SYNTHESIS AND LUMINESCENCE OF A FOLIC ACID-EUROPIUM CHELATE CONJUGATE

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We have synthesized a folic acid–europium complex conjugate which shows promise for biomedical applications. We have studied the absorption spectra, the luminescence spectra, and the luminescence excitation spectra of folic acid–spacer–amino-substituted phenanthroline and folic acid–spacer–europium chelate conjugates, and also of the individual components of the synthesized triads. All the spectral luminescence data obtained confirm that a folic acid–europium complex conjugate is formed. Binding of the synthesized conjugate to a folate receptor on HeLa tumor cells is demonstrated.

***Keywords:** folic acid, organic complexes of europium, folic acid–europium complex conjugate, absorption spectrum, luminescence spectrum, luminescent tag.*

Introduction. Complexes of some rare-earth elements (lanthanides (Ln)), in particular europium (Eu^{3+}), with organic chelating addends[**] have specific luminescent properties, making it possible to use them as highly sensitive luminescent probes in biomedical studies (see, for example, [1]). The major feature of lanthanide probes is their prolonged luminescence in the millisecond range. This makes it possible, using a time-resolved technique for detecting the signal from the luminescent probe, to practically completely eliminate the background luminescence of the analyte objects and the medium. Such detection enhances the sensitivity of luminescent analysis of biosystems by 3–5 orders of magnitude compared with detection without time resolution. The afterglow time (the time until it has practically completely decayed) for intrinsic fluorescence of biological objects is 50–100 ns [2]. An important feature of the luminescence of organic complexes of lanthanides is the lack of quenching by oxygen. There are two reasons for this: 1) the luminescence of the lanthanide ion itself is not sensitive to oxygen, since the optical transition occurs within the $4f$ shell, shielded by the outer electrons of the atom; 2) energy transfer from the addends to Ln^{3+} occurs from the triplet level T_1 with a fast rate constant, while shortening the lifetime of the T_1 state also makes it insensitive to the effect of dissolved oxygen.

An important direction in modern medicine is development and synthesis of luminescent probes selective for cells of pathologically altered tissues [3]. Such selectivity can be accomplished using probes that can bind to specific receptors expressed by these cells. Available literature data suggests that for cancers, in particular for many epithelial tumors, elevated expression of folate receptors by tumor cells is observed [4]. This is due to the fact that the folate receptor enables accumulation in the cells of folates, participating in synthesis of nucleic acids and proteins [5] needed by cells when their proliferative activity increases. Consequently, determination of the level of expression of folate receptors by cells can be used as a highly sensitive approach to diagnosis of a tumor process, estimation of the tumor growth rate and the individual sensitivity of cells to chemotherapeutic drugs.

This work is the first step toward development of applications of time-resolved luminescent analysis in oncology by designing luminescent probes selectively binding to proliferatively active cells, based on conjugates of folic acid (FA) with lanthanide tags.

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**We intentionally do not use the more common term "ligand" to describe complexation, because ligand has a different meaning in the biochemistry of folic acid.

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Objects of Investigation and Experimental Technique. We studied an FA–spacer–europium chelate conjugate we synthesized (FA–(Phen)Eu(BTA)$_3$, where Phen = 1,10-phenanthroline, Eu(BTA)$_3$ is a complex of europium with benzoyl trifluoroacetone). To synthesize the conjugate, we used folic acid from Sigma. The rest of the commercial reagents (Eu(NO$_3$)$_3$, benzoyl trifluoroacetone, phenanthroline, organic solvents) were purified before the experiment.
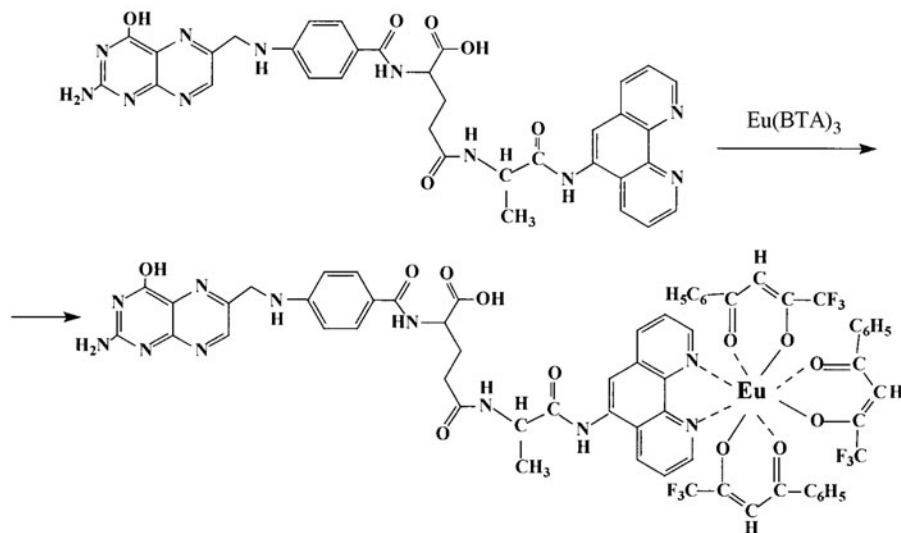
We studied binding of the conjugate to cells on malignantly transformed human cervix epithelial cells (HeLa). The cells were cultured in complete nutrient medium 199 supplemented with 5% fetal calf serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. This medium contains folic acid in low concentration ($2 \cdot 10^{-8}$ mol/L). The cells were incubated in culture bottles at 37°C in a humid atmosphere with 5% CO$_2$. The cells were passaged after three days maintained in the log growth phase.

The luminescence and luminescence excitation spectra were measured on an SDL-2 automatic spectrofluorimeter, consisting of an MDR-12 wide-aperture excitation monochromator and an MDR-23 detection monochromator. The absorption spectra were recorded on a Cary 500 Scan UV-Vis-NIR spectrophotometer. All the spectral measurements were made at room temperature.

Results and Discussion. In order to design selective methods for time-resolved luminescent microanalysis based on a complex of the Eu^{3+} ion with sensitizer addends (in particular, β-diketones, intensely absorbing light and efficiently transferring excitation energy to the lanthanide ion), we used the specific affinity of folic acid for proliferatively active cells, where the folic acid is chemically bound to the luminescent complex as a result of conjugate formation. As the luminescent tag, we selected the complex Eu(BTA)$_3$. However, for such luminescent complexes of europium with β-diketones in aqueous medium, nonradiative transfer of the excitation energy occurs to the vibrational levels of the water molecules, which enter the first coordination sphere of the complex, leading to strong quenching of luminescence. This problem is solved by filling the coordination sphere with a non-quenching agent: Phen, which forms a stable complex with europium, (Fen)Eu(BTA)$_3$.

The conjugate FA-(Phen)Eu(BTA)$_3$ was synthesized using a special spacer (L-alanine), to which the components of the triad formed were "grafted" on both sides. In the first step of the synthesis of the lanthanide-containing conjugate, we obtained a conjugate of the folic acid molecule covalently bound through a hydrocarbon bridge (the spacer) to amino-substituted phenanthroline (NH$_2$-Phen). The spacer prevents interaction between the lanthanide tag and the biological object. The peripheral amino group NH$_2$ is needed for "grafting" the Phen to the spacer. The NH$_2$-Phen was synthesized by the familiar procedure in [6].

The F–spacer–amino-substituted Phen conjugate (FA-Phen) was synthesized by the procedure we used earlier in synthesis of a conjugate of folic acid with the dimethyl ester of chlorin e_6 [7]. The FA-(Phen)Eu(BTA)$_3$ conjugate was synthesized by vigorous mixing of stoichiometric amounts of the FA–Phen conjugate and the Eu(BTA)$_3$ complex in dimethylsulfoxide. After the bright yellow crystals of the conjugate precipitated, they were centrifuged, washed with water and alcohol, and then dried. The structures of the FA–Phen and FA–(Phen)Eu(BTA)$_3$ conjugates obtained have the form:
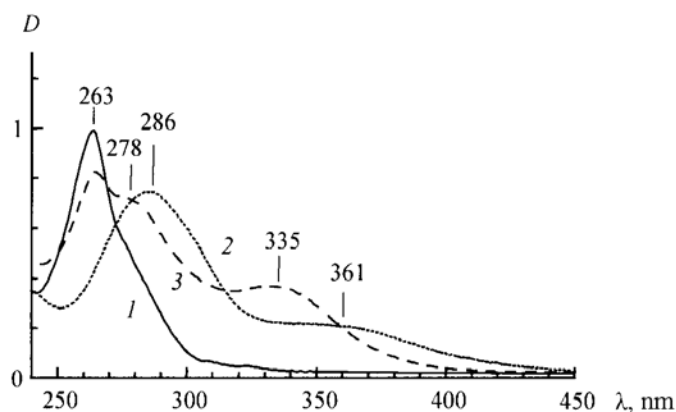
Fig. 1. Absorption spectra of NH$_2$-Phen (1), folic acid (2), and the folic acid–Phen conjugate (3) in ethyl alcohol.

Figure 1 shows the absorption spectra of NH$_2$-Phen, folic acid, and the FA-Phen conjugate in ethyl alcohol. The absorption spectrum of NH$_2$-Phen has an intense band at 263 nm with a long-wavelength wing descending down to ~330 nm. The absorption spectrum of folic acid in the UV region has maxima at 286 nm and 361 nm. As we see, the spectrum of the product obtained as a result of the reaction carried out between folic acid and NH$_2$-Phen (curve 3) is quite different from the absorption spectra of the starting materials, which is evidence for the formation of the FA-Phen conjugate. For the conjugated molecule, the long-wavelength absorption band undergoes a hypsochromic shift to 335 nm (compared with the folic acid band at 361 nm). The second absorption band of folic acid (286 nm) is similarly shifted, which in the spectrum of the conjugate is found at 278 nm. In the absorption spectrum of the conjugate, there is a band analogous to the absorption band for unbound NH$_2$-Phen (263 nm).

In order to interpret the spectral characteristics of the conjugate FA–(Phen)Eu(BTA)$_3$, first we studied the fluorescent properties of the conjugate FA+Phen. Figure 2 (curve 1) shows the absorption spectrum of FA+Phen in dimethylformamide (DMF). Note that the absorption band at 336 nm has increased relative intensity and half-width compared with the analogous band in ethanol. In the fluorescence spectrum at λ_{ex} = 283 nm (curve 2), two bands are identified: an intense band with maximum at 333 nm, and a broad low-intensity band with maximum in the ~475 nm region. We should point out that for free folic acid molecules in aqueous solution, a fluorescence maximum at 445 nm is characteristic [8]. From analysis of the absorption and fluorescence spectra shown, we can draw the following conclusions: the weak and very broad band with maximum at ~475 nm belongs to the fluorescence of unbound folic acid molecules, the long-wavelength absorption band of which extends to 450 nm; the absorption band recorded at ~336 nm (curve 1) is the superposition of the absorption of free folic acid and the absorption of the conjugate FA+Phen. A substantial contribution to formation of the spectral contour of the bands at 283 nm and 330 nm in the fluorescence excitation spectrum for λ_{det} = 475 nm might come from absorption of the conjugate FA+Phen: in this region, the absorption spectrum of the conjugate FA+Phen has corresponding bands. The short-wavelength intense fluorescence band at 333 nm is a fluorescence band of the conjugate, which undergoes a small Stokes shift relative to the long-wavelength absorption band of the conjugate at 330 nm.

The absorption spectrum of the conjugate FA–(Phen)Eu(BTA)$_3$ in DMF (Fig. 3, curve 1) differs from the absorption spectrum of the conjugate FA+Phen (Fig. 2, curve 1) in the increase in the relative intensity of the band at 326 nm, which corresponds to absorption of the organic addends of the europium ion (BTA). Excitation at λ = 283 nm leads to the appearance of a complex luminescence spectrum (Fig. 3, curve 2): we simultaneously observe very weak luminescence of free folic acid with maximum at ~475 nm, weak luminescence with maximum at 333 nm (luminescence of the conjugate FA+Phen), and intense luminescence of the europium ion in the 580–710 nm region with an intense narrow band at 613 nm. The luminescence excitation spectrum for λ_{det} = 613 nm (curve 3) completely corresponds to absorption by the organic ligands of the europium ion (absorption by the BTA), although the band at 326 nm does not have a symmetric contour (a shoulder is observed on the long-wavelength edge). The latter may be
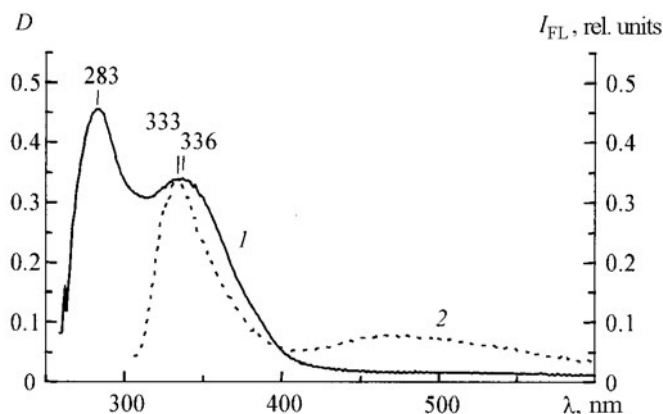
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Fig. 2. Absorption spectrum (1) and fluorescence spectrum for λ_{ex} = 283 nm (2) for the folic acid–Phen conjugate in DMF.
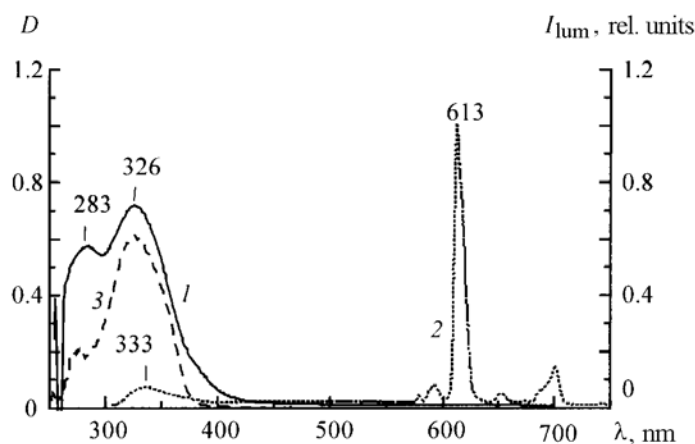

Fig. 3. Absorption spectrum (1), luminescence spectrum for λ_{ex} = 283 nm (2), and luminescence excitation spectrum for λ_{det} = 613 nm (3) for the folic acid–(Phen)Eu(BTA)$_3$ conjugate in DMF.

evidence for excitation of the europium ion via absorption by the conjugate FA+Phen, confirming chemical bonding between the europium chelate and the conjugate FA+Phen, i.e., formation of the conjugate FA–(Phen)Eu(BTA)$_3$.

In order to test the ability of the conjugate obtained to bind to folate receptors expressed by cells, we studied accumulation of the conjugate in cells after its addition to the incubation medium for a cell monolayer. We used a monolayer with 70–90% confluence and conjugate concentration of $5 \cdot 10^{-7}$ mol/L. The cells were incubated with the conjugate for 3 h, washed with cold buffered physiological saline and lysed in 0.1% Triton X-100. Binding of the conjugate by the cells was determined from the intensity of europium luminescence in the cell lysates, using the calibration curves for luminescence of the conjugate in the lysing solution vs. its concentration. We established that under the conditions used, the cell bind the conjugate to a significant extent ($\approx 4 \cdot 10^6$ conjugate molecules per cell). This binding dramatically decreases (by 68%) in the presence of exogenous folic acid ($1 \cdot 10^{-4}$ mol/L), which suggests participation of the folate receptor localized on the surface of the HeLa cells in the binding of the conjugate. The assignment of the luminescence to the europium ions within the conjugate, bound to the cell, is confirmed by luminescence kinetics measurements, where the duration of luminescence is ~110 µs.

Conclusion. Our studies are the first step in using the specific affinity of folic acid for proliferatively active cells and the millisecond duration of luminescence of europium complexes to design selective methods for time-resolved luminescent microanalysis based on conjugates of folic acid with europium complexes.

We synthesized a conjugate of folic acid with an organic chelate of europium and studied its spectral luminescence parameters in solutions. In an *in vitro* system, we have demonstrated binding of the conjugate obtained to proliferatively active HeLa tumor cells. We have established the determining role in binding of the conjugate by cells played by the folate receptor expressed by the cells.

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