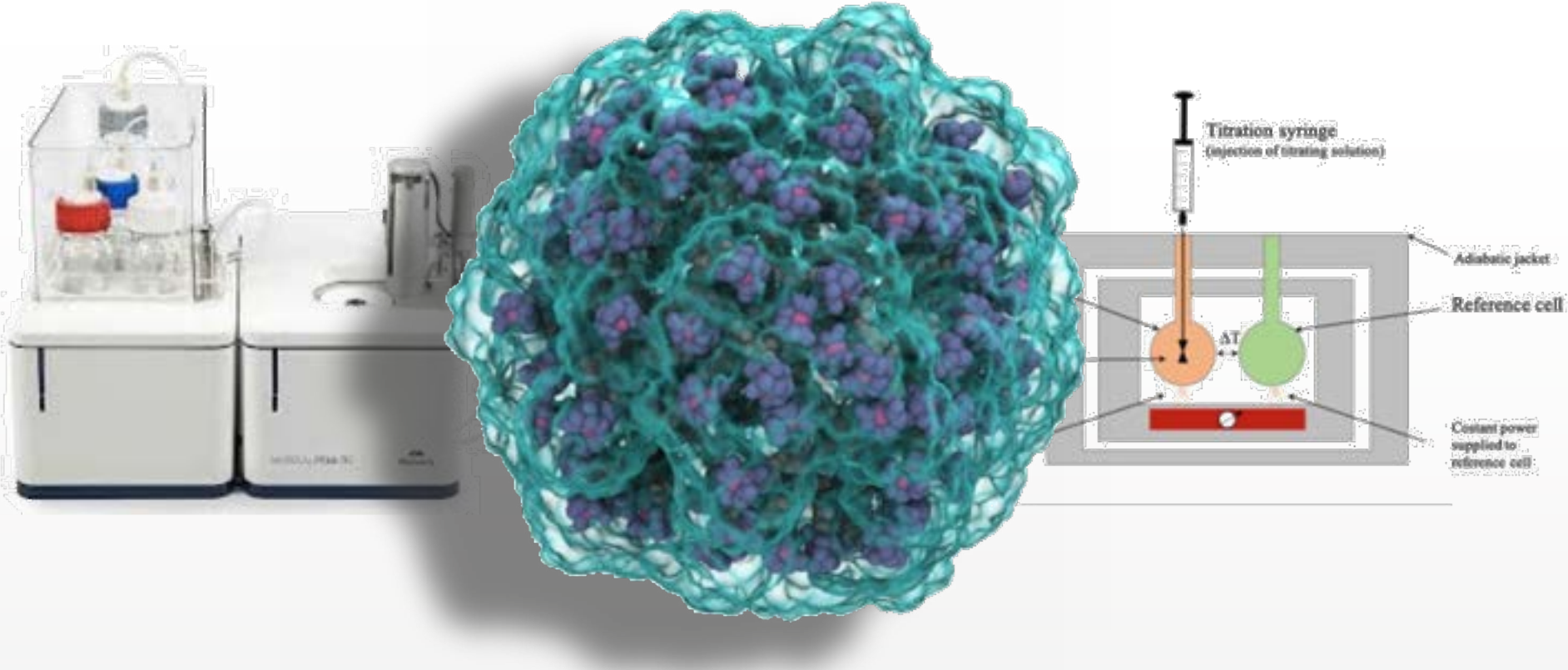

Isothermal Titration Calorimetry (ITC) in cancer nanomedicine

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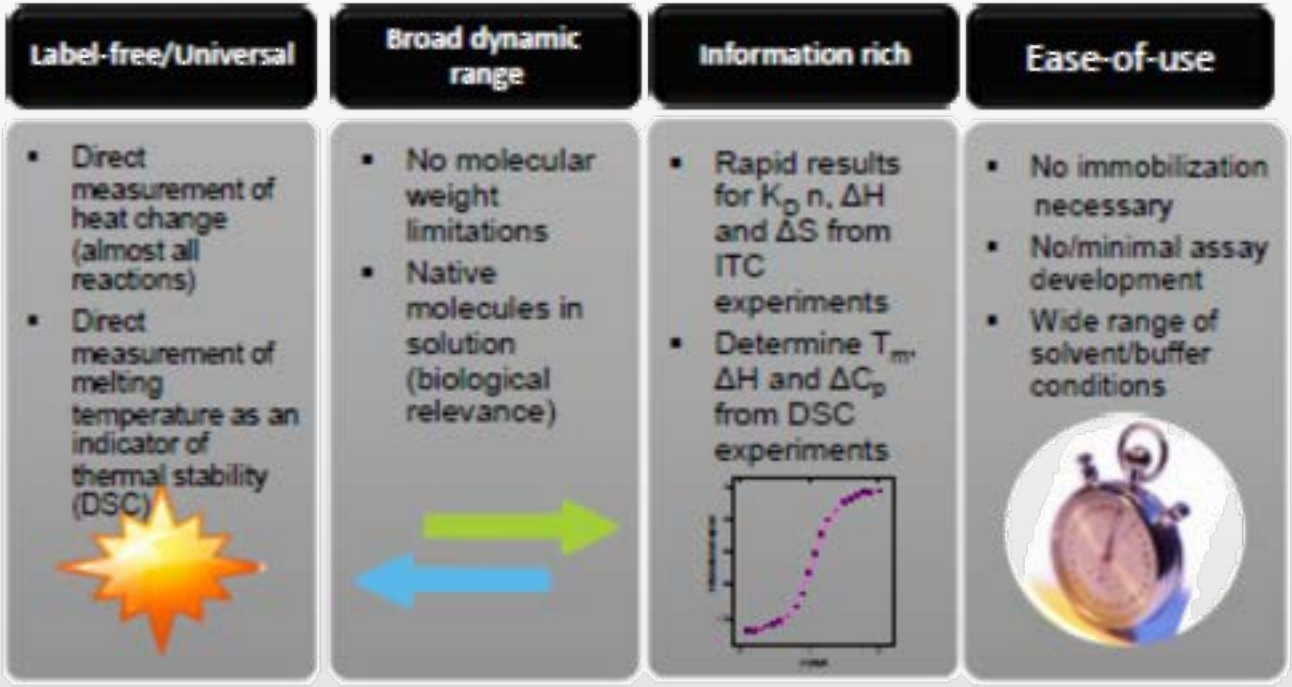
Schedule:

- ITC – Why?
- ITC – Principles
- ITC – Case studies:
  - ITC-1 Anticancer drug / HSA binding study
  - ITC-2 Self-assembling ADs for imaging
  - ITC-3 AM Self-Assembly Study
- ITC – Technical Notes

ITC – Why?

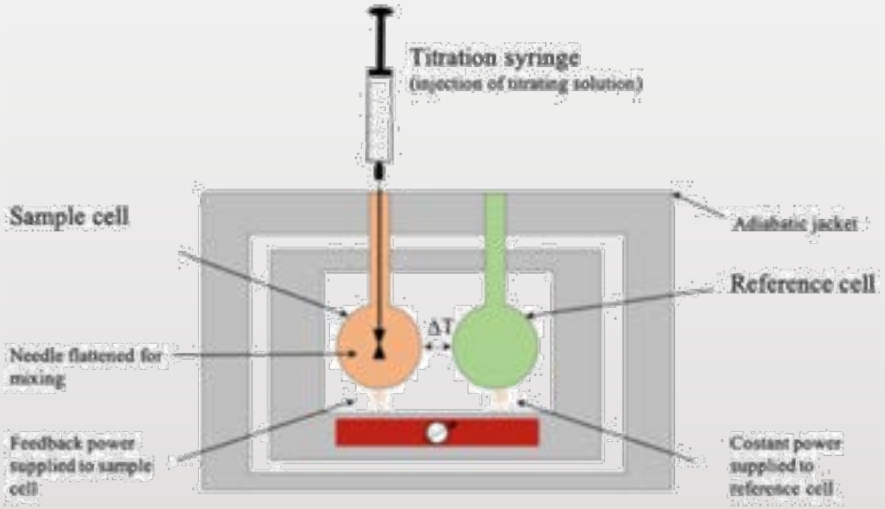
ITC can provide a direct measurment of the heat from the interaction between two molecules in solution.



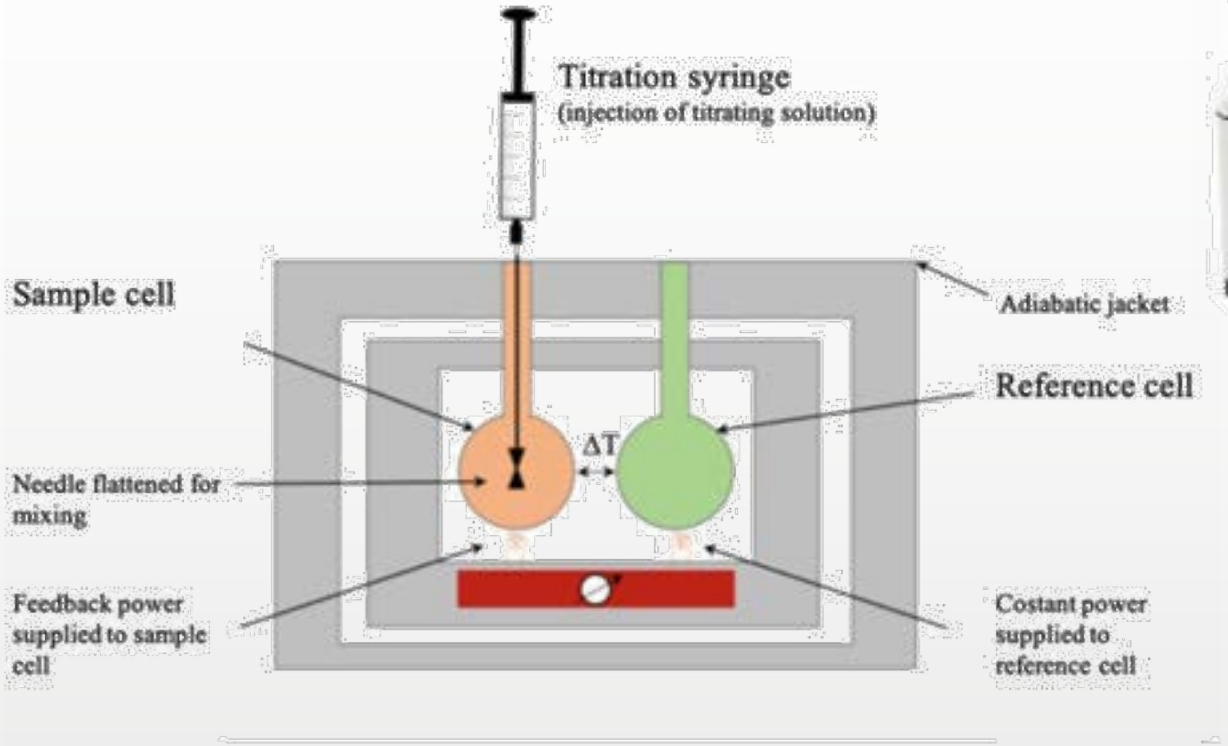
ITC – Principles

- Calorimetric principles:
  - Heat Flux
  - The heat is allowed to flow out (or into) the cell. The potential difference is recorded as a function of time, and the signal will come back to the baseline (thermal equilibrium).

- Power compensation
  - The sample and reference cells are heated permanently by separate heaters (very low intensity) and there is a feedback system that controls and varies the power supplied to each heater to keep the temperature difference between the cells ≈ 0. When an event happens on the sample cell, the power is increased (decreased) to keep $\Delta T \approx 0$.



ITC – Principles



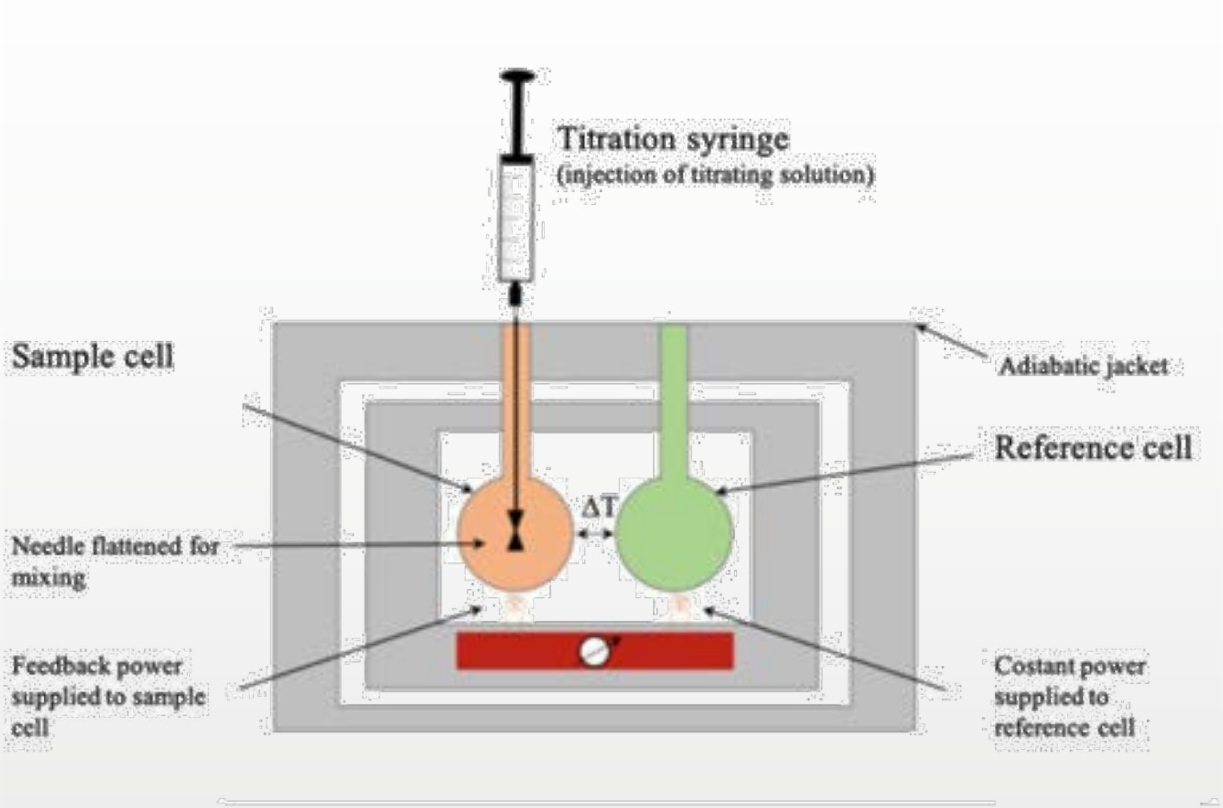
Principal components:

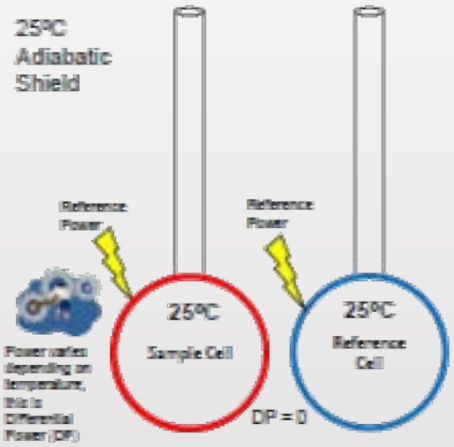
(i) Titration syringe (ligand solution);

(ii) Sample cell (protein solution);

(iii) Reference cell (water or buffer solution)

ITC – Principles

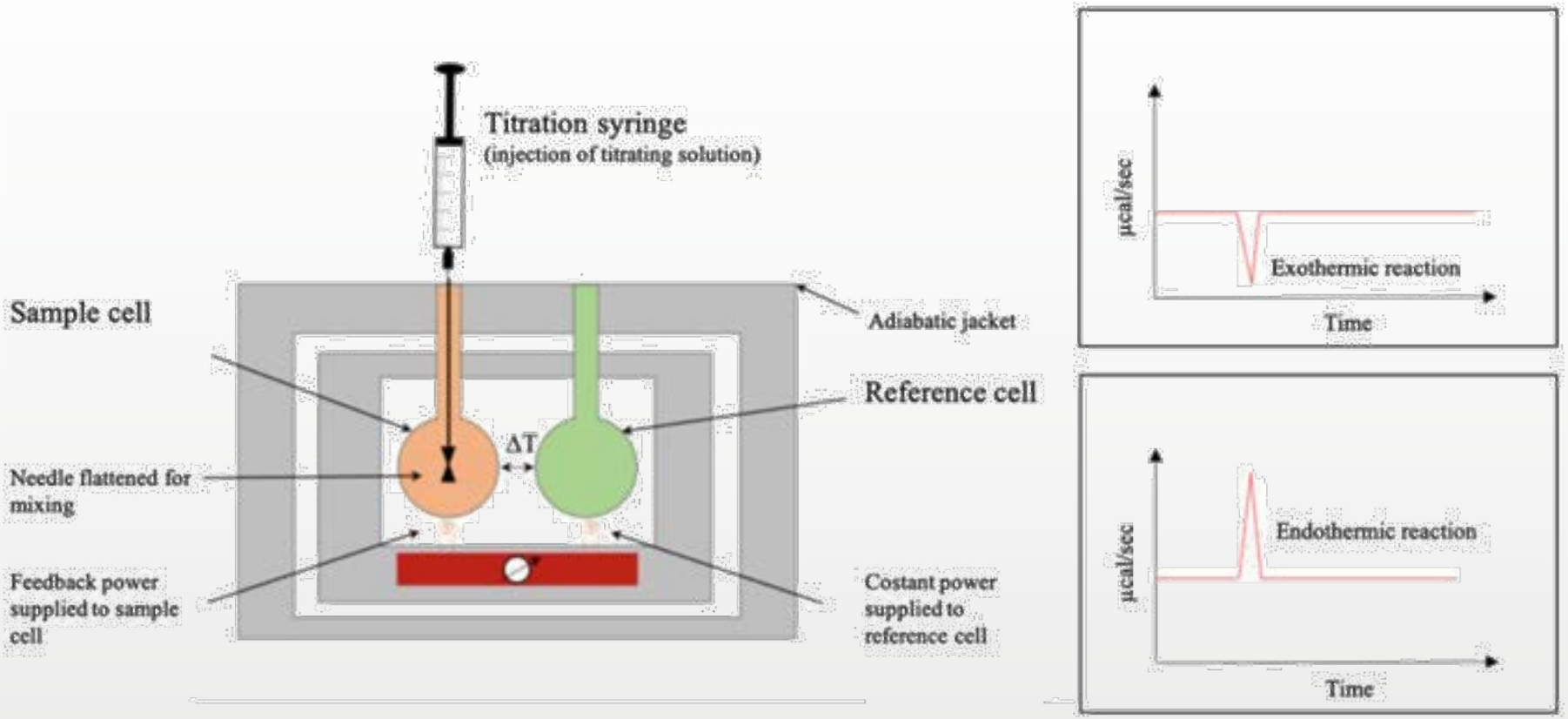




How does it works?

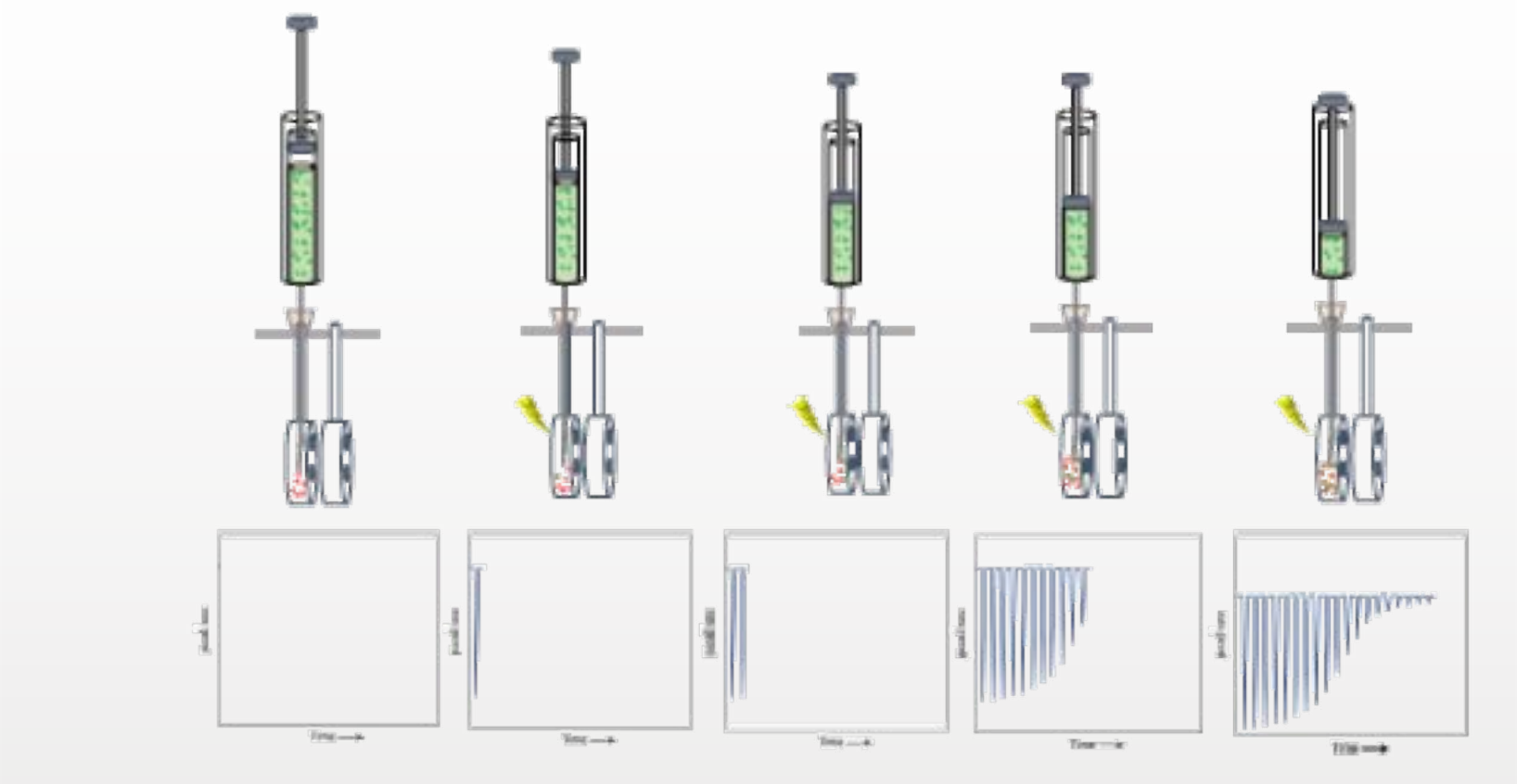
- Heat flux (µcal or µJ / s);
- Differential power (DP) calculation;
- Sample cell and reference cell in thermal equilibrium ($\Delta T° = 0$)

ITC – Principles



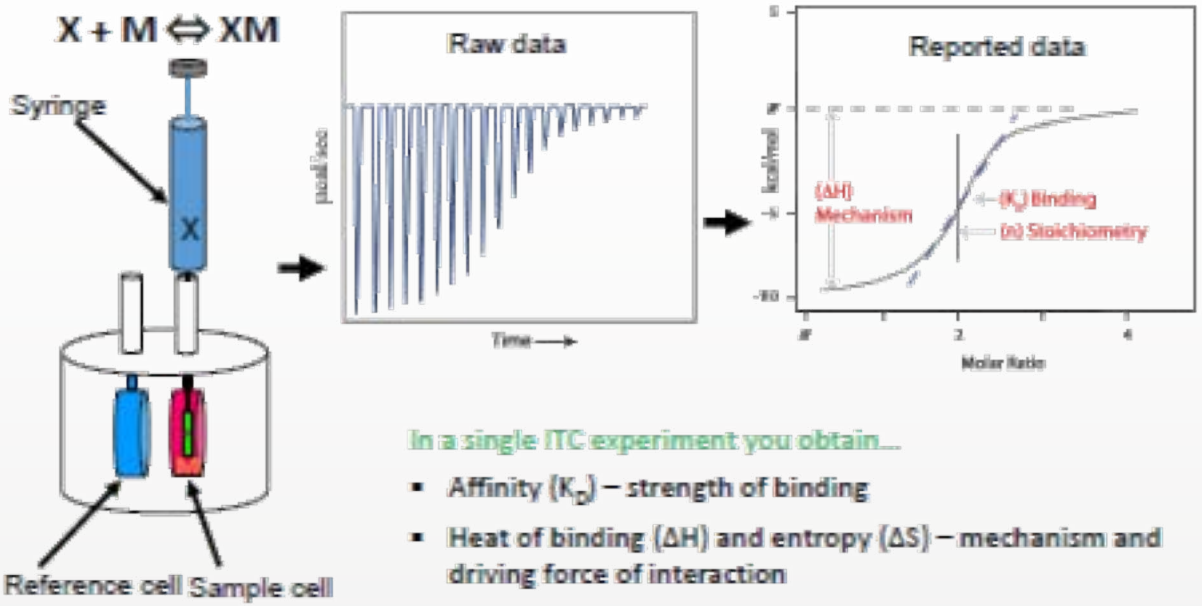
- Reference power is applied to both cells → power compensation;
- Heat release → exothermic reaction;
- Heat absorption → endothermic reaction.

ITC – the thermogram (raw data)



- restoring the heat-flux to baseline;
- toward the end of the titration, the heat signal becomes very low;
- saturation by the titrant → only background heat due to unspecific phenomena (i.e., ligand dilution or liquid friction) is observed.

ITC – Data integration



- Direct results :
  - K_d and n (stoichiometry) = inflection point
  - ΔH_{bind} = differences between lower and upper plateaus
- Indirect results :
  - $\Delta G_{bind} = RT\ln K_d$
  - $-T\Delta S_{bind} = \Delta G_{bind} - \Delta H_{bind}$

ITC – Binding mechanism

Interaction

- $P + L \leftrightarrow PL$
- $K_D = [P][L]/[PL]$
- $K_A = [PL]/[P][L]$
- K_D is inverse of K_A

Thermodynamics

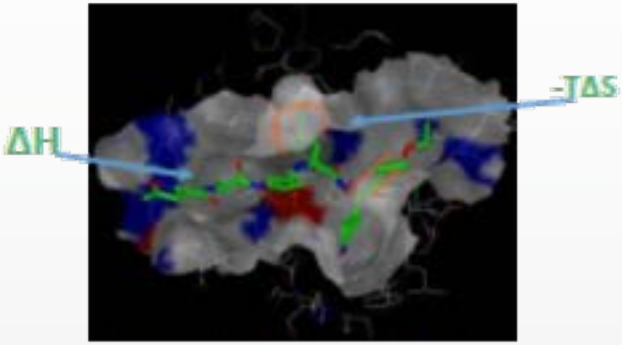
- $\Delta G = RT\ln K_D$
- $\Delta G = \Delta H - T\Delta S$

ΔG is Gibbs free energy change

R is gas constant

T is temperature (Kelvin)

Overall binding affinity K_D correlates with IC_{50} or EC_{50}.
This is directly related to ΔG, the total free binding energy



- ΔH, enthalpy is indication of changes in hydrogen and van der Waals bonding
- -TΔS, entropy is indication of changes in hydrophobic interaction and conformational changes
- n, stoichiometry indicates the ratio of ligand-to-macromolecule binding

- Primary Enthalpic Contributions
  - Hydrogen bonding and van der Waals interactions
- Primary Entropic Contributions
  - Hydrophobic effect-water release (favorable)
  - Conformational changes and reduction in degrees of freedom (unfavorable)

ITC – Binding mechanism

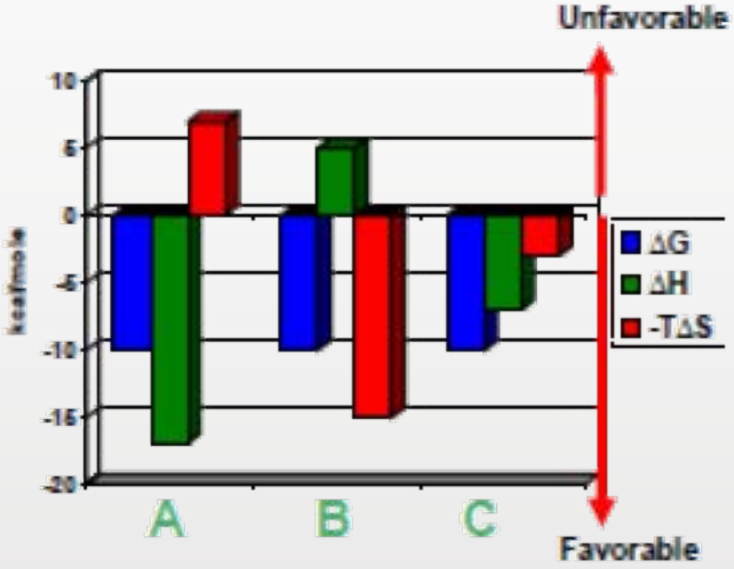
Same affinity, different energetics

ITC results are used to gain insights into the mechanism of binding

A. Good hydrogen bonding with unfavorable conformational change

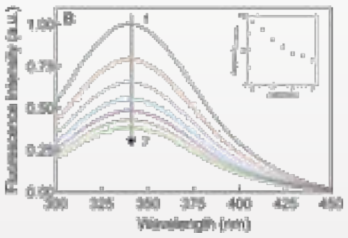
B. Binding dominated by hydrophobic interaction

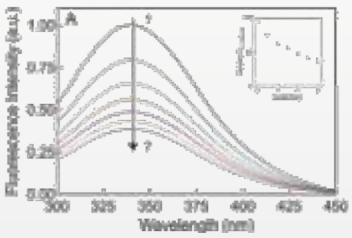
C. Favorable hydrogen bonds and hydrophobic interactions

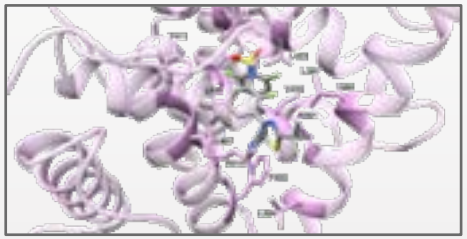


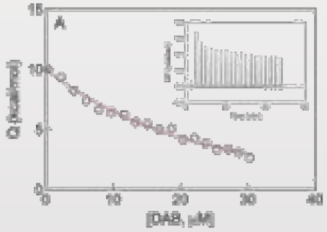
ITC-1 Anticancer drug / HSA binding study

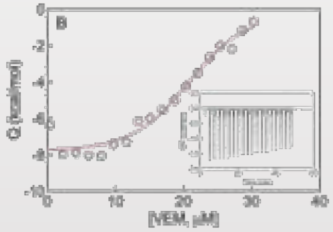
- Drug binding to HSA significantly affect biological activity
- Binding mechanism of the two B-Raf inhibitors dabrafenib and vemurafenib to HSA
- combined strategy = fluorescence spectroscopy + ITC + Molecular Modeling
- Thermodynamics and kinetics information

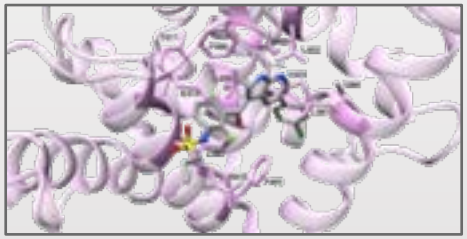








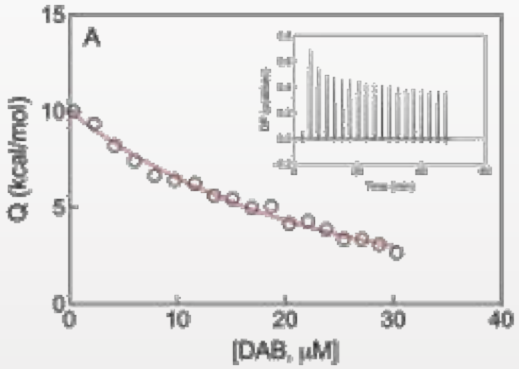


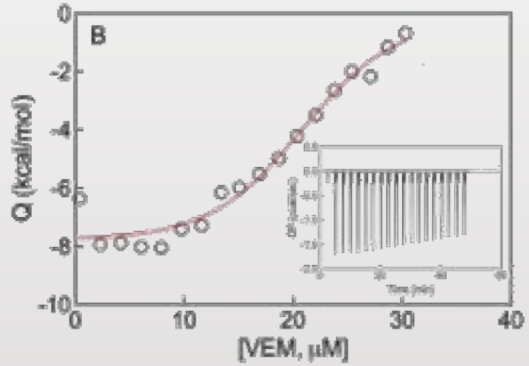


- **K_a**
- **$\Delta G, \Delta H, T\Delta S$**
- **K_{off}**
- **K_{on}**
- ***t_r***

ITC-1 Anticancer drug / HSA binding study

- 1:1 stoichiometry + comparable affinity
- within the same binding pocket (subdomain IIIA)
- dabrafenib/HSA complex is more entropically driven
- vemurafenib/HSA assembly is prevalently enthalpic in nature





	ΔG kcal/mol	ΔH kcal/mol	-TΔS kcal/mol	k_a 10^5 M^{-1}	k_{on} 10^4 $M^{-1}s^{-1}$	k_{off} 10^{-2} s^{-1}	t_r s
DAB	**-7.19**	**+5.12**	**-12.31**	**1.86**	**1.57**	**8.44**	**11.8**
VEM	**-7.25**	**-5.27**	**-1.98**	**2.06**	**1.12**	**5.42**	**18.5**

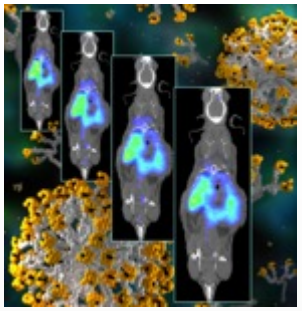
$$k_a = k_{on}/k_{off}$$

$$t_r = 1/k_{off}$$

- dabrafenib/HSA has short residence time
- vemurafenib/HSA is provided with a slightly greater residence time.

ITC-2 Self-assembling ADs for imaging

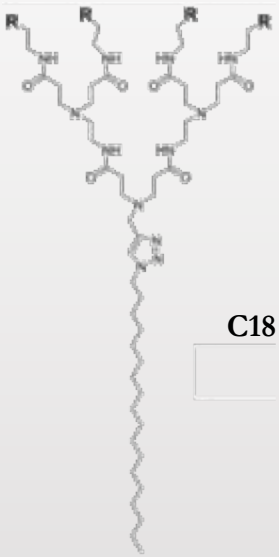
- efficiently deliver contrast or imaging agents
- better and more precise imaging:
  - Improving imaging sensitivity and specificity
  - Reducing toxicity
- an innovative nano-system for:
  - positron emission tomography (**PET**) imaging
  - single photon emission computed tomography (**SPECT**) imaging
  - **C18** single tail PAMAM-based AD
  - surface of ADs decorated with different radionuclide
    - Ga3+ or Gd3+ or In3+
  - complexed within different macrocyclic chelator
    - NOTA or DOTA cage



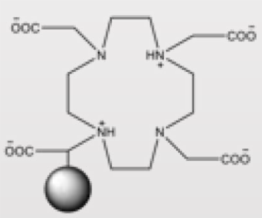
Proc Nat Acad Sci USA 2018; 115:11454-11459.

Chem.Comm 2020; 56:301-304.

Small 2020; 2003290.

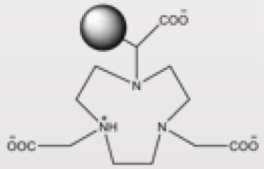


C18 AD



DOTA

1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid

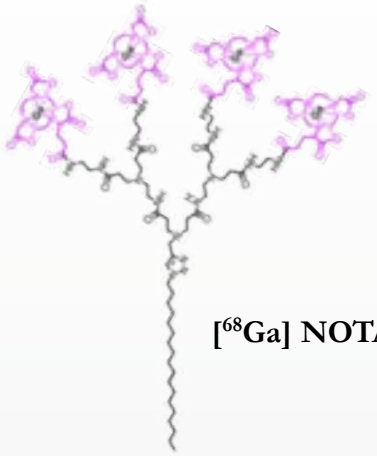


NOTA

1,4,7-triazacyclononane-1,4,7-triacetic acid

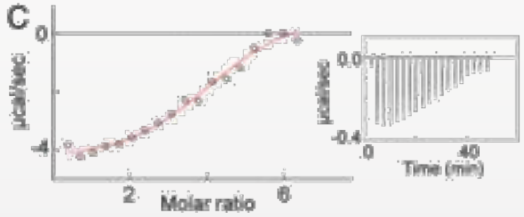
ITC-2 Self-assembling ADs for imaging

- **gallium-68** as **PET** radioisotope with half-life of 68 min
- NOTA as Ga3+ chelator
  - optimal size
  - geometry
  - denticity



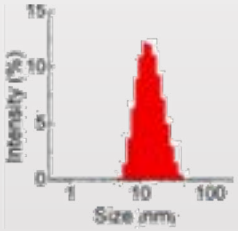
[^{68}Ga] NOTA NV

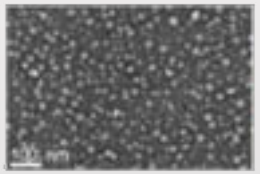
- favorable enthalpic and entropic contributions to the chelation
- ITC confirmed:
  - gallium binding thermodynamics
  - occupied NOTA site (n = 4)

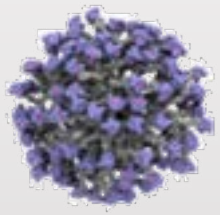


ΔG kcal/mol	ΔH kcal/mol	-TΔS kcal/mol
-6.99	-4.05	-2.94

- DLS, TEM and MD simulation provided:
  - self-assembly confirmation
  - size (14 nm) and shape (spherical micelles)

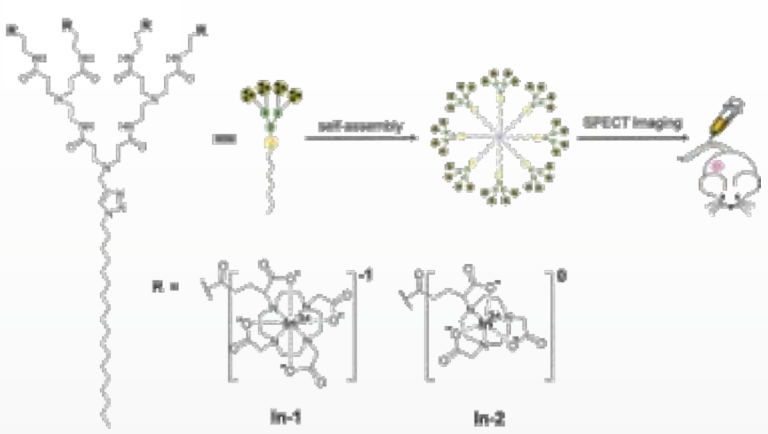




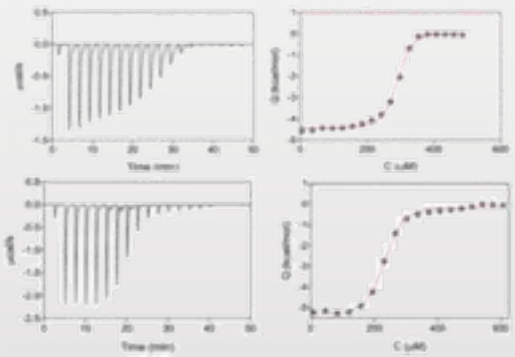


ITC-2 Self-assembling ADs for imaging

- **Indium-111** as SPECT radioisotope
- DOTA (**In-1**) or NOTA (**In-2**) as chelator
- Characterization:
  - **ITC**
  - DLS
  - TEM
  - Computational Studies

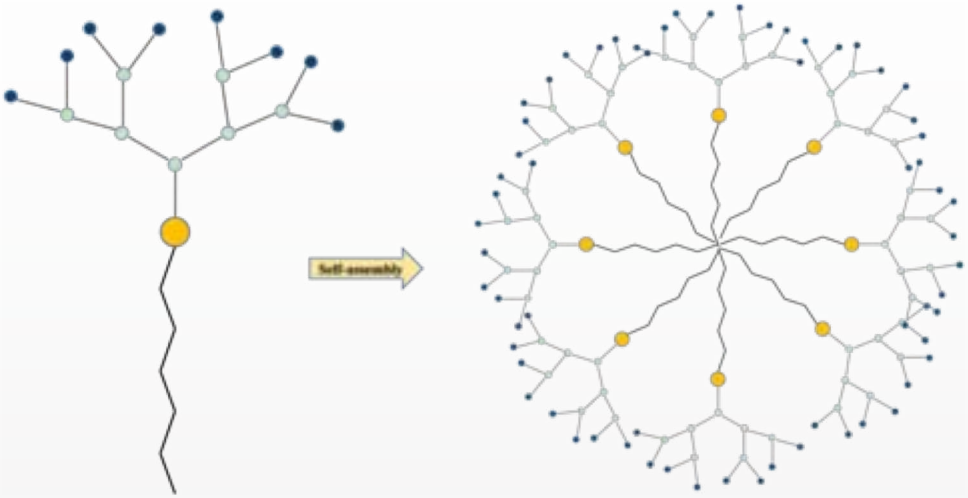


	ΔG kcal/mol	ΔH kcal/mol	-TΔS kcal/mol
In-1	-7.86	-5.25	-2.61
In-2	-7.64	-5.43	-2.21



- similar thermodynamics
- both enthalpic and entropic contribution fovorable
- complete site occupation (n=4)

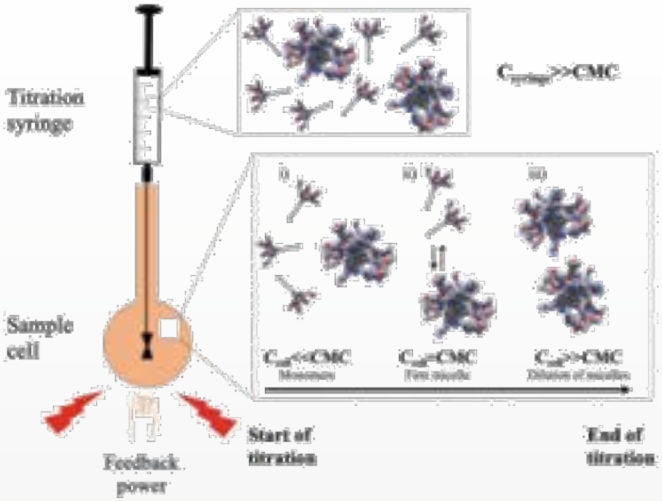
ITC-3 AM Self-Assembly Study



- Gene therapy
- Drug delivery

- Complete thermodynamics of the process:
  - ΔG → spontaneity
  - ΔH → mechanism
  - $T\Delta S$ → entropic contribution
- CMC : Critical Micellar Concentration
- N_{agg} : Aggregation Number

ITC-3 AM Self-Assembly Study



- Syringe: SLN of AM at concentration >> CMC.
- Sample Cell: solvent → H_2O or buffer
- Investigation of the demicelization process → ΔH_{demic}.
- self-assembly is reversible process

→ $\Delta H_{demic} = -\Delta H_{mic}$

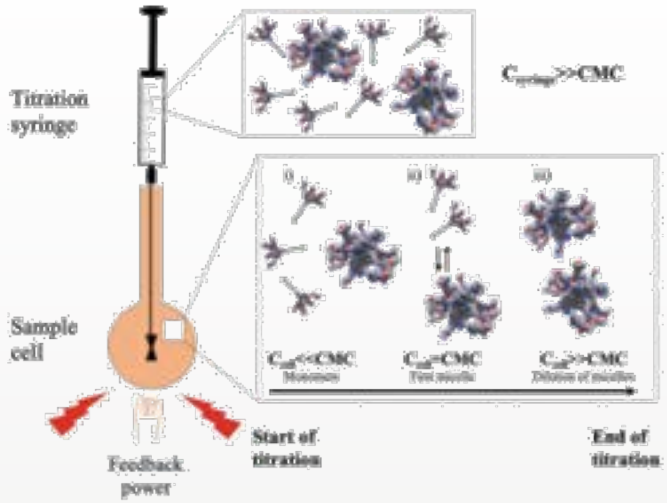
The ITC thermogram of a demicellization process is characterized by 3 distinct phases:

(i) micelles dissociate into monomers → the heat of the dilution of the monomer is recorded;

(ii) [AM] increases in the sample cell until reaching the CMC value → formation of micelles;

(iii) once the self-assembly process is finished → only the micellar dilution heat.

ITC-3 AM Self-Assembly Study



From the sigmoidal profile I can exstract:

(i) Inflection point → CMC;

(ii) First derivative Q vs C → more precise CMC;

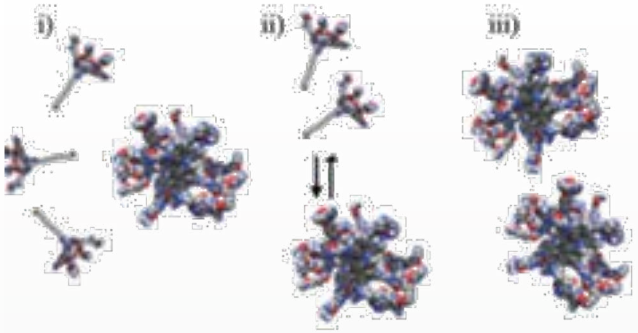
(iii) Difference between plateaus → $-\Delta H_{mic}$.

ITC-3 AM Self-Assembly Study

CMC validation:

- Pyrene Assay
- Conductimeter Assay
- ! " !#$%&

ITC-3 AM Self-Assembly Study



- N_{agg} = the average number of monomers in a spherical micelle;
- characterized by the two-state reaction model→

$N_{agg} * S = MIC_{Nagg}$

- S = amount of monomer
- MIC_{Nagg} = amount of micelles

- by regression analysis of the normalized ITC integrated data =

$$\frac{dln\left(\left(\frac{d[AM]}{dAM_{TOT}}\right)^{-1} - 1\right)}{dln[AM_{TOT}]} = \frac{N_{agg} - 1}{N_{agg}} + \frac{\left(N_{agg} - 1\right)^2}{N_{agg}} \frac{d[AM]}{d[AM_{TOT}]}$$

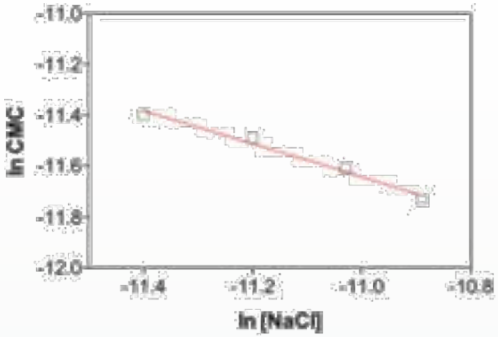
- [AM] = is the concentration of the AM molecules in their monomeric state
- $[AM_{TOT}]$ = total concentration of AM during ITC experiement

- → From the principle of mass conservation : $[AM_{TOT}] = [AM] + N_{agg} * [MIC_{N_{agg}}]$
- *Sum of Squares due to Regression* (SSR) methods is applied for retrieve N_{agg} from regression analysis

ITC-3 AM Self-Assembly Study

- $\Delta G_{mic} \sim -RT * \ln (1/CMC)$

Warning! Only for neutral AM!



For charged AM:

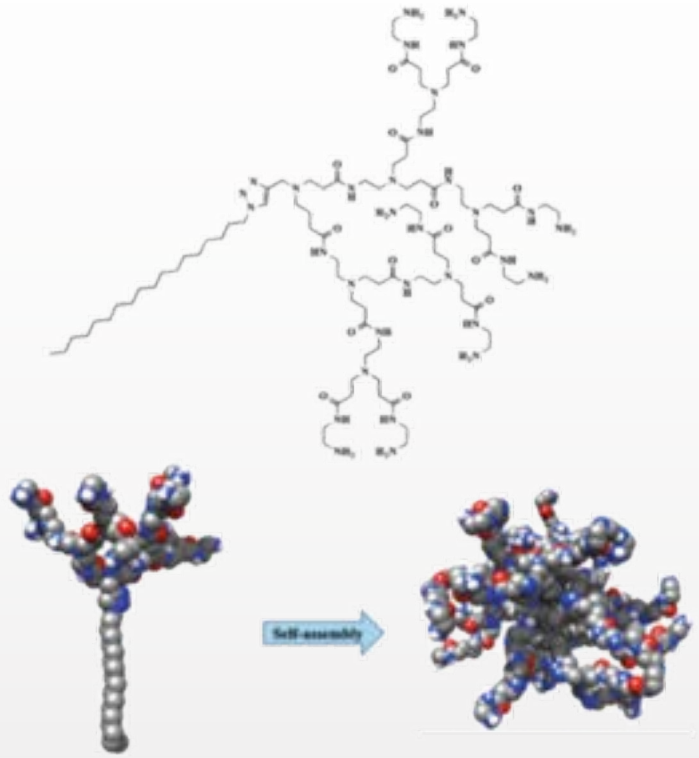
- $\Delta G_{mic} = - (1+\beta)RT * \ln (1/CMC)$
- β = degree of counterion binding
  - Repeat experiments to calculate CMC at different [NaCl] → ionic strength
  - Plot the log of the obtained CMC values vs log [NaCL]

    There is a linear relationship ($\ln CMC = -\beta * \ln[Cl^-] + K$)
  - → the slope of the data linear regression= β

$$\Delta G_{mic} = \Delta H_{mic} - T\Delta S_{mic}$$

ITC-3 AM Self-Assembly Study

ΔH_{mic}	$T\Delta S_{mic}$	ΔG_{mic}
kcal/mol	kcal/mol	kcal/mol
18.67	**28.84**	**-10.17**
CMC	β	N_{aggf}
μM^1	(-)	(-)
11.2	**0.65**	**10.07**

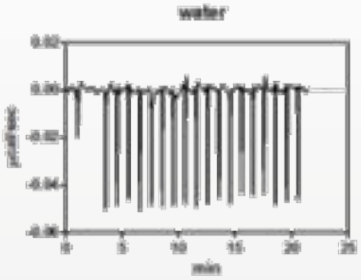


ITC – Technical Notes

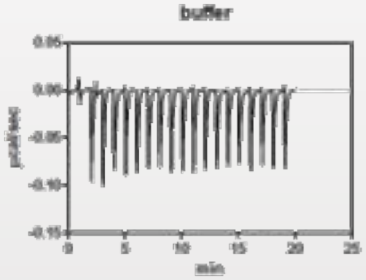
background heat due to unspecific phenomena

SIDE EXPERIMENTS:

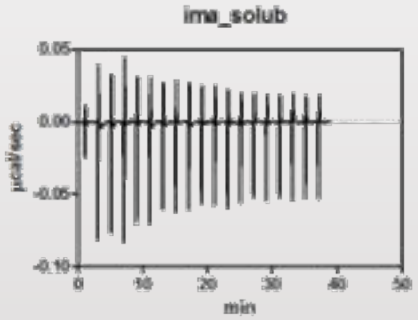
- water-in-water titration: heat from stirring



- buffer-in-buffer titration: heat from the protic dissociation



- titrant solubilization: heat from the solubilization of the titrant in the solvent:
    - titrant in syringe (at the same [M] of the ”normal” ITC
    - only buffer in the sample cell
    - point by point subtraction



ITC – Technical Notes

Cleanliness rules!!!

<u>The strength of ITC is that you can see everything</u>

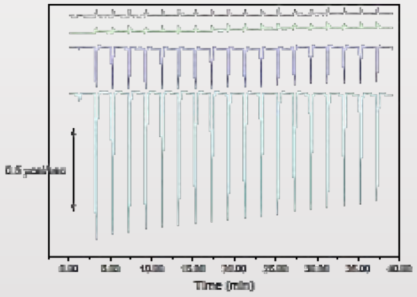
<u>The weakness of ITC is that you can see everything</u>

- 6 out of 11 solving problems in the Troubleshooting Guide for ITC best practice suggest: clean cell and syringe thoroughly
- clean module (automatic)
- Washing cell and syringe after each ITC experiments (10 min)
- Soaking at 65°C the sample cell weekly!

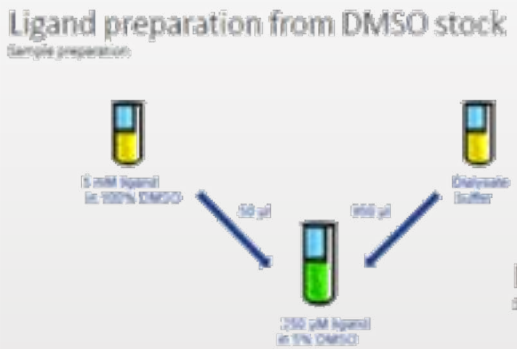
ITC – Technical Notes

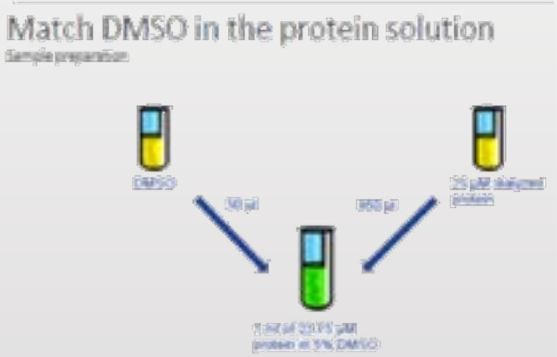
Buffer mismatch

- Different types of buffers can be used (PBS, HEPES, etc.) as ITC is compatible with all aqueous buffers in a range of pH 2–12
- the buffers in the sample cell and in the titration syringe MUST be identical
- otherwise the total heat measured might account for undesired contributions due to buffer mixing and dilution effect



Buffer into buffer
5% DMSO into 5% DMSO
5% DMSO into 4.5% DMSO
5% DMSO into 4 % DMSO





ITC – Technical Notes

Critical parameters

- Input concentrations are strictly related to the heat integration and the data fitting → precise determination of initial concentration in the sample cell and in the syringe

- Required volumes for a single ITC experiment:
  - 208 µL for the cell (300 µl for a good manual filling)
  - 36 µL for the syringe (automatic filling)

- Required concentrations for 1:1 stoichiometry complex:
  - 5 – 50 µM for the macromolecule in the sample cell
  - 50 - 500 µM for the ligand in the syringe

- Required concentrations for self-assembly study:
  - depend from CMC value (inflection point of the sigmoid)
  - can be optimized

ITC – Technical Notes

Critical parameters

- ITC temperature = set in the range of 2 – 85 °C

- Injection number: it is a compromise parameter!
    - 12 (3 µL each) / 18 (2 µL) / 36 (1 µL) / 72 (0.5 µL)
    - more volume = more interaction heat
    - more injections = best fitting

- Injection spacing: the time between each injection
    - 60 – 600 s: the signal have to return to the baseline
    - mandatory for an optimal heat integration of the peak

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Thank you for your kind attention!