Fluorescence imaging of the nanoparticles modified with indocyanine green

K G Gareev¹, K Y Babikova¹, V N Postnov²,³, E B Naumisheva²,³, D V Korolev³,⁴,⁵

¹Saint Petersburg Electrotechnical University "LETI", Saint Petersburg, 197376, Russia
²Saint Petersburg State University, Saint Petersburg, 199034, Russia
³Federal Almazov North-West Medical Research Centre, Saint-Petersburg, 194156, Russia
⁴First Pavlov State Medical University of Saint Petersburg, Saint-Petersburg, 197101, Russia
⁵Saint Petersburg National Research University of Information Technologies, Mechanics and Optics, Saint Petersburg, 197101, Russia

Abstract
The comparative research of silica, the magnetite and magnetite-silica nanoparticles modified with fluorescent dyes using gas-phase and liquid-phase methods was conducted. At the content of fluorescent dye comparable in size a particular spectrophotometric method, nanoparticles with fluorescein have up to 1000 times larger overall luminous efficiency. It is revealed that magnetic nanoparticles are characterized by a smaller light efficiency in comparison with silica particles, at the same time particles of a magnetite are most effective at modification with fluorescein, and magnetite-silica particles – at modification with indocyanine green.

1. Introduction
History of fluorescent visualization in medicine contains more than 50 years. At the same time only two fluorophores are allowed for use in many countries of the world today, it is indocyanine green (ICG) or as it is called still, cardiogreen and various modifications of fluorescein (FS) [1]. Today, these two fluorophores are used mainly for receiving angiograms of a retina of an eye which demand rather high dose of substance. At the same time there can be side effects, such as hypersensitivity reactions, however, any specific toxicity it was revealed not [2]. For molecular visualization much lower dosage of a fluorophore having minimum toxicity is used [1].

Now the fissile are used and the activated composite fluorescent constructions [3]. The fissile constructions represent the fluorescent dyes conjugated with nanoparticles, monoclonal antibodies, enzymes and so on [4]. The activated constructions contain a fluorophore the secondary radiation of which is extinguished in a routine state. To receive induced radiation it is necessary to suppress bloom clearing with chemical [5], specific to this case. A classic example of such extinguishing of a fluorescence is formation of dimers in aqueous solutions of ICG and solution activation by an albumin [6].

In this work the possibility of application for fluorescent visualization of various types of nanoparticles (NPs) modified via various methods with two fluorophores was investigated: fluorescein and indocyanine green.

2. Experimental methods
In present micrographs of NPs were obtained with a use of the transmission electron microscope (TEM) JEM-1400 STEM cathode (JEOL, Japan).

In this work the NPs of following types were used: industrially produced silica nanoparticles - Aerosil A-200 of the Evonik Degussa GmbH, NPs of Aerosil are spherical, of magnetite - a needle-form (MNP-
NPs of magnetite-silica (MNP-2) were obtained according to [7]. All nanoparticles had the average size about 10 nanometers. NPs of A-200 represented particles in a form the close to spherical, MNP-1—particles of a needle form, MNP-2—separate particles and their agglomerates. The average form factor of A-200 and MNP-2 was equal to 1, of MNP-1—3.5.

For an immobilization of fluorophores on a surface of NPs amination was carried out. Two methods of amination were used: gas-phase and liquid-phase. The gas-phase technique as these NPs initially were in a type of dry powder was applied to NPs of Aerosil, and there was an opportunity to carry out synthesis in the pseudo-boiling gas-phase mode. The NPs received in the form of suspensions — magnetite and magnetite-silica — amination in the fluid environment. In both cases 3-aminopropyltriethoxysilane (APTES) was used as the amination agent.

For carrying out gas-phase modification with NPs amino groups the technique including APTES chemisorption from a gas phase in the flowing reactor was applied. The design of the reactor provided evaporation of reagent at a temperature 220 °C, a chemisorption, removal of excess of reagent and side products of reaction. As gas carrier nitrogen was used. The technique included physical removal of the binded water from a NPs surface at a temperature of 250 °C within 2 hours, chemisorption of APTES at a temperature of 250 °C within 1 hour, removal of excess of reagents and side products of reaction at the same temperature within 2 hours [8].

Liquid-phase amination of magnetic NPs was carried out with two methods: from organic solvent and from an aqueous medium.

For amination from organic solvent the aqueous suspension containing 50 mg of NPs in 2 ml of water was centrifuged for 5 minutes at a speed of 3000 min⁻¹. Supernatant merged, and the insoluble precipitate was five times washed out with ethanol. Then the NPs dispersion was carried out with use of UZD-2 ultrasound disperser (Ultrasound Technics, Russia) within 5 minutes in ethanol.

Liquid-phase amination was carried out as follows. The suspension containing 50 mg of nanoparticles in 2 ml of ethanol or distilled water was placed in the polypropylene tube with a volume of 15 ml then 200 μl of APTES was added and mixed on LS-220 shaker (LOIP, Russia) within 30 minutes at a speed of 300 min⁻¹ at room temperature. Then suspension was centrifuged within 5 minutes at a speed of 3000 min⁻¹ and washed out 5 times with distilled water with centrifugation.

Chemisorption of fluorescent dyes on aminated NPs was carried out. For all NPs types operation was performed identically.

Solution of fluorescent dye with concentration of 1 mg/ml in a volume of 1 ml and distilled water in a volume of 1 ml were added to the suspension containing 50 mg aminated nanoparticles in 2 ml of water. The sorption was carried out in 15 ml polypropylene test-tubes on LS-220 shaker at a speed of 300 min⁻¹ within 2 hours. Then solution was centrifuged within 5 minutes at a stirring speed of 3000 min⁻¹ and washed out 5 times with distilled water and centrifuged. The presumable mechanism of chemisorption of FLN is shown in the figure 1a, ICG — in the figure 1b.

The amount of sorbed fluorescent dye was defined after sorption. 0.1 N Solution of sodium hydroxide in a volume of 10 ml was added for this purpose to a washed precipitate and then desorption was carried out within 15 minutes. Then solution was centrifuged and 1 ml of supernatant liquid was taken. The received solution was analyzed on the content of fluorescent dye with a spectrophotometric method.

The wavelength of absorption maximum was defined with a use of a spectrophotometer Unico 2802s and equaled 485 nanometers for FLN and 700 nanometers for ICG.

Calibration of dependence of attenuation range of aqueous solutions on the maintenance of both fluorophores was carried out at these wavelengths. Since the maximum of a wavelength of absorption of FLN is blocked by the line of absorption of cations of iron, and alkali in turn partially dissolves MNP for MNP-FLN samples the content of fluorescent dye with this method did not manage to be estimated. For MNP-ICG samples, on the contrary, cation of iron does not prevent determination of content of a fluorophore.
Table 1. Properties of the modified nanomaterials

| Sample No. | Type of NPs | Fluorophore | Method of amination | Fluorophore content, mg/ml |
|------------|-------------|-------------|---------------------|---------------------------|
| 1.0        | —           | FLN         | —                   | 0.005                     |
| 1.1        | A-200       | FLN         | Gas-phase           | 0.013                     |
| 1.2        | MNP1        | FLN         | Water               | 0.005                     |
| 1.3        | MNP1        | FLN         | Ethanol             | 0.020                     |
| 1.4        | MNP2        | FLN         | Water               | 0.005                     |
| 1.5        | MNP2        | FLN         | Ethanol             | 0.020                     |
| 2.0        | —           | ICG         | —                   | 0.005                     |
| 2.1        | A-200       | ICG         | Gas-phase           | 0.007                     |
| 2.2        | MNP1        | ICG         | Water               | —                         |
| 2.3        | MNP1        | ICG         | Ethanol             | —                         |
| 2.4        | MNP2        | ICG         | Water               | —                         |
| 2.5        | MNP2        | ICG         | Ethanol             | —                         |

Samples (Table 1) which then were used in an experiment on fluorescent imaging were synthesized. For comparison of samples FLN and ICG solution in distilled water were prepared (samples 1.0 and 2.0 respectively).

For a research of a possibility of the synthesized samples in vitro fluorescence the IVIS Lumina LT Series III (Perkin Elmer, the USA) device was used. Researches were carried out in the tablet with the small holes in which with a use of a batcher with removable tips was put on 100 μl of the studied solution. The colloidal solution of NPs in distilled water was prepared from beforehand the washed out samples by means of an ultrasonic disperser, concentration for NPs labeled FLN was 4 mg/ml, for NPs labeled ICG – 2 mg/ml. For induction of fluorescence embedded light filters for FLN, 465 nanometers, for ICG, 745 nanometers, were used. Registration was carried out with the embedded FITS and ICG filters respectively having the characteristics suitable for selection of induced radiation of the corresponding fluorescent dyes according to documentation on the device.
3. Results and discussion

Aminated samples were investigated using TEM. The analysis did not allow to estimate APTES shell’s thickness. It means that a layer of the amination agent rather thin to be identified by means of this method. Also lack of agglomeration in comparison with initial samples was revealed that in turn testifies to applicability of all three methods of amination for the studied NPs.

Data of two channels of visualization of samples on both fluorophores are shown on the Figure 2. Numerical characteristics of light return of samples fluorescent radiation are provided in Table 2.

The overall light return of samples with FLN nearly three orders higher than for samples with ICG. However at the same time the area of a fluorescence of this dye coincides with an autofluorescence of many materials and living tissues that is the known fact [9]. It limits use of dyes on the basis of FLN. At the same time the maximum level of a fluorescence in comparison with control (sample 1.0) is observed at an sample 1.1 in which dye is immobilized on A-200 surface. The under level of a fluorescence of samples based on the magnetic nanoparticles apparently is bound to an overlap of iron and FLN absorption ranges. Despite it samples 1.2 and 1.3 based on MNP also show good level of fluorescence suitable for practical application.

Absolutely other picture is observed when using ICG. Samples 2.1, 2.4 and 2.5 on the basis of A-200 and MNP2 were suitable for practical use. Apparently it is bound to the fact that when using MNP1 the effect of extinguishing of fluorescence on magnetite surface takes place. It is absolutely true that such fact is excluded at NPs based on A-200. MNP2 have an envelope from silica which isolates a fluorophore from magnetite. More high level of fluorescence of samples 2.4 and 2.5 in comparison with the sample 2.1 apparently is bound to method of amination of a surface. A thin covering is formed at gas-phase amination. NPs are covered with thicker APTES polymer containing respectively larger

![Figure 1. Scheme of synthesis: a — amination of nanoparticles; b — a fluoresceine immobilization; c — an immobilization of indocyanine green](image-url)
quantity of amino groups at liquid-phase amination. The experiment shows that the number of available groups \(-\text{NH}_2\) for fluorescent dye is also higher in this case.

![Figure 2](image_url) *In vitro* testing of the received exemplars by means of a fluorescent visualizer of IVIS Lumina III LT

### Table 2. Measured values of output fluorescent radiation of samples

| Sample No. | Overall light output [p/s] / [µW/cm²] ×10⁸ | Average light output [p/s/cm²/sr] / [µW/cm²] ×10⁸ | Standart deviation [p/s/cm²/sr] / [µW/cm²] ×10⁸ | Min light output [p/s/cm²/sr] / [µW/cm²] ×10⁸ | Max light output [p/s/cm²/sr] / [µW/cm²] ×10⁸ |
|------------|------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| 1.0        | 2583,000                                 | 950,000                                       | 180,100                                       | 605,600                                       | 1300,000                                     |
| 1.1        | 2418,000                                 | 644,400                                       | 109,300                                       | 408,700                                       | 934,700                                      |
| 1.2        | 564,900                                  | 153,300                                       | 25,490                                        | 97,890                                        | 224,700                                      |
| 1.3        | 596,500                                  | 137,100                                       | 22,220                                        | 80,980                                        | 200,800                                      |
| 2.0        | 26,550                                   | 9,256                                         | 1,733                                         | 6,008                                         | 13,060                                       |
| 2.1        | 4,763                                    | 1,904                                         | 0,415                                         | 1,169                                         | 3,197                                        |
| 2.4        | 7,445                                    | 2,694                                         | 0,568                                         | 1,412                                         | 4,540                                        |
| 2.5        | 14,840                                   | 6,473                                         | 1,384                                         | 2,934                                         | 8,923                                        |

### 4. Conclusions

Nanoparticles of three types: Aerosil, magnetite and magnetite-silica were investigated as possible agents for fluorescent imaging. For the purpose of modification of nanoparticles surface with molecules of fluorescein and indocyanine green fluorescent dyes amination of particles with use of liquid-phase and gas-phase methods was provided. Comparison of results of amination on the content of fluorescent dye showed that for modification of magnetic nanoparticles of both types application of a liquid-phase method of amination from ethanol solution is the most efficient. Nanoparticles modified with the molecules of fluorophores were compared for light output efficiency.

The overall light output of nanoparticles with fluorescein is up to three orders higher than a light output of nanoparticles with indocyanine green. Nevertheless, application of such particles is limited to a presence of lines of an autofluorescense of other substances and living tissues at a fluorescein fluorescence wavelength range. Magnetite particles showed the highest level of the overall light output (among magnetic nanoparticles) using modification with fluorescein, and magnetite-silica NPs showed the highest overall light output at modification with indocyanine green.

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