

AGGREGATION OF PROTEIN NANOPARTICLES TESTING BY OPTICAL SPECTROSCOPY

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Abstract. In this contribution we report on the inverse optical problem solution for characterizing protein nanoparticles aggregation by optical spectroscopy: absorption spectra, integral static (time average) light scattering spectra and intensity of differential static light scattering. The measurements are compatible, non-destructive and can provide information about the processes in "ill-defined" three-dimensional disperse systems (3D DS) with nanoparticles.

1. Introduction

Protein molecules in water can be considered as three-dimensional (3D) disperse system (DS) with nanoparticles as a disperse phase in a dispersive medium [1]. The 3D DS with nano- and micro- particles are so called "ill defined" systems [1]. Light scattering is sensitive to the parameters and to size distributions of particles in systems [1, 2]. There are several modifications of light scattering method such as integral static (time average) light scattering, differential static light scattering, differential dynamic light scattering, etc. The solution of inverse optical problem for light scattering is "ill-conditioned" task (i.e. there are many particle size distributions that correspond to similar scattering signal) [1, 2]. That is why no one modification of characterization by light scattering is absolute, but as sensitive tool this method can be used for "ill defined" 3D DS control [3-10].

2. Materials and methods

Water solutions (dispersions, 3D DS) of bovine serum albumin (BSA, Reachim and Fluka) were studied at different concentrations (in mg per ml) prepared by weighting. Extinction of light due to absorption and integral static light scattering was measured at spectrophotometer (SP-26, LOMO, St. Petersburg, Russia) at wavelengths from 200 nm up to 700 nm and at 4 degrees of aperture angle of a photo receiver. Diaphragms were used for obtaining size distributions of particles [5]. For solution of the inverse optical problem for integral light scattering the "turbidity spectrum method"[1] was used. Differential (at detector angles from 60 up to 120 degrees) laser (wavelength 632.8 nm) static light scattering was measured at self-made installation relatively to scattered standard at ambient conditions.

3. Results and discussion

Optical density (D) spectra of BSA dispersions with concentration 1 mg/ml are presented in Fig. 1. The dependence $D(\lambda)$ is shown (where λ is the wavelength from 260 nm up to 600 nm)

for water 3D DS with nanoparticles of BSA (curve 1 – Reachim, curve 2 - Fluka). In general, using a spectrophotometer it is possible to measure transmittance of light - T:

$$T = (I_t / I_0) \cdot 100\% \quad (1)$$

where I_t and I_0 are the intensity of transmitted (outgoing through a cuvette) and incident light, respectively. The optical density D (other names: absorbency A , extinction of light E) and turbidity τ are connected with T by the formulas:

$$D = -\lg T = \lg (I_t / I_0) \quad (2)$$

$$\tau = -\ln T / l = 2.3 d / l \quad (3)$$

where l is the length of optical way (length of a cuvette, usually in cm).

The light extinction due to the scattering at all angles (except the aperture angle of a photo receiver) is the measure of the integral static (time average) light scattering. At the interval of λ , where there is no absorption of light, $D(\lambda)$ can be considered as the measure of integral light scattering $D_{sc}(\lambda)$. For absorbing nanoparticles with a diameter less than λ , at first approximation:

$$D(\lambda) = D_{sc}(\lambda) + D_{abs}(\lambda) \quad (4)$$

where $D_{sc}(\lambda)$ and $D_{abs}(\lambda)$ are the optical densities connected with scattering and absorption, correspondingly.

In Fig. 2 the optical density spectra (presented in Fig. 1) are shown in a bilogarifmic scale. The slope of linear part of spectrum (there is no absorption of light from 400 up to 600 nm) is so called [1] wavelength exponent $n(\lambda)$:

$$n(\lambda) = -\partial \lg D(\lambda) / \partial \lg \lambda \quad (5)$$

The function $n(\lambda)$ is the characteristic function [1] of the relative size of particles α and the relative refractive index of particles m :

$$\alpha = \pi d \mu_0 / \lambda \quad (6)$$

$$m = \mu_p / \mu_0 \quad (7)$$

where d is the mean effective equivalent diameter of particles; μ_0 and μ_p are the refractive indexes of dispersive medium and disperse phase (particles), correspondingly; λ is the wavelength in the middle of a considered interval. For particles in water as dispersive medium $\mu_0 = 1.33$ and d :

$$d = 0.24 \alpha \lambda \quad (8)$$

From the calculations of the characteristic function $n(\alpha, m)$ [1], it is possible to determine α and d . In the interval: $2 < n < 4$, n does not depend on m (the result of Mie's theory in this range). If μ_p is known, from $D_{sc}(\lambda)$ one can calculate N_{sc} , the mean effective equivalent number of scattered particles, and the mean effective equivalent disperse phase mass M [1].

Numerical calculations according to Mie's theory for model gamma-distributions of particles and experiments with bimodal latex suspensions have shown that the mean effective particle radius determined by the turbidity spectrum method from $n(r)$ calibrations for monodisperse spherical particles (r_n) corresponds with a minimal error to the relationship [1]:

$$\bar{r}_n = \sqrt{r^{n+4} / r^{n+2}} \quad (9)$$

Analysis of spectra $D(\lambda)$ in the bilogarithmic presentation (Fig. 2) according (4) gives the absorption spectra $D_{abs}(\lambda)$ and the integral light scattering spectra $D_{sc}(\lambda)$. Results of both spectra analysis are shown in Figs. 3–6. Concentration dependences of the main integral light scattering parameters, $D_{sc}(500)$ and $n(500)$, for BSA (Reachim) are presented in Fig. 3.

In Fig. 2 the value $D_{abs}(280)$ at λ corresponding to the maximum D_{abs} equals to 0.93 for BSA (Reachim) and to 0.89 for BSA (Fluka), which are close values. If to suppose that for defatted 1% BSA solution [11] $D(280) = 6.7$, it is possible to conclude that about 28 % for

BSA (Reachim) and 25 % for BSA (Fluka) of $D_{\text{abs}}(280)$ are corresponded to the absorption of lipid impurities which also have absorption at this range of λ . From the other side, the lipid impurities are well known aggregation factor. Analysis of $D_{\text{sc}}(\lambda)$ can help to study the aggregation process in protein 3D DS.

In Fig. 2 the values of integral light scattering parameters are the following: for BSA (Reachim) $D_{\text{sc}}(280) = 0.620$ and $n(500) = 2.06$; for BSA (Fluka) $D_{\text{sc}}(280) = 0.110$ and $n(500) = 2.79$. From these data it is possible to conclude that there are aggregates at both dispersions and the mean size of particles for BSA (Reachim) is larger. About the larger mean size of particles in water BSA 3D DS (Reachim) in comparison with BSA (Fluka), one can also conclude from the dependence of differential static light scattering intensity on an angle presented in Fig. 4.

Further optical measurements with diaphragms [5] for these dispersions at sedimentation allowed at first approximation to divide each of them on 2 modes (Fig. 5) – conditionally ‘small aggregates or associates’ (mode ‘a’ in Fig. 5) and ‘large aggregates or associates’ (mode ‘b’ in Fig. 5).

At spherical approximation, the diameter of BSA molecule [11] is 14 nm. One can conclude for the first mode ‘a’ of BSA (Fluka, 2a in Fig. 5) from $n(500) = 3.81$, $\alpha = 0.42$ and the mean equivalent effective diameter $d = 50.4$ nm that in the first mode of size distribution there are aggregates or associates of molecules, in average about 20 - 30 molecules per aggregate [6] according to ‘ideal’ scheme of aggregation [1].

For the first mode of BSA (Reachim, 1a in Fig. 5) with $n(500) = 2.96$, $\alpha = 1.20$ and the mean equivalent effective diameter $d = 144$ nm, it is possible to conclude that there are also aggregates or associates of molecules in this mode.

If to suppose that for aggregates $\mu_p = 1.05$ [6], the following results can be obtained: for the second modes:

- 1) BSA (Reachim, 1b in Fig. 5) – $n(500) = 1.79$, $\alpha = 14.5$ and $d = 1740$ nm;
- 2) BSA (Fluka, 2b in Fig. 5) – $n(500) = 1.46$, $\alpha = 22.1$ and $d = 2650$ nm;

for the initial dispersions:

- 1) BSA (Reachim) – $n(500) = 2.06$, $\alpha = 6.2$ and $d = 744$ nm;
- 2) BSA (Fluka) – $n(500) = 2.79$, $\alpha = 1.4$ and $d = 168$ nm.

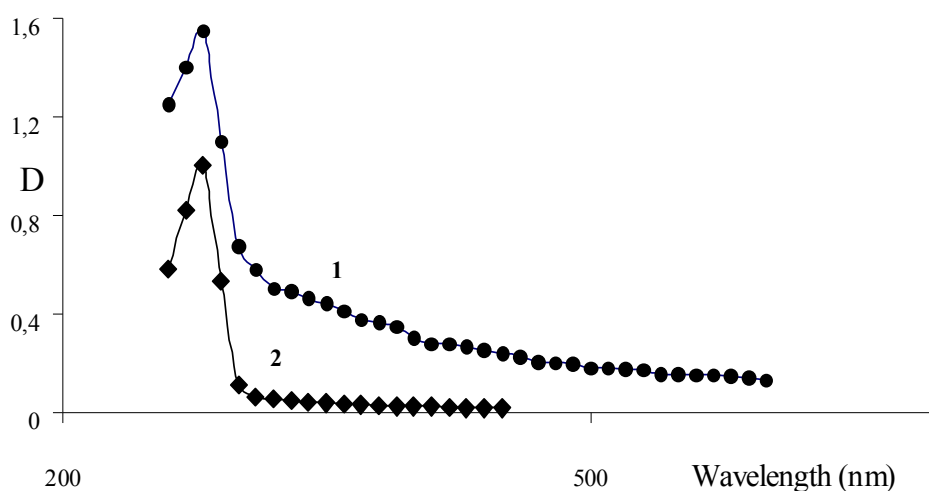


Fig. 1. Optical density spectra for bovine serum albumin (BSA) water dispersions (3D DS) at concentration 1 mg/ml. 1 - BSA (Reachim). 2 - BSA (Fluka).

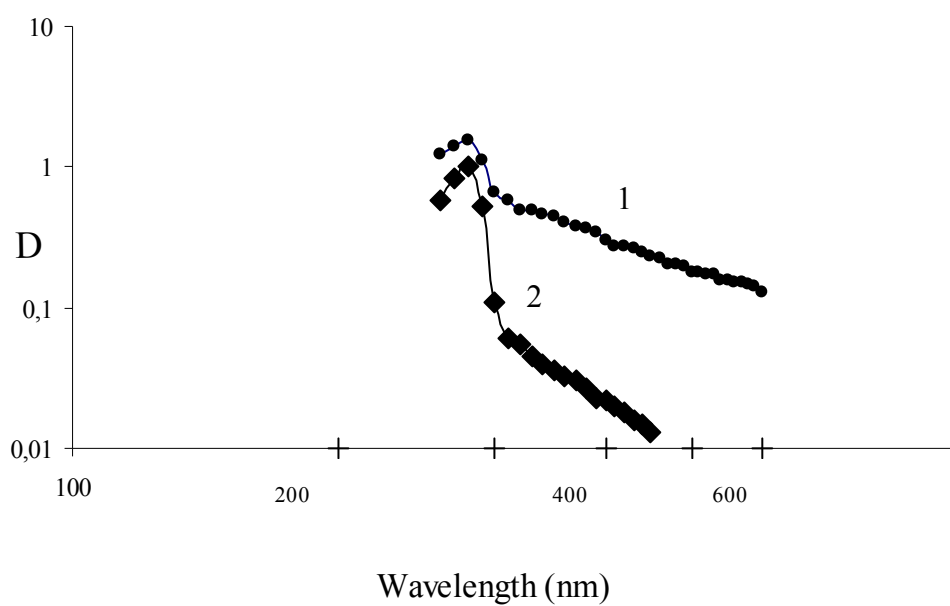


Fig. 2. Bilogarithmic plot of optical density spectra presented in Fig. 1 for BSA in concentration 1 mg/ml. 1 - BSA (Reachim). 2 - BSA (Fluka).

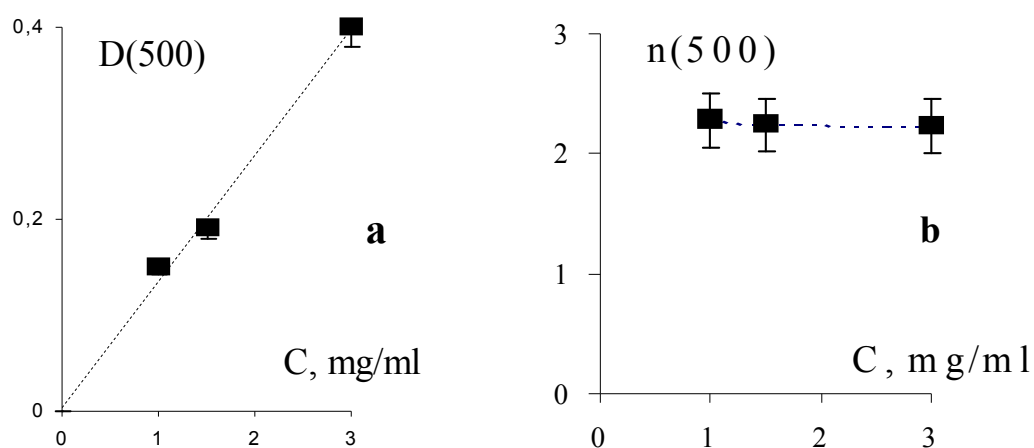


Fig. 3. Concentration dependence of parameters for BSA (Reachim):
a – $D(500) = D_{sc}(500)$; b – $n(500)$.

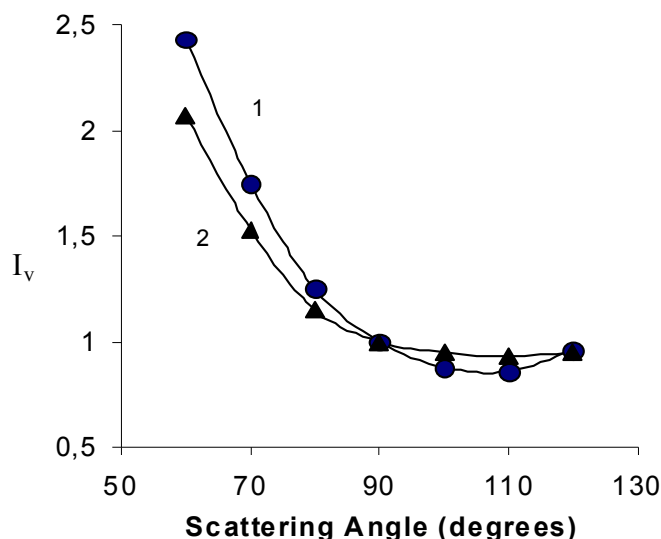


Fig. 4. Normalized for 90 degree differential light scattering intensity of vertically polarized laser light with $\lambda = 632,8$ nm (I_v) at angles from 60 up to 120 degrees for BSA in concentration 1 mg/ml. 1 - BSA (Reachim). 2 - BSA (Fluka).

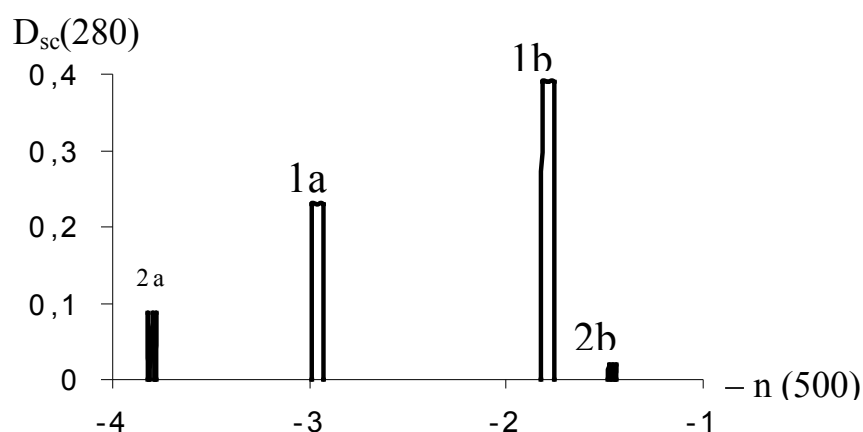


Fig. 5. Bimodal presentation (as first approximation) of BSA integral light scattering parameters:

$n(500)$ at $\lambda = 500$ nm and $D_{sc}(280)$ at $\lambda = 280$ nm (maximum of absorption spectra).

1 - BSA (Reachim). 2 - BSA (Fluka).

a – the first mode (small aggregates or associates);

b – the second mode (large aggregates or associates).

In general, polycomponent content and polymodality are inherent properties of many natural 3D DS with nano- and micro- particles. BSA solutions (dispersions) are used in this study as an example of multiparametric optical analysis application for polycomponent polymodal systems. BSA 3D DS are bicomponent (protein molecules and impurities – mainly lipids) polymodal 3D DS, which particle size distribution can consist of the following modes: 1) BSA molecules; 2) lipid molecules; 3) aggregates (associates) of BSA molecules; 4) aggregates (associates) of lipid molecules; 5) aggregates (associates) of BSA molecules with lipid molecules. The probability of each mode existence can be different; some of them can not show itself as real modes. In many cases, size distribution can be approximated as gamma-distribution [1]. Optical parameters can reflect most changes in the state of such complex systems.

3D DS state differentiation can be made by multiparametric analysis of optical data, which includes: a) simultaneous measurements of dispersions by different compatible non-destructive optical methods such as refractometry, absorbency, fluorescence, light scattering (integral and differential, static and dynamic, unpolarized and polarized), and b) solution of the inverse optical problem by different methods among which are modern technologies of data interpretation by information-statistical theory. In our research we have investigated different 3D DS with nano- and micro- particles [3-10]. The analysis of experimental data by compatible optical methods allows draw the conclusion that there are three classes of parameters.

The first class parameters refer to measured optical values under certain conditions, for example, the optical density connected with integral light scattering $D_{sc}(\lambda)$ (for BSA – Fig. 1, Fig. 2, Fig. 3a and Fig. 5). The second class parameters can be calculated from experimental optical data (without any ‘a priori’ information about disperse phase), for example, wave exponent (for BSA – Fig. 3 b and Fig. 5). The second class parameters are mainly independent of particles concentration (Fig. 3 b) if the state of the 3D DS does not change with the concentration. The third class parameters are the parameters of 3D DS state, which can be obtained after solution of the inverse optical problem for experimental data, for example, the mean effective equivalent diameter d .

The experience [3-10] suggests that the set of parameters of the second class is unique [3, 8] for each 3D DS. In other words each 3D DS can be characterized by N -dimensional vector in N -dimensional space of the second class optical parameters. The optical parameter vectors can reflect in an implicit form all the peculiarities of 3D DS and changes in their state (Fig. 6).

In Fig. 6 the bilogarithmic plots of optical data presentation in the spaces of second class optical parameters for dispersions of BSA (Reachim) are shown. The optical measurements for BSA (5 dispersions) were made at the same conditions with the uncertainties about 10%. In Fig. 6a the positions of 2-dimensional (2D) vectors $\mathbf{P} \{P_k, P_n\}$ in 2D space of optical parameters for dispersions with concentrations 1 mg/ml, 1.5 mg/ml, 3 mg/ml and for the second mode 1b are near and these parameters (P_k and P_n) can be considered as not so informative for differentiation of 3D DS general state of aggregation. In Fig. 6b the positions of 4-dimensional (4D) vectors $\mathbf{P} \{P_i, P_j, P_l, P_m\}$ in 4D space of optical parameters for dispersions 1, 1.5 and 3 are near to each other, but the positions of dispersions 1a and 1b differs from them significantly. In Fig. 6c the 6-dimensional (6D) vectors $\mathbf{P} \{P_i, P_j, P_k, P_l, P_m, P_n\}$ in 6D space of optical parameters indicate that the general state of dispersion with concentration 3 mg/ml is less aggregated at the moment of measurements than the states of dispersions with concentrations 1 and 1.5 mg/ml. These data are obtained without any ‘a priori’ information about particles and particle size distributions in dispersions. It is possible to suggest other vectors $\mathbf{P} \{P_1, P_2, \dots, P_9, P_{10}, \dots\}$ for increase of differentiation sensitivity [3-10].

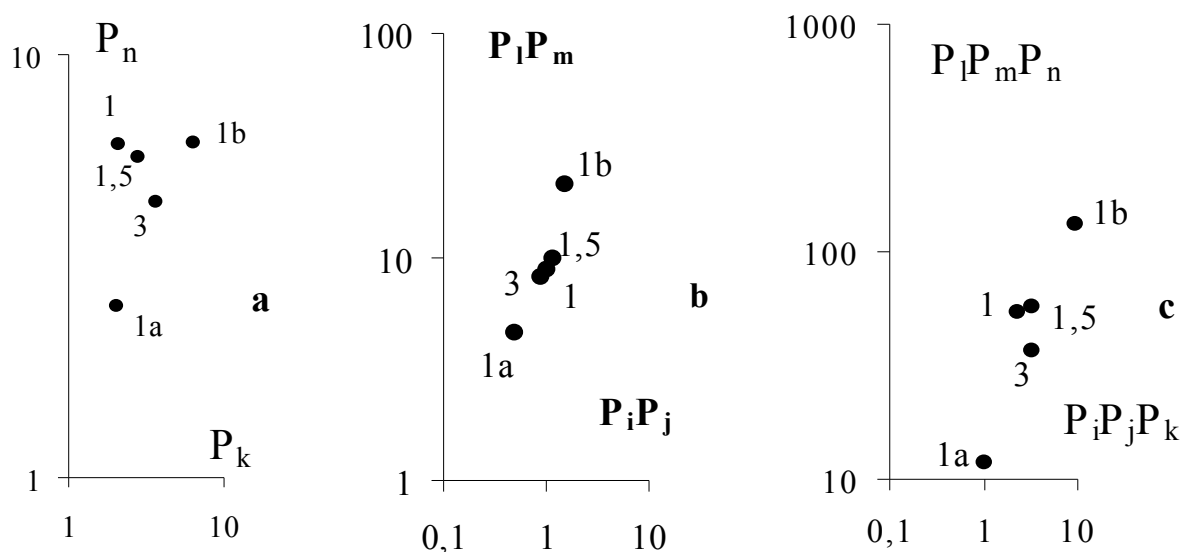


Fig. 6. Bilogarithmic plots as examples of optical data presentation in spaces of ‘second class’ optical parameters for dispersions of BSA (Reachim) with different states of 3D DS (different particle aggregation states and different size distributions of particles at the moment of measurements):

- a) as 2-dimensional (2D) vectors $\mathbf{P} \{P_k, P_n\}$ in 2D space of optical parameters;
- b) as 4-dimensional (4D) vectors $\mathbf{P} \{P_i, P_j, P_l, P_m\}$ in 4D space of optical parameters;
- c) as 6-dimensional (6D) vectors $\mathbf{P} \{P_i, P_j, P_k, P_l, P_m, P_n\}$ in 6D space of optical parameters (without any ‘a priori’ information about structure and size distribution of particles).

Optical measurements for BSA (5 dispersions) were made at the same conditions; the uncertainty is about 10%. Figures near points denote concentration of dispersions in mg per ml (Fig. 3 and Fig. 4),

1a and 1b correspond to dispersions in Fig. 5.

4. Conclusions

The inverse problem incorrectness [1, 2] can often arise from a rather wide problem statement. Making the solution unambiguous by means of additional conditions and restrictions is referred to as regularization of the inverse problem [1]. For example, smoothing the oscillating functions serves as the aim of regularization of the inverse problem of the turbidity spectrum method. In this method there is *a priori* refuse from full characterization of the 3D DS in order to obtain information on averaged properties, such as the mean size of particles, the numerical and mass-volume concentration of a dispersed phase, etc., that, as the experiment suggests, is quite sufficient to solve a number of scientific and technological problems [1].

The information–statistical approach [12] to the inverse problem solution suggests a refuse from any regularization (refuse from any *a priori* information about the state of 3D DS). This approach can help to increase the possibility of 3D DS differentiation at *on-line* optical measurements. Combination of information–statistical method with other methods can help to investigate the processes in 3D DS such as aggregation, disaggregation, inclusion [9, 10], coalescence, geteroaggregation, sedimentation, flotation, etc. Due to the fusion of various optical data and by information statistical theory, it is possible to increase sensitivity of optical measurements for 3D DS state differentiation, to find the set of the most informative parameters, to solve the inverse optical problem on the presence of a component of interest in mixtures and on the nature of particles. In this case, the polymodality of particle size distributions is not an

obstacle. This approach can be considered as an integral one for the study of a system at the moment of measurements, as a single whole intact nondestructive unity with the minimum interference. It can demonstrate an awareness of potential applications for polymer science, bio- and nano- technology, medicine and for environment protection.

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