ORIGINAL ARTICLES

SPECTROSCOPIC STUDY OF METHYLENE BLUE PHOTOPHYSICAL PROPERTIES IN BIOLOGICAL MEDIA

Pominova D.V.[1,2], Ryabova A.V.[1,2], Romanishkin I.D.[1], Markova I.V.[2], Akhlustina E.V.[2], Skobeltsin A.S.[1,2]
[1]Prokhorov General Physics Institute of Russian Academy of Sciences, Moscow, Russia
[2]National Research Nuclear University MEPhI (Moscow Engineering Physics Institute), Moscow, Russia

Abstract

A spectroscopic study of the photophysical properties of methylene blue (MB) in aqueous solutions was carried out. Absorption and fluorescence spectra as well as fluorescence lifetime were recorded. The concentration dependence of the intensity and shape of the spectra allowed establishing the ranges of MB concentrations for *in vitro* and *in vivo* studies at which aggregation is not observed (up to 0.01 mM, which corresponds to 3.2 mg/kg). Studies of photodegradation in biological media showed that photobleaching of more than 80% in plasma and culture media is observed already at a dose of 5 J/cm^2, while in water at this concentration and dose photobleaching is not yet observed, and at a dose of 50 J/cm^2 photobleaching of MB is about 30%. It was found that in media containing proteins and having an alkaline pH, photobleaching occurs significantly faster than in neutral aqueous media. The ionic strength of the solution has no effect on the photobleaching rate. Such photobleaching is caused by the photodegradation of MB rather than the transition to the leucoform.

The efficiency of singlet oxygen generation and photodynamic activity were evaluated *in vitro*. In the investigated range of MB concentrations, the efficiency of singlet oxygen generation is rather low, because positively charged MB binds to negatively charged cell membranes, which leads to a change in the type of photodynamic reaction. The emergence of other reactive oxygen species (ROS), different from singlet oxygen, in cells has been demonstrated. The generation of ROS and the low quantum yield of singlet oxygen generation indicate the tendency of MB to provide the type I photosensitization mechanism (electron transfer with the formation of semi-reduced and semi-oxidized MB$^+$ radicals) rather than to the type II mechanism (energy transfer to oxygen with the formation of singlet oxygen) in biological media and *in vivo*.

Keywords: methylene blue, spectroscopy, absorption, fluorescence, photobleaching.

Contacts: Pominova D.V., e-mail: pominovadv@gmail.com

For citations: Pominova D.V., Ryabova A.V., Romanishkin I.D., Markova I.V., Akhlustina E. V., Skobeltsin A.S. Spectroscopic study of methylene blue photophysical properties in biological media, *Biomedical Photonics*, 2023, vol. 12, no. 2, pp. 34–47. doi: 10.24931/2413–9432–2023–12-2-34–47.

СПЕКТРОСКОПИЧЕСКОЕ ИССЛЕДОВАНИЕ ФОТОФИЗИЧЕСКИХ СВОЙСТВ МЕТИЛЕНОВОГО СИНЕГО В БИОЛОГИЧЕСКИХ СРЕДАХ

Д.В. Поминова[1,2], А.В. Рябова[1,2], И.Д. Романишкин[1], И.В. Маркова[2], Е.В. Ахлюстина[2], А.С. Скобельцин[1,2]
[1]Институт общей физики им. А. М. Прохорова Российской академии наук, Москва, Россия
[2]Национальный исследовательский ядерный университет «МИФИ», Москва, Россия

Резюме

Проведено спектроскопическое исследование фотофизических свойств метиленового синего (МС) в водных растворах и биологических жидкостях. Зарегистрированы спектры поглощения и флуоресценции, а также времена жизни флуоресценции. По зависимости интенсивности и формы спектров от концентрации удалось установить диапазоны концентраций МС для исследований *in vitro* и *in vivo* при которых не наблюдается агрегация (до 0,01 мМ, что соответствует 3,2 мг/кг).

Исследовано фотообесцвечивание МС под действием лазерного излучения. Исследования фотодеградации в биологических средах показали, что фотообесцвечивание более чем на 80% в плазме и культуральной среде наблюдается уже при дозе 5 Дж/см^2, в то время как в воде при такой концентрации при дозе 5 Дж/см^2 фотообесцвечивания еще не наблюдается, а при дозе 50 Дж/см^2 фотообесцвечивание МС составляет порядка 30%. Установлено, что в средах, содержащих белки и обладающих щелочным pH, фотообесцвечива-

ние происходит существенно быстрее, чем в нейтральных водных средах. Ионная сила раствора не оказывает влияния на скорость фотообесцвечивания. Такое фотообесцвечивание вызвано фотодеградацией МС, а не переходом в лейкоформу.
Проведена оценка эффективности генерации синглетного кислорода и фотодинамической активности *in vitro*. В исследуемом диапазоне концентраций МС эффективность генерации синглетного кислорода достаточно низкая, так как положительно заряженный МС связывается с негативно заряженными мембранами клеток, что приводит к изменению типа фотодинамической реакции. Продемонстрировано возникновение в клетках других активных форм кислорода (АФК), отличных от синглетного кислорода. Генерация АФК и невысокий квантовом выход генерации синглетного кислорода свидетельствуют о склонности МС к механизму фотосенсибилизации I типа (перенос электрона с образованием полувосстановленных и полуокисленных радикалов $МВ^+$), а не к механизму II типа (перенос энергии к кислороду с образованием синглетного кислорода) в биологических средах и *in vivo*.

Ключевые слова: метиленовый синий, спектроскопия, флуоресценция, поглощение, фотообесцвечивание, АФК, синглетный кислород.

Контакты: Поминова Д.В., e-mail: pominovadv@gmail.com

Для цитирования: Поминова Д.В., Рябова А.В., Романишкин И.Д., Маркова И.В., Ахлюстина Е.В., Скобельцин А.С. Спектроскопическое исследование фотофизических свойств метиленового синего в биологических средах // Biomedical Photonics. – 2023. – Т. 12, № 2. – С. 34–47. doi: 10.24931/2413–9432–2023–12–2-34–47.

Introduction

Photodynamic therapy (PDT) is a very promising therapeutic method for treatment of tumors and non-malignant diseases. The method is based on use of photosensitizer (PS), which is selectively localized in target tissue, and irradiation with light. The PS absorbs the light and can then transfer the energy to molecular oxygen and create singlet oxygen (type II photochemical reaction), which is extremely phototoxic, causing photodamage and subsequent cell death. Another way is the participation of PS in electron-transfer reactions initiating formation of hydroxyl radicals and hydroperoxides and radical-induced damage in biomolecules (type I photochemical reaction) [1, 2]. For the vast majority of clinically approved PS, singlet oxygen is the main cytotoxic agent that determines the mechanism of photodynamic effect and causes cell death in PDT [3]. The type I photochemical reaction may be preferable for therapy of tumors which are hypoxic [1]. However *in vivo* it is difficult to establish without doubt which of these mechanisms is more prevalent, both processes can ultimately lead to cell death [4], but the knowledge how these processes are affected by biological environments is important [5].

Due to the popularity of fluorescence diagnostic methods and PDT [6–9], there is interest in the study of various dyes that can be used as PS. One of the actively studied PS is methylene blue (MB) – a heterocyclic aromatic phenothiazine dye discovered in 1876 by Heinrich Caro. In the dry state MB appears as odorless dark blue crystals, soluble in water, chloroform, and alcohol. Its molecular weight is 319.85 g/mol [10]. In its oxidized state, MB is blue in color because the phenothiazine molecule strongly absorbs in the 600-700 nm region. In aqueous solutions, the wavelength λ_{max} of the absorption band maximum for monomer is given in various works as 668 nm, 664 nm [11], 660 nm [12, 13]; λ_{max} for dimer is 614 nm [12, 13], 605 nm [11]; λ_{max} for trimer is 580 nm [11]. In the UV region for the monomer λ_{max} = 292 nm [13]. The oxidized form can be easily reduced to colorless leucomethylene blue (LMB) in the absence of oxygen [14] or by interaction with NAD(P)H [15, 16] or reduced glutathione [17]. LMB predominantly absorbs in the UV region (256 nm).

MB redox properties are important because, depending on its form, it can be both an electron donor and an electron acceptor, changing rapidly from one state to another [18]. For example, MB interacts directly with the mitochondrial electronic circuit, donating electrons to complexes I and III and/or providing partial restoration of the Krebs cycle [19], whenever NADH is oxidized by MB or even resuscitation of the mitochondrial electronic circuit. The redox properties of MB are of great interest to researchers and make it possible to use it to treat various pathologies, for example, to treat methemoglobinemia by reducing iron to its divalent state [20–23], to relieve septic shock [24, 25], to treat lactoacidosis [26], and as an antidote for carbon monoxide poisoning [27] or cyanide [28, 29]. MB also increases oxygen consumption by tissues with aerobic glycolysis and tumors, while the effect of MB is approximately proportional to the enzymatic capacity of tissues [30], which is promising in terms of photodynamic therapy because the efficiency of singlet oxygen generation obviously depends on oxygen concentration.

The ability of MB to fluoresce is used to label tumors and other blobjects in order to visualize them [31]. For a long time, MB has been actively used for photodynamic therapy of neoplasms [32–36], photodynamic inactivation of pathogens [37–40], including antibiotic-resistant microflora [41–44].

The photophysical properties of MB in solutions are actively studied. Although its photochemical properties in isotropic solution are well established, its effect *in vivo* needs further study. Most of the results reported in the literature are obtained for MB in ethanol or water. Many papers report that efficient intersystem crossing is observed with quantum yields around 0.5 [2, 45], but it should be noted that this value was obtained in isotropic

ethanol solutions [46]. The quantum yield and generation of $^1O_2$ decreases to values close to zero if MB dimerizes due to a fast nonradiative decay (3–4 ps) of the excited dimer population [47, 48], favoring electron transfer reactions and consequent generation of radical species [45, 49, 50]. Dimerization could also lead to fluorescence quantum yield decrease [45, 50]. Another parameter which affects the singlet oxygen generation efficiency is the pH. According to literature data the production of singlet oxygen is approximately five times more efficient in alkaline than in acidic medium [51]. The ratio of monomer to dimer depends significantly on the solution composition as well as MB concentration [52, 53, 53–60]. According to literature data, in 20 μM aqueous solution only MB monomers are present, at higher concentration dimerization was observed, however at concentrations below ~20 ppm [61] or ~70 ppm [57] the existence of trimeric or tetrameric aggregates can be neglected.

The state of MB aggregation in aqueous solution is also sensitive to the ionic strength [52], it was shown that the increase of the inert salts concentration indicates the decreasing tendency of the MB molecules to undergo aggregation. The concentration of surfactant also influences the dimerization. The polarity of the solvent, concentration of surfactants [50], binding of molecules in membranes and interaction with surfaces [62] can cause the changes in ratio of monomer to dimer as well as the singlet oxygen production.

A large number of interrelated factors affecting the photophysical and photochemical properties of methylene blue make it difficult to study and implement in clinical practice, despite a number of unique properties that can be very useful for improving the effectiveness of photodynamic therapy. For clinical use, it is important to study the photophysical and photochemical properties of MB under conditions closest to real biological ones. In this regard, in this work, the absorption, fluorescence, photobleaching, and singlet oxygen generation efficiency of MB in biological media (serum and RPMI medium for cell cultivation) were studied using modern spectroscopic methods. The range of concentrations was chosen based on literature data on MB concentrations in blood after a typical daily dose: 19 μM after oral administration of 500 mg, 10 μM after intravenous administration of 100 mg [63, 64]. The data obtained for biological media were compared with aqueous solutions to determine the main mechanism of photodynamic activity.

Materials and methods

Materials

Methylene blue solution purchased in a pharmacy was used for the studies: Methylene blue, 1% aqueous solution, the active substance methylthioninium chloride (Samaramedprom, Russia). Spectroscopic studies were performed for aqueous MB solutions with concentrations in the range of 0.001–0.05 mM, which corresponds to 0.32–16 mg/kg.

Measurements were performed in distilled water (PanEco, Russia), blood serum (newborn calf blood serum, PanEco, Russia), cell culture medium RPMI (PanEco, Russia) with 10% calf blood serum added, and saline (0.9% sodium chloride, infusion solution, Grotex, Russia). pH of the solutions was controlled using indicator paper (Johnson universal indicator paper, pH 1–11). For water and saline, the pH was 7, for cell culture medium RPMI—8, for plasma—9. To analyze the effect of pH on the spectroscopic properties of MB we also used water with pH adjusted to 8 using 1M NaOH solution.

Absorption spectroscopy

Absorption spectra in the range 200-1000 nm were recorded using a Hitachi U3400 spectrophotometer (Hitachi, Japan) in quartz cuvettes with optical path lengths of 1 cm (for water and NaCl) and 2 mm (for plasma and RPMI medium). To compare the results obtained with each other, all optical density values were multiplied by the corresponding coefficients (0.1 and 0.5 for the centimeter and 2-mm cuvette, respectively) and scaled to the optical density value for a 1-mm cuvette. The analysis of absorption spectra allows studying the transition of the basic form of MB into its reduced form (LMB) under the influence of external factors, photodegradation, and aggregation of MB.

To analyze aggregation, the absorption spectrum shape was approximated in the 500-800 nm range by two Gaussian peaks with maximums at wavelengths corresponding to dimers and monomers, then the ratio of areas under these peaks was considered as a characteristic of dimerization. A linear approximation of the baseline was performed to account for scattering.

Fluorescence spectra and lifetime measurements

The fluorescence spectra of MB aqueous solutions were measured using a LESA-01-Biospec fiber spectrometer (Biospec, Russia) equipped with a longpass edge filter (LP640) in the wavelength range of 650-850 with HeNe laser excitation, 15 mW power output from the fiber.

To investigate the lifetime of aqueous MB fluorescence solutions, a system of Hamamatsu streak camera and streak scope (C9300 and C10627-13) was used to carry out measurements with picosecond resolution.

Study of photobleaching in biological media

To study the laser effect on the solutions, irradiation was performed in 1 × 1 cm optical cuvettes. The surface of the solution was uniformly irradiated with an LED source with a power density of 100 mW/cm^2 and a wavelength near the MB absorption maximum of 664 nm. The light source was controlled using a programmable timer, which enabled studying the dependence of the effect on the light dose in increments of 0.5 to 5 J/cm^2. The irradiation was performed in cyclic mode, the fluorescence intensity

was measured in pauses between irradiation windows when excited by a 632.8 nm laser. The MB photobleaching rate was estimated by the decrease in integral fluorescence intensity (area under the fluorescence peak in the wavelength range of 650–800 nm) as a function of light dose density. Fluorescence intensity was determined using a LESA-01-BIOSPEC fiber optic spectrometer (Biospec, Russia).

Assessment of photodynamic activity of MB in vitro

The photodynamic activity of MB was determined by measuring the oxygen content in erythrocyte solution with MB by the hemoglobin oxygenation level [65]. We measured the deoxygenation rate of PDT with MB on erythrocyte suspension with registration of MB fluorescence under 660 nm laser irradiation at a power density of 250 mW/cm^2 (10 min=150 J/cm^2). As a result of the type II photosensitization mechanism, the formed $^1O_2$ reacts irreversibly with biological molecules, resulting in a reduction of dissolved O_2 in the sample. We calculated the relative quantum yield of singlet oxygen generation for MB concentrations of 1–100 mg/kg by comparing with experimental data for aluminum phthalocyanine with the known quantum yield of singlet oxygen generation in aqueous medium from the literature ($\phi\Delta = 0.38$) [66].

Assessment of singlet oxygen generation efficiency in PDT with MB using the Singlet oxygen sensor green

Singlet oxygen sensor green (SOSG, Invitrogen, USA) reagent was dissolved in methanol to make a stock solution of 500 μM. SOSG (with final concentration of 50 μM) was added to 10 mg/l MB aqueous solution, irradiated with a 660 nm laser at a power density of 250 mW/cm^2. SOSG fluorescence was measured with a LESA-01-BIOSPEC fiber spectrometer in the range of 550–600 nm with 532 nm laser excitation. To quantify the generation of singlet oxygen, we compared it with the experimental data for the 1 mg/l of aluminum phthalocyanine.

Evaluating the efficiency of MB generation of reactive oxygen species in vitro

6-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Carboxy-H2-DCFDA, Lumiprobe RUS Ltd, Russia) was used as an indicator of reactive oxygen species (ROS is specific for hydrogen peroxide H_2O_2 and other ROS such as superoxide-anion O2·-, hydroxyl radical ·OH, hydroperoxides ROOH, and peroxynitrites ONOO-, but with much lower sensitivity compared to H_2O_2) in living cells after PDT with MB. HeLa cells were grown on RPMI-1640 medium (PanEco, Russia) supplemented with 10% fetal bovine serum (FBS, BioSera, Nuaille, France), 100 U/mL penicillin and 100 μg/mL streptomycin (Life Technologies, Carlsbad, California, USA) in standard conditions (37°C, 5% CO_2). Cells were subcultured every third day. For confocal microscopy, cells were seeded in a POC-R2 glass-bottomed Petri dish (PeCon GmbH, Erbach, Germany) at a density of 100 × 10^3/cm^2 one day before the experiment. Twenty-four hours later, MB at a concentration of 20 mg/L was added to the cells 120 minutes before the microscopic examination. 30 minutes before the microscopic examination Carboxy-H2-DCFDA was added at a concentration of 25 μM in FBS at 37°C, 5% CO_2. The cells were washed three times with prewarmed phosphate-buffered saline, replaced with fresh culture medium, and examined with a laser scanning fluorescence microscope. Sensor fluorescence was excited with a 514 nm laser and detected in the 530–600 nm range. MB fluorescence was excited with a 633 nm laser and detected in the 650–730 nm range.

Results and discussion

Concentration effects on absorption, fluorescence, and fluorescence lifetime of MB in various biological media

Absorption spectra of aqueous solutions of MB as a function of concentration are shown in Fig. 1.

Characteristic peaks are observed in the red (664 nm—monomer, shoulder at 615 nm—aggregates) and ultraviolet (250, 290, and 320 nm) parts of the spectra. Additional peaks in the absorption spectra of MB in plasma and medium are associated with their absorption (415 nm—plasma, 560 nm—phenol red in culture medium). There was also strong absorption in the UV region for plasma and culture medium, which makes it impossible to analyze the MB absorption peaks in this range.

To analyze the aggregation, the absorption spectra were normalized by their maximum, to observe how the spectrum shape changes with increase in concentration, Fig. 2.

The shape of the absorption spectrum changes at concentrations above 0.01 mM (3.2 mg/kg) for water and NaCl and at 0.03 mM (9.6 mg/kg) for serum and RPMI media, which appears as a dimer peak of MB in the spectral region of 600–630 nm.

To quantify and compare aggregation in different solutions, the spectrum was decomposed and the relationship between the areas under the peaks corresponding to monomers and dimers was determined depending on the concentration, Fig. 3.

In water, the most rapid increase in aggregation with increasing concentration is observed. At the same time, at low MB concentrations, the degree of aggregation is higher in serum solutions.

Fluorescence spectra of MB in water as a function of concentration are shown in Fig. 4.

To analyze aggregation and reabsorption, the fluorescence spectra in water and biological media were normalized to the fluorescence maximum (694 nm), Fig. 5.

The obtained spectra show that at a concentration of 0.01 mM (3.2 mg/kg) there is a shift of the maximum

to the long-wavelength region, which indicates the presence of aggregation as well as reabsorption at higher concentrations. The shape of the spectrum does not change in this case. More rapid shift of the fluorescence maximum to the long-wavelength region in case of serum confirms higher aggregation at low concentration.

Comparison of the MB fluorescence spectra registered in various biological media shows that the most intense fluorescence is observed in saline and in water, Fig. 6.

The decreased fluorescence intensity of MB in culture medium and in plasma may be due to both interaction with plasma proteins and more alkaline pH. At the same time, the fluorescence intensity in culture medium is higher (pH = 8) than in pure plasma (pH = 9). The shape of the fluorescence spectrum did not change in the studied media. Analysis of the integral fluorescence intensity

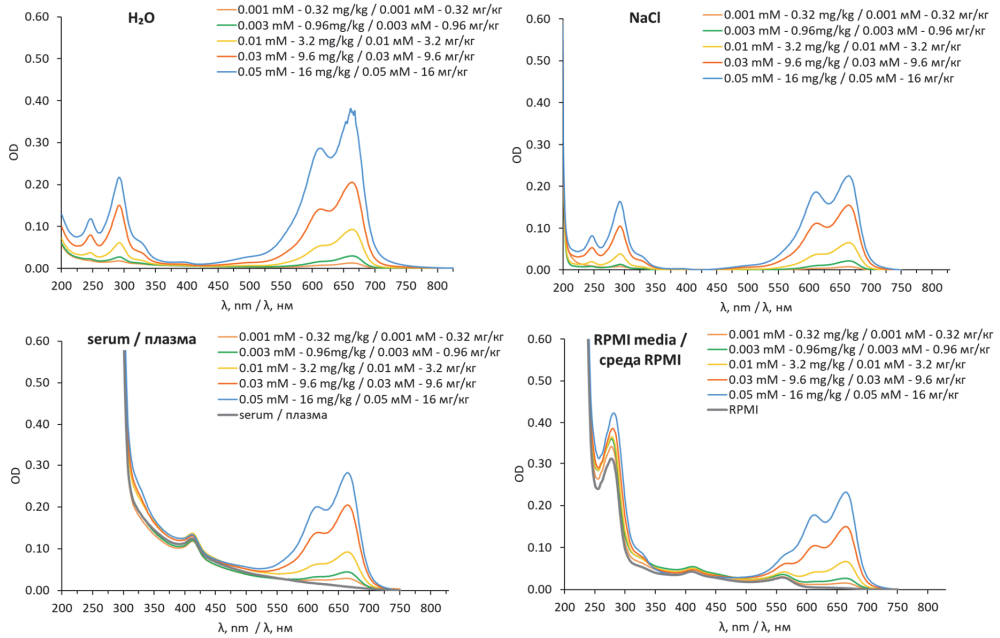

Рис. 1. Спектры поглощения МС в воде и биологических средах в зависимости от концентрации.
Fig. 1. Absorption spectra of MB in water and biological media depending on the concentration.

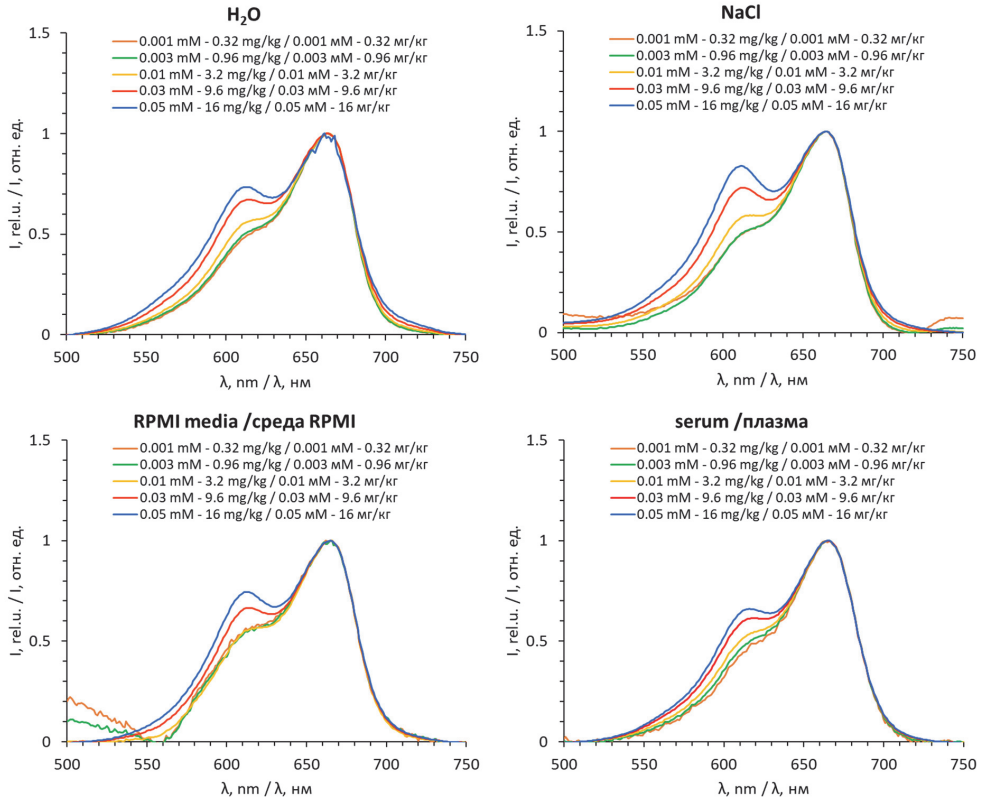

Рис. 2. Нормированные на максимум спектры поглощения МС в воде и биологических средах в зависимости от концентрации.
Fig. 2. Normalized by their maximum absorption spectra of MB in water and biological media as a function of concentration.

(estimated as the area under the fluorescence peak in the 650-800 nm range) shows that in all the studied media the dependence on concentration is nonlinear, a linear section is observed up to the concentration of 0.01 mM for all media, except for plasma, in which a deviation from the linear dependence is observed already at 0.003 mM, which presumably is associated with a large number of dimers in plasma at low concentrations. At concentrations of 0.01-0.03 mM, there is a sublinear section followed by saturation, which could be associated with reabsorption of excitation in solution as well as with small penetration depth of laser irradiation in solution.

A study of the MB fluorescence lifetime depending on concentration was carried out. No significant differences in lifetime were observed for all investigated concentrations (Table 1), which may indicate that the aggregates formed at high concentrations of MB do not fluoresce.

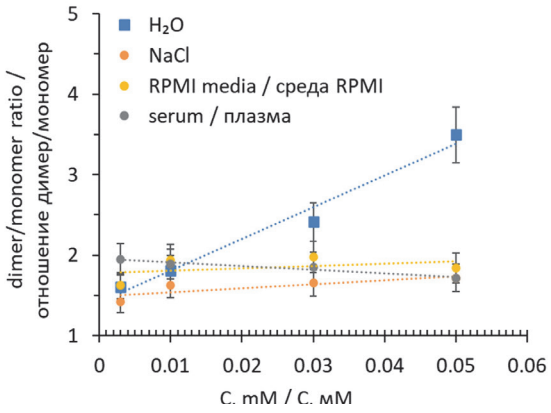

Рис. 3. Зависимость отношения площадей под пиками соответствующими димерам и мономерам (отношение димер/мономер) от концентрации.
Fig. 3. Dependence of the ratio of the areas under the peaks corresponding to dimers and monomers (dimer/monomer ratio) on concentration.

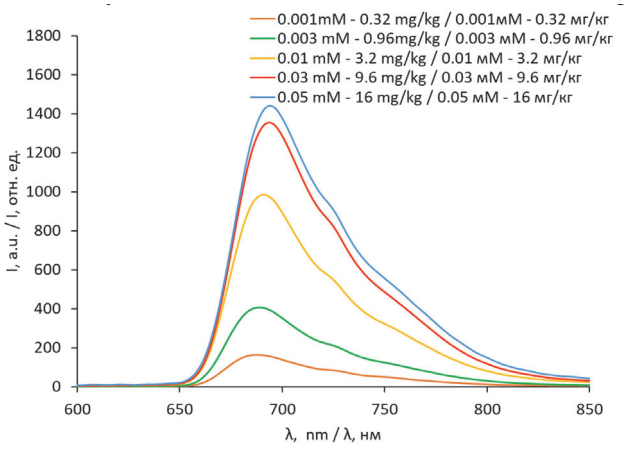

Рис. 4. Спектры флуоресценции МС в воде в зависимости от концентрации.
Fig. 4. Fluorescence spectra of MB in water as a function of concentration.

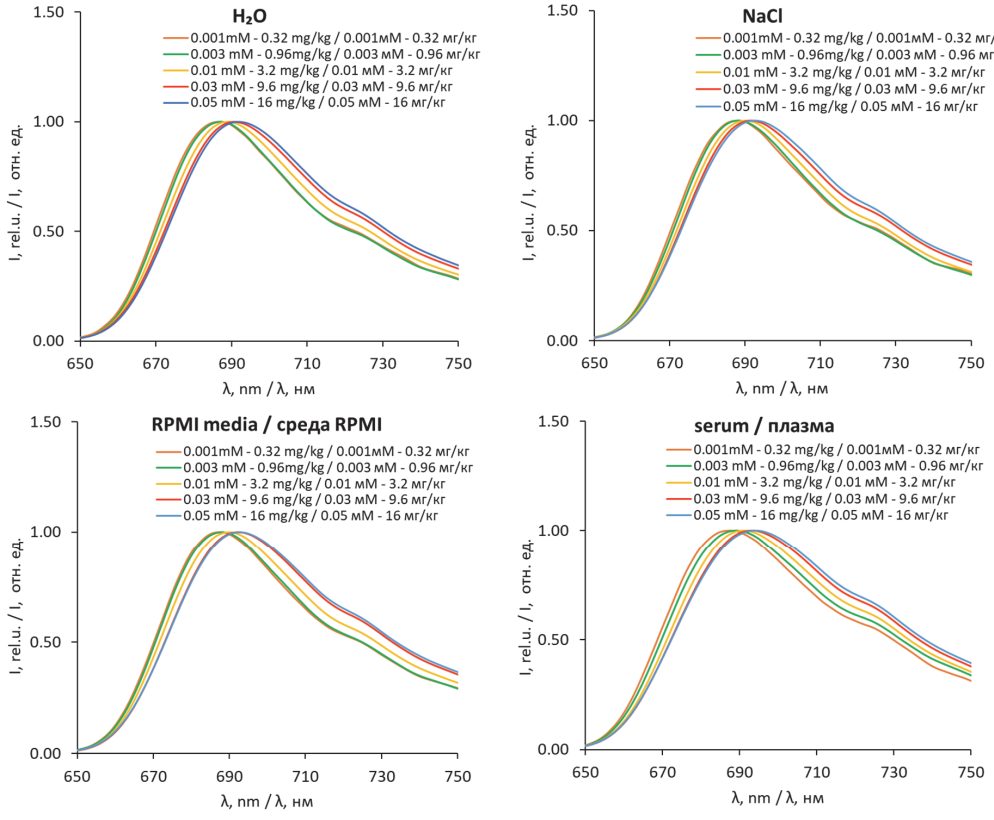

Рис. 5. Нормированные спектры флуоресценции МС в воде и биологических средах в зависимости от концентрации.
Fig. 5. Normalized spectra of MB fluorescence in water and biological media as a function of concentration.

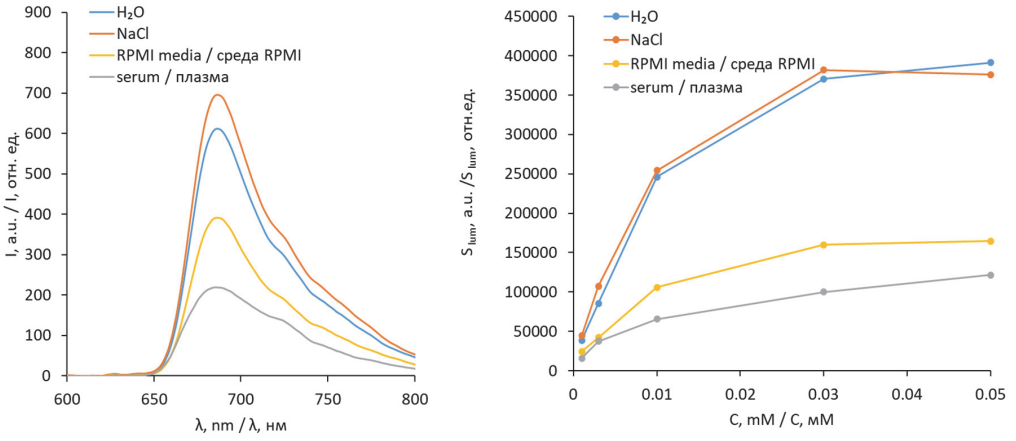

Рис. 6. Слева – спектры флуоресценции МС в различных биологических средах. Справа зависимость интегральной интенсивности флуоресценции (площади под пиком флуоресценции в диапазоне 650–800 нм) от концентрации МС.
Fig. 6. Left – fluorescence spectra of MB in various biological media. Right—the integral fluorescence intensity (area under the fluorescence peak in the range of 650–800 nm) dependence on MB concentration.

Таблица 1
Времена жизни флуоресценции МС в водных растворах в зависимости от концентрации.

Table 1
Fluorescence lifetime of MB in aqueous solutions as a function of concentration.

C, mM / C, мМ	τ, ns / τ, нс
0.001	0.3687
0.003	0.3700
0.01	0.3696
0.03	0.3696
0.1	0.3707

Influence of laser exposure on absorption and fluorescence

We studied the dependence of MB fluorescence intensity in biological media on the light dose density for MB concentration of 10 μM (3.2 mg/kg). The studies of photodegradation in biological media showed that photobleaching by more than 80% in plasma and culture medium was already observed at the dose of 5 J/cm^2, while in water at such concentration there was no photobleaching yet at the dose of 5 J/cm^2, and at the dose of 50 J/cm^2 the photobleaching of MB was about 30%, Fig. 7.

The results of previous studies also show that MB is photostable in water, the photodegradation of molecules when irradiated with doses up to 50 J/cm^2 does not exceed 40% [43], while for lower concentrations of MB photobleaching occurs more rapidly, which is presumably due to the large volume of the irradiated area at low concentrations, at high concentrations of the drug is absorbed most of the radiation in a thin layer due to high optical density. Due to the small amount of oxygen in the thin layer, the photobleaching rate at high concentrations slows down because it is limited by the oxygen diffusion rate.

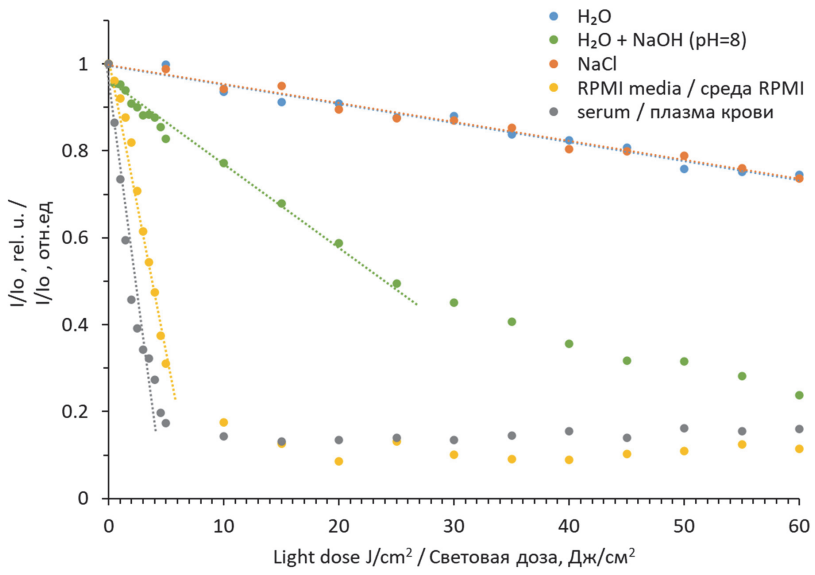

Рис. 7. Снижение интенсивности флуоресценции МС в зависимости от плотности дозы излучения за счет фотодеградации в различных средах.
Fig. 7. Decrease of MB fluorescence intensity as a function of radiation dose density due to photodegradation in different media.

The photobleaching rate in physiological solution is the same as in water, and in the RPMI medium is the same as in serum, indicating that the ionic strength of the solution has no effect on the photobleaching rate.

The higher rate of photobleaching in plasma and culture media may be due to a more alkaline pH: according to the published data, the efficiency of MC singlet oxygen generation is 5 times higher at pH 9 than at pH 7 [51].

However, based on a comparison of the photobleaching rate of an aqueous solution with the addition of NaOH to pH 8, we can conclude that the change in pH is not the only reason for the increased photobleaching rate.

The photobleaching rate in plasma as well as in water [43] depends on concentration, and at high concentrations is slower, Fig. 8.

The obtained dependencies indirectly indicate that photobleaching in plasma occurs due to interaction with singlet oxygen; the rate of photobleaching is limited by the rate of oxygen diffusion in the medium.

To check the effect of interaction with plasma proteins on the spectroscopic properties of MB over time without laser exposure, the absorption spectrum of MB in plasma was recorded 30 minutes after preparation, and no change in the spectrum was observed. Absorption spectra recorded after photobleaching suggest that the photobleaching is caused by photodegradation of MB and is not related to the transition to the leucoform, Fig. 9.

Assessment of photodynamic activity and efficiency of singlet oxygen generation in vitro

The value of photodynamic activity determined by irreversible quenching of singlet oxygen which is

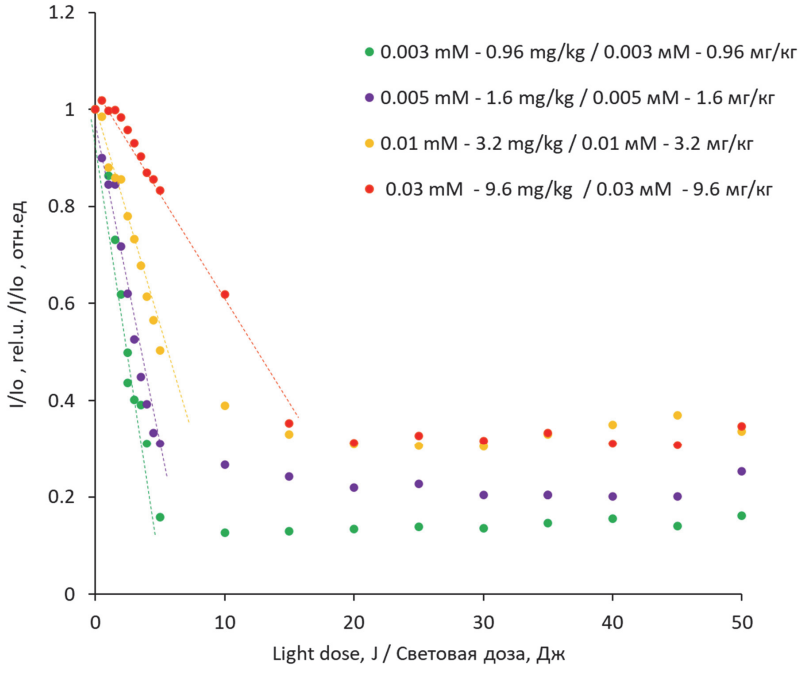

Рис. 8. Снижение интенсивности флуоресценции МС за счет фотодеградации в зависимости от плотности дозы излучения для различных концентраций МС в плазме.
Fig. 8. Decrease in MB fluorescence intensity due to photodegradation as a function of radiation dose density for different concentrations of MB in plasma.

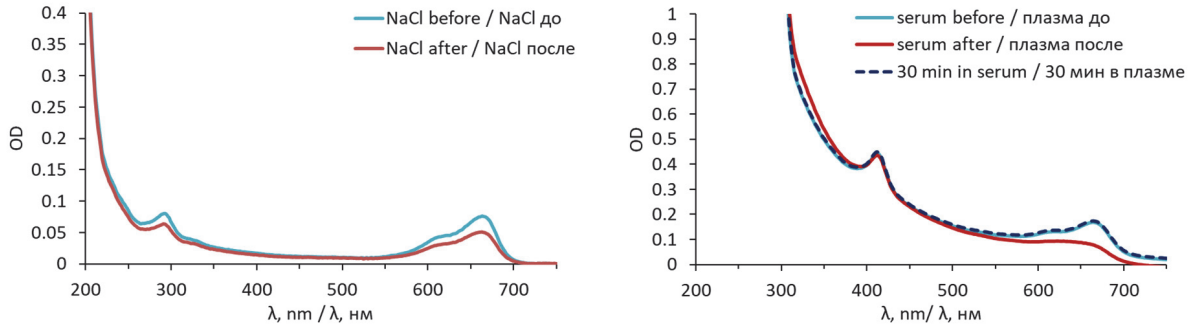

Рис. 9. Спектры поглощения МС до и после облучения в различных биологических средах.
Fig. 9. Absorption spectra of MB before and after irradiation in various biological media.

equivalent to the quantum yield of singlet oxygen generation for MB in the concentration range of 1-100 mg/kg relative to aluminum phthalocyanine ($\phi\Delta$ = 0.38) was 0.0065 ± 0.0014. In the investigated range of MB concentrations, the efficiency of singlet oxygen generation is rather low.

The measured quantum yield of singlet oxygen generation using the SOSG sensor in water demonstrated a similar value for the MB concentration of 10 mg/kg — $\phi\Delta$ 0.0028. Thus, it is shown that the photodynamic reaction *in vitro* in the presence of erythrocytes for MB proceeds according to type I, when not singlet oxygen but other reactive oxygen species are formed. We assume that this is caused by the positively charged MB binding to negatively charged cell membranes, which leads to a change in the type of photodynamic reaction [36].

This is verified by the ROS generation test performed on the cell culture (Fig. 10).

After incubation with MB, cells show staining for ROS diffusely in the cytoplasm and brightly in some vesicles. After irradiation of cells with accumulated MB at 660 nm, 50 J/cm^2, the intensity of the ROS sensor in vesicles becomes brighter. In control cells without MB, the ROS sensor is not detected, even after irradiation.

The results on ROS generation and low quantum yield of singlet oxygen generation confirm the tendency of MB to shift from type II photosensitization mechanism (energy transfer to oxygen with formation of singlet

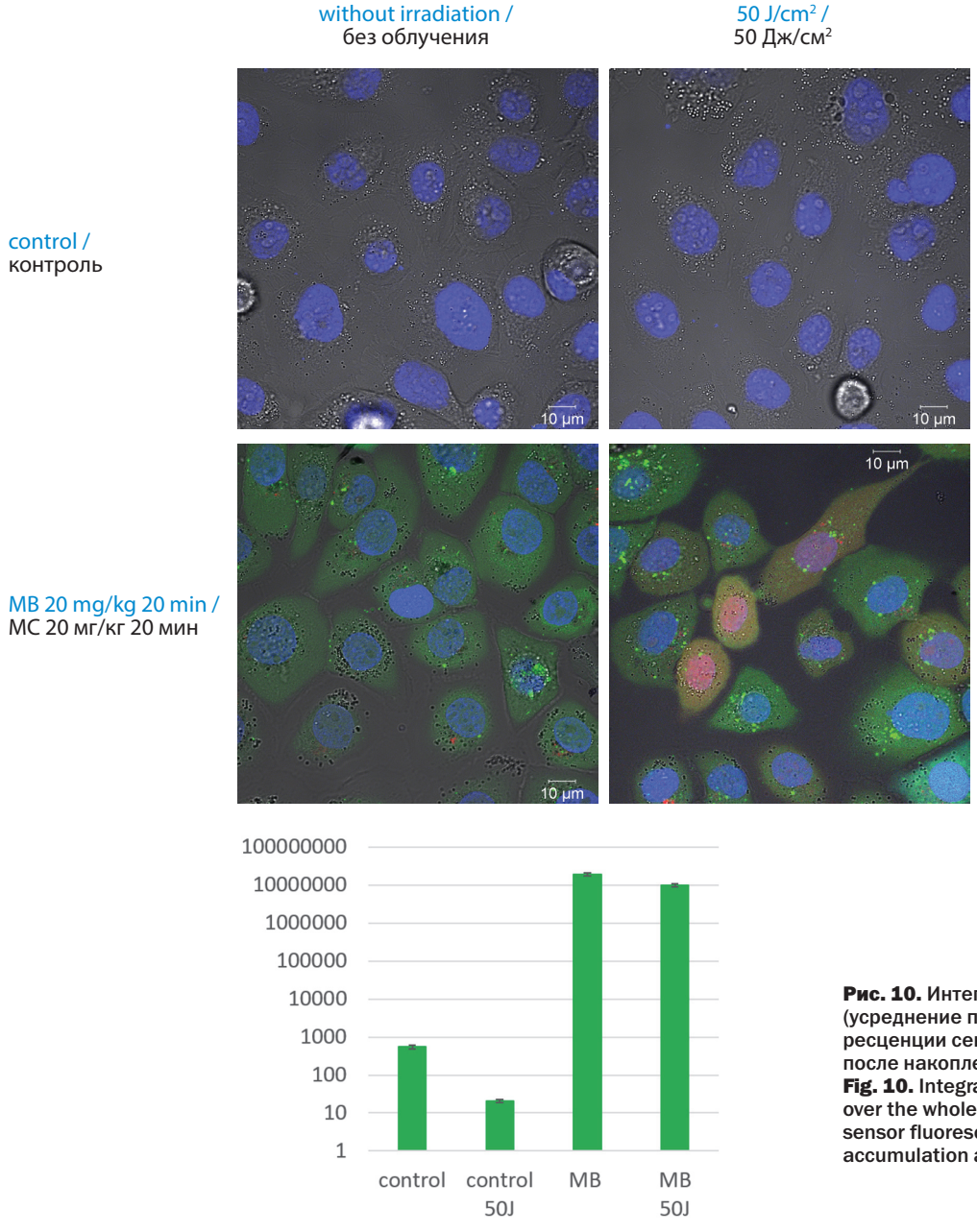

Рис. 10. Интегральная интенсивность (усреднение по всей картинке) флуоресценции сенсора на АФК в клетках после накопления МС и облучения.
Fig. 10. Integral intensity (averaging over the whole picture) of the ROS sensor fluorescence in cells after MB accumulation and irradiation.

oxygen) to type I mechanism (electron transfer with formation of MB^+ semi-reduced and semi-oxidized radicals) [50].

Conclusion

Absorption and fluorescence spectra in water, saline, blood plasma and cell-culturing medium RPMI were measured for MB concentrations 0.001–0.05 mM, fluorescence lifetime was determined for different concentrations. The analysis of the spectrum shape indicates weak aggregation at concentrations above 0.01 mM (3.2 mg/kg), which manifests in the appearance of the MB dimer peak in the spectral region of 600–630 nm.

The obtained spectra of MB fluorescence in aqueous solutions show that at a concentration of 0.01 mM (3.2 mg/kg) there is a shift of the maximum to the long-wave region, indicating the presence of reabsorption and aggregation at higher concentrations. The shape of the spectrum in this case does not change. Comparison of MB fluorescence spectra recorded in various biological media shows that the most intense fluorescence is observed in saline and in water. The decrease of MB fluorescence intensity in the culture medium and in plasma may be due to both the interaction with plasma proteins and more alkaline pH. At the same time, the fluorescence intensity in culture medium is higher (pH 8) than in pure plasma (pH 9). The shape of the fluorescence spectrum does not change in the studied media. The analysis of integral fluorescence intensity dependence on MB concentration shows that in all the studied media it is non-linear, the linear section is observed up to the concentration of 0.01 mM for all the media, except for plasma, in which the deviation from linear dependence is observed up to 0.003 mM (presumably due to large number of dimers in plasma at low MB concentrations in comparison to water). At concentrations of 0.01–0.03 mM, the sublinear section of the dependence begins followed by saturation, which could be associated with reabsorption of excitation in solution as well as with small penetration depth of laser irradiation in solution.

A concentration-dependent MB fluorescence lifetime study was performed. No differences in the lifetime were observed for all studied concentrations, which may indicate that the aggregates formed at high concentrations of MB do not fluoresce.

Studies of photodegradation in biological media showed that more than 80% photobleaching in plasma and culture media is already observed at a dose of 5 J/cm^2, while in water at this concentration at a dose of 5 J/cm^2 there is no photobleaching yet, and at a dose of 50 J/cm^2 the photobleaching of MB is about 30%.

The photobleaching of MB under the effect of laser radiation was investigated. For lower concentrations of MB the photobleaching occurs more rapidly, which is presumably associated with a larger volume of irradiated area at low concentrations: at high concentrations of the drug most of the radiation is absorbed in a thin layer due to high optical density. The obtained relations indirectly indicate that photobleaching in plasma occurs due to interaction with singlet oxygen; the rate of photobleaching is limited by the diffusion rate of oxygen in the medium.

In media containing proteins with alkaline pH, photobleaching is significantly faster than in neutral aqueous media. The ionic strength of the solution has no effect on the photobleaching rate. The absorption spectra recorded after photobleaching suggest that the photobleaching is not due to the transition to the leucoform but is caused by the photodegradation of MB.

The efficiency of singlet oxygen generation and photodynamic activity were evaluated *in vitro*. In the investigated concentration range the efficiency of singlet oxygen generation is quite low. We assume that this is because the photodynamic reaction for MB proceeds according to type I, when not singlet oxygen is formed, but other reactive oxygen species. This is because the positively charged MB binds to negatively charged cell membranes, which leads to a change in the type of photodynamic reaction. It is demonstrated that in addition to the generation of singlet oxygen, the presence of other ROS in the cells is observed. After incubation with MB, cells show staining for ROS diffusely in the cytoplasm and brightly in some vesicles. After irradiation of cells with accumulated MB at 660 nm, 50 J/cm^2, the sensor intensity for ROS in vesicles becomes brighter. In control cells without MB the ROS sensor is not detected even after irradiation.

Acknowledgment

The study was funded by a grant from the Russian Science Foundation (project N 22-72-10117).

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