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Studies on the Synthesis of Fluorescein-5-isothiocyanate: A Fluorescent Nanomarker for Biosensors

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Abstract—A review of the methods of synthesis of fluorescein is given. An improved method of synthesis of a chromatographically pure fluorescein-5-isothiocyanate nanomarker for labeling immunoglobulins is developed, which will allow creation of high-quality biosensors. A unique method of synthesis of 5-aminofluorescein, a precursor of fluorescein-5-isothiocyanate, was also developed. The properties of fluorescein-5-isothiocyanate were studied to find that it exists in two forms having different solubility.

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INTRODUCTION

At present fluorescein and hydroxyxanthene dyes are finding growing use both in traditional and new fields of chemistry and related sciences.In particular, fluorescein dyes are used in sensors, Langmuir–Brodgett monolayers and films, microchips for immune analysis, molecular lighthouses, nanochemistry, and supramolecular chemistry. New perspectives are associated with the use of the photophysical and photochemical properties of earlier scarcely studied fluorescein derivatives and analogs [1]. Recently biochemical, including immunological, applications of fluorescein dyes have recently been reported. The strong fluorescence of conjugates in the range of the maximum human visual sensitivity, different fluorescence and autofluorescence colors of microorganisms and tissues, as well as ability to dissolve in weakly alkaline make Fluorescein-5-Isothiocyanate (FITC) raise it to the first place among known protein biomarkers.

Urgency in Developing the Technology for the Production of a Practically Important Nanomarker Fluorescein-5-isothiocyanate

The outbreaks of infectious diseases, appearance of new types of contagious diseases, and recent comeback of the so-called “old” infections are responsible for an epidemiologically troubling situation in the world. The genetic variability of circulating strains, hospital infections, bacteria carrying, and difficulties in providing and applying immunobiological preparations make demand for enhanced effort in the field of immunoprophylaxis and immunotherapy. Insufficient attention to this problems will inevitably increase the risk of infectious diseases. A powerful technique for rapid diagnosis of viral infections is immunofluorescence staining.

The antigene–antibody interaction forms the basis of many high-sensitivity immunological assays. This is explained by unique specificity of the produced antibodies to a specific antigene, i.e. the antibodies to a specific antigen with bind exclusively with this antigen (antigenic determinant, epitope) [2].

The antigene–antibody interaction is based on the principle of mutual recognition, underlied by their conformational complementarity. The active site of the antibody is strictly complementary to the antiginic determinant, which ensures their binding.

The immunofluorescence interaction is based on the ability of tissue antigenes treated with fluorochrome-labeled immune serums to fluoresce under the UV light of a luminescent microscope. A fluorochrome-labeled antibody forms an antibody–antigen complex which can be observed by a microsocope in the UV light which excites fluorescence. Direct immunofluorescence (DIF) assays use the interactions of fluorochrome-labeled antibodies with antigenes on cells, in cells, or in tissues. Therewith, not infrequently we deal with the covalent binding of fluorophores to

biomolecules via the NCS group of fluorescein isothiocyanate.

Fluorescein-labeled immunoglobulins have found wide application because of high quantum yields and resistance to photobleaching [3]. Fluorescein isothiocyanate (FITC) makes possible express analysis of biomaterials, including those using flow cytometry. The essence of the latter technique is as follows: a suspension of cells labeled with fluorescing monoclonal antibodies or fluorescent dyes is introduced into a stream of fluid passing through a flow cell. Conditions are chosen so that cells form a line. When a cell crosses the laser path, the detectors measure:

– small-angle light scattering (from 1° to 10°) (gives information on cell size);

– 90° light scattering (gives information on the core/cytoplasm ratio and cell inhomogeneity and granularity);

– fluorescence intensity in several fluorescence channels (from 2 to 18–20) (gives information about the subpopulation composition of the cell suspension).

Over the past decade, there has been a great research interest in biosensors [4–11]. The use of the above reagent allows determination of the concentration of pathogenic microorganisms in a sample within a short time (10–15 min), and in certain cases the time of analysis can be reduced to 1 s and shorter. It should be noted identification of the pathogen takes 10^{-3} to 10 s.

One of the basic components of a biosensor is an ion-conducting memebrane containing biologically active components (for example, fragments of sheep antibodies to mouse immunoglobulin A, labeled with microbiological dyes).

It is worth noting that FITC was used in sensors for the detection of organophosphorus compounds in the workplace air at chemical weapons destruction facilities [12]. It was found in the cited work that the highest sensitivity of biochemical biosensors can be reached with fluorescence detection. The biologically active component in the described device was FITC-labeled eel electric organ acetylcholinesterase (AChE), immobilized on quartz.

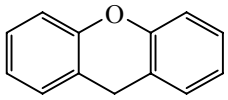
To develop and commercialize novel domestic biosensors on the basis of cheap domestic components is not only quite an urgent but also a timely task.

Due to its properties, FITC can be used in diagnostics in different fields of medicine, specifically, in immunology for cell cytotoxicity assessment, in oncology for monitoring patients at risk, in cytology for activity assessment of intracellular enzymes, in hematology for diagnosis of acute leukemia, in pharmacology, and for identification of a series of special danger infections.

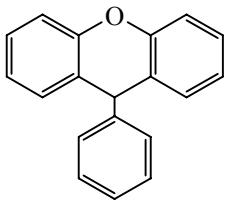
In view of the aforesaid, research into the synthesis of such a practically important fluorescent nanomarker as fluorescein-5-isothiocyanate with the aim to secure a market for a Russian reagent for domestic biosensors production.

Note that an essential drawback of foreign commercial FITC samples is their unstable quality due to the presence of impurities. As a rule, such samples are contaminated with intermediate synthesis products, including those capable of reacting with proteins, and the resulting background fluorescence may complicate antigen identification in immunofluorescent analysis.

Fluorescein-5-isothiocyanate is a xanthene dye. Xanthene dyes are derivatives of the heterocyclic compound xanthene [13]:



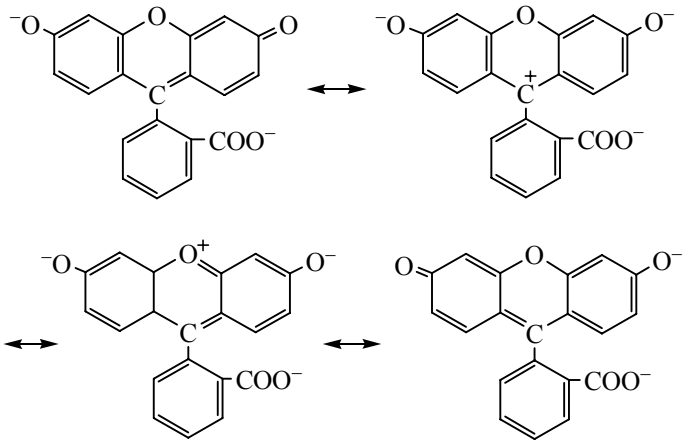
Of practical interest are derivatives of 9-phenylxanthene (structurally related to triarylmethane dyes):



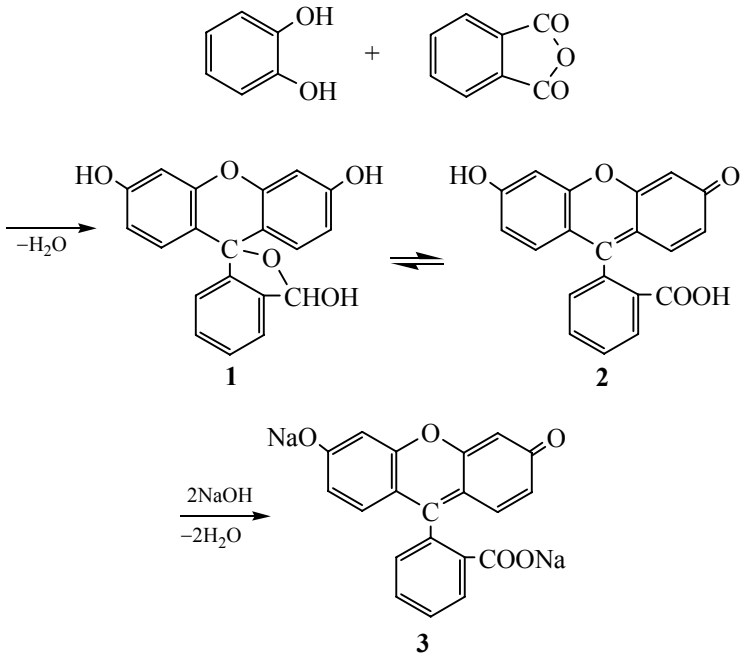
The molecules of xanthene dyes contain no less than two hydroxy or amino groups in the para position to the central carbon atom and frequently contain a carboxy group in the benzene ring not linked to the other rings via the oxygen atom, in the ortho position to the central carbon atom.

Fluorescein is the simplest representative of dihydroxyxanthenes. This strongly fluorescing dye was first synthesized by the German chemist Adolf von Baeyer in [1]. Since that time fluorescein and its derivatives have been among the most widely dyes in chemistry and related fields. Fluorescein exhibits a strong green fluorescence in solutions in a fully dissociated form, specifically, a doubly charged anion which is presented as a number of boundary structures (Scheme 1).

Scheme 1.



Scheme 2.



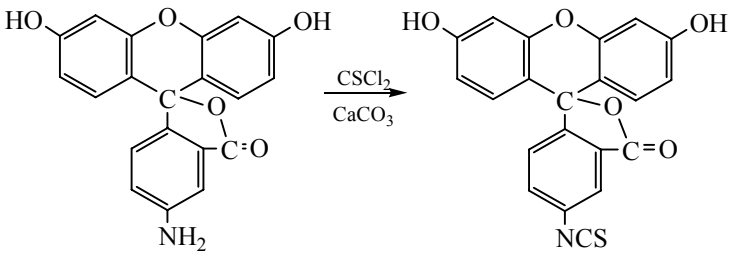
Fluorescein is prepared by the condensation of phthalic anhydride with resorcinol (Scheme 2).

Dihydroxyfluorane (**1**) is a colorless lactone form of fluorescein which undergoes tautomeric transformation to a yellow quinoid form (**2**). The disodium salt form of fluorescein is known as uranine (**3**) which was previously used to dye wool and silk to a bright yellow color. Alkaline solutions of fluorescein have a yellowish red color and exhibit a strong green fluorescence in reflected light. Fluorescence is visible at a very strong dilution with water (1 : 40000000), and, therefore, the dye is used to study stream currents and to detect leaks in industrial sewage pipes. Uranine is also used for painting marine markers seen at a long distance due to bright daylight fluorescence.

Synthesis of Fluorescein-5-isothiocyanate

Fluorescein-5-isothiocyanate was first prepared as hydrochloride in 1958 by the reaction of aminofluorescein and thiophosgene in acetone. However, only neutral form of FITC can be used as a microbiological marker. To obtain a neutral FITC, Boldyreva et al. [14] introduced calcium carbonate, amides, or tertiary amines into the synthesis (Scheme 3).

Scheme 3.



There has been reported a number of syntheses of isothiocyanate. In [15, 16], the decomposition of S-(ethoxycarbonyl)dithiocarbamates, thiophosgene method, and decomposition of thiourea derivatives were studied.

The synthesis of aryl isothiocyanates (mustard oils) can be divided into three stages: (1) formation of dithiocarbamate salt **5** from amine **4**, carbon sulfide, and a base; (2) formation of *S*-(ethoxycarbonyl)–dithiocarbamate **6** by treatment of salt **5** with ethyl chlorocarbonate; and (3) decomposition of compound **6** to form isothiocyanate **7**.

$$\underset{\mathbf{4}}{R{-}NH_2} + CS_2 + B \rightarrow \underset{\mathbf{5}}{:R{-}NHCS_2^-} + BH^+$$

$$R{-}NHCS_2^- + Cl{-}CO{-}OC_2H_5 \rightarrow \underset{\mathbf{6}}{R{-}NH{-}CS{-}S{-}CO{-}OC_2H_5}$$

$$\rightarrow \underset{\mathbf{7}}{R\text{-}NCS} + COS + C_2H_5OH.$$

The first stage is more difficult to realize with aromatic amines compared aliphatic because of the lower basicity of the former. Aryl dithiocarbamates should be synthesized in anhydrous solvents, such as benzene or ether, and a strong organic base.

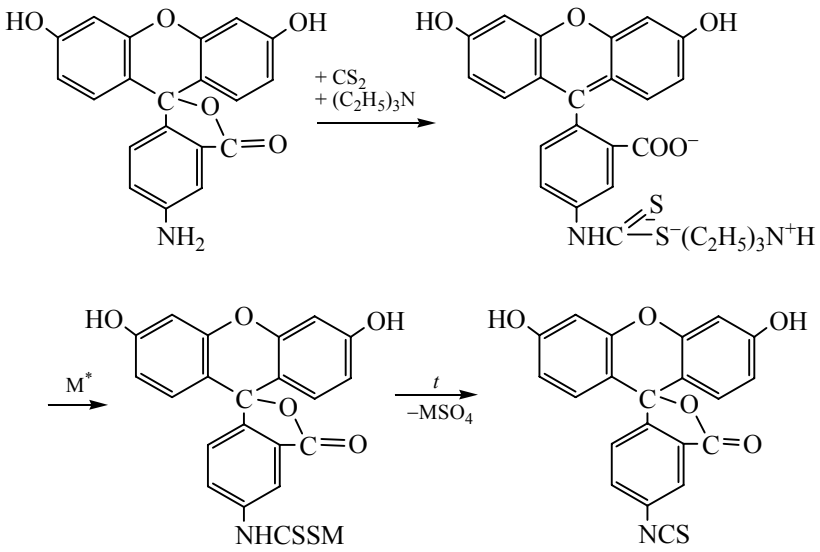
We found that the quality of 5-aminofluorescein (5-AF), a precursor of FITC, is the key factor responsible for the chromatographic purity of the resulting dye. In this connection our further research on the development of an improved synthesis of FITC focused first of all on the preparation of a pure of 5-AF.

Procedures of Synthesis of 5-Aminofluorescein and Fluoresceina-5-isothiocyanate

We showed that the neutral dye is best prepared by thermolysis of heavy metal salts of fluorescein-5-dithiocarbamic acid (Scheme 4).

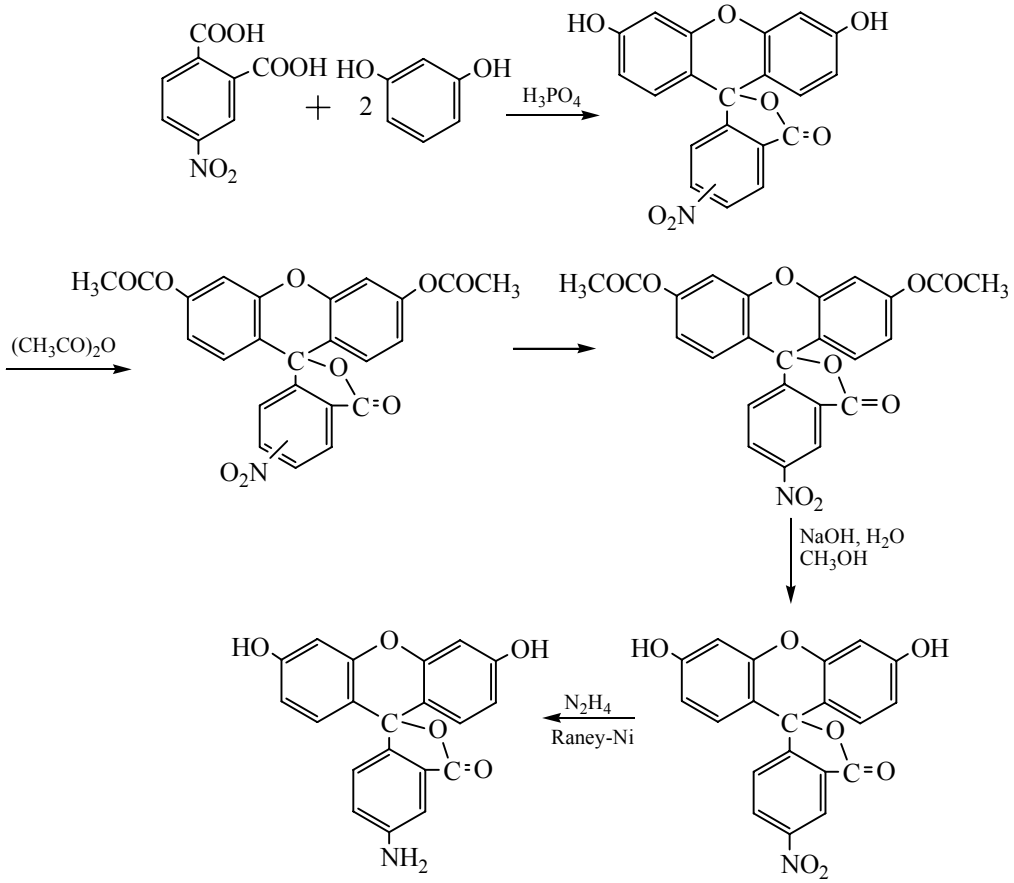
The solution of triethylammonium fluorescein-5-dithiocarbamate was added to a solution of a heavy metal salt, the corresponding precipitated metal fluorescein-5-dithiocarbamate was dried and refluxed in acetone, after which the solution was filtered, evapo-

Scheme 4.



M = Pb(II), Cu(II), Co(II), Ni(II),Fe(II), Zn(II), Cd(II), Ag(I).

Scheme 5.



rated until a precipitate started to form, cooled down, and the resulting fluorescein-5-isothiocyanate was filtered off. The yield of the product was 80%.

A great number of works have been published, where the authors underlined how important is that FITC for luminescence labeling of immunoglobulins be as pure as possible [11]. At the same time, no other methods of FITC have been reported than its fractional precipitation from acetone solutions with petroleum ether [12]. However, even this evidently inefficient method could not always be used, because such purification was associated with losses of the main substance.

The strange contradiction between the desire of researchers to obtain pure FITC and lack of effort on its purification is probably explained by the fact that this dye was considered as extremely unstable.

Thus, some authors recommended storage of FITC over P_2O_5 under nitrogen at –30°C in the dark.

We are the first to show that FITC can be purified by precipitation from anhydrous solvents or recrystallization. The most efficient of the methods we tried is chromatography on silica in anhydrous solvents; it gives a 99.8% pure FITC.

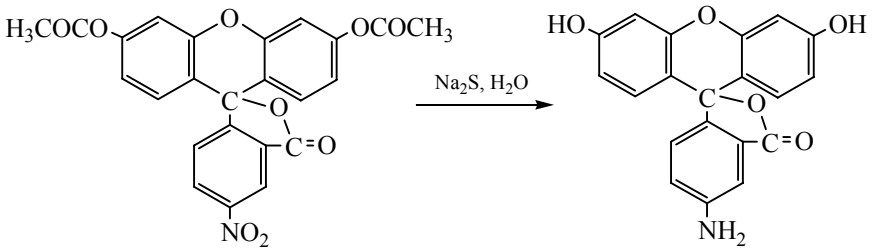
We previously developed a method for the determination of amino derivatives of xanthene dyes in their isothiocyanates [17]. It was found by this method that the synthesized fluorescein-5-isothiocyanate contains no starting 5-aminofluorescein.

To prepare pure 5-AF, we improved its classical synthesis first reported in 1942 [18] (Scheme 5).

The first stage of the synthesis involves the condensation of 4-nitrophthalic acid with resorcinol to form a mixture of 5- and 6-nitrofluoresceins.

The task we set ourselves in the work on improving this stage was to found an efficient catalyst for the condensation reaction. The best results were obtained with orthophosphoric acid as both a condensing agent and a solvent. By varying the acid concentration, as well as other process parameters (temperature, order of reagent mixing, reaction times) we found optimal

Scheme 6.



conditions, specifically, heating of the starting materials in 70% orthophosphoric acid at 135–140ºC for 4 h. The yield of the target product was 85–98 %.

The method differs from that proposed by Coons et al. [19] by that it is facile and not time-consuming, because the reaction occurs in solution. Moreover, orthophosphoric acid creates favorable conditions for the formation of the required isomer, which is evidenced by the high yield of 5-nitrofluorescein diacetate obtained at the second stage of the process after separation of isomers. The yield f 5-nitrofluorescein diacetate was 67%, mp 219–220ºC.

The acetyl groups were saponified with alcoholic alkali, and the resulting 5-nitrofluorescein was reduced in the alcoholic alkali solution with hydrazine hydrate on a Raney nickel.

However, later we developed a unique method of synthesis of 5-AF, which gives the best quality product as a result of direct reduction of 5-nitorfluorescein diacetate with sodium sulfide in water; the saponification of the acetyl groups with sodium hydroxide released in the synthesis occurs simultaneously with reduction [20] (Scheme 6).

A solution of 7.9 g (0.033 mol) of sodium sulfide in 20 mL of distilled water was added to a solution of 4.6 g (0.01mol) of 5-nitrofluorescein diacetate in 50 mL of distilled water. The solution was refluxed for 2 h, cooled, and, after sulfur had been filtered off, acidified with acetic acid. The precipitated 5-aminofluorescein was filtered off. The yield of the product was 80–85%. After one run of purification by reprecipitation of the hydrochloride of technical amine, the purity of the product was 98–99%. On thin-layer chromatography on Silufol UV-254 plates in a 5 : 3 benzene–acetone mixture 5-AF appeared as a fluorescent orange spot at R_f = 0.27; the spot of 5-nitrofluorescein was lacking from the chromatogram. The molar extinction coefficient of 5-AF at 490 nm was 86500.

Synthesis of Pure Fluorescein-5-isothiocyanate and Its Solubility Study

As mentioned above, to be used for luminescent labeling immunoglobulins for biosensor applications FITC should be as pure as possible. We managed to find an efficient method of synthesis and purification of this dye, due to which we obtained more than 99% pure neutral fluorescein-5-isothiocyanate.

However, it should be noted that chromatographic purification is efficient only with FITC synthesized using carbon sulfide as a reagent, because in this case the main substance is eluted earlier before all admixtures. In the FITC samples synthesized with thiophosgene contain an admixture which elutes before the main substance.

Characteristics of fluorescein-5-isothiocyanate samples

Purity, %	Crystal form	Solubility, mg/mL (dissolution time, min)	Relative luminescence intensity in phosphate buffer (10^{-5} M) with respect to fluorescein
99.3	Plates with small spherical inclusions	1.2 (15)	81
99.1	Opaque balls	2.5 (5)	67
99.5	Opaque balls	2.5 (5)	73
98.0 (with thiophosgene)	Mixture of transparent plates and opaque balls	1.0 (15)	72

One of the requirements to high-grade FITC is that it should readily dissolve in weakly alkaline buffers (phosphate, carbonate), where the fluorochrome is conjugated with proteins. It was found that FITC dissolves at different rates. The dependence of the quantity of dissolved substance on sample weight for prolonged shaking of different weights of the same sample with the same volume of phosphate buffer had the shape of a broken line with an inflection point. We suggested that the samples were inhomogeneous due to the existence of different crystal forms of FITC.

Microscopy of well purified samples of the dye showed that, indeed, some of the samples were a mixtures of different crystals. One of the crystal forms looked like weakly colored transparent plates and the other, like orange opaque balls. These two forms could not be manually separated under microscope. The characteristics of the studied fluorescein-5-isothiocyanate samples are presented in the table.

X-ray phase analysis gave evidence showing that we deal here with different crystal forms, and solid-phase IR spectroscopy revealed differences in their molecular structure. The stretching vibration band of the carbonyl group at 1760 cm^{-1} in the IR spectrum of the plate form suggests a lactone structure, whereas spherical crystals have a quinoid structure.

CONCLUSIONS

A new method of synthesis of nitrofluorescein is developed. Orthophosphoric acid as both a catalyst and solvent offers the advantage of a facile and fast condensation in solution and a high yield of nitrofluorescein.

A simple and efficient method of reduction of 5-nitrofluorescein diacetate in water, bypassing the stage of saponification in alcoholic alkali, is developed.

An efficient method of production of a high-quality neutral FITC is developed. Research into the purification of FITC allowed us to reveal two crystal forms with different solubility.

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