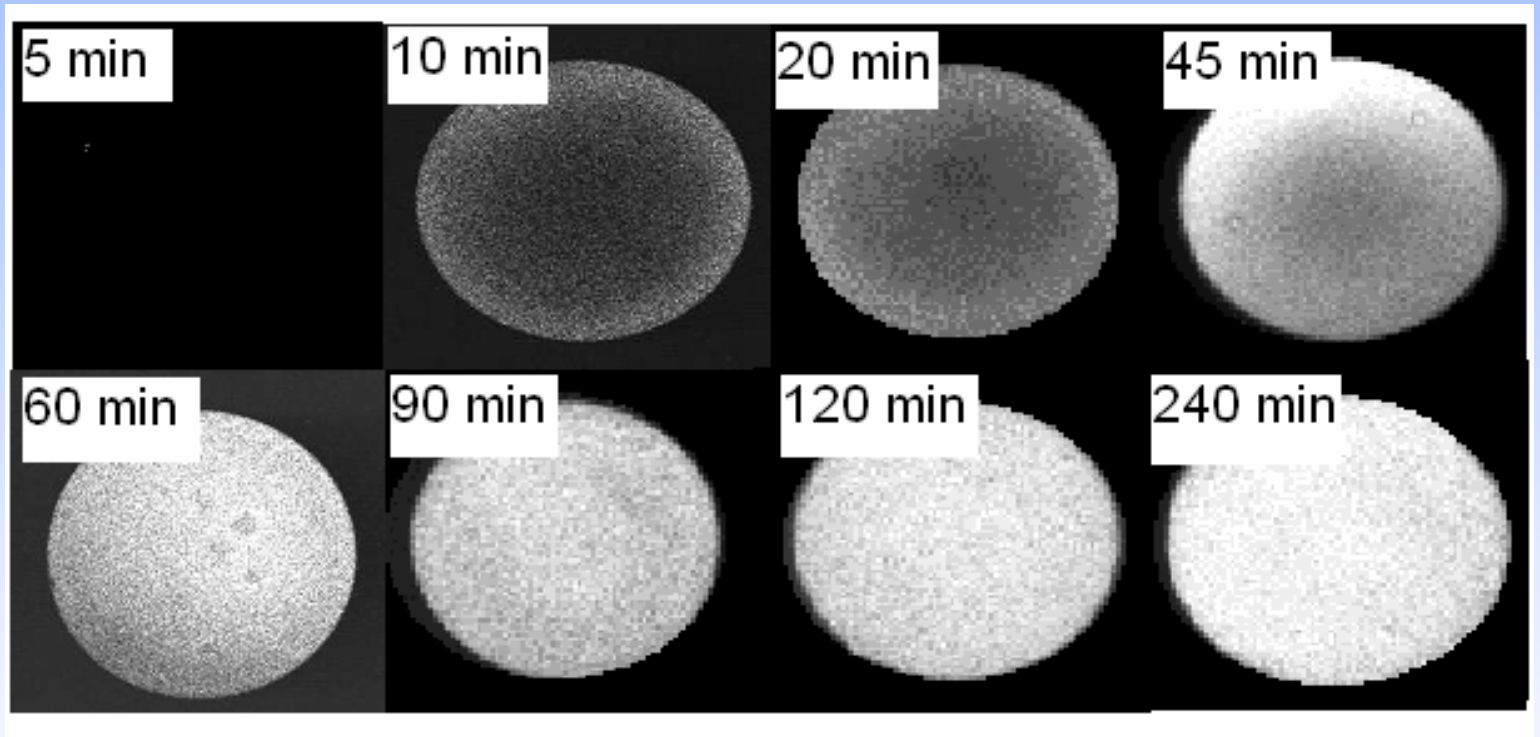
For kinetic studies, it would be ideal to resolve sites of reaction in bead

- Needs some kind of imaging method

Two-photon microscopy allows spatially resolved kinetics on solid phase substrates



Thermolysin catalysed hydrolysis of Fmoc-Phe-Phe-PEGA, examined by dansyl labelling of released amino groups and two-photon microscopy, giving optical sections

Annie Bosma et al, 2003

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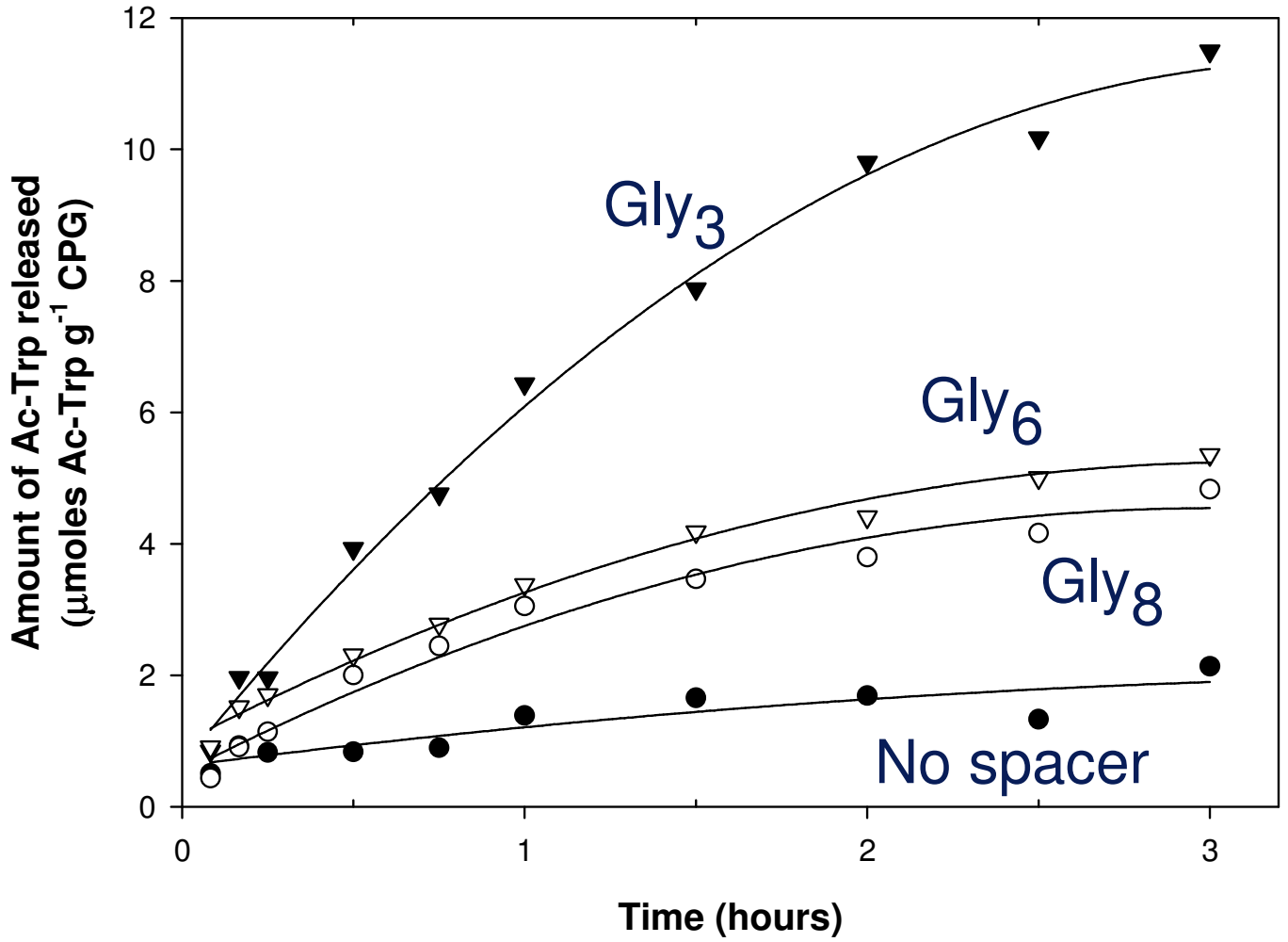
Release of Ac-Trp from CPG



Joe Deere, Antonia Lalaouni, Laura Solares et al, 2008

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Spacer length effects

- In absence of any spacer, see enzyme preference for amine leaving group structure
- Gly_1 or Gly_2 sufficient for maximal rate with PEGA, probably because of flexibility of PEG chains
- Optimum at Gly_4 with CPG
- Decline with longer spacers may reflect known structure change in oligoglycines

Enzyme reactions on solid-phase substrates

- Can get large and synthetically useful shifts in equilibrium compared to equivalent reaction in solution
- Methods being developed to study effects on kinetics with spatial resolution
- Spacers are important for optimal rate, but best length depends on support type

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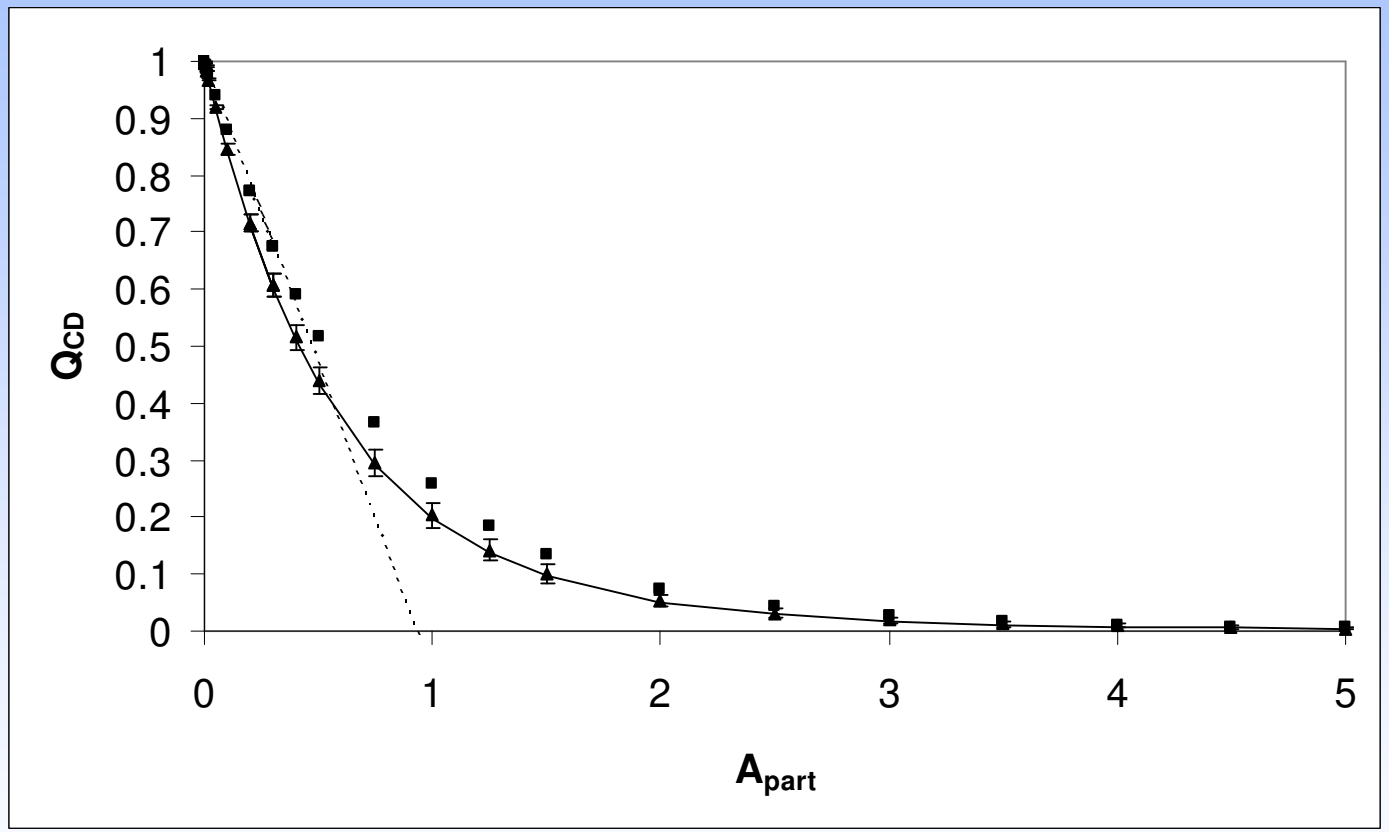
Most physico-chemical techniques used to study dissolved proteins suffer problems with suspensions

- Light scattering affects/prevents optical methods
- May need mixing to keep in suspension
- Molecular scale physics altered (e.g. NMR relaxation)

Mathematical models of absorption or CD flattening

- Series of literature treatments (since Duysens, 1956!)
- All require some approximations or assumptions, e.g.
  - spherical particles
  - uniform size
  - very low volume fraction
- Give flattening coefficient, observed divided by unflattened signal – e.g. Q_{CD}

Flattening coefficient for CD estimated by simulation



Simulations for pathlength twice particle size, volume fraction 0.01 (triangles) or 0.1 (squares). A_{part} is the absorbance along a diameter through one particle. Lines are for literature analytical models. Halling (2009).

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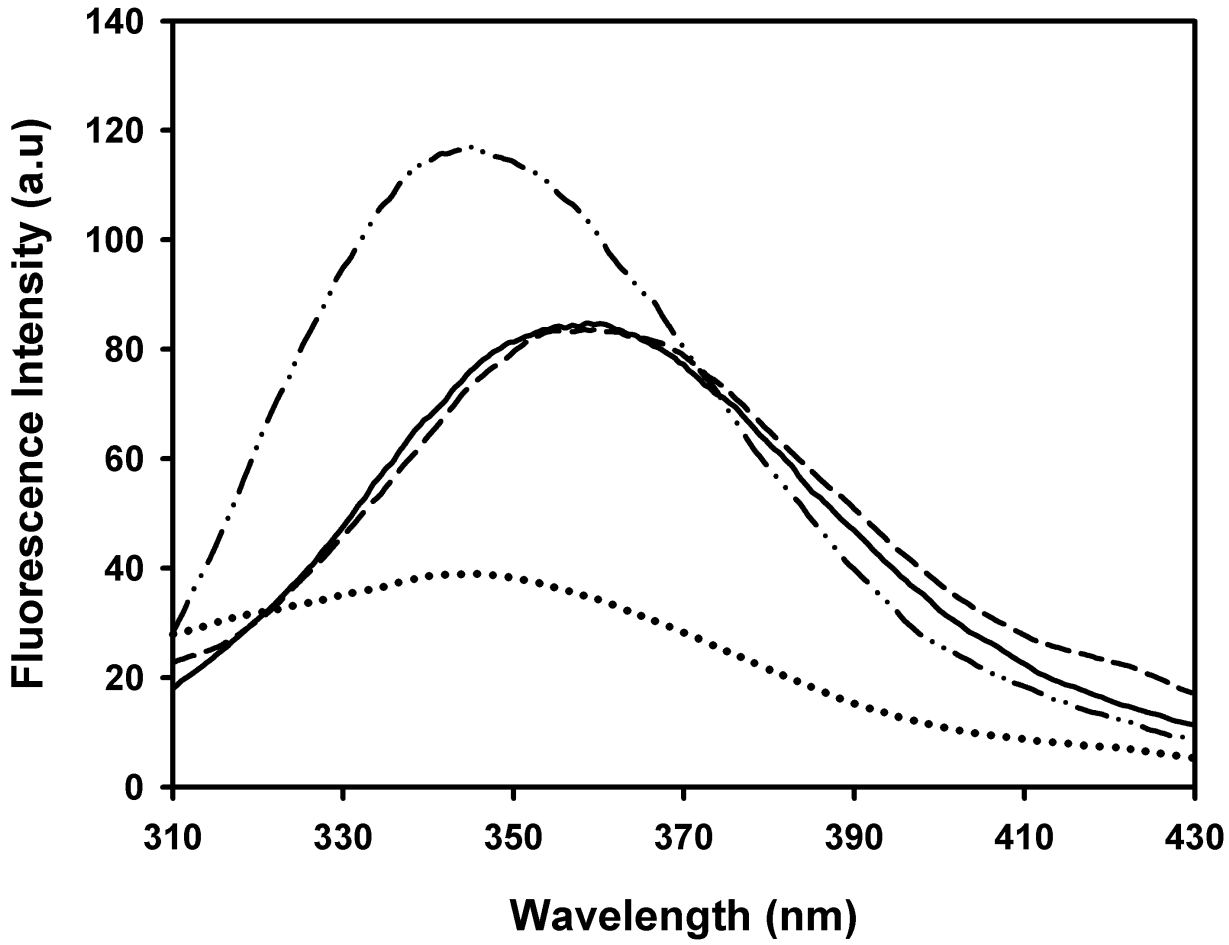
Simulation is readily adapted to handle particle size range

		Q_{CD}	
Particle volume fraction	Pathlength/ Particle size	monodisperse	size range
0.01	2	0.213 ± 0.014	0.174 ± 0.010
0.01	200	0.200 ± 0.009	0.186 ± 0.007
0.1	2	0.253 ± 0.004	0.207 ± 0.004

Simulation results for absorbance = 1 through particle diameter, with either monodisperse particles, or standard deviation 20% of mean size. Halling (2009).

Fluorescence of endogenous Trp greatly enhanced in inactivated biocatalyst escapes intramolecular quenching



Excitation at 295 nm.
Aqueous solution (—), silica-subtilisin in aqueous (- - -), in ACN (· · · ·), and inactivated (-···-···-)

Ashok Ganesan et al, 2009.

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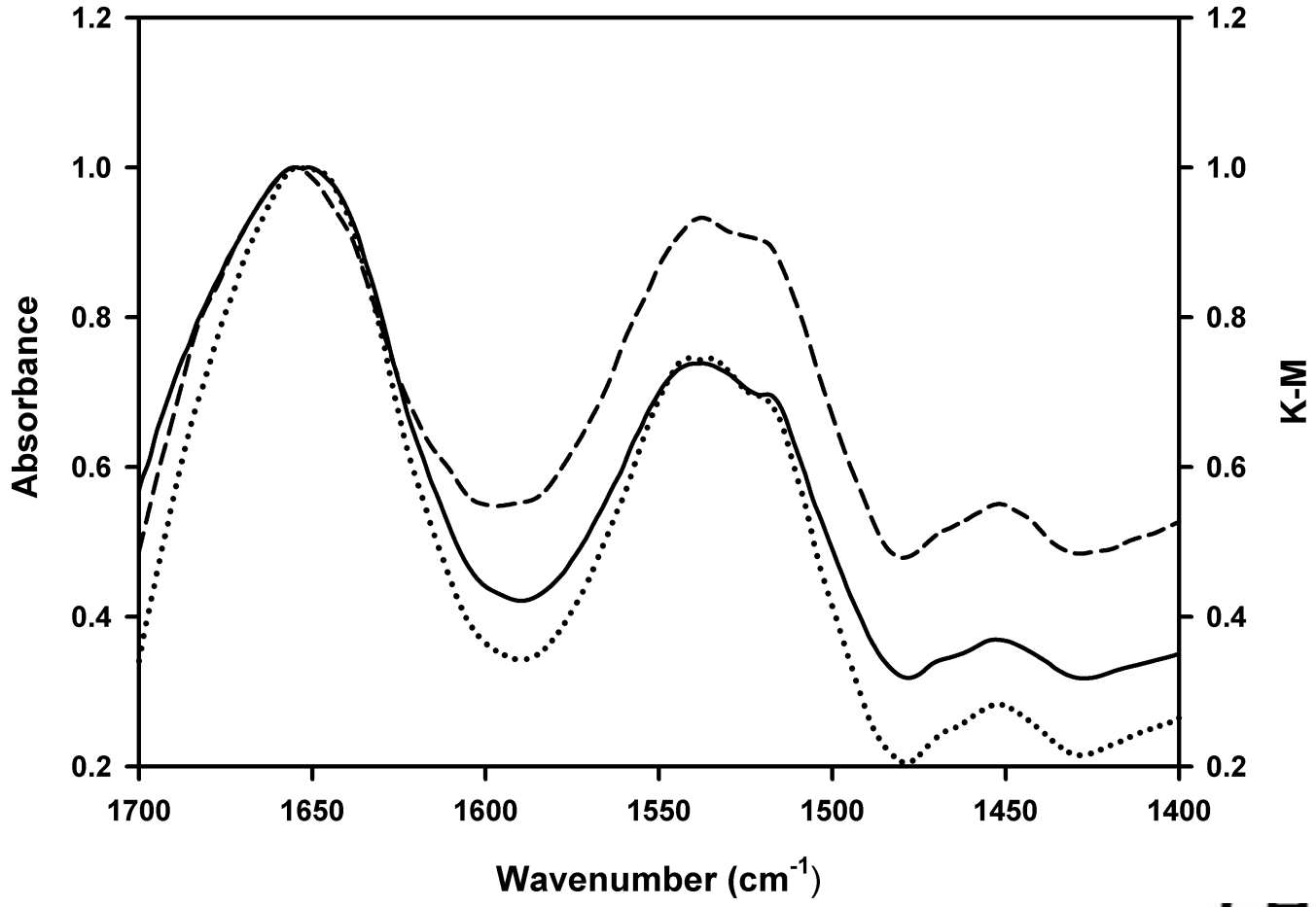
Pressing discs for transmission IR may perturb protein structure, can be avoided by DRIFT



Spectra of lyophilised subtilisin, measured in pressed KBr discs by transmission (—) or DRIFT (·····), in mix with KBr by DRIFT (- - -).

Ashok Ganesan et al, 2009

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Experimental requirements for proper CD flattening correction

- Volume fraction of particles – usually should be known
- Particle size distribution – should be measurable
- Correct treatment of scattering contribution to measured absorbance – wide angle collection used anyway to reduce differential scattering contribution
- Parallel measurement of absorbance of CD sample suspension – should be obtainable from data for photomultiplier voltage