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Novel Biomaterial for NCT—"Rigid" Particles of (DNA-Gadolinium) Liquid-Crystalline Dispersions

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ABSTRACT

The formation and physico-chemical properties of biomaterial, based on double-stranded (ds) DNA molecules and bearing high concentration of gadolinium, is described. This "rigid" biomaterial demonstrate a few unique properties: 1) *the ds DNA molecules forming complexes with gadolinium are fixed in the spatial structure of "rigid" particles*, 2) *an abnormal negative band in the circular dichroism spectrum permits to follow the formation of this biomaterial*; 3) *local concentration gadolinium in the content of biomaterial can reach* 40%. *These properties show that we are dealing with a novel type of biomaterial strongly enriched by gadolinium. This opens a gateway for practical application of this biomaterial for neutron-capture reactions. A first attempt to apply this material for neutron-capture reaction in combination with neutron generator of thermal neutron flux was performed. Positive result obtained at destruction of CHO cells allows one to state that the advantages of this biomaterial are a simple manipulation with it, a possibility to adjust its gadolinium content, long-term stability of its physico-chemical properties, as well as a reduced cost of neutron-capture experiment.*

Keywords: *Liquid-Crystalline DNA Dispersions, Cholesterics, Circular Dichroism Spectrum, Gadolinium, Neutron-Capture Therapy*

1. Introduction

Biological molecules, in particular nucleic acid molecules, are becoming increasingly popular as polyfunctional object for nanobiotechnology [1].

Deliberate and controlled variation in the properties of these molecules provides possibilities for formation of various types of nanoconstructions (nanostructures, nanobiomaterials, etc.) allowing their wide application in biotechnology and medicine. For instance, nanoconstructions, formed by double-stranded (ds) DNA molecules fixed in structure of cholesteric liquid-crystalline dispersions (CLCDs) and cross-linked by nanobridges, were used as biosensing units for biosensor devices [1]. Besides, DNA nanoconstructions may be used as "carriers" for genetic material or as a "reservoir" for various biologically active compounds embedded in the composition of these structures.

Among recently studied nanoconstructions, "rigid" nanoconstruction ("rigid" DNA particles) are of special practical importance and interest due to their unique physicochemical properties [2].

Recently possibilities of application of the "rigid" particles of (ds DNA-Gd) complexes for neutron-capture therapy (NCT) were hypotesized [3]. Neutron capture therapy (NCT) is a cancer cells treatment that utilizes nuclear neutron capture reaction (NCR) of radiation producing elements administrated *in vivo* by thermal neutron flux generated, as a rule, by nuclear reactor [4].

The current attention to radiation-producing elements is essentially focused on gadolinium for its favorable properties, even though extensive studies have been reported on the other elements. It is known that naturally existing gadolinium consists of several stable isotopes. Among them ^{157}Gd (15.6% in natural sample) has most broad medical application. Indeed, gadolinium neutron capture therapy (Gd-NCT) utilizes the following nuclear capture reaction (NCR) of ^{157}Gd by thermal neutron irradiation [5]:

^{157}Gd + *n*th$\rightarrow$^{158}Gd + γ-rays + internal conversion electrons$\rightarrow$^{158}Gd + γ-rays + Auger electrons + character-

istic X-rays.

According to accepted point of view, the success of gadolinium neutron capture therapy depends, first, on a high accumulation of Gd in the tumor [6].

Therefore, the first problem here—a sufficient concentration of gadolinium could be retained in the tumor tissue during neutron irradiation after intratumoral injection. The second problem is the toxicity of free gadolinium, because gadolinium ion is strongly toxic even at low doses at its administration to tissues [7,8]

For this reason, various kinds of "carriers" (ligands) capable of forming stable complexes with Gd^{3+} before it is administrated to patients have been constructed.

Indeed, the chelate complexes of gadolinium with polymeric molecules (see, for instance [9]), nanoparticles of various origin [10,11] have been produced as potent biomaterials (bioconjugates) for targeting a site and controlled release of a drug. Biodegradable, biocompatible chitosan nanoparticles [4,11] have recently received considerable attention as systems capable of retaining gadolinium in the tumor tissue during a Gd-NCT trial.

This facts show that the developers of NCT are interested in particular molecular constructions (bioconjugates, biomaterials, etc.), which can retain a sufficient gadolinium concentration.

Thus, a key factor in the success of the current Gd-NCT trial is the use of biomaterials by means of which Gd can be delivered efficiently and retained inside tumor tissues and/or cells during thermal neutron irradiation.

The aim of this study was to investigate whether the "rigid" particles of the (ds DNA-Gd) complexes can be practically used as a biomaterial for Gd-NCT. For this purpose "rigid" ds DNA particles, highly loaded with gadolinium, were obtained and firstly tested in Gd-NCT for disintegration of the living cells. Our preliminary attempt showed that this biomaterial has a good potential as a gadolinium carrier for modern NCT.

2. Materials and Methods

2.1. Preparation of the "Rigid" Particles of the CLCD of (Double-Stranded DNA-Gadolinium) Complexes

The formation of the CLCDs based on the phase exclusion of ds DNA from PEG-containing water-salt solutions was performed according to the Method that was described previously in detail [12]. In the physicochemical sense, the system under investigation is particles of the CLCD of the ds DNA that are distributed isotropically in the water-salt solution of PEG. These particles do not exist in the absence of high osmotic pressure of the solvent (induced by high PEG concentrations).

The formed CLCD was treated with $GdCl_3$ solutions to induce formation of (ds DNA-gadolinium) complexes. The efficiency of formation of these complexes was checked by the measuring the CD spectra.

The gadolinium salts (99.99% of purity) were purchased from the Institute "Giredmet" (Moscow, Russia). The absorption spectra of all solutions were taken on a spectrophotometer ("Cary100", Varian, USA) and the CD spectra were recorded by a portable dichrometer SKD-2 (manufactured by the Institute of Spectroscopy of the RAS, Troitzk, Moscow Region). In all cases, the quartz cells with 1 cm optical path were used.

The morphology of the dsDNA CLCD particles treated by $GdCl_3$ was examined using Atomic Force Microscope P47-SPM-MDT (produced by NT-MDT, Russia). To isolate these particles, the solution in which they were formed was filtered through a poly(ethyleneterephtalate) (PETP) nuclear membrane filter with size of pores of 150 nm (produced by the Institute of Crystallography of the RAS), that allowed us to immobilize particles; filters were dried in air for no less than 1 h.

To estimate the number of Gd^{3+} ions in the content ds DNA CLCD particles treated by $GdCl_3$, their magnetic properties have been measured and total magnetic moment of Gd^{3+} ions was determined. The magnetic properties of the samples were measured at magnetic field of 71.29 mT (712.9 Oe) at sample position by the superconducting interferometer device (SQUID-magnetometer) produced by D. Mendeleev University (Moscow, Russia) [13].

The concentration of the gadolinium in the content of ds DNA CLCD particles treated with $GdCl_3$ was additionally checked by the neutron activation analysis [14].

The fluorescence of SYBR Green (an intercalating fluorescent dye, "Invitrogen" USA, Lot 666062) was measured by the fluorescence spectrophotometer ("Cary Eclipse", Varian, USA).

The particles of CLCD formed by (ds DNA-Gd) complexes were sedimented as a result of low speed centrifugation, the pellet was re-suspended in 0.3 ml of H_2O and added to 5 ml of the standard Dulbecco's MEM medium.

2.2. Gd-NCT with "Rigid" Particles of CLCD Formed by (ds DNA-Gd) Complexes

2.2.1. Cell Line Cultivation

Chinese hamster ovary (CHO) adherent cells were maintained in plastic flasks containing standard Dulbecco's MEM medium supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mg/ml streptomycin and 100 units/ml penicillin in a humidified atmosphere containing 5% carbon dioxide at 37˚C.

For cell line passage cell monolayer was washed with PBS (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), standard solution of trypsine-EDTA (Sigma) was added, then plates with cells were placed in a 5% CO_2 atmosphere at 37˚C for 3-5 minutes, and then medium DMEM with FCS was added, cells were suspended and were plated in flasks at necessary concentrations.

CHO cells, forming monolayers, were seed in 4 cultural flasks (set A - N1 and N2; set B- N3 and N4; see **Figure 1**) with 25 cm^2 square 500000 cells per flask. (Monolayers formed by these cells were in their original state within whole time of experiment). In 20 hours medium in two flasks (set A - N2 and set B- N4) was changed with fresh one, and in two flasks (set A - N1 and set B - N3) with DMEM medium containing "rigid" particles of CLCD formed by (ds DNA-Gd) complexes (the amount of the "rigid" particles per 1 CHO cell in these flasks was equal to about 5×10^4, that provides "close enough contacts" between the cells and "rigid" particles). Then, in 2 hours all four flasks were closed hermetically and two of them (one with CHO cells plus "rigid" particles of CLCD formed by (ds DNA-Gd) complexes (in set A - N1), and the other one - CHO cells only with DMEM medium (in set A - N2) were irradiated with thermal neutrons (see **Figure 1**).

Two flasks (set B-N3 and N4), which were not irradiated (see below), were incubated during 1h in thermostated (37˚C) polymeric box located nearby neutron generator.

2.2.2. Gd-NCT with "Rigid" Particles of CLCD

In contrast to classical neutron source such as huge nuclear reactors, we first attempted to use more smaller in size, more cheaper neutron generator NG-400 (France) with the neutron energy about 14 MeV and the total intensity of the order of 10^{11} neutrons/sec for the thermal neutron capture inside "rigid" particles the (ds DNA-Gd) complex and generation of the secondary irradiation outside of these particles.

The thermal neutrons were produced by the moderator system consisting of a tungsten converter and poly (ethylene) block (20 × 20 × 20 cm, called as "phantom") assembled in neutron generator. The earlier estimations showed that the conversion electrons, X-rays and gamma rays (range in tissue about 5×10^4 nm), which are generated as a result on the gadolinium thermal neutron capture reaction, can cause the ds DNA double-strand breaks, inducing their killing.

Two flasks - one with "rigid" particles and CHO cells (set A - N1, see above) and the other one - CHO cells with DMEM medium (set A - N2) were placed in the rectangular hole (10 × 5 × 5 cm) inside a "phantom".

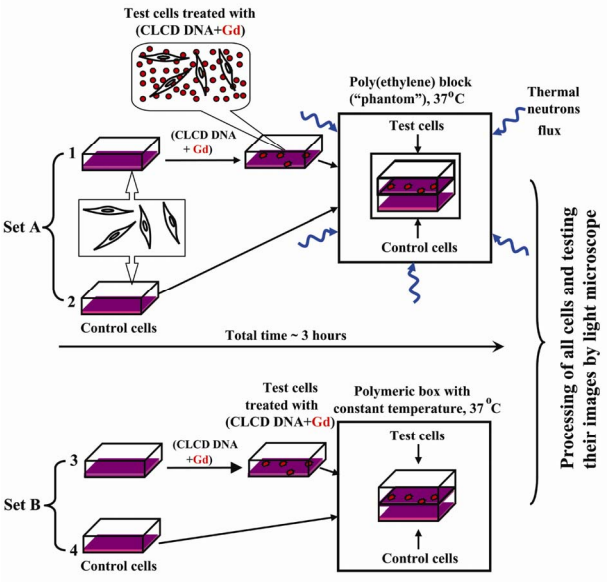


Figure 1. Principal scheme of the Gd-NCT experimental set-up with the use of the "rigid" DNA particles as carriers for gadolinium. Set B was used as a control. The numbers from 1 to 4 in sets A and B denote the monolayers of initial CHO cells.

These flasks (samples) were fixed at the depth of 5 cm inside the "phantom" and exposed at 37˚C to thermal neutrons generated by NG-400.

The irradiation time was about 1 h and the thermal neutron fluence was about $5 \cdot 10^{11}$ neutron/cm^2. The thermal and fast neutron fluxes were estimated by method of activation analysis [15].

3. Results

3.1. Formation and Properties of "Rigid" Particles of CLCD of (ds DNA-Gd) Complexes

Figure 2 compares the CD spectrum of the CLCD (curve 1) formed by initial ds DNA molecules in water-salt PEG-containing solution to the CD spectra for CLCDs (curves 2-5) treated with $GdCl_3$ solution.

The formation of ds DNA dispersion is clearly accompanied by an appearance of an intense negative band in the CD spectrum in the region of the spectra, where the DNA nitrogen bases absorb.

One can remind that every particle of CLCD contains about 10^4 ds DNA molecules fixed on distances within 2.5 - 5.0 nm (depending on osmotic pressure of the solvent [12]. Note that the particles of the low-molecular mass ds DNA dispersions are "microscopic droplets of concentrated DNA solution", which cannot be "taken in hand" or "directly seen".

According to theoretical calculations, the appearance

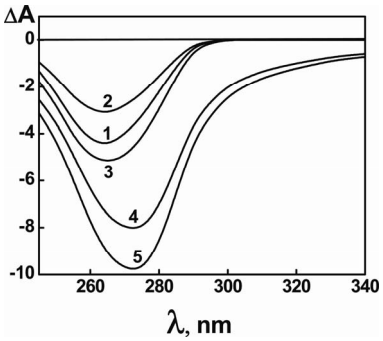


Figure 2. The CD spectra of the ds DNA CLCD in the absence (curve 1) and in the presence (curves 2-5) of $GdCl_3$ in solution: Curve 1 – gadolinium r_{total} = 0; 2 – 1.65; 3 – 16.5; 4 – 32.7; 5 – 95; C_{DNA} = 0.01 mg/ml; C_{NaCl} = 0.3 M; $C_{initial\ GdCl3}$ = 0.1 M; C_{PEG} = 170 mg/ml; $\Delta A = A_L - A_R$ (× 10^{-3} opt. units). r_{total} – is the ratio of total $GdCl_3$ molar concentration to the molar concentration of the DNA nitrogen bases.

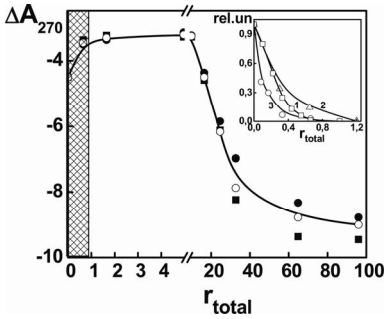


Figure 3. The dependence of the amplitude of the abnormal negative band in the CD spectrum of the CLCD formed by (ds DNA-Gd) complexes upon gadolinium r_{total} value. C_{DNA} = 0.01 mg/ml; C_{NaCl} = 0.3 M; C_{PEG} = 170 mg/ml; ΔA (λ 270 nm) = $A_L - A_R$ (× 10^{-3} opt. units). Insert: Curve 1. The dependence of the relative amplitude of the band (λ 280 нм) in the CD spectra of the linear ds DNA treated with $GdCl_3$ upon gadolinium r_{total} value. C_{DNA} = 0.01 mg/ml; C_{NaCl} = 0.003 M. Curve 2. The dependence of the relative amplitude of the abnormal negative band (λ 280 nm) in the CD spectra of the CLCD formed by (ds DNA-Gd) complexes upon gadolinium r_{total} value. C_{DNA} = 0.01 mg/ml; C_{NaCl} = 0.3 M; C_{PEG} = 170 mg/ml. Curve 3. The dependence of the Bragg peak relative amplitude of the CLCD formed by (ds DNA-Gd) complexes upon gadolinium r_{total} value. C_{NaCl} = 0.3 M; C_{PEG} = 170 mg/ml; gadolinium r_{total} – is the ratio of total $GdCl_3$ molar concentration to the molar concentration of the DNA nitrogen bases.

of this band unequivocally testifies the macroscopic cholesteric twist of neighboring DNA molecules in particles of dispersion. The negative sign of the band in the CD spectrum proves the left-handed cholesteric twist of the right-handed DNA molecules (B-form) in these particles. The intense band in the CD spectrum (**Figure 2**, curve 1) located in the absorption region of the nitrogen bases of the DNA molecule, in our case negative, is the direct evidence for the formation of the CLCD characterized by helically twisted spatial structure [16-18], or a so-called cholesteric structure of particles of LCD and the term CLCD (cholesteric liquid-crystalline dispersion) was used to signify these particles.

The gadolinium-concentration dependence of the amplitude (ΔA_{270}) of an abnormal negative band in the CD spectrum of the ds DNA CLCD particles is shown in **Figure 3**. One can see that the amplitude of the abnormal band in the CD spectrum of particles of the CLCD is changed, albeit slightly, at low concentrations of $GdCl_3$ (gadolinium $r_{total} \leq 0.5$).

Insert to **Figure 3** compares both the diminishing in the amplitude of *low-intensity band* in the CD spectra of *original linear* ds DNA (curve 1) and the decrease in the amplitude of an intense negative band in the CD spectrum of ds DNA CLCD at low concentration of gadolinium (curve 2).

The state of the DNA secondary structure under conditions of high concentration of gadolinium cations was checked by application of an "external chomophore" approach based on theoretical consideration of the peculiarities of the CLCDs CD spectra [19,20]. As an "external chromophore" we have used SYBR Green—an intercalating fluorescent dye [21].

Figure 4 demonstrates the obtained results. One can see that in agreement with theoretical predictions [19] the

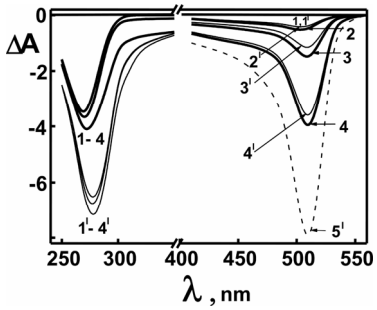


Figure 4. The comparison of the CD spectra of the ds DNA CLCD treated with SYBR Green (SG; curves 1 - 4) to the CD spectra of CLCD formed initially by (ds DNA-Gd) complexes and then treated with SG (curves 1^I - 4^I). Dotted curve 5^I- the expected CD spectrum of the CLCD formed by (ds DNA-Gd) complexes and treated with SG under condition of the homogeneity in the DNA secondary structure. SG r_{total} in the case of curves 1 and 1^I = 0; in the case of 2 and 2^I – 0.033; 3 and 3^I – 0.2; 4 and 4^I – 0.66; 5^I – 0.66; C_{DNA} = 0.01 mg/ml; C_{PEG} = 170 mg/ml; C_{NaCl} = 0.3 M; C_{GdCl3} = 0.003 M; $\Delta A = A_L - A_R$ (× 10^{-3} opt. units). SG r_{total} – is the ratio of total SG molar concentration to the molar concentration of the DNA nitrogen bases.

treatment of CLCD particles formed by (ds DNA-Gd) complexes with SYBR Green, used as an "external chromophore", is accompanied by an appearance of an additional abnormal negative band in the CD spectrum.

Figure 5 compares the changes in the emission intensity of SYBR Green intercalated between nitrogen bases of initial, linear ds DNA with homogeneous secondary structure (curve 1), to ds DNA in the content of CLCD (curve 2) as well as ds DNA in the content of CLCD treated with gadolinium (curve 3).

Figure 6(a) displays the 2D-AFM images of "rigid" CLCD particles formed by (ds DNA - Gd) complexes immobilized onto the surface of a nuclear membrane filter.

It is evident that these particles exist as independent individual objects, which are easy to visualize. **Figure 6(b)** demonstrates the size distribution of these particles as well as the pores in the filter.

3.2. Gd-NCT with "Rigid" Particles of CLCD Formed by (ds DNA-Gd) Complexes

We used biomaterial, *i.e.*, "rigid"(ds DNA-Gd) particles as a potential platform for Gd-NCT. For this purpose effect of radiation as a result of Gd-NCR on culturated CHO cells was measured. These cells were exposed to thermal neutrons flow from neutron generator in the presence and in the absence of "rigid" (ds DNA-Gd) particles (see **Figure 1**).

After irradiation of the cells in the flasks in set A (see **Figure 1**) all four flasks from set A and set B were maintained in a 5% CO_2 atmosphere at 37˚C within 16 hours. Then medium in all flasks was changed with fresh one without "rigid" particles, and the images of CHO cells in monolayers were analyzed by light microscope (Leica DMI4000). The images were taken in 20, 60 and 120 hours after irradiation

One can remind that the time of experiment with Gd-NCT was about 3 hours. Hence, the first important question to be answered is the penetration of "rigid" particles containing Gd into CHO cells within this time.

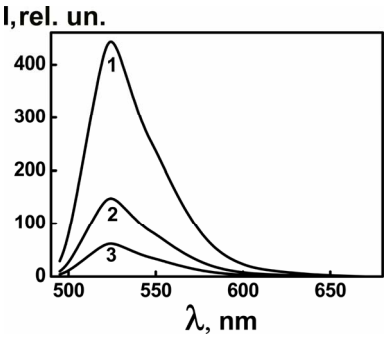
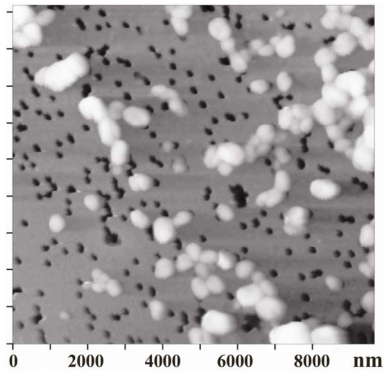
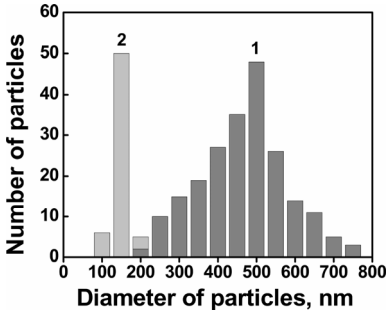


Figure 5. The fluorescence spectra of the initial, linear ds DNA and CLCD formed by ds DNA as well as the spectrum of CLCD of (ds DNA-Gd) complexes treated by SYBR Green (SG, curves 1, 2, and 3, respectively). C_{DNA} = 0.01 mg/ml; C_{NaCl} = 0.3 M; C_{PEG} = 170 mg/ml; $C_{SG} = 9.73 \times 10^{-6}$ M; C_{GdCl3} = 0.003 M.



(a)



(b)

Figure 6. (a) 2-D AFM image of the "rigid" particles immobilized onto the surface of the nuclear membrane filter (PETP). C_{DNA} = 0.001 mg/ml; C_{NaCl} = 0.03 M; C_{PEG} = 17 mg/ml; C_{GdCl3} = 0.0023 M. (The dark spots are "pores" in the nuclear membrane filter); (b) Size distribution of the ds DNA CLCD particles treated by $GdCl_3$ (1) and the pores (2) in the membrane filter. C_{DNA} = 0.001 mg/ml; C_{NaCl} = 0.03 M; C_{PEG} = 17 mg/ml; C_{GdCl3} = 0.0023 M.

Control experiment showed, that before irradiation, the penetration of "rigid" (ds DNA-Gd) particles into CHO cell was practically absent. This means that in our case the "rigid" particles, which are located outside the CHO cells, were used for NCT.

Figure 7(a) shows the image typical of initial CHO cells without irradiation (set B N4).

Figure 7(b) represents the image of CHO cells in monolayers corresponding to flask N2 (set A), *i.e.*, the image for CHO cells irradiated by thermal neutrons in the absence gadolinium carrier.

Figure 7(c) shows the image for CHO cells irradiated by thermal neutrons in the presence of "rigid" gadolinium carrier (set A N1).

4. Discussion

4.1. Formation and Morphology of "Rigid" Particles of CLCD of (ds DNA-Gd) Complexes

Figure 2 shows that a quite sharp increase in the ampli-

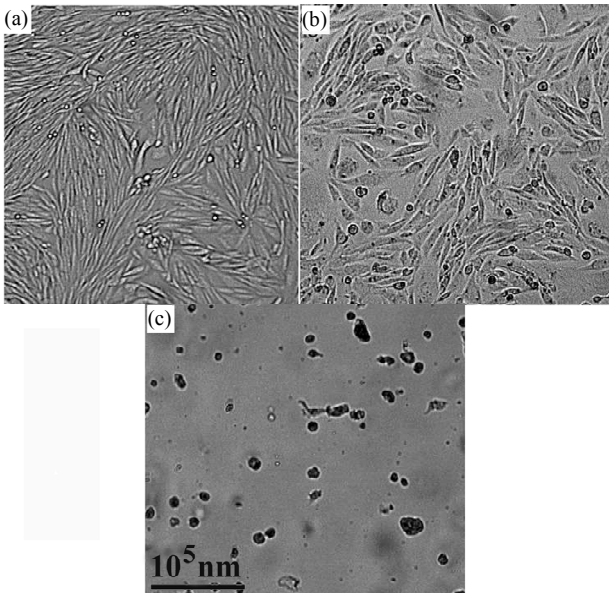


Figure 7. The images of CHO cells monolayers taken after 120 hours of cell processing by light microscope (Leica DMI4000). (a) the image without the thermal neutron irradiation and without gadolinium carrier; (b) the image with thermal neutron irradiation without gadolinium carrier; (c) the image after 1 hour of the thermal neutron irradiation with gadolinium carrier.

tude of the intense negative band in the CD spectrum of the CLCD of the (ds DNA-Gd) complexes occurs at a large concentration of gadolinium cations in the solution ($r_{total} > 20$) and its maximum is shifted by 10 nm toward long wavelengths (inset to **Figure 2**, curves 1-5).

As it was shown previously [3,22-24] when rare earth cations, and in particular, Gd cations, are bonded to *linear* ds DNA, noticeable alterations in the CD spectra of these molecules were observed at gadolinium r_{total} values close to 0.5 [22,24]. This effect was explained as a deformation (alteration) of the ds DNA secondary structure [25,26]. This deformation can be associated with the conformational transition of B → Z type [25,27]. Besides, the junctions between B-DNA and Z-DNA fragments contain extruded bases providing the sites with modified local properties. Combination of these two effects is accompanied by breaking of the regular, homogeneous character of the ds DNA secondary structure. The "modified" ds DNA molecules can be separated into alternating fragments differing in conformations, for instance, of B → Z – Z → B – B → Z → B → –Z– type. Hence, even low concentrations of gadolinium cations are capable of inducing the breaking of regular character of the ds DNA secondary structure of *initial linear* ds DNA molecules. Because formation of ds DNA CLCD does not result in change in the parameters of the DNA secondary structure, one can expect that the breaking process happens in the case of ds DNA molecules packed in CLCD particles.

To check the character of the secondary structure of ds DNA molecules ordered in the particles of CLCD treated with various concentrations of gadolinium salt, the small-angle X-ray scattering (SAXS) curves of these objects were recently obtained [28].

The most important observation detected by SAXS is that structural changes in the ds DNA molecules in the content of CLCD particles occur while the concentrations of the gadolinium salt are very low. At gadolinium r_{total} = 0.66 the characteristic Bragg peak in X-ray scattering curves has completely disappeared (**Figure 3**, curve 3). Therefore, if the fragments of the neighboring molecules of the complexes of the DNA with gadolinium or even all molecules packed in particles of CLCD acquire an inhomogeneous secondary structure, the translational order of these fragments (molecules) is broken and the small-angle reflection on X-ray scattering curves must disappear and this disappearance is observed experimentally [28]. Hence, the analysis of the SAXS spectra. confirms the assumption that the treatment of particles of the CLCD by gadolinium cations leads to the appearance of the modified secondary structure of the DNA molecules.

Taking into account the suggestion above that "modified" DNA molecules are separated into alternating fragments differing in conformations (e.g., B – Z – B – B – Z – Z etc.), the existence of ds DNA fragments with the B-form was checked by the method of "external chromophore". As an external chromophore we have used intercalating drug—SYBR Green, which is highly selective for native B-form of ds DNA molecules with regular secondary structure.

Figure 4 shows that in the CD spectrum of (ds DNA-Gd) complexes there are two bands. One occurs in the absorption region of the DNA nitrogen bases ($\lambda \sim 270$ nm) and the other lies in the absorption region of SYBR Green chromophores ($\lambda \sim 510$ nm). Under binding of SYBR Green with ds DNA molecules in content of CLCD particles formed by (ds DNA-Gd) complexes both bands have negative signs despite of SYBR Green concentration. The identical signs of two bands in the CD spectra unequivocally mean that SYBR Green molecules are fixed in quasinematic DNA layers. The amplitude of the band in the CD spectrum in the region of SYBR Green absorption grows with increasing number of its molecules bound to DNA, although the amplitude of the band in the region of DNA absorption remains practically constant. The shown CD spectra mean that the orientation of SYBR Green molecules coincides with the orientation of the nitrogen base about the DNA axis and SYBR Green molecules intercalate into DNA so that the angle between SYBR molecule and the long axes of the DNA is ~90˚.

However, the experimentally measured amplitudes (compare, for instance, curve 4' to theoretically calculated curve 5') are 2 times smaller then expected ones (if one can take into account the correlation between the amplitudes of abnormal bands in the DNA and "external chromophores" absorption regions [19]. The detected difference shows that, indeed, that at high gadolinium concentration the breaking of the ds DNA secondary structure in the CLCD particles of (ds DNA-Gd) complexes takes place.

The measurements of the SYBR Green fluorescence in the content of CLCD particles formed by (ds DNA-Gd) complexes speak in favor of this point of view. **Figure 5** demonstrates that the fluorescence intensity of SYBR Green is decreased in the case of both ds DNA CLCD and (ds DNA-Gd) CLCD. The most important facts consist in the following: 1) condensation of ds DNA molecule and formation of the ds DNA CLCDs is accompanied by drop in the intensity of fluorescence of SYBR, and 2) there is difference in fluorescence of SYBR Green intercalated between nitrogen bases pairs in the content of ds DNA molecules ordered in initial CLCD and in the content of CLCD treated with gadolinium. Since the solvent used for measurements of curves (2) and (3) was not changed and because SYBR Green molecules bind with regular B-form of ds DNA, the difference between curves (2) and (3) confirms ones more the statement that neighboring ds DNA molecules, packed in particles of the CLCD (ds DNA-Gd) complexes, acquire an inhomogeneous secondary structure.

Figure 3 shows that at high gadolinium concentration in solution ($r_{total} > 20$) the CD spectrum of CLCD formed by ds DNA changes dramatically. The observed increase in the amplitude of the negative band and the change in the shape of the CD spectrum of the DNA CLCD are similar to changes in the CD spectra of this CLCD upon cross-linking of neighboring DNA molecules due to the formation of nanobridges between them [29]. The formation of such nanobridges leads both to decrease in solubility of initial CLCD structure and to the disappearance of the "liquid" character in the location of DNA molecules in the particles of the CLCD [1].

This allows one to suppose, that interaction of gadolinium with ds DNA molecules is accompanied by decrease in solubility of these molecules as well. In addition, the inhomogeneous chemical nature of the nitrogen bases in ds DNA molecules leads to the fact that the interaction between gadolinium cations and ds DNA molecules is accompanied by nanoscale conformational changes (similar to the changes that are appeared at B → Z transition) only in the fragments of these molecules. Because separation of chains of the ds DNA molecules in the content of particles of the LCD is impossible due to the sterical reasons [30,31], the alteration of the ds DNA secondary structure, induced by gadolinium treatment, can be "transformed" into the change in the mode of spatial packing of the neighboring DNA molecules in these particles. Because ds DNA molecules cannot "leave" the physical volume of CLCD particles, due to the fixed osmotic pressure of PEG-containing solution, the loss of solubility of individual neighboring ds DNA molecules combined with an increase in the interaction between their fragments with different conformations initiates the transition of the overall structure of CLCD particles from a "liquid" to a "rigid" state [32].

It is known, that a "liquid" mode of spatial location of ds DNA molecules in the particles of CLCD dispersions prevents their immobilization on the surface of membrane filters. However, if poorly soluble CLCD particles consisting of molecules of the (ds DNA–Gd) complexes are formed, the immobilization of particles on the surface of the nuclear membrane filter becomes possible and the size and shape of these particles can be investigated.

Figure 6(a) shows the AFM image of ds DNA CLCD particles after their treatment with $GdCl_3$ and immobilization on nuclear membrane filter. **Figure 6(b)** demonstrates the size distribution of these particles as well as the pores in the filter.

One can see that these particles exist as independent, individual objects.

The presence of single particles (**Figure 6(a)**) testifies that at treatment of particles of ds DNA CLCD by $GdCl_3$, the "liquid" character of the DNA packing in these particles is disappeared and the particles have a rigid spatial structure.

Therefore, particles of the CLCD of the ds DNAs whose phosphate groups are neutralized by gadolinium ions become poorly soluble and can exist in the absence of osmotic pressure of the PEG-containing solution and the osmotic pressure of the water-salt PEG-containing solution is not required for supporting the spatial structure of CLCD particles formed by (DNA-gadolinium) complexes.

The mean size of particles makes 4500 - 5000 A˚, *i.e.* the mean diameter of the ds DNA CLCD particles after gadolinium treatment coincides with the mean diameter of initial ds DNA CLCD particles [12,33]. The particles have shape of the spherocylinders and the diameter of particles is close to their height. The obtained result is important, because it allows one to suggest that the average packing density of the DNA molecules in particles of the CLCD of the (ds DNA–Gd) complex is quite close to the packing density of the DNA molecules in particles of the CLCD formed from initial DNA molecules. In this case, the mean concentration of chromophores (nitrogen bases) of the DNA in particles of the CLCD of the

DNA–gadolinium complex must also retain not only high [34], but sufficient for holding the abnormal optical activity of these particles.

The visualization of single particles indicates that, when particles of the CLCD of ds DNA are treated by the $GdCl_3$ solution, the liquid character of packing of DNA molecules in these particles is indeed disappeared and particles acquire a rigid spatial structure. Such a structure is presented not only the decrease in the solubility of DNA molecules, but also the presence of strong interaction between the fragments of neighboring DNA molecules, because gadolinium ions can be nonuniformly distributed.

Hence, the treatment of the ds DNA CLCD by $GdCl_3$, is accompanied not only by neutralization of phosphate groups of the DNA molecules by Gd^{3+} ions, but by a significant attraction between the neighboring DNA molecules. Disappearance of the fluidity of the ds DNA CLCD particles proves a short-range attractive interaction between the charged DNA molecules arising from interlocking Gd^{3+} ions, sometimes called as "counterion cross-links". An existence of independent particles speaks in favor of an appearance of noncompensated positive surface charge on the CLCD particles. This, in turn, prohibits the coalescence of these particles.

In addition, nonuniform distribution of gadolinium ions over the surface of DNA molecules having the inhomogeneous secondary structure is accompanied by irregular interaction between the fragments of the neighboring DNA molecules in quasinematic layers. Because each phosphate group of the DNA molecules, carrying one "effective" negative charge, is neutralized by Gd^{3+} ion [35], that carries three positive charges, this means that the altered surface charge distribution makes an additional contribution to the chiral interaction between adjacent (ds DNA-Gd) complexes in the particles [22,19]. Under these conditions, the interaction between the modified DNA molecules can induce change in the twisting of the cholesteric helical structure of the DNA molecules. In this case one can expect that the pitch (P) of the spatial twist of cholesteric structure formed by the ds DNA is changed as a result of interaction of gadolinium ions with these molecules.

Indeed, the change in the twist angle between neighboring quasinematic layers of CLCD is supported by results of the theoretical calculations [3,32,36] according to which an increase in the twist angle (decrease in P value) of the spatial structure of (ds DNA-Gd) CLCD particles [36] determines a drastic increase in the amplitude of an abnormal negative CD band in the absorption region of DNA nitrogen bases, when the CLCD particles are transformed from "liquid" to into "rigid" state.

The results above allows one to suggest the scheme of "liquid-rigid" structural transition of ds DNA CLCD shown in **Figure 8**.

One can see, that here the spatial ordering of neighboring DNA molecules in quasi-nematic layers is practically absent; besides, under these conditions the twist angle between neighboring DNA quasi-nematic layers is increased (the P value is decreased, right structure). According to [32,37] in the presence of large excess of gadolinium cations, these cations can displace the sodium ions, initially bounded to the phosphate groups of the ds DNA. The gadolinium ions neutralizing the negative charges of the phosphate groups of the ds DNAs make particles of the CLCD of (ds DNA-Gd) insoluble in PEG-salt-aqueous solutions. It is worth noting that, when Gd^{3+} ions are bonded to polyphosphates, poorly soluble Gd-polyphosphate is formed (solubility constant is equal to about 10^{-12} M) [38,39]. Since ds DNA molecules have polyphosphate nature, these molecules in the presence of saturating gadolinium concentrations become poorly soluble in poly(ethyleneglycol)-water salt solutions. Under high concentration of gadolinium, the stable spatial structure of dispersion particles is formed and the presence of poly(ethyleneglycol) is not required to stabilize the structure of particles of the CLCD. Moreover, gadolinium ions, neutralizing the charges of the phosphate groups of the DNAs, create an excess positive surface charge on particles of the CLCD and aggregation of these particles. This behavior is corroborated by the atomic force microscopic data according to which these gadolinium-ion-treated particles of the CLCD of the DNAs are existing as single independent objects (see **Figure 6**). According to this scheme the amplification of the abnormal negative band ($\lambda \sim 270$ nm) in the CD spectrum of CLCD particles formed by ds DNA molecules treated with high concentration of gadolinium cations confirms the formation of "rigid" CLCD particles of (ds DNA-Gd)

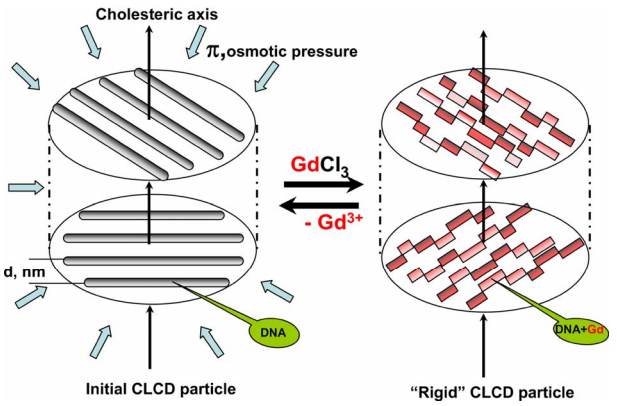


Figure 8. Sheme of transition from "liquid" to "rigid" structure of the particle of the CLCD induced by high Gd^{3+} concentration.

complex (**Figures 2** and **3**) under used conditions.

In addition, the results of low-temperature magnetometric study and neutron activation analyses showed [3, 15] that in the case of "rigid" particles formed at $r_{total} > 20$, one gadolinium cation is bounded approximately to one DNA phosphate group. The evaluations showed that local concentration gadolinium in the content of these particles can reach 40%, *i.e.*, we are dealing with a novel type of biomaterial containing a very high local concentration of gadolinium.

The obtained "rigid" CLCD particles strongly enriched by gadolinium open a possibility for various manipulations with them, for instance, their application as "Gd-carriers" at neutron capture therapy (NCT).

4.2. Gd-NCT with "Rigid" Particles of CLCD Formed by (ds DNA-Gd) Complexes

A potential of the "rigid" (ds DNA-Gd) particles as biomaterial for Gd-NCT, is based on a few facts:

1) the formation of these particles can be easily checked by the CD spectroscopy or by the AFM;

2) a long-term stability of the physicochemical properties of these particles allows one to manipulate with these particles;

3) this biomaterial has a higher concentration of ^{157}Gd compared to other known Gd-carriers such as, for instance, Gd-chelate complexes [4,6,9,11].

Comparison of **Figure 7(b)** to **Figure 7(a)** shows that irradiation of CHO cells by the thermal neutron fluence (5×10^{11} neutron/cm^2) within 1 h results only in minor (if any!) changes in the CHO cells and does not influence their ability to grow. Even after 120 hours of cell processing, these cells, irradiated in absence of (ds DNA-Gd) particles grow as initial cells and form monolayer**.** This signifies that thermal neutron irradiation of intact CHO cells under our conditions does not influence strongly the proliferation ability of these cells.

Figure 7(c) shows the image for CHO cells irradiated by thermal neutrons in the presence of "rigid" gadolinium carrier (set A N1). It can be seen, that after 120 hours of cell processing, the image of CHO cells differs from that of cells irradiated without "rigid" gadolinium carrier. The efficiency of cells proliferation is reduced and cells don't form colonies. The growth of CHO cells administrated with the "rigid" (ds DNA-Gd) particles and then irradiated with thermal neutrons was significantly suppressed compared to that in control cells. Besides, the cell debris in cultural medium begins to appear after 60 hours of cell processing (data not shown). The amount of cell debris after 120 hours shows that significant part of cells was disintegrated. Finally, the irradiation of CHO cells in presence of "rigid" particles results in full absence of alive CHO cells.

Taking into account that flasks N1 and N2 (set A) were irradiated by thermal neutrons simultaneously the difference in the cell killing efficacy for these samples (**Figure 1**, set A) might be due to the thermal NCR induced only by gadolinium in the content of "rigid" particles containing (ds DNA-Gd) complexes. Hence, CHO cells in the sample with gadolinium were killed, while the cells in control samples survived under the same conditions of irradiation [40].

The presence of strongly deformed cells (collapsed cells) **Figure 7(c)** allows one to suppose, that although concrete reasons for an appearance of these cells were not investigated carefully, that irradiation of CHO cells in presence of (ds DNA-Gd) "rigid" particles is accompanied by a few processes:

1) disintegration of genetic material of these cells;

2) deformation and destruction of lipoprotein membrane as a result of possible retention of Gd-containing particles in lipoprotein membrane of CHO cells and

3) penetration of small fraction of (ds DNA-Gd) "rigid" particles into the CHO cells inducing additional destruction of these cells.

It is necessary to stress that according to known data [5,7,8,40] in the case of other Gd-carries the cell growth was inhibited until to 10 days after the neutron irradiation.

Considering the reasons for the effects shown in **Figure 7(c)**, one can remind the following. When bombarded with thermal neutrons, ^{157}Gd releases photons and electrons with energies up to 7.9 MeV [5,41]. Previous studies on CHO cells have shown a significant enhancement of lethal effects induced by Gd-NCR [5]. During the Gd-NCR, the emission of γ-rays is followed by the internal conversion and subsequent emission of Auger electrons [41] and these electrons play an important role in cell killing. As the range of these electrons is extremely limited in tissue, gadolinium distribution in the cells, particularly with respect to the genetic material, is crucial in determining the extent of biological effects induced by Gd-NCT. Indeed, it was shown in [42,43] that in the thermal neutron irradiation of plasmid DNA/gadolinium mixture the extent of double-strand breaks to be considerably reduced by sequestering the gadolinium from DNA, suggesting the effect to have been mainly due to Auger electrons.

However, the ranges of high-energy electrons and photons produced in the gadolinium NCR [41] are sufficiently long within tissue for cell inactivation to occur even if Gd is present in the vicinity of the cell. Indeed, it was demonstrated that mouse ascites cells in the peritoneal cavity to be inactivated by the radiation released from ^{157}Gd contained in microcapsules, suggesting the proximity of ^{157}Gd to cellular genome not to be critical.

Taking into account the fact that gadolinium in microcapsules was effective in suppressing murine ascites tumor, most of the effects observed apparently resulted from the radiation of high-energy internal-conversion electrons having adequate ranges and photons from Gd. This result permits to suppose that the intracellular presence of Gd is, indeed, not critical in Gd-NCT**.**

It is known as well that the energy of conversion electrons is equivalent to the path inside biological tissue more then 50 μm [44]. It was demonstrated earlier [45], that the conversion electrons (the energy ≥ 7 KeV) and γ-rays are not significantly absorbed inside the initial "rigid" (ds DNA-Gd) CLCD particles. Besides, the calculations showed that more than 70% of the conversion electrons can penetrate into the CHO cells and create the radiation dose, requested for cell destruction. Taking into account that the (ds DNA-Gd) CLCD particle density was about of $5 \cdot 10^4$ particles per cell and the thermal neutron fluence was of the order of 5×10^{11} neutron/cm^2, this results in 100 neutron captures in each particle. In that case the secondary electrons and photons, with the probability of about 100%, can induce the DNA double-strand breaks in the cell nucleus if the (ds DNA-Gd) CLCD particles located nearby the surface of CHO cells. This means that we can expect that the secondary irradiation from "rigid" (ds DNA-Gd) particles can hit the material of cell nucleus, inducing its disintegration. Indeed, comparison of the images shown in **Figure 7** in combination with the value of neutron fluence equals to 5×10^{11} neutron/cm^2 and high Gd-concentration in "rigid" particles used in our study permits one to state, that highly-gadolinium-loaded DNA "rigid" particles have potential usefulness for Gd-NCT.

We are clearly understanding that for practical medical application, the optimization of physico-chemical parameters of "rigid" (ds DNA-Gd) particles, including concentration of Gd, as well as the time before irradiation and the time of irradiation is required. One can suppose that the efficiency of application of these particles in Gd-NCT can be even increased in the case of forced penetration of DNA "rigid" particles inside CHO cells. Besides, it is necessary to perform "the size of "rigid" particles—the dose" calculations [46,47] because, as it was shown in [46,47], the doses from neutrons, prompt γ-rays and internal conversion electrons, and the dose distribution to be a function of strongly-interrelated parameters such as tumor size, gadolinium concentration and its spatial distribution through the tumor, tumor-to-normal tissue concentration ration and tumor site.

In conclusion, one can say that the obtained results (despite of their noncomplete character) showed that "rigid" (ds DNA-Gd) particles have a potential as a novel biomaterial with high concentration of gadolinium for NCT. In this case appears a possibility to use simpler (in comparison to nuclear reactors) devices for NCT realization. Our results demonstrate as well that the intracellular presence of Gd-carrier is not an obligatory condition for effective disintegration of CHO cells at Gd-NCT.

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