PROBING

BIOVISCOSITY **VIA** FLUORESCENCE

Invited talk

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What is bioviscosity ?

Lack of precise definition.

Possible answers:
1) macroscopic viscosity of biological fluids like blood or plasma (*biorheology*)
and/or
2) apparent viscosity (or ***microviscosity***) manifesting itself in the rates of ***molecular motions*** (*molecular biophysics*).

We shall mostly deal with the second aspect of bioviscosity.

Some fundamental relations concerning microviscosity

Classical review of the subject:
A.H.Alwattar, M.D.Lumb and J.B.Birks (1973) in "*Organic Molecular Photophysics*", vol.1, J.B.Birks Ed.,Wiley, New York, chapter 8.

In hydrodynamics, viscosity is a measure of the internal friction of a fluid.

early XVII century
Sir Isaac Newton:
Viscosity **η** as a **proportionality factor** between the velocity gradient in the direction perpendicular to the flow direction **(shear rate)** and the force per unit area required to maintain the flow **(shear stress)**:

$$F/S = \boldsymbol{\eta} \cdot dv/dx. \qquad (1)$$

The fundamental unit of viscosity is **Pascal-second** (Pa·s).
1 Pa·s =103 **centipoise** (cp).

1851

Sir George Gabriel Stokes:

The **force exerted by a fluid on a macroscopic spherical body** of a radius r moving in it with a constant velocity v:

$$F = 6\Pi\eta rv. \qquad (2)$$

(It was also Stokes, who named and explained the phenomenon of ***fluorescence*** in 1852).

The **friction coefficient** for the **translational motion** of a spherical object:

$$f_t = 6\Pi\eta r. \qquad (3)$$

The friction coefficient for the **rotational motion** of a sphere:

$$f_r = 8\Pi\eta r^3. \qquad (4)$$

Albert Einstein ((1906) *Ann.der Physik* **19**, 371-381):

Relation between the **diffusion coefficient** D of a **microscopic sphere** and the friction coefficient f:

$$D = kT/f, \qquad (5)$$

For the **rotational diffusion**

$$D = kT/6\eta V, \qquad (6)$$

V - volume of the sphere.
In terms of the rotational correlation time $t_r = 6D^{-1}$

$$t_r = V\eta/kT. \qquad (7)$$

The **solvent** is treated as a **continuous medium** and the velocity of the solvent at the surface of the sphere is zero relative to it (**"stick" boundary condition**).

SED (Stokes-Einstein-Debye) model.

Francois Perrin ((1934) *J. Phys.Rad.,Ser.VII* **V**, 497-511):

General theory of the **rotational motion of an ellipsoidal particle with 3 different orthogonal semiaxes** $\rightarrow$ 3 rotational friction coefficients $\rightarrow$ 3 diffusion coefficients.

Problem:

Molecules with diameters on the order of 1 nm or less usually diffuse more rapidly than predicted by the above theories.

Solution:

Introduction of the **"slip" boundary condition** and for some solute-solvent systems a mixture of "stick" and "slip".
To account for a) **variable boundary conditions** and b) **different molecular shapes** the expression 6 must be modified accordingly (B. Kovert and D. Kivelson (1976) *J.Chem.Phys*.**64**, 5206-5217):

$$D_i = kT/6\eta V f_i g_i \quad , \quad i = 1,2,3 \qquad (8)$$

where **f** is the **coupling factor** (f =1 for "stick" and f<1 for "slip" boundary conditions) and **g** is the **shape factor**.

Problem:

The rotational reorientation dynamics of **charged molecules**

usually cannot be adequately described by the SED model.

Solution:

An additional frictional force resulting from the induced polarization of the surrounding solvent must be included in the theory - the **dielectric friction model** (.L.A.Philips, S.P.Webb and J.H.Clark (1985) *J.Chem.Phys*. **83,**5810-5821)

Other refinements of the microviscosity theory

A.Gierer and K.Wirtz ((1953) *Z.Naturforsch*.**8a**, 532-538)
- the **discontinuous** character of the solvent shell and the **free volume effects**.

P.B.Macedo and T.A.Litovitz ((1965) *J.Chem.Phys.***42**, 245-256) - viscosity as a function of **temperature** and the free volume (solvent molecules treated as hard spheres):

$$\eta = aT\exp(bV_0/V_f + E/kT), \qquad (9)$$

a and b - constants,
V_0 - the van der Waals volume of the solvent molecule,
V_f - the free volume per solvent molecule,
E - the activation energy.
The increase of viscosity following the pressure increase may be regarded as a result of the free volume reduction.

Is the notion of viscosity applicable to ordered and/or inhomogeneous media such as biological molecular systems ?

Yes, but...

1) It has been established that biological fluids like blood and cytoplasm exhibit non-Newtonian behaviour (R.L.Evans, R.B.Kirkwood and D.G.Opsahl (1971), *Biorheology* **8**,125-128), i.e. eq.1 is not valid for them.
2) Microviscosity can be unrelated to bulk viscosity and restrictions to diffusion may be also completely unrelated to viscosity.
3) Often, the dynamics of biomolecular systems can be less ambiguously described using **diffusion coefficients D** than **viscosity** η. The two quantities are related by the equations of the type (8) in which molecular dimensions are involved.
4) In many instances one does not look for the value of viscosity itself but rather for the time constant characterizing the studied type of molecular motion (for rotational diffusion it is the **rotational correlation time** $\tau_r \sim \eta$)

The *literally* vital importance of diffusion and viscosity for living **cells**

- the homeoviscous adaptation mechanism maintains a well-defined range of lipid membrane **fluidity** (fluidity = 1/viscosity) in stress conditions
- the upper limit on the size of cells is determined by the rate of molecular diffusion in the gel-like cytoplasm

Lipid membranes - regions of increased viscosity in a cell.
Membrane viscosity - a property which depends on the type of diffusing molecules.
Ion channels - regions of reduced viscosity for particular ions (Na^+, K^+, Cl^-, Ca^{2+}). Such "viscosity" could be probed via **fluorescence quenching** by these ions.

The diffusive motion does not account for all of the transmembrane movements of ions or molecules.
Mediated transport systems - the translocation of a substance through the membrane requires the conformational change of the **carrier protein** which is induced by the approach of this substance (the process involves binding, conformational change and dissociation of the substance on the other side of the membrane). Here dynamic properties of carrier proteins (like their **segmental mobility** and **flexibility**) and membrane lipids (viscosity) play a role, too.

A single viscosity parameter is not sufficient to describe the hindrance to the motion of a molecular probe in a lipid membrane. Consider, for instance, the lateral motion. In general, the viscous drag on a molecule moving along and across the lipid acyl chains is expected to be different. Such a case of the **"viscosity anisotropy"** was discussed by M.Shinitzky and I.Yuli ((1982) *Chemistry and Physics of Lipids* **30**, 261-282)

Viscosity dependence of fluorescence decay

Example:
For a two-state adiabatic excited-state process (e.g.proton transfer or twisted intramolecular charge transfer) the fluorescence decay rate is

$$k_f = k_r + k_{nr}(\eta) + k_{12}(\eta). \qquad (10)$$

For molecules exhibiting a high degree of structural flexibility, (e.g.triphenylmethane dyes), the internal conversion rate k_{nr} was found to be viscosity-controlled, leading to the viscosity dependence of the fluorescence quantum yield F (N.Tamai,M.Ishikawa,N.Kitamura and H.Masuhara (1991) *Chem.Phys.Lett***.184**,398-40). Usually

$$F \sim \eta^x, \text{ where } 0.5<x<1 \qquad (11)$$

(W.Rettig (1986) *Angew.Chem.Int.Ed.Engl*.**25**, 971-988)

Viscosity dependence of spectra

General:
Excitation of solute molecules ⇒ temporary thermodynamic **non-equilibrium** of the system consisting of **excited molecules** and the surrounding **solvent** ⇒ **viscosity-dependent relaxation**

Example:
Two interconverting excited-state species, e.g. monomers and excimers ⇒ appearance of a second fluorescence band.
Time-resolved emission spectroscopy allows to monitor the evolution of the excited-state populations and determine the **viscosity-dependent relaxation rates**.
The **steady-state** fluorescence **spectra** are then also viscosity-dependent

Viscosity dependence of polarization

For pulsed excitation, the evolution of the **fluorescence emission anisotropy** (EA)

$$r(t) = \frac{I_{\parallel}(t) - I_{\perp}(t)}{I_{\parallel}(t) + 2I_{\perp}(t)} \qquad (12)$$

is due to the **relaxation of the angular distribution** of the photoexcited molecules (their **emissive transition moments**, to be precise) following the initial, highly anisotropic one.
The rate at which the EA approaches its equilibrium value r ($t=\infty$) is viscosity-dependent.
For an isotropic molecular rotor of a volume V, the EA decays according to

$$r(t) = r_0 \exp(-t/t_r), \qquad (13)$$

and the steady-state EA is

$$r = r_0/(1 + \tau/t_r). \qquad (14)$$

The plot of 1/r vs T/η (**Perrin plot**) should be a straight line and the slope is k/Vr_0.
The Perrin plot can be used for estimating bioviscosity (**not in ordered systems !)** by calibration with isotropic solutions of differing viscosities.
U.Cogen, M.Shinitzky, G.Weber and T.Nishida (1973) *Biochemistry* **12**, 521-528
The steady-state EA can be influenced **indirectly** by viscosity due to the fluorescence **decay time** dependence on viscosity, for instance when **diffusion-controlled dynamic quenching** takes place in a system of interest.

Models of the molecular reorientation include the cases of :

- **homogeneous** and **isotropic solutions** (the probe senses solvent viscosity directly - **unrestricted reorientation**)
- **heterogeneous**, **isotropic solutions** (the probe is embedded in a biomolecule - **restricted reorientation** superimposed on a relatively slower **reorientation of the biomolecule** itself)
- **ordered systems**.

When no restrictions on the orientation of a probe are imposed, the EA at infinite time is $r(\infty) = 0$, otherwise $r(\infty) \neq 0$.

For a review see:
A.Kawski, *Critical Rev. Anal.Chem*. (1993) **23**, 459-529

Example:
A classical theory of T.J. Chuang and K.B.Eisenthal ((1972) *J.Chem.Phys*.**57**, 5904 -5097) for a hydrodynamic SED model of unconstrained rotational diffusion of ellipsoidal molecules predicts

$$r(t) = 0.3\{4p_xp_yq_xq_y\exp[-3(D+D_z)t] + 4p_yp_zq_yq_z\exp[-3(D+D_x)t] + 4p_zp_xq_zq_x\exp[-3(D+D_y)t] + (\beta+\alpha)\exp[-2(3D+\Delta)t] + (\beta-\alpha)\exp[-2(3D-\Delta)t]\} \quad (15)$$

where $\alpha = q_x^2p_x^2 + q_y^2p_y^2 + q_z^2p_z^2 + 1/3,$

$\beta = (D_x/\Delta)(q_y^2p_y^2 + q_z^2p_z^2 - 2q_x^2p_x^2 + q_x^2 + p_x^2) + (D_y/\Delta)(q_z^2p_z^2 + q_x^2p_x^2 - 2q_y^2p_y^2 + q_y^2 + p_y^2) + (D_z/\Delta)(q_x^2p_x^2 + q_y^2p_y^2 - 2q_z^2p_z^2 + q_z^2 + p_z^2) - (2D/\Delta),$

and $D = (1/3)(D_x + D_y + D_z),$

$\Delta = (D_x^2 + D_y^2 + D_z^2 - D_xD_y - D_yD_z - D_zD_x)^{1/2}$.

D_k (k=1,2,3) - three rotational diffusion coefficients (**inversely proportional to viscosity)**.

p_x, p_y, p_z and q_x, q_y, q_z - direction cosines for absorption and emission transition moments.

The maximum number of rotational correlation times is:
theoretically - **5,**
experimentally resolved to date - **3** (for Y_t -base in propylene glycol at 10^oC)
(I. Gryczyński, H. Cherek and J.R. Lakowicz (1988) *Biophys.Chem*.**30**,271-277).

A realistic interpretation of the EA decay data in terms of the multiexponential EA decay function (15) is far from obvious and relevant algorithms are just beginning to emerge (J.Szubiakowski, W.Nowak, A.Balter and A.A.Kowalczyk (1995), *Computers&Chemistry*, in print).

Individual **correlation times** are combinations of diffusion coefficients and are expected to be **linear functions of viscosity**. Indeed, such a dependence is often observed.
A **monoexponential EA decay** (eq.13) is expected either for an **isotropic (spherical) rotor** or a **symmetric top** rotor with at least one of the **transition moments aligned with the symmetry axis.**

Examples:

BTBP
(*N*,*N'*-bis(2,5-di-*tert*-butylphenyl)-3,4,9,10-perylenetetracarboximide)
in **n-alkanes, n-alcohols, ethanol/glycerol** and **paraffin oil/dodecane** mixtures **-** a single exponential decay of the EA and a **linear** dependence of **tr vs** η in the **0.5-150 cP** range.
A.M.Williams and D.Ben-Amotz (1992) *Anal.Chem*.**64**, 700-703

Perylene and 3,9-dibromoperylene in glycerol
- a biexponential EA decay of and a **linear** dependence between glycerol viscosity and each of the **two rotational correlation times** in the **7-60 P** (perylene) and **4-60 P** (3,9-DBP) ranges.
Improper choice of the EA decay model may cause significant deviations from linearity.
A.Balter and J.Szubiakowski (1993) *J.Fluorescence* **3**, 247-249

Required viscosity probe properties

1. **Minimum disturbance of the investigated system** - obvious
Use either intrinsic probes (like tryptophan in proteins) or extrinsic probes which closely resemble the molecular environment to be monitored.
Recommended:
Comparative studies using **different probes** in order to **exclude probe-specific effects**.
2. Good knowledge of probe's **photophysical** and **photochemical properties** in well **controlled environments** i.e. isotropic liquids and model ordered systems.
Ideally, non-bound (free) viscosity probes should have:
(a) well defined symmetry,
(b) no specific binding sites,
(c) well defined directions of transition moments.
Requirements **(a)** and **(b)** are necessary to provide a well defined hydrodynamic shape. The requirement **(c)** facilitates the analysis of the EA decay in terms of eq.15.

There are still some **unresolved basic (!) problems** concerning the properties of the popular probes like **DPH**
(U.A.van der Heide, M.J.Zandvoort, E. van Faassen, G. van Ginkel and Y.K.Levine (1993) *J.Fluorescence* **3**, 271-279)
or **perylene**
(J.Szubiakowski, A.Balter, W.Nowak, A.Kowalczyk, K.Wiśniewski and M. Wierzbowska, to be published).

Example:
The **initial anisotropy deficit** of **perylene** in glycerol
r0=0.34 instead of 0.40 expected for the S0→S1 excitation
- when interpreted in terms of an angle γ between the absorption and emission transition moments, leads to γ **= 190**.

Out-of-plane deformation of perylene ?

Probing viscosity of specific molecular systems

a) Membranes

Microviscosity properties of biological membranes can be discussed in terms of various degrees of motional freedom available to their constituents.
The **fluorescence recovery after photobleaching** technique (R.Peters and M.Scholz (1991) in R.J.Cherry,Ed. *New Techniques of Optical Microscopy and Microspectroscopy*, CRC Press p.199)
provides **translational diffusion** coefficients of lipids in the lateral membrane plane.
Typical values: $\mathbf{D_{trans} \cong 10^{-8} cm^2 s^{-1}}$.
The **fluorescence quenching, excimer formation and photodimerization** techniques may also be used to provide the lateral diffusion coefficients, but they may **overestimate the diffusion coefficient** because of contributions from static nonrandom distributions of the probe.

Spontaneous transversal movement of phospholipids (**"flip-flop"**) occurs on a time scale of several hours but can be accelerated by some proteins. This has been studied by monitoring **nonradiative energy transfer** (R.Pagano and K.Longmuir (1985) *J.Biol.Chem.***260**, 1909) and **pyrene excimer fluorescence** (B.W. Van der Meer, R.D.Fugate (1989) *Biophys.J.***56**, 935).
Internal motions of lipid acyl chains have been extensively studied, particularly using the **EA** technique with probes such as **DPH, anthroyloxy fatty acids and parinaric acid** which are often referred to as **membrane fluidity probes**.
Membrane fluidity probes are sensitive to only the **angular reorientation** of lipid acyl chains - a process which does **not necessarily correlate** with other dynamic processes such as **lateral diffusion**.

For references see:
R.P.Haugland (1992) *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes Inc.

b) Micelles

Example:
Probing of the internal viscosity of **SDS micelles** by investigating the rotational behaviour of **tetracene** using the **frequency-domain** technique of the EA decay measurement.
D.A.Piasecki and M.J.Wirth (1993) *Anal.Chimica Acta* **271**, 183-193
Result: *the addition of alcohol allows tetracene to rotate faster within the micelle, however the chain length of the alcohol molecule has little effect on the reorientation behaviour which is well approximated by a hydrodynamic model.*

c) Proteins

Fluorescence spectroscopy can be used to monitor both the **viscosity** of the **protein interior** and the viscosity of the **medium** in which the protein molecule floats.

The depolarizing rotations of the protein-bound fluorophore are a superposition of the probe **segmental mobility** and the **overall protein rotation**.

Diffusional quenching of buried tryptophan fluorescence by **oxygen** may be used to estimate the viscosity of the protein matrix interior. The bimolecular quenching constants k_Q for the oxygen quenching of tryptophan fluorescence in proteins range from 20% to 80% of the diffusion controlled values in water (~ 10^{10} $M^{-1}s^{-1}$). One may expect that the **rate constant for the diffusion controlled reaction** will be **proportional to T/η,** therefore **viscosity** of the **protein interior** may be **up to 5 times larger than that of the surrounding aqueous medium**.

J.R.Lakowicz, B.P.Maliwal, H.Cherek and A.Balter (1983) *Biochemistry* **22**, 1741-1752, J.R.Lakowicz and B.P.Maliwal (1983) *J.Biol.Chem*.**258**,4794-4801

The problem of molecular "shape"

Do fluorescence anisotropy decay experiments provide information about the molecular "shape" ?

When using a **general ellipsoid model** in the data analysis, we should bear in mind that this "shape" refers to the **rotational diffusion tensor**.

In many instances it is not possible to detect the anisotropy of rotation **not because the molecule really behaves like an isotropic rotor,** but because the **transition moments** are so **aligned** that the fluorescence anisotropy decay is **monoexponential**, just as for a spherical rotor.

Carrying experiments at **various excitation wavelengths** (when transition moments change their orientation with respect to the molecular reference frame) may help to reveal the anisotropy of rotation.

Molecules which exhibit a high degree of rotational anisotropy

(e.g.perylene) are particularly well suited to probe the ordered systems. Since, however, the multiexponential decay data analysis for such molecules is not quite straightforward, one may prefer a viscosity probe whose EA decay is just monoexponential. One such probe is seemingly BTBP which can hardly be regarded as a spherical molecule.

Membrane fluidity probes are often classified as **rod-like** (DPH and derivatives, derivatives of parinaric acid and anthroyloxy fatty acids.) and **disc-like** (pyrene, perylene and derivatives, coronene).

And what about **sphere-like** probes ?

C60 buckminsterfullerene - could it be used as an **ideally spherical fluorescence probe** ?

Spectroscopic properties (in toluene at room temperature) :

Absorption - between 430 and 620 nm

Fluorescence - in the 650-800 nm range

Lifetime - about 1ns

Fluorescence quantum yield - only about $2 \cdot 10^{-4}$.

C70 has similar properties.

D.Kim,M.Lee,Y.D.Suh, S.K.Kim(1992)*J.Am.Chem.Soc*.**114**,4429- 4430.

Study of the rotational dynamics of spherical buckminsterfullerenes is a very challenging issue.

^{13}C-NMR studies of C60 in deuterated toluene at 303K (Jones and Rodriguez) revealed the rotational correlation time of about **17 ps**. Similar results were obtained by other authors in other solvents.

Doraiswamy, however, failed to detect any fluorescence anisotropy in his time-resolved studies of C60 and C70 in toluene (in the 191-296 K range). The reason for this is not clear since at the same time the steady-state value of the EA of C60 was found to be 0.05 ± 0.003.

V.KJones and A.A.Rodriguez (1992) *Chem.Phys.Lett*. **198**, 373-378

M.R.S.Doraiswamy (1994) *Chem.Phys.Lett*.**225**, 181-185

Conclusions

Bulk viscosity of isotropic liquids is usually measured with **mechanical viscometers**, but in monitoring
local microviscosity in heterogeneous and ordered molecular systems (not necessarily biological),
fluorescence viscometry appears to be a method of choice.
A great advantage of **optical viscosity monitoring** is that it can be performed with **extremely small samples, remotely** and **under on-line conditions**.
Coupling of optical viscometry with fluorescence microscopy $\Rightarrow$ determination of **microviscosity in various cell compartments**.
Viscosity imaging as a variant of the FLIM method (J.R.Lakowicz, H.Szmaciński, K.Nowaczyk, K.W.Berndt and M.Johnson (1992) *Analytical Biochemistry* **202**, 316-330)

At present, fluorescence can hardly be recommended as a technique for absolute viscosity measurements, because of problems with boundary conditions and molecular shapes. However, this situation may change with the appearance of new specially designed e.g. spherical fullerene-like fluorescent viscosity probes.