CORSO DI LAUREA IN BIOLOGIA PER LA SOSTENIBILITÀ

BIOCHIMICA APPLICATA (6 CFU)

LEZIONE 8

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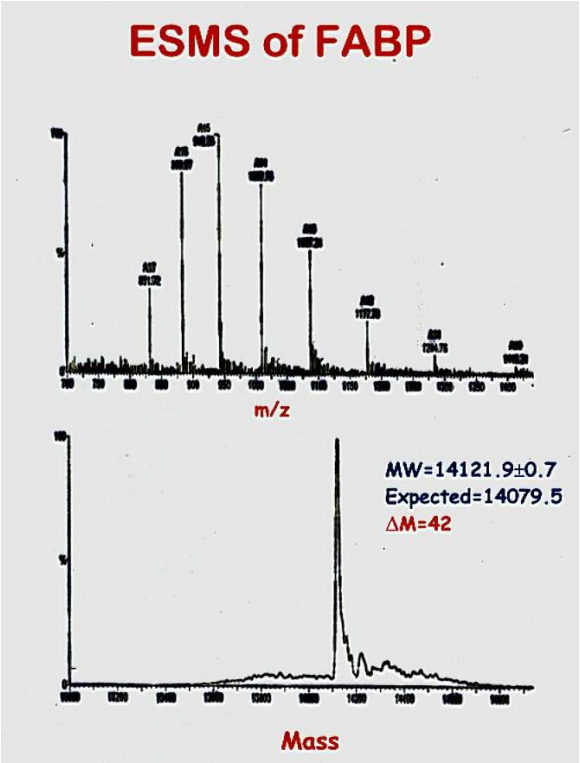
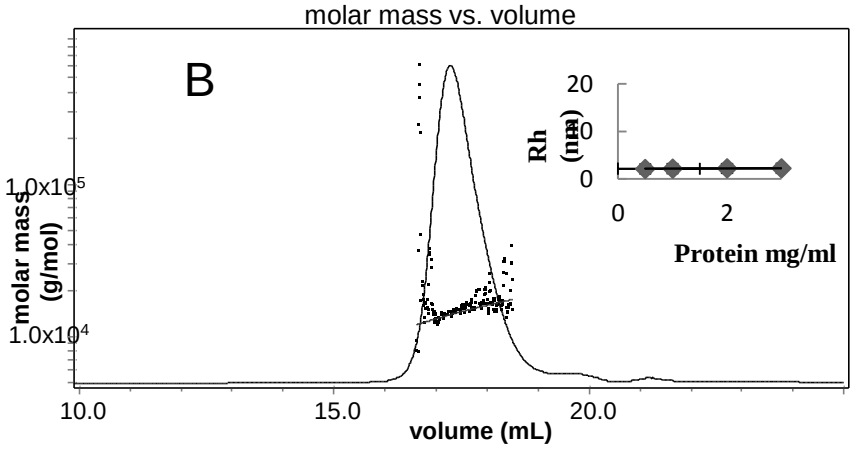
Determinazione massa molecolare; numero e peso molecolare delle subunità

✓Light scattering



✓Spettrometria di massa



Light scattering: diffusione della luce

Diffusione ottica (scattering): consiste nella deflessione in molte direzioni, differenti da quella del raggio incidente, di una radiazione incidente su un sistema costituito da particelle più o meno disperse e di grandezza variabile.

La diffusione della luce solare è un fenomeno dovuto alla presenza di particelle disperse in un gas, in un liquido o in un solido.

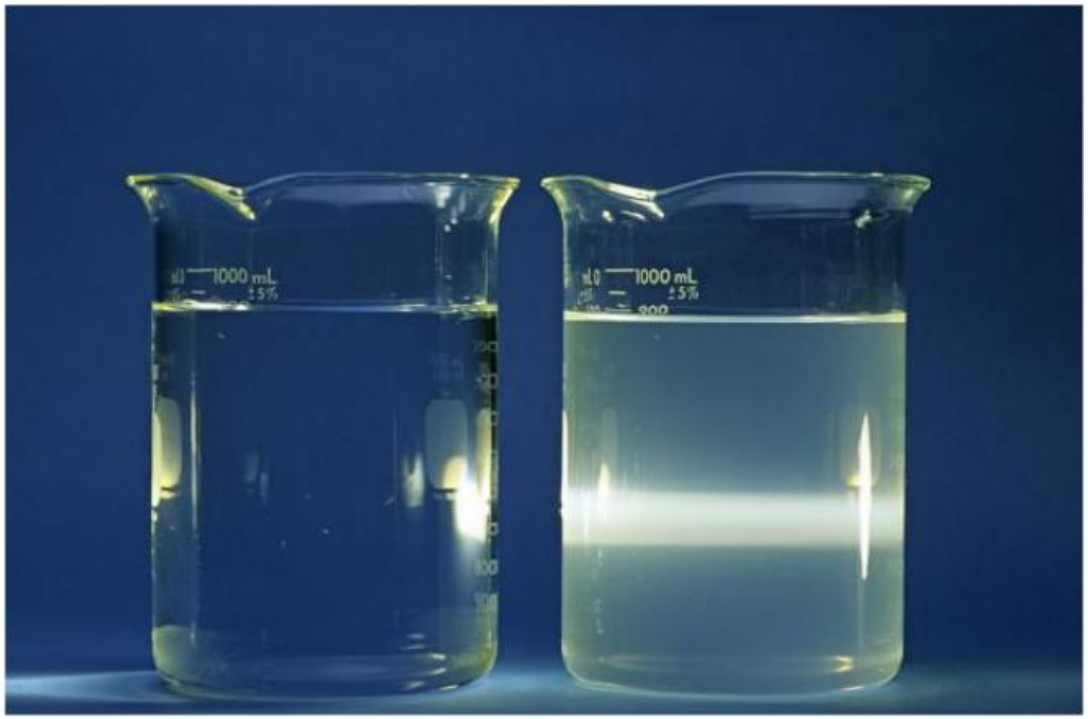
La dispersione avviene in modo non uniforme nello spazio e con diversa intensità secondo la lunghezza dell'onda.

L'intensità della luce globalmente diffusa è minore o al più uguale a quella della radiazione incidente.

La luce diffusa può avere la stessa lunghezza d'onda della luce incidente o di poco diversa.

EFFETTO TYNDALL

Il fenomeno della diffusione della luce o light-scattering fu descritto per la prima volta nel 1868 da John Tyndall, che osservò il light scattering in sospensioni colloidali (effetto Tyndall), nelle quali il diametro delle particelle è maggiore della lunghezza d'onda della radiazione incidente



EFFETTO TYNDALL

L'effetto Tyndall si osserva anche quando le particelle di polvere disperse nell'aria sono investite da un raggio di luce solare che penetra attraverso le fessure di una finestra, diventano quindi visibili ed appaiono come punti brillanti.
L'intensità del fenomeno è linearmente proporzionale alla concentrazione delle particelle sospese ed al cubo delle loro dimensioni.

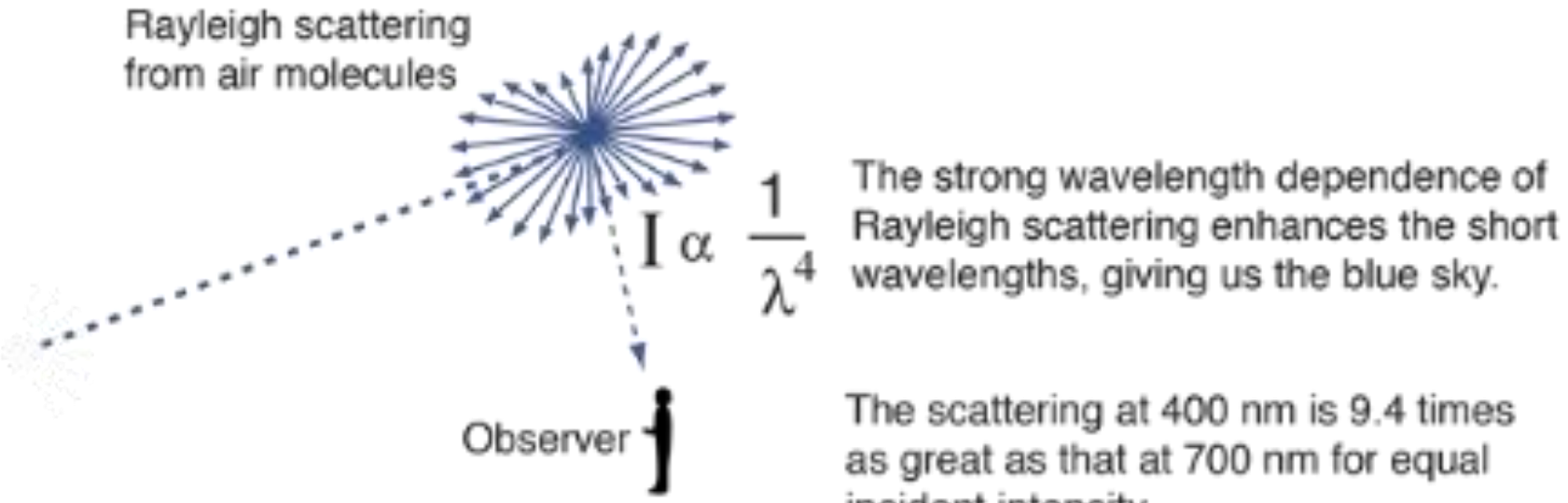
SCATTERING DI RAYLEIGH

Subito dopo le osservazioni di Tyndall, Lord Rayleigh descrisse il fenomeno di light-scattering causato da particelle che hanno un diametro minore della lunghezza dell'onda della radiazione incidente (scattering di Rayleigh, 1871)

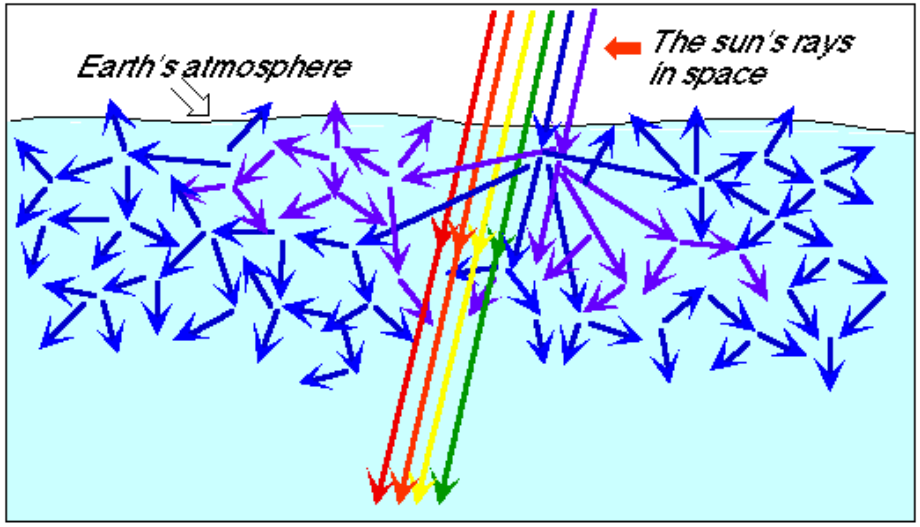
Tale osservazione spiega che la colorazione blu del cielo è il risultato dello scattering della luce da parte delle particelle presenti nell'atmosfera e dell'indice di rifrazione del mezzo che gioca un ruolo fondamentale nel fenomeno della diffusione della luce

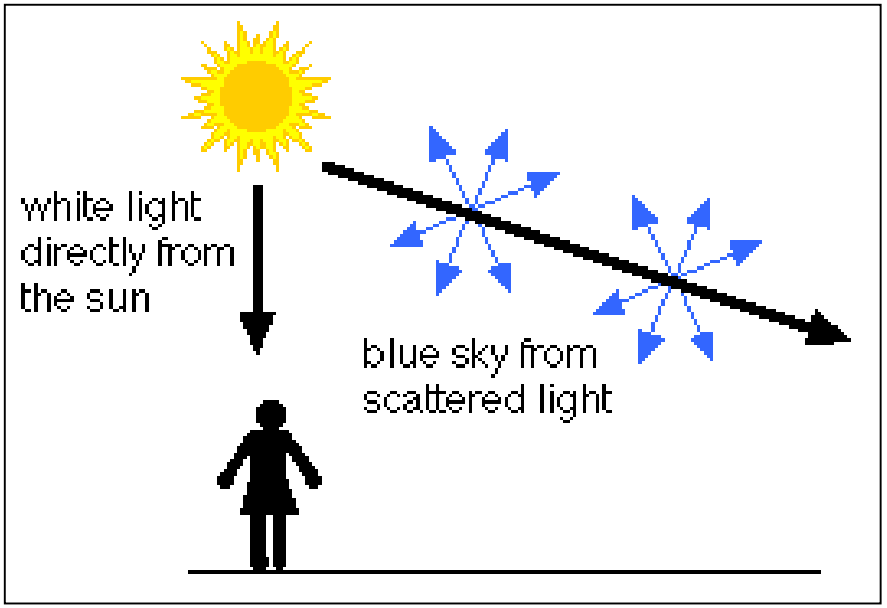
SCATTERING DI RAYLEIGH

Lo scattering di Rayleigh, detto anche scattering elastico, dipende fortemente dalla lunghezza d'onda della luce incidente: l'intensità della radiazione riflessa è inversamente proporzionale alla quarta potenza di λ



Blue Sky





Il risultato più generale della teoria di Raylegh è che le radiazioni a lunghezza d'onda più corta (**blu/violetto**) sono diffuse più efficacemente rispetto a quelle con lunghezza d'onda maggiore (**rosso/giallo**).

Rayleigh Scattering: Blue Sky

- Blue light scatters more than red light
- This is the reason that the sky is blue
- The light from the sun contains all visible wavelengths
- It scatters from particles in the atmosphere
- Blue light scatters more than red, so we see predominantly blue light when we look at the sky
- This is also the reason that sunsets are red
- At sunset, light from the sun has to travel through more of the atmosphere, and the blue light scatters away before the light reaches our eyes

SCATTERING DI MIE

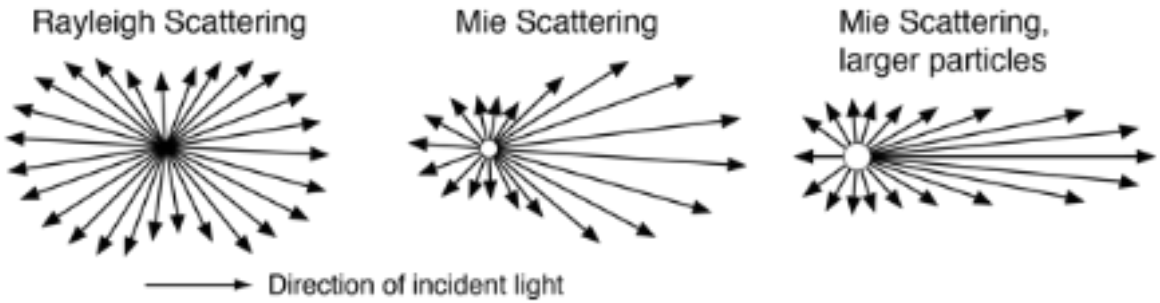
Nobel per la fisica 1904

Nel caso di particelle di dimensioni maggiori della lunghezza d'onda della radiazione incidente il fenomeno prevalente è lo scattering di Mie.

Nel caso in cui le particelle responsabili dello scattering della luce siano sfere perfette esiste una soluzione matematicamente rigorosa per le equazioni che regolano lo scattering singolo detta soluzione di Mie.

In contrast to Rayleigh theory, Gustav Mie (1908) described a theory (Mie theory) to study the scattering of light from absorbing and non-absorbing particles that are large compared to the wavelength of light by taking into account particle shape and the difference in refractive index between particles and the medium the particles are present in.

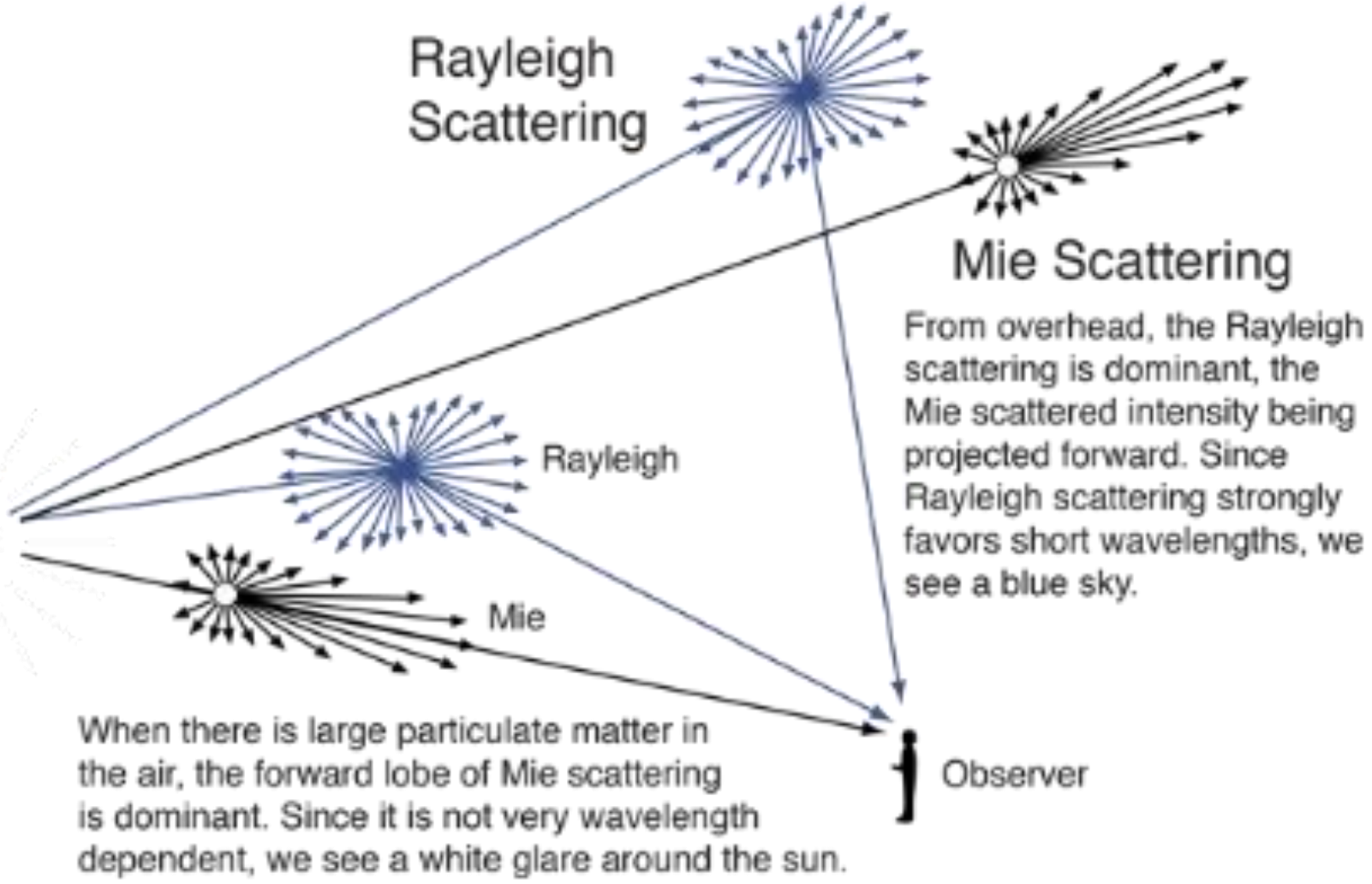
SCATTERING DI MIE

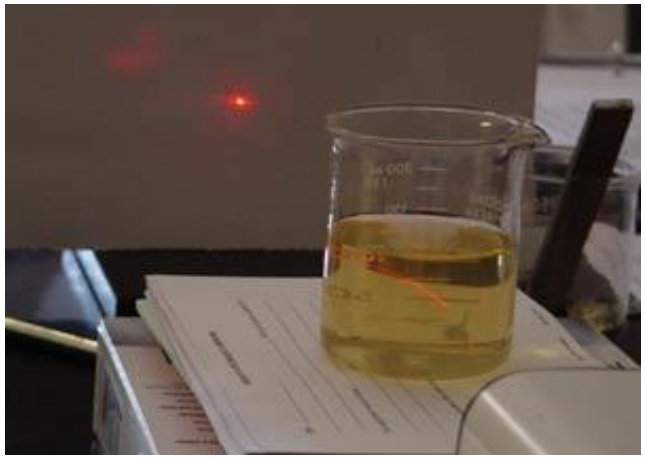


Nobel per la fisica 1904

Mie scattering is not strongly wavelength dependent and produces the almost white glare around the sun when a lot of particulate material is present in the air. It also gives us the white light from mist and fog.

Greenler in his "Rainbows, Haloes and Glories" has some excellent color plates demonstrating Mie scattering and its dramatic absence in the particle-free air of the polar regions.


Rayleigh
Scattering
Mie Scattering
From overhead, the Rayleigh scattering is dominant, the Mie scattered intensity being projected forward. Since Rayleigh scattering strongly favors short wavelengths, we see a blue sky.
Rayleigh
Mie
When there is large particulate matter in the air, the forward lobe of Mie scattering is dominant. Since it is not very wavelength dependent, we see a white glare around the sun.
Observer



quando un raggio luminoso investe una soluzione, la attraversa direttamente:
notare il puntino rosso del laser che colpisce lo schermo e la traccia del laser (per diffusione trasversale) nella soluzione.

quando un raggio luminoso investe una dispersione, la luce viene diffusa in tutte le direzioni:
notare che il puntino rosso del laser non colpisce lo schermo

Turbidometria e nefelometria

Utilizzate per determinare la concentrazione di sospensioni diluite.

Nella turbidometria viene misurato l'assorbimento apparente della radiazione quando attraversa una sospensione una soluzione colloidale o un precipitato finemente disperso in un liquido. La legge di Lambert e Beer non è applicabile.

Non avviene assorbimento ma fenomeni di dispersione in cui la luce è diffratta dalle particelle sospese. Si usa sempre lo spettrofotometro.

APPLICAZIONI

Misura di sospensioni di microrganismi (batteri, lievito) lettura a 600 nm.

La dimensione delle particelle che provocano torbidità è dell'ordine o superiore al micrometro

NEFELOMETRIA

permette di dosare la concentrazione di sostanze finemente disperse in un liquido misurando comparativamente la quantità di luce dispersa da una sospensione colloidale molto diluita che produce un effetto Tyndall l'intensità della luce Tyndall risulta, entro certi limiti, proporzionale alla concentrazione della fase dispersa.

particelle di più piccole dimensioni, dell'ordine di decine o centinaia di nanometri

Light Scattering

Questa tecnica permette di determinare la massa di una macromolecola in modo non distruttivo.

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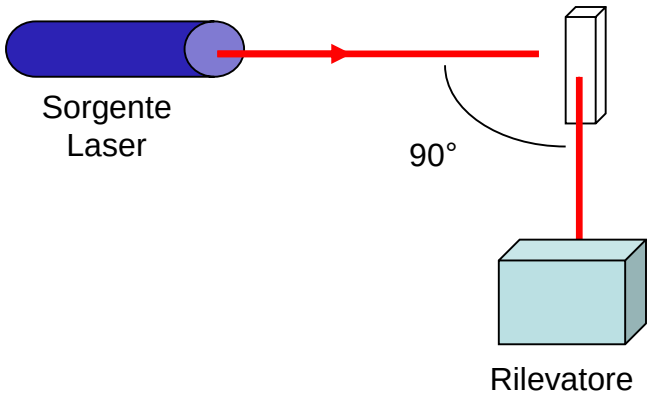
Verifica della struttura oligomerica delle proteine



Why Light Scattering?

- The scattering intensity is a function of the molecular weight and concentration.
- Non-invasive technique, giving information on the size, mass, and charge of a protein sample.
- Monitors the properties of macromolecules in solution.
- Provides information about the oligomeric state of the protein.
- Light scattering is extremely sensitive to the presence of small amounts of aggregates.

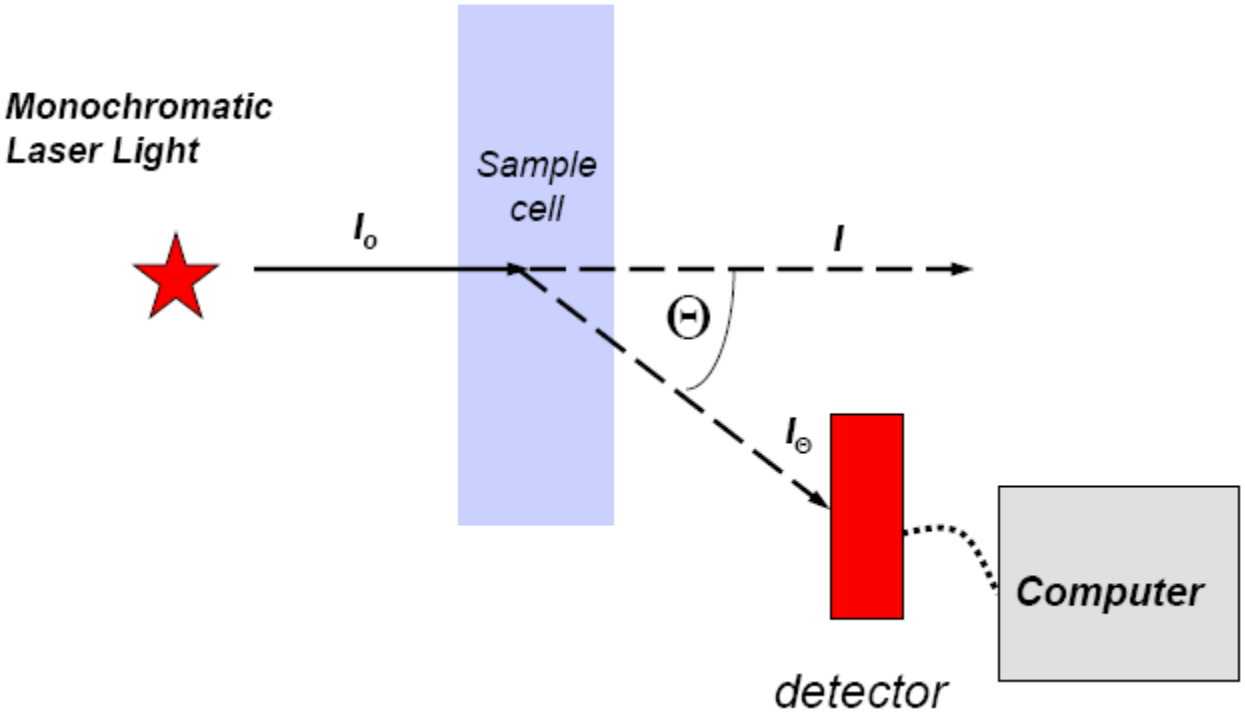
Light Scattering

The scattering signal may be analysed by several methods:

- Average signal strength: **static**, 'classical Light Scattering'

- Fluctuations of signal: **dynamic Light Scattering**, quasi-elastic

Light Scattering Experiments



Classical light scattering
"static" or "Rayleigh" scattering
MALLS

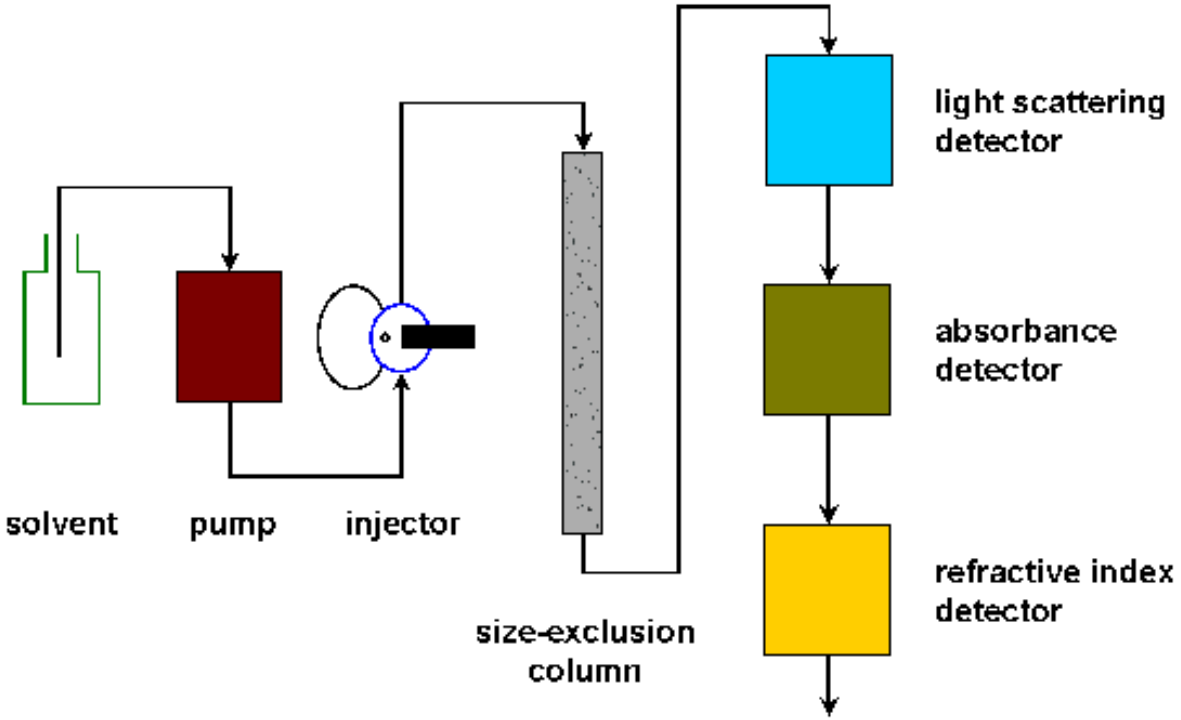
Static light scattering involves measuring the amount of light scattered by a solution at some angle relative to the incident laser beam. For globular proteins smaller than ~500 kDa, the intensity of the scattered light is uniform in all directions, so it is only necessary to measure scattering at a single angle (usually 90 degrees).

The intensity of this scattered light will be proportional to the product of the protein concentration (in mg/ml) times its molecular mass.

It also can be used for measuring the stoichiometry of complexes between different proteins (*e.g.* receptor-ligand complexes or antibody-antigen complexes).

MALLS= "multi-angle laser light scattering" simultaneous measurements at several angles relative to the direction of incident light

MALLS technique is generally best used on-line in conjunction with size-exclusion chromatography (SEC-MALLS) (HPLC/FPLC), as shown in the diagram.



MALLS= "multi-angle laser light scattering"

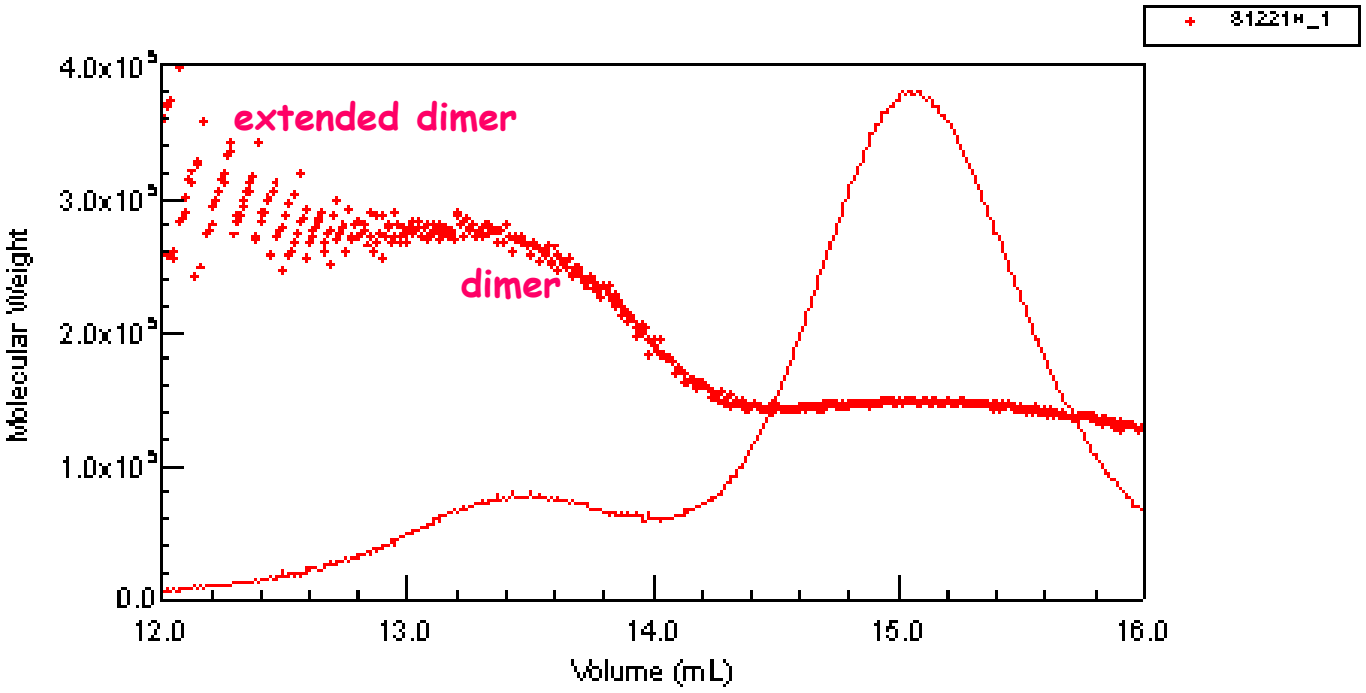
In SEC-MALLS, proteins are first separated by size (hydrodynamic radius, not simply mass) and then passed through the MALLS detector where laser light scattered by the macromolecules is recorded by photodiodes placed at multiple angles around a flow cell (Wyatt 1993).

The concentrations of each eluted component (or time slice) are measured by a differential refractive index (DRI) detector located downstream from the MALLS detector.

Since the signal from the light-scattering detector is directly proportional to the molecular mass of the protein times the concentration (mg/ml), by combining this signal with that from a concentration detector (refractive index or absorbance) it is possible to measure the molecular mass of each peak coming off the column.

Unlike conventional SEC methods, these molecular masses from light scattering are independent of the elution volume. Thus this technique can be used with **"sticky"** proteins that elute unusually late as a result of their interactions with the column matrix, and also with highly **elongated** proteins which elute unusually early for their molecular mass. The molecular masses derived by this technique are generally accurate to 3% or better.

results from some studies of an antibody sample



The individual data points (+) show the molecular weight at each point in the chromatogram, while the solid line shows the elution profile as detected by the refractive index (RI) detector.

The data proved that the peak eluting around 13.4 ml is a **dimer**. However, the shoulder of material eluting before the dimer was unexpectedly found to also contain **dimer**, rather than trimer or tetramer as had been assigned based on elution position. Based on these data it was possible to show that there are **two different conformations of dimer** in this material.

Dynamic Light Scattering (DLS)

✓Measures the time dependence of the light scattered from a very small region of solution, over a time range from tenths of a microsecond to milliseconds.

✓ The fluctuations in the intensity of the scattered light are related to the rate of diffusion of molecules in and out of the region being studied (Brownian motion of molecules) which is directly related to their hydrodynamic size (which depends on both mass and conformation)

✓Data are usually converted to a distribution of hydrodynamic radius graph

✓excellent for detecting trace amounts (0.01% to <1 p.p.m. of very large aggregates

✓covers extremely large range of sizes at one time

✓works in wide range of solvent conditions

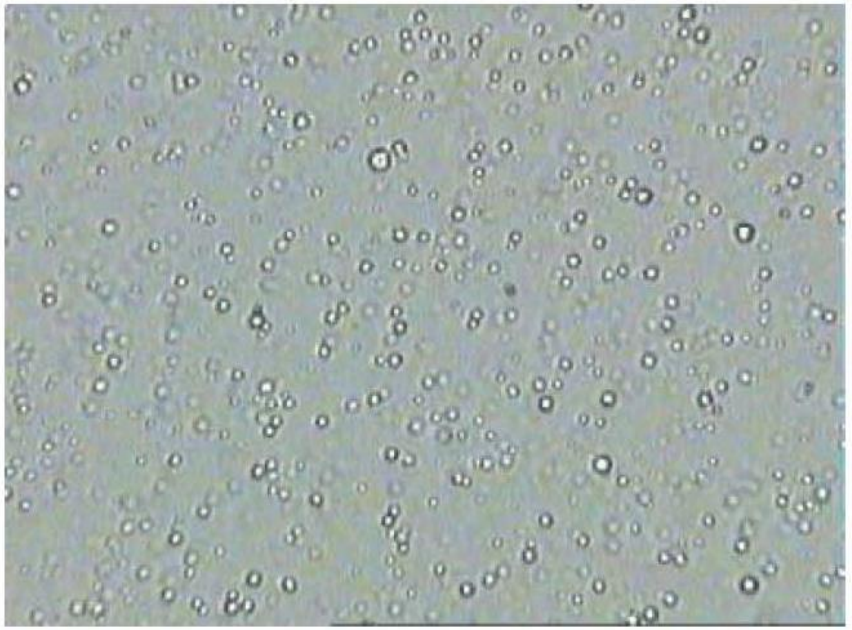
✓useful far continuous monitoring of aggregation at elevated temperatures (accelerated stability)

Dynamic light scattering technology offers the following advantages:

- Accurate, reliable and repeatable particle size analysis in one or two minutes.
- Measurement in the native environment of the material.
- Mean size only requires knowledge of the **viscosity** of the liquid.
- Simple or no sample preparation, high concentration, turbid samples can be measured directly.
- Size measurement of sizes < 1nm.
- Size measurement of molecules with MW < 1000Da.
- Low volume requirement (as little as 2μL).

Brownian Motion

- Random movement of particles due to the bombardment by the solvent molecules that surround them



Analysis of these intensity fluctuations yields the velocity of the Brownian motion and hence the particle size using the Stokes-Einstein relationship.

Brownian Motion

Temperature must be
. accurately known (viscosity)
. stable (otherwise convection present)

- The larger the particle the more slowly the Brownian motion will be
- The higher the temperature the more rapid the Brownian motion will be
- Hydrodynamic Radius (d_H): the diameter of a sphere that has the same translational diffusion coefficient as the particle (D)

Stokes-Einstein Equation

$$d_H = \frac{kT}{3\pi\eta D}$$

d_H = hydrodynamic diameter (m)
k = Boltzmann constant (J/K=kg·m2/s2·K)
T = temperature (K)
η = solvent viscosity (kg/m·s)
D = translational diffusion (m^2/s) coefficient – velocity of Brownian Motion

The three key strengths of dynamic scattering are:

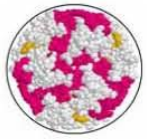
1. it can analyze samples containing very broad distributions of species of widely differing molecular masses (*e.g.* a native protein and various sizes of large aggregates)

2.it can detect very small amounts of the higher mass species (<0.01% in many cases).

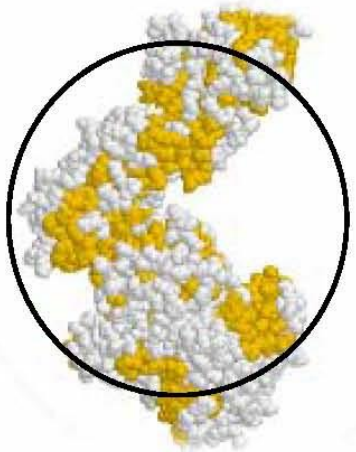
3.because there is no chromatographic separation or dilution involved (it is a batch-mode measurement), one does not have to worry that protein aggregates are being lost within a chromatographic column or from dissociation by dilution.

Comparative Protein R_H Values

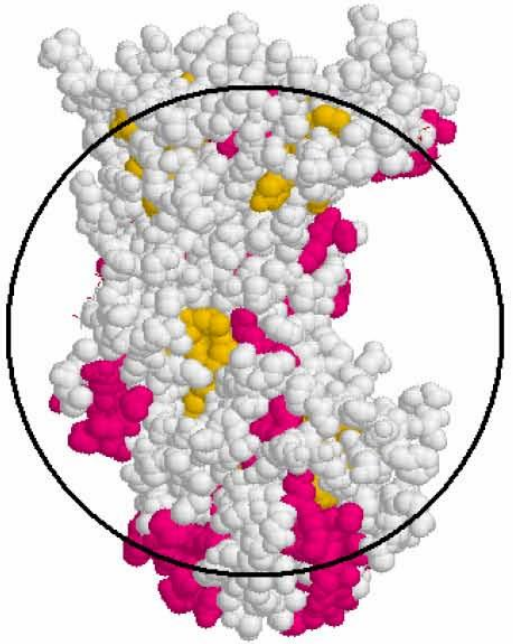
Lysozyme
M_W=14.5 kDa
R_H=1.9 nm



Insulin - pH 7
M_W=34.2 kDa
R_H=2.7 nm



Immunoglobulin G
M_W=160 kDa
R_H=7.1 nm

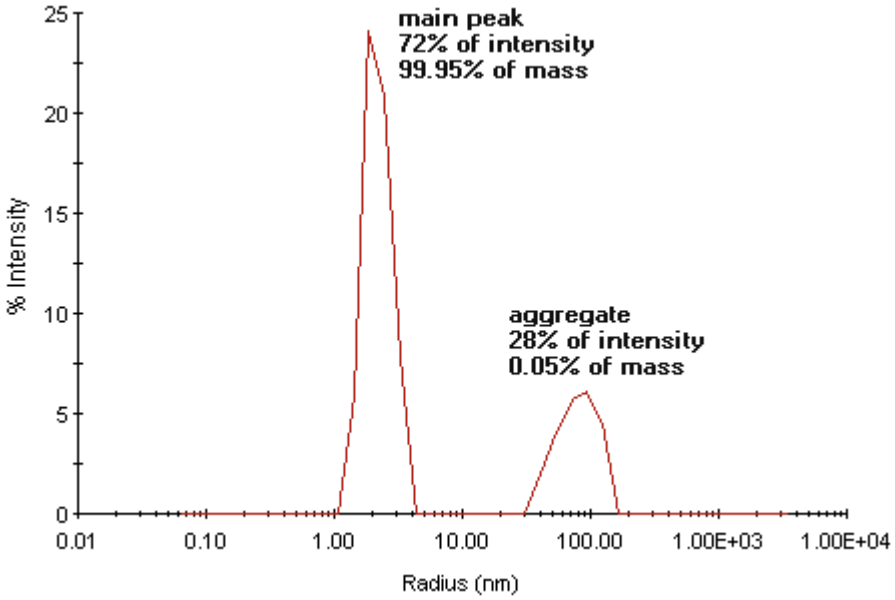


Thyroglobulin
M_W=650 kDa
R_H=10.1 nm

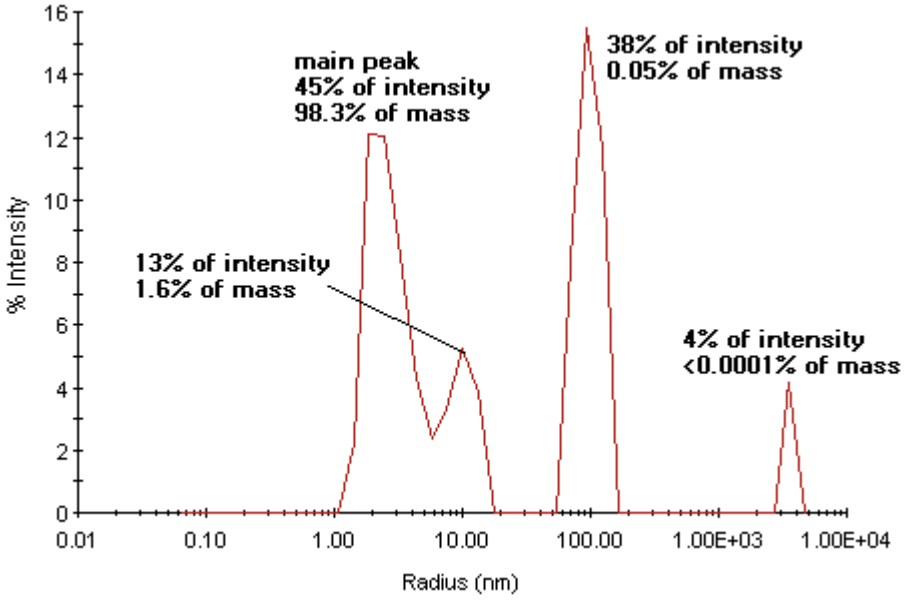
5 nm

The DLS measured radius is the radius of a hypothetical hard sphere that diffuses with the same speed as the particle under examination. This definition is somewhat problematic with regard to visualization however, since hypothetical hard spheres are non-existent.

In practice, macromolecules in solution are non-spherical, dynamic, and solvated. As such, the radius calculated from the diffusional properties of the particle is indicative of the *apparent* size of the dynamic hydrated/solvated particle. Hence the terminology, 'hydrodynamic' radius.



An example of a sample with a main component at ~2.2 nm and a clear, well-resolved aggregate peak at ~80 nm. Although this aggregate peak represents 28% of the total scattered light, because that intensity is proportional to molecular mass, and mass increases as (radius)^3, the amount of mass represented by the aggregate is only ~0.05% of the total!

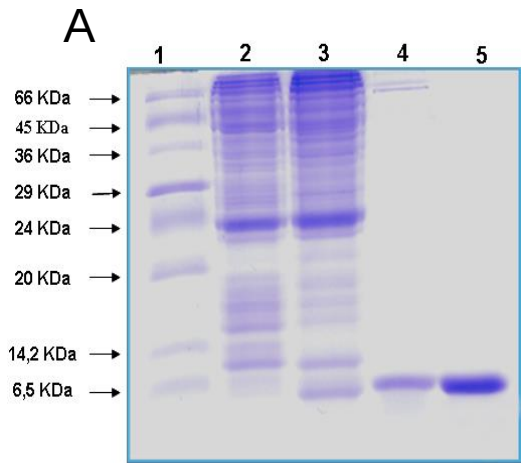


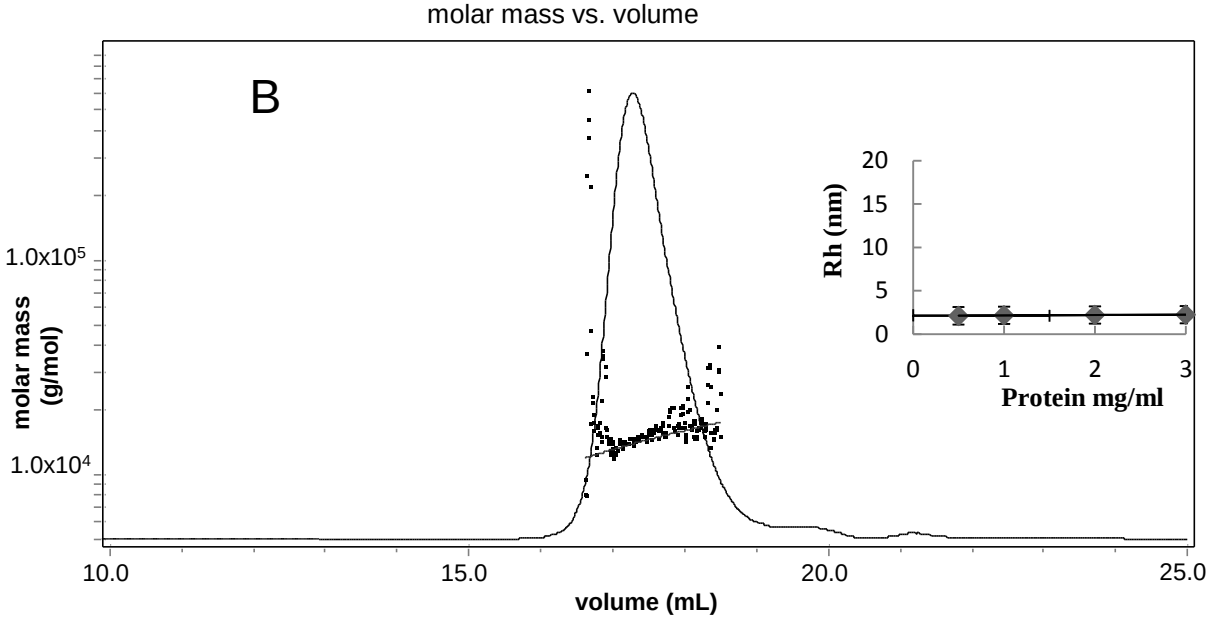
The size distribution for a particularly 'bad' lot of this material, showing additional aggregate peaks at ~10 nm and ~4,000 nm, and a shift of the intermediate-size aggregate peak to somewhat larger sizes. The largest aggregate represents less than 1 part-per-million of the total mass!

Purification of recombinant C68 and determination of its quaternary structure.

C68 from the *Sulfolobus islandicus* plasmid–virus pSSVx is a novel member of the AbrB-like transcription factor family

Patrizia CONTURSI*†[1,2], Katia D'AMBROSIO†[1], Luciano PIRONE†, Emilia PEDONE†[2], Tiziana AUCELLI*, Qunxin SHE‡, Giuseppina DE SIMONE† and Simonetta BARTOLUCCI*

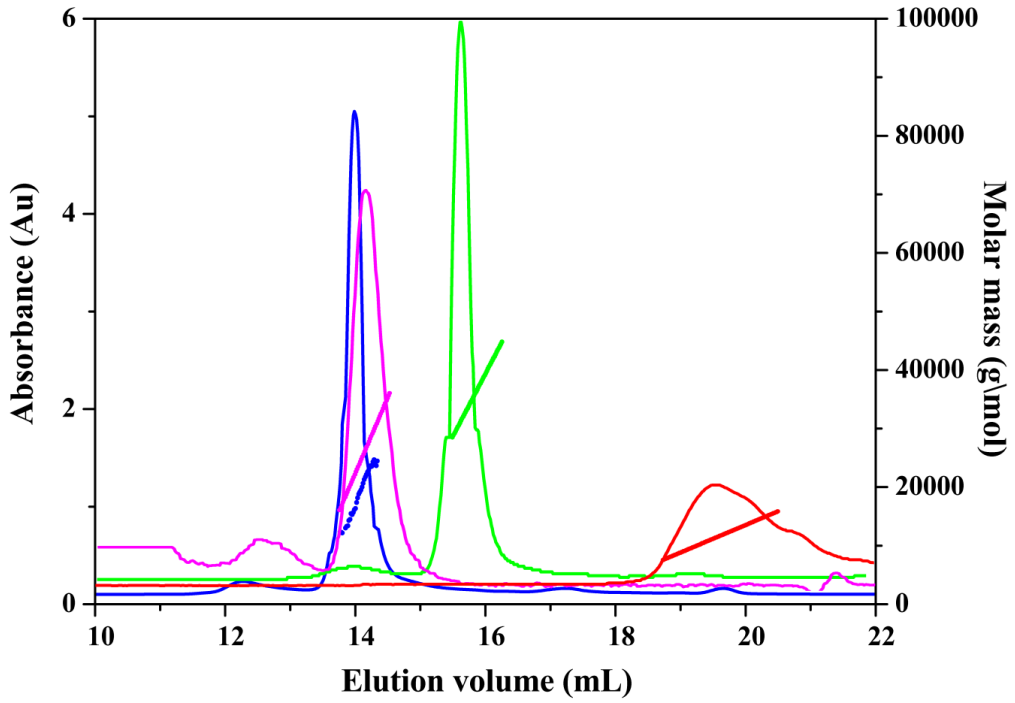




(**A**): SDS-PAGE analysis of protein extracts at various stages of C68 purification. Lane 1: molecular weight markers; lane 2: crude extracts from *E.coli* BL21(DE3)/pET30-*orfc68* not induced with IPTG; lane 3: crude extracts from *E.coli* BL21(DE3)/pET30-*orfc68* induced with 0.5 mM IPTG; lane 4: the sample after cationic-exchange chromatography; lane 5: the sample after gel-filtration chromatography.
(**B**): A graph of the molar mass versus the elution volume of C68
(**B** insert): the dependence of the C68 hydrodynamic radius (Rh) on the protein concentration, as measured by Dynamic Light Scattering.

C68 has a molecular mass of 14470 (0.9%) Da, which indicates that the protein is a dimer

Light-scattering measurements



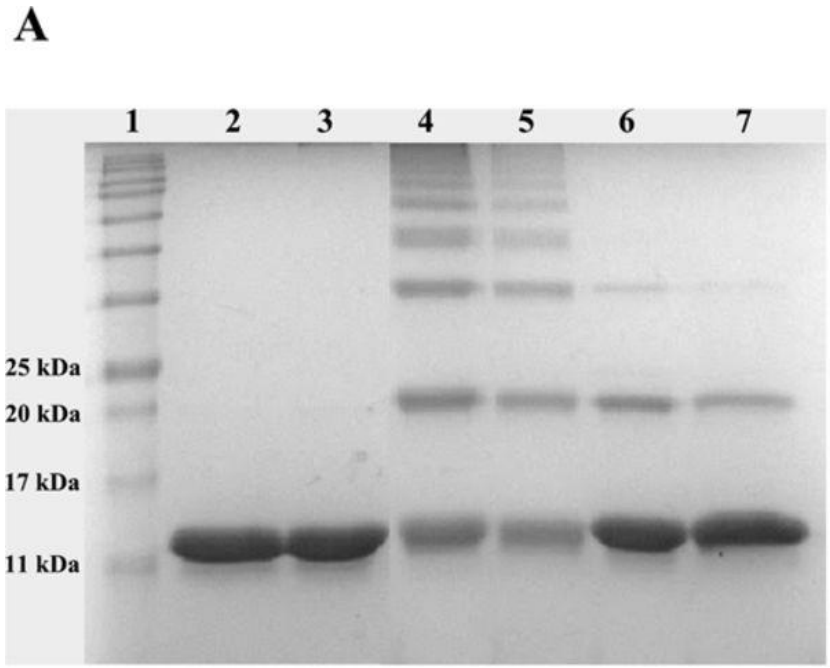
A graph of the molecular mass and absorbance at 280 nm versus the elution volume. In red is reported Stf76; in blue 35 bp-site A; in purple the complex of Stf76 and 35 bp-site A (molar ratio 1:1), in green the complex of Stf76 and 35 bp-site A (molar ratio 2:1).

A single peak (green), with a molecular weight corresponding to a 2:1 (protein:DNA) complex, was obtained with no indication of excess of either protein or DNA. In the molecular ratio 1:1 (purple) a single peak with a molecular weight corresponding to a 1:1 complex was observed indicating that the protein is also able to bind DNA as monomer. The 1:1 complex presents an elution volume similar to free DNA (blue), while remarkably 2:1 complex an elution volume greater. This behavior could be justified from the existence of a more compact specie

Contursi P et al. Nucl. Acids Res. 2014;nar.gku215

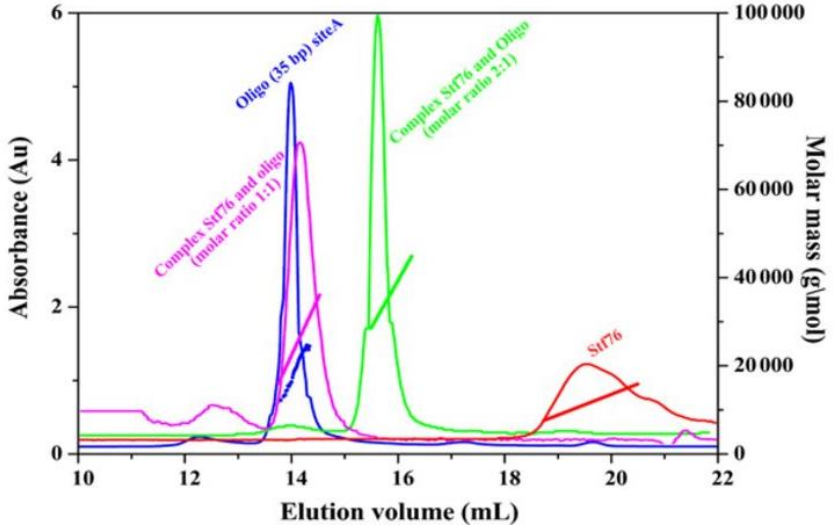
Nucleic Acids Research

Chemical cross-linking of Stf76 with BS3



(**A**) Chemical cross-linking of Stf76 with BS3[Bis (sulfosuccinimidyl suberate)]. Stf76 (10 μM) was incubated with and without cross-linker and analysed on SDS-PAGE. Lane 1: molecular weight markers; lane 2: recombinant Stf76; lane 3: Stf76 in the presence of DNA (10 μM); lanes 4 and 5: Stf76 in the presence of BS3 (100 μM and 200 μM, respectively); lanes 6 and 7: Stf76 in the presence of BS3 and 35 bp-site

A Stf76 tendency to oligomerize was observed in chemical cross-linking experiments performed with the homobifunctional amino-reactive reagent BS3



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Dynamic light scattering