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EXAM I COURSE TFY4310 MOLECULAR BIOPHYSICS

Suggested solutions

Friday, 16 December 2016

Time: kl. 09.00 - 13.00

All questions have the same weight. None of the questions require lengthy answers so answer as precisely and concisely as possible. Good luck!

Exercise 1.

Justify **five** (5) of the following sentences:

1. In the presence of trivalent ions, long DNA chains adopt more condensed conformations than those predicted by a simple neutralization of the phosphate charges along the chain.

Answer: DNA is a polyelectrolyte with a relatively high charge density. When trivalent ions associate with it via electrostatic interactions, these can correlate with the trivalent ions in other parts of the (large) DNA that will be in the proximity. Such (ion) correlations lead to an attractive interaction between the two “DNA surfaces”, which results in a decrease in the overall DNA chain dimensions that goes beyond that of a neutral chain.

2. The width of the probability distribution of the end-to-end distance ($P(R_{ee})$) of a polymer chain is larger taking into account the freely jointed model than the rotationally-hindered statistical model, but the root-mean-square of the end-to-end distance $\langle R_{ee}^2 \rangle^{1/2}$ is lower.

Answer: The freely-jointed model consists in describing a polymer based solely on the number of bonds and their length. The rotationally-hindered chain model introduces restrictions on both the values of the bond angle and those of the dihedral (or torsion) angles. Such restrictions lead to an increase in the average dimensions of the polymer, $\langle R_{ee}^2 \rangle^{1/2}$, since the bond angle is generally taken as 109° , and so all random walks based on angles smaller than this are removed. Likewise, the dihedral angles that are taken into account in the rotationally-hindered chain, excludes dihedral angles that contribute to smaller conformations (excluded due to chain overlapping effects). On the other hand, such restrictions lead fewer possible polymer conformations and thus, a narrower size distribution.

3. When a cross-linked rubber is stretched by a dead load and is heated its extension decreases.

Answer: The stretching of a cross-linked rubber is entropic in nature. When a rubber is stretched the polymer chains within the network are stretched out into conformations that have a lower entropy and therefore are not very favourable from a free energy point of view. When the rubber is heated up, the polymer chains the entropy in the system increases which leads to more favourable less extended polymer conformations. This leads then to the shrinking of the rubber band.

4. Brownian dynamics is a suitable modelling technique to follow adsorption of long polymers to oppositely charged surfaces.

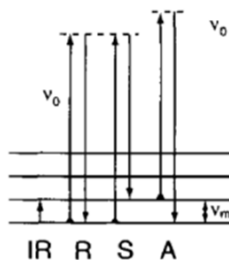
Answer: If we want to follow the adsorption process of a polymer to an oppositely charged surface we need to use a modelling technique that allows us to follow the dynamics of the process. If we want to study long polymers, and assuming that the specific chemistry of the polymer and/or surface are not important, the most convenient procedure is to coarse-grain the system and use a implicit solvent approach. A Brownian Dynamics technique that solves the Langevin equation, for example, which include Newton's equation of motion and the viscosity of the medium and a random force that includes collisions with the solvent molecules, would be a good approach.

5. In a common transient electric birefringence set-up the analyser is oriented 90° relatively to the polariser.

Answer: With transient electric birefringence one can measure the rotational diffusion coefficient of long (and somewhat stiff) macromolecules. The set-up is built so that the macromolecules are firstly oriented in solution, using an electric field. When the the electric field is switched off, the molecules relax to a random orientation in the sample, which is measure in terms of changes in birefringence. The birefringence is detected by using an optical system with two crossed polarisers. If there is no birefringence in the sample the intensity coming out of the analyzer will be zero. If there is birefringence there will be a phase difference between light polarised in y-direction and x-direction, which will be detected, in the form of intensity reaching the detector.

6. In Raman scattering, the Stokes lines are stronger than the anti-Stokes lines, at room temperature.

Answer: Raman scattering is a technique that probes the vibrational levels of a molecule by exciting electrons (typically) from the electronic ground state to virtual states and measuring the emitted photon as the electrons returns to the ground state. In most cases the excitation and emitted photon will have the same frequency (Rayleigh scattering) but if there is a change in polarizability with the equilibrium separation of



the nuclei, Stokes (S) and anti-Stokes (A) lines can be observed. The former refers to transitions that start as the ground vibrational level and end in the excited vibrational level, and the latter corresponds to transitions that start in the first vibrational state and finish in the ground vibrational level (see scheme above). At room temperature, it is more likely to find electrons in the ground state than in the excited state. The Stokes lines are thus expected to be stronger.

7. Considering that the reciprocal of the scattering vector is $2\pi/q$, X-ray scattering ($\lambda = 0.1$ nm) radiation with a scattering angle of 0.1° is better suited to study proteins with around 60 nm in diameter than visible light ($\lambda = 400$ nm) at a scattering angle of 50° .

Answer: In the described X-ray experiment the scattering angle will be

$$q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right) = \frac{4\pi}{0.1} \sin\left(\frac{0.1}{2}\right) = 0.11 \text{ nm}^{-1}$$

which leads to distances of about

$$d = 2\pi/q = 2\pi/0.1 = 62 \text{ nm.}$$

which is within our scale of interest. Using the light scattering source and experimental geometry leads to $q = 0.013 \text{ nm}^{-1}$ and dimensions of about 473 nm.

8. Small angle neutron scattering (SANS) is a powerful tool to study the structure of DNA-protein complexes such as the nucleosomes.

Answer: We can only observe scattering if there is a source of “contrast” in the sample. In SANS the contrast is given by the scattering length density which varies with the nuclei composition. As seen in the table in the end of the exam, the scattering density of water and deuterated water are quite different, and the scattering density of the major classes of biological molecules are also different and conveniently placed between the two solvent. This means that by choosing different H_2O to D_2O compositions one can selectively match the contrast of, say, proteins to the solvent and make them “transparent”. In this case one would be able to obtain the form and or structure factors of the nucleic acid only, and vice-versa.

Exercise 2.

1. Shortly describe the three forces that contribute to the van der Waals force.

Answer: The van der Waals forces gather three types of forces. These are the Keesom or orientation interaction that describes the interaction between two freely rotation dipoles, the Debye or induced interaction which describes the interactions between a (freely rotating) dipole and a apolar molecule (induced dipole), and finally the London or dispersion forces that describe the interaction between two apolar molecules, or induced dipole - induced dipole interaction.

In all these, the potential of free energy varies with the inverse sixth power of the distance, and together contribute to the total van de Waals interaction between atoms and molecules.

2. van der Waals interactions are predicted to decrease with increasing ϵ of the medium, however the strength of the interaction between two small apolar molecules increases when these are placed in water. Justify.

When apolar molecules are placed in water, the water is unable to form hydrogen bonds with it. The water will thus “organize” itself around the polar molecules to preserve the hydrogen bonds with the neighbours. This will lead to an increase in the average H-bond per water molecule resulting in an increase in the energy but a large decrease in the entropy. To minimise this entropy loss, the two molecules will be pushed together, decreasing the overall surface area exposed to the solvent and thus the number of water molecules that participate in the so-called clathrates. This apparent attraction between the apolar molecules is called hydrophobic interaction, it is entropic in nature and is the reason why apolar molecules associate in water.

3. Figure 1(a) shows a lipid membrane in water and the lateral pressure (or stress) profile. Taking into account the pressure profile, discuss the forces that act within the bilayer.

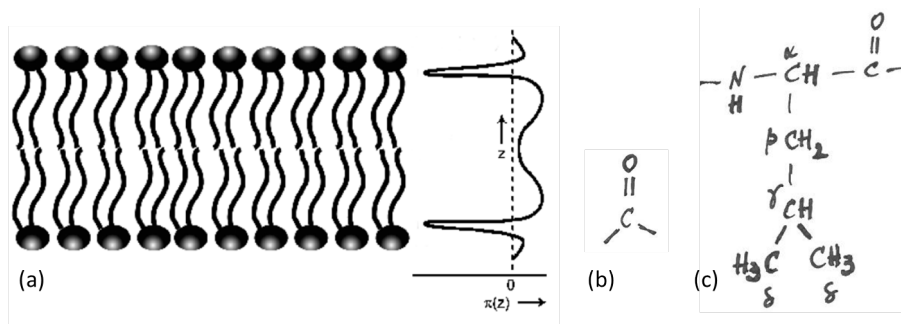


Figure 1: (a) Scheme of a lipid bilayer and lateral pressure profile (question 2.3). (b) Molecular structure of the carbonyl group (question 3.1). (c) Molecular structure of leucine (question 3.3).

Answer: Starting from the top we can see that the pressure is positive due to repulsions between the headgroups of the lipids. This can be due to steric (entropic) effects or electrostatic repulsions, if the headgroups of the lipids are charged. In the interface between the hydrophilic headgroups and the hydrophobic tails, the pressure is negative (due to the interfacial tension), indicating the attraction between the lipid tails due to hydrophobic interactions. As one moves towards the middle of the bilayer the pressure becomes again positive due to the steric (entropic) repulsions of the lipid tails inside the membrane, in the middle of the bilayer the pressure goes nearly to zero since the density is also lower here.

4. Assuming that the headgroups of the lipids are negatively charged, that there is one lipid molecule per 0.8 nm^{-2} at 293 K, and that the concentration of ions in the bulk tends to zero, what will be the surface density of counterions at the membrane surface? Assume these occupy a thickness of 0.2 nm.

Answer: The charge density of the lipid membrane will be $\sigma = -1.6 \times 10^{-19} \text{ [C]} / 0.8 \times 10^{-18} \text{ [m}^2\text{]} = 0.2 \text{ C m}^{-2}$.

The density of counterions at the surface is given by:

$$\rho_s = \rho_0 + \frac{\sigma^2}{2\epsilon\epsilon_0 k_B T} = 0 + \frac{(-0.2)^2 \text{ [C}^2\text{m}^{-4}\text{]}}{2 \cdot 78.4 \cdot 8.854 \times 10^{-12} \cdot 1.38 \times 10^{-23} \cdot 293 \text{ [C}^2\text{J}^{-1}\text{m}^{-1}\text{JK}^{-1}\text{K]}}$$

$$\rho = 3.56 \times 10^{28} \text{ counterions/m}^3$$

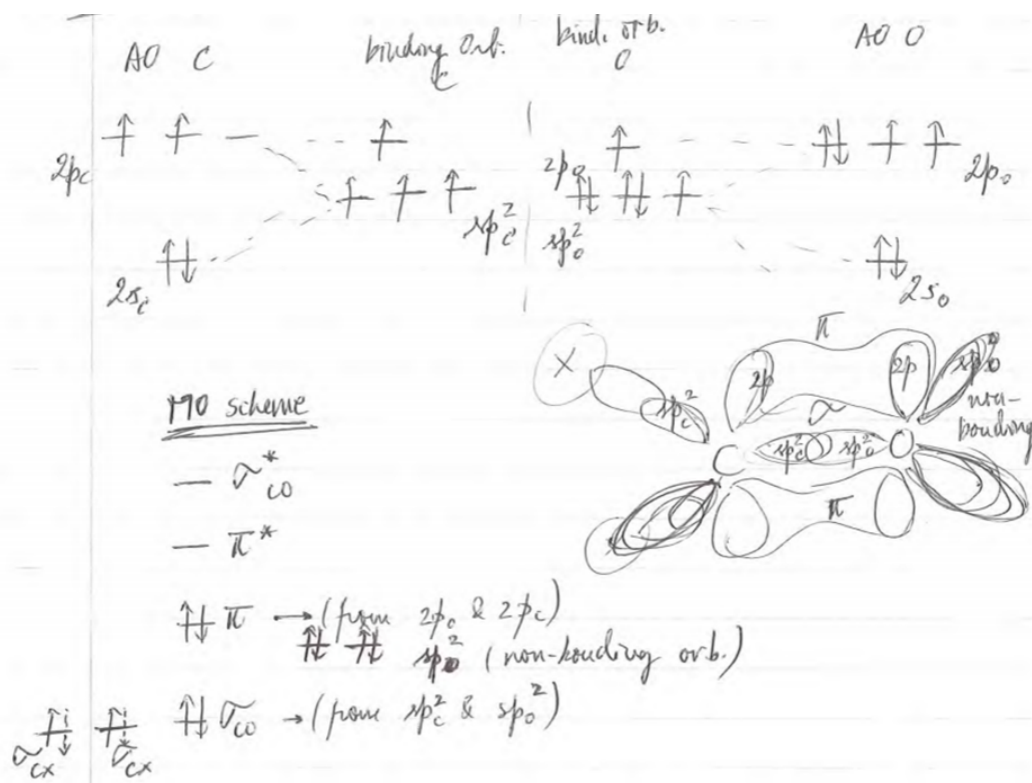
To calculate the density we simply multiply for the thickness the counterions occupy:

$$\sigma_{\text{ctions}} = 3.56 \times 10^{28} \cdot 0.2 \times 10^{-9} = 7.12 \times 10^{18} \text{ counterions/m}^2 = 1.1 \text{ C/m}^2$$

Exercise 3.

1. Draw the molecular orbital diagram of the carbonyl group in the peptide bond (Figure 1(b)) and discuss the hybridization scheme of the carbon and oxygen atoms.

Answer: The hybridization of both carbon and oxygen atoms is sp^2 . The atomic, binding and molecular orbital diagrams are:



For full score one needs to indicate that the π orbitals arise from the overlap of the 2p orbitals.

2. Why does the extinction coefficient (ϵ) of a molecule depend on the wavelength?

Answer: The extinction coefficient contains the wavelength or frequency dependence of the absorption spectrum of a particular molecule, that is, it describes how a particle absorbs light. A molecule absorbs light when an electronic transition occurs, which happens when the wavelength of light corresponds to the energy gap of two electronic states, and the transition selection rules are met. Each molecule, with its own chemical structure, will have its own electronic structure, and thus a different wavelength-dependent extinction coefficient.

3. The molecular structure of the amino acid leucine is depicted in Figure 1(c). Protons α to δ give rise to NMR peaks at chemical shifts around 3.3, 2.4 and 2.6 (each β proton experiences a different chemical environment due to the chiral group they are attached to), 1.9 and 1.0, respectively.

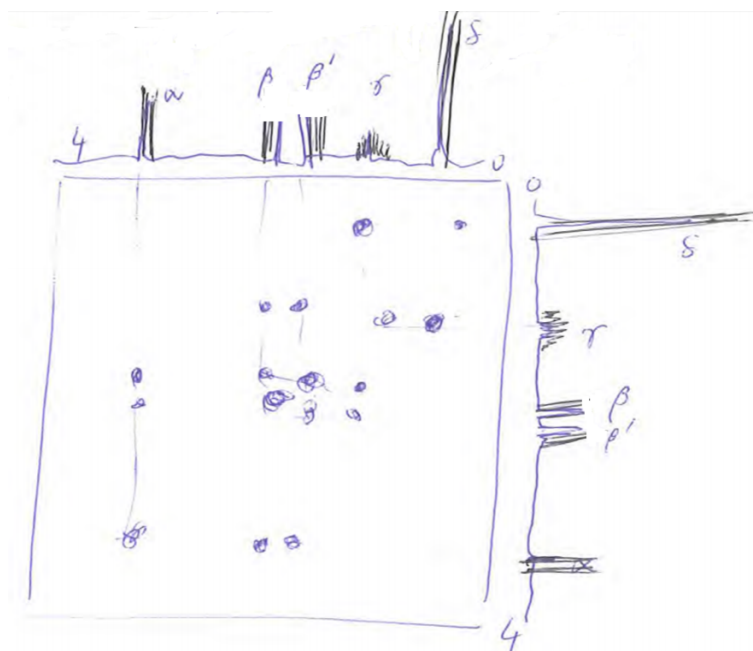
- i. Which multiplicity do you expect to observe for each peak?

Answer: Taking into account the given structure and taking into account only the marked protons, one expects the α proton peak, at 3.3, to have two doublets, since protons β possess different chemical shifts, each of the β protons should be three doublets (from the α , other β and γ), γ proton would give a complicate peak which would include a two doublets (from protons β and a septet (7 peaks) from the δ protons. Finally protons δ would be expected to show a peak splitted in two.

Many did not take into account the differences in the chemical shift of the neighboring protons, that is, the answer was given as: triplet (α), quadruplets for each β protons, a nonet (9 peaks) for the γ proton and a doublet for the δ protons. 10 % was drawn for the question, in this case.

- ii. Draw the two-dimensional COSY spectrum for leucine taking into account solely the indicated protons.

Answer: The scheme of the 2D COSY is as follows:



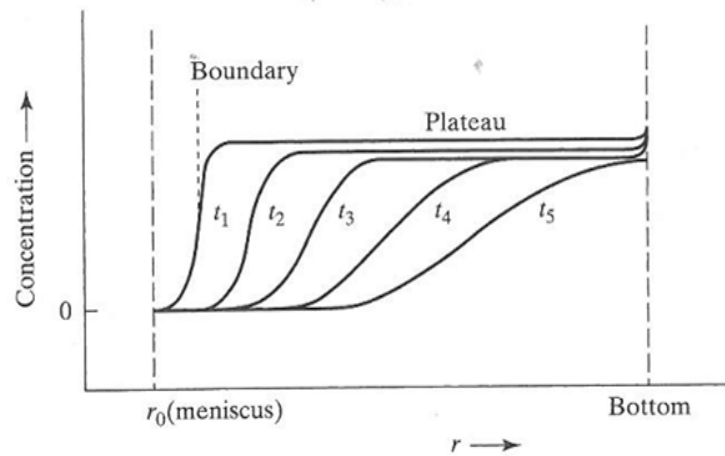
Exercise 4.

The variation in solute concentration with distance from the rotation axis r and time is given by the Lamm equation:

$$\frac{\partial c(r, t)}{\partial t} = D_T \left(\frac{\partial^2 c(r, t)}{\partial r^2} + \frac{1}{r} \frac{\partial c(r, t)}{\partial r} \right) - s\omega^2 \left(r \frac{\partial c(r, t)}{\partial r} + 2c(r, t) \right)$$

1. Draw the evolution of the solute concentration along the cell with time.

Answer: The evolutions of the solutie concentration along the cell with time in sedimentation centrifugation is:



To plot the evolution of concentration along the cell with time, using equilibration centrifugation would have also been final.

- Starting from the Lamm equation, explain how the sedimentation coefficient, s , is obtained experimentally.

Answer: Looking at the above drawn evolution of the solute concentration along the cell with time we can see that in the plateau region the concentration is constant in relation to r , that is both first and second derivative with C with r in the Lamm equation will be zero. We can thus simplify the Lamm equation to:

$$\frac{\partial c_p(t)}{\partial t} = -2s\omega^2 c_p(t)$$

This equation has solution

$$c_p(t) = c_0 \exp \{-2s\omega^2 t\} \quad (1)$$

$$s = \frac{\ln \{c_0/c_p(t)\}}{2\omega^2 t}. \quad (2)$$

This last expression is well suited for experimental determination of s if we have an analytical ultracentrifuge which is equipped with an optical detection system that directly determines the $c(r, t)$.

- The data given below describe the variation of the sedimentation coefficient and diffusion coefficient for protein A as a function of pH. Explain what happens to the protein at low and high pH, assuming that it is in the native (functional) state between pH 5 and 6.

pH	$s_{20,w} (\times 10^{-13} \text{ s})$	$D_{20,w} (\times 10^{-7} \text{ cm}^2\text{s}^{-1})$
3	3.02	4.85
4	3.89	4.85
5	4.41	3.58
6	4.40	3.59
7	4.15	3.40
8	3.60	2.95
9	2.25	1.85

Answer: Both sedimentation coefficient and translational diffusion coefficient are inversely proportional to the friction coefficient, and thus dimension and shape of the

protein, but the sedimentation coefficient is also depend on the molecular weight. For high pH values both s and D decrease and their ratio is nearly constant. This indicates that the molecular weight remains constant and the shape of the molecule changes, that is the protein is probably denaturated at above pH 6. As low pH values we can see that, again, both s and D change but the ratio between them is not kept so there will be a variation in the molecular weight of the protein (and maybe also on the shape). The fact that the translational diffusion coefficient becomes larger means that the protein become smaller, which suggest that the native proteins is composed of at least two units and these dissociate at low pH.

4. Calculate the molecular weight of protein A ($\bar{V}_1^{(S)} = 0.72 \text{ cm}^3/\text{g}$) at pH 5.

Answer: To calculate the sedimentation coefficient we use the Svedber equation:

$$s = \left(1 - \bar{V}_1^{(S)} \rho\right) \frac{M_1}{N_{\text{Av}} f}$$

$$M_1 = \frac{s N_{\text{Av}} f}{1 - \bar{V}_1^{(S)} \rho}$$

The diffusion friction coefficient f is calculated using

$$f = \frac{k_B T}{D_T} = \frac{1.30 \times 10^{-23} [\text{m}^2 \text{Kg s}^{-1} \text{K}^{-1}] 293 [\text{K}]}{3.58 \times 10^{-11} [\text{m}^2 \text{s}^{-1}]} = 1.129 \times 10^{-10} \text{ Kg s}^{-1}$$

The molecular weight is calculated according to:

$$M_1 = \frac{4.41 \times 10^{-13} [\text{s}] 6.022 \times 10^{23} [\text{mol}^{-1}] 1.129 \times 10^{-10} [\text{Kg s}^{-1}]}{1 - 0.72 [\text{cm}^3 \text{g}^{-1}] 1 [\text{g cm}^{-3}]} = 107.1 \text{ Kg mol}^{-1}$$

5. Calculate the hydrodynamic radius of protein A at pH 5.

Answer: The hydrodynamic radius is calculated using the Stokes formula:

$$f = 6\pi\eta R_h$$

$$R_h = \frac{f}{6\pi\eta} = \frac{1.129 \times 10^{-10} [\text{Kg s}^{-1}]}{6\pi 0.01 \times 10^{-3} [\text{Kg cm}^{-1} \text{s}^{-1}]} = 5.99 \times 10^{-7} \text{ cm} = 5.99 \text{ nm}$$

6. Protein A was additionally studied using small-angle X-ray scattering, where X-rays of $\lambda = 0.154 \text{ nm}$ were used, and the following data recorded:

θ (mrad)	$\ln I_s$
1.41	70.76
2.50	70.71
3.40	70.66
4.00	70.61

Calculate the radius of inertia (gyration). The data showed that the proteins have a nearly spherical shape, what is their diameter?

Answer: To calculate the radius of inertia we resort to the Guinier approximation given by:

$$I_s(q) = I_0 \exp\left(-\frac{1}{3} q^2 R_G^2\right)$$

where q , the scattering angle is defined as:

$$q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right).$$

With the information given we see that a graph of $\ln(I_s(q)/I_0)$ vs. q^2 gives a straight line with a slope of $-\frac{1}{3}R_G^2$, from where we can calculate the R_G .

To calculate the slope lets us take two values from the table, say the first and the last.

$$q(\theta = 1.41 \text{ [mrad]}) = \frac{4\pi}{0.154 \text{ [nm]}} \sin\left(\frac{1.41 \times 10^{-3}}{2}\right) = 0.0575 \text{ nm}^{-1}$$

$$q(\theta = 4.00 \text{ [mrad]}) = 0.163 \text{ nm}^{-1}$$

$$\text{Slope} = \frac{\Delta y}{\Delta x} = \frac{70.61 - 70.76}{0.163^2 - 0.0575^2} = -6.45 \text{ nm}^2$$

$$R_G = \sqrt{3 \times 6.45} = 4.40 \text{ nm}$$

To calculate the diameter of the particle we take the relation:

$$R_{G,\text{sphere}} = \sqrt{3/5}R_s$$

and get

$$R_s = R_G/\sqrt{3/5} = 4.40/0.774 = 5.68 \text{ nm}.$$

The diameter of the sphere is thus 11.36 nm.

7. Comment on the values obtained in question 5 and 6.

Answer: We can see from the previous exercises that the hydration radius of the protein is larger than the radius obtained used small-angle X-ray scattering. There could be two reasons for the discrepancy in these two quantities, the shape and the hydration of the protein. SAXS data indicates that the protein is nearly spherical and so we are left with the hydration. Based on the obtained results we can calculate an hydration factor of 1.54 for this protein.

The following formulas and data may or may not be of use in answering the preceding questions. You do not need to derive any of the formulas but all parameters must be defined, if used.

Electron charge: $e = 1.602 \times 10^{-19} \text{ C}$

Avogadro constant: $N_{\text{Av}} = 6.022 \times 10^{23} \text{ mol}^{-1}$

Boltzmann constant: $k_{\text{B}} = 1.380 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}, \text{ J K}^{-1}$

Permittivity in vacuum: $\varepsilon_0 = 8.854 \times 10^{-12} \text{ C}^2 \text{ J}^{-1} \text{ m}^{-1}$

Properties of water at 20 °C:

$$\varepsilon = 78.4; \quad \eta = 0.01 \text{ g cm}^{-1}\text{s}^{-1}; \quad \rho = 1.02 \text{ g/cm}^3$$

Temperature: $[\text{K}] = [^\circ\text{C}] + 273.15$

Atomic orbitals: H: $1s^1$; C: $[\text{H}]2s^2 2p_x^1 2p_y^1$; N: $[\text{H}]2s^2 2p_x^1 2p_y^1 2p_z^1$; O: $[\text{H}]2s^2 2p_x^2 2p_y^1 2p_z^1$

Atomic weights: $A_r(\text{H}) = 1.0$; $A_r(\text{C}) = 12.0$

Thermodynamics $G = H - TS$ $A = U - TS$ $\vec{F} = -\vec{\nabla}A$
 $S = k_B \ln W$

Statistical chain molecules $\langle R_{ee}^2 \rangle = Q^2 n$

$$\langle R_{ee}^2 \rangle = Q^2 n \left(\frac{1 - \cos \theta}{1 + \cos \theta} \right)$$

$$\langle R_{ee}^2 \rangle = Q^2 n \left(\frac{1 - \cos \theta}{1 + \cos \theta} \right) \left(\frac{1 + \langle \cos \phi \rangle}{1 - \langle \cos \phi \rangle} \right)$$

Coulomb potential $V(r) = \frac{z_1 z_2 e^2}{4\pi\epsilon_0 \epsilon r}$

Screened Coulomb potential

$$V(r) = \frac{z_1 z_2 e^2}{4\pi\epsilon_0 \epsilon r} \exp\left(-\frac{r}{\lambda_D}\right)$$

Debye screening length $\lambda_D^2 = \frac{\epsilon k_B T}{\sum_i (eZ_i)^2 n_{i\infty}}$

Density of ions at a charged surface

$$\rho_s = \rho_0 + \frac{\sigma^2}{2\epsilon\epsilon_0 k_B T}$$

Friction coefficients $\vec{F} = -f\vec{v}$, $\vec{M} = -\xi\vec{\omega}$

Stokes formula $f = 6\pi\eta R_h$, $\xi = 8\pi\eta R_h^3$

For long chains and the random walk model

$$\langle R_{ee}^2 \rangle = 6 \langle R_G^2 \rangle$$

Radius of gyration of a sphere

$$R_{G,\text{sph}} = \sqrt{3/5} R_{\text{sph}}$$

Hydrodynamic volume

$$v_{h,1} = \left(\bar{V}_1^{(S)} + \delta\bar{V}_0^{(S)} \right) \frac{M_1}{N_{\text{Av}}}$$

Specific volume (per mass) $V_1^{(S)} = v_1 \left(\frac{N_{\text{Av}}}{M_1} \right)$

Fick's laws $\frac{\partial c}{\partial t} = -\vec{\nabla} \cdot \vec{J}$, $\vec{J} = -D_T \vec{\nabla} c$, $\frac{\partial c}{\partial t} = D_T \frac{\partial^2 c}{\partial x^2}$

Nernst-Einstein relations $f D_T = k_B T$, $\xi D_R = k_B T$

Lamm-equation
$$\frac{\partial c(r, t)}{\partial t} = D_T \left(\frac{\partial^2 c(r, t)}{\partial r^2} + \frac{1}{r} \frac{\partial c(r, t)}{\partial r} \right) - s\omega^2 \left(r \frac{\partial c(r, t)}{\partial r} + 2c(r, t) \right)$$

Svedberg equation
$$s = \left(1 - \bar{V}_1^{(s)} \rho \right) \frac{M_1}{N_{Av} f}$$

Equilibrium centrifugation:
$$m_1(r) = m_1(r_m) \exp \left\{ \frac{M_1 (1 - \bar{V}_1^{(s)} \rho) \omega^2 (r^2 - r_m^2)}{2RT} \right\}$$

Electrically-induce birefringence
$$I(t) = \frac{I_0}{4} \delta_0^2 \exp(-12D_R t)$$

Raman spectroscopy
$$P = \alpha_0 E_0 \cos 2\pi\nu_0 t + \frac{1}{2} \left(\frac{\partial \alpha}{\partial q_i} \right)_0 q_{i0} [\cos(2\pi(\nu_0 + \nu_m)t) + \cos(2\pi(\nu_0 - \nu_m)t)]$$

Nuclear spin
$$\vec{m} = \gamma \vec{L}, \quad (\vec{m})^2 = \gamma^2 \hbar^2 \ell(\ell + 1), \quad m_z = m_\ell \gamma \hbar$$

Gyromagnetic ratio

Nucleus	¹ H	² H	¹³ C	¹⁴ N	¹⁹ F	³¹ P
$\gamma \left(10^7 \frac{\text{rad/s}}{\text{T}} \right)$	26.753	4.107	6.728	1.934	25.179	10.840

Larmor frequency
$$\nu = \frac{\gamma}{2\pi} B_0$$

Small-angle scattering
$$q = \frac{4\pi}{\lambda} \sin \left(\frac{\theta}{2} \right)$$

Guinier approximation
$$I_s(q) = I_0 \exp \left(-\frac{1}{3} q^2 R_G^2 \right)$$

Discrete identical homogeneous particles
$$\langle I_s(q) \rangle = N b^2(0) P(q) S(q)$$

Static light scattering RGD regime
$$\frac{\langle I_s(q) \rangle}{I_0} = c M \kappa \frac{1}{R^2},$$

Large systems
$$\frac{\kappa c}{R_\theta} = \frac{1}{M} \left[1 + \frac{16\pi^2}{3\lambda^2} R_G^2 \sin^2 \frac{\theta}{2} \right] \cdot [1 + 2B_2 c],$$

Dynamic light scattering Siegert relation
$$g^{(2)}(q, \tau) = 1 + [g^{(1)}(q, \tau)]^2$$

$$g^{(1)}(q, \tau) = \exp(-q^2 D_T \tau)$$

Scattering length density

Substance	H ₂ O	D ₂ O	proteins	nucleic acids	lipids
$\rho \text{ (} 10^{-4} \text{ nm}^{-2}\text{)}$	-0.55	6.36	3.11	4.44	-0.01

