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Simultaneous Antigen Detection Using Multiplex Dyes

K. Galla,[1] J. Arden-Jacob,[2] G. Deltau,[2] K. H. Drexhage,[2] M. Martin,[1] M. Sauer,[1] J. Wolfrum,[1] and S. Seeger[1,3]

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To detect several antigens simultaneously, antibodies directed against different antigens were immobilized on a quartz surface. The antigens were tagged with multiplex dyes, which show different fluorescence lifetimes but similar excitation and emission spectra. The antigens were detected by recognizing the characteristic fluorescence lifetime. Furthermore, the effect of labeling of the dye on the antigen molecules was examined.

KEY WORDS: Multiplex dyes; coumarin; rhodamine; time resolved fluorescence spectroscopy; immunoassay.

INTRODUCTION

Highly specific detection of molecules via a noncovalent linkage to receptor molecules immobilized on a surface is widely used in medical diagnostics, food, and environmental analysis. Of particular importance in biodiagnostics is the antibody–antigen interaction [1,2]. An antibody molecule—a protein with a molecular weight of 150,000 or more—has two binding sites, which are determined by the three-dimensional structure of the peptide chains. This structure recognizes a specific complementary structure, the antigen molecule. Due to this high specificity of the antibody–antigen reaction, antibodies are widely used as receptor molecules. Usually, one component is immobilized, and a second antibody is tagged with a label, which can be detected easily by a "sandwich assay" or by a "competitive assay," i.e., using tagged antigen molecules to compete with the real antigens for the antibody binding sites [1].

Although presently the most immunoassays are performed with antibodies tagged with radionuclides or enzymes, fluorescent dyes are now becoming more important as labels. By first labeling the antibody with suitable fluorescent dyes, the antibody–antigen complex is detected due to intrinsic fluorescence of the dye. Using dyes with different emission wavelengths, different antigens can be detected simultaneously using a broadband light source or different lasers and different filters for discrimination of the excitation light from fluorescence [3]. Because the spectra of the various dyes overlap, only a small number, i.e., three or four different colors can be separated with high sensitivity. In many cases, e.g., in tumor diagnostics, the presence of more than three or four antigens in the patient's blood has to be tested. Using only the color information, different assays have to be performed. In order to extend the number of antigens which can be identified in a sample, an additional characteristic property of fluorescent dyes has to be found. In addition to the spectrum, the lifetime of the excited state is a characteristic feature of fluorescent dyes. Introducing the fluorescence lifetime as a characteristic parameter of dyes, the concept of simultaneous detection can be extended [4]. Hence, fluorescent dyes whose absorption and emission spectra are nearly identical, but which differ in fluorescence lifetime have to be designed [4,5].

These so-called "multiplex dyes" can be linked to

[1] Physikalisch-Chemisches Institut, Ruprecht-Karls-Universität, Im Neuenheimer Feld 253, 69120 Heidelberg, Germany.
[2] Institut für Physikalische Chemie, Universität Gesamthochschule, Adolf-Reichwein-Straße 3, 57068 Siegen, Germany.
[3] To whom correspondence should be addressed.

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antibodies or other biomolecules by *in situ* activated succinimido esters of the dyes, which react with the amino functions of the basic lysine residues. With antibodies labeled in this way, a simultaneous detection of different antigens by time-resolved fluorescence spectroscopy is possible. Because the lifetimes of the dyes are known, the measured lifetimes only have to be compared with the possible ones (pattern recognition technique) [6,7]. Hence, the number of photons which have to be collected are not as high as for measuring unknown lifetimes.

First investigations with coumarine and rhodamine dyes demonstrate the principle. A heterogeneous immunoassay is presented, showing the detection of different antigens by one measurement at one wavelength by time-resolved fluorescence spectroscopy.

EXPERIMENTAL

Carboxycoumarin or -rhodamine derivatives were activated by succinimido esters and coupled to the aliphatic amino functions of the lysine residues of the antibody in phosphate buffered saline (PBS) pH 8.

The coumarins 7-amino-4-methylcoumarin-3-acetic acid succinimidyl ester and 7-methoxycoumarin-3-carboxyclic acid succinimidyl ester were purchased from Molecular Probes. $6{,}85 \cdot 10^{-8}$ mol of activated dye was dissolved in DMF and linked to $2{,}7 \cdot 10^{-9}$ mol goat-anti-rat-antibodies, respectively, mouse-anti-rabbit-antibodies (Dianova) in borate-buffered saline (BBS) pH 8,5. Unbound dye was separated from conjugates by gelfiltration (Sephadex G-25) [8].

$4 \cdot 10^{-8}$ mol of the rosamines DR-317-succinimidylester and JA-65-succinimidylester were prepared from the corresponding carboxyclic acids with N,N,N',N'-tetramethyl-succinimido-uronium-tetrafluoroborat (TSTU) [9], and coupled to $2{,}7.10^{-9}$ mol goat-anti-rat-antibodies in BBS 8,5 [8]. Unbound dye was removed by gelfiltration (Sephadex G-25). To retain maximum binding specificity, only conjugates with a dye/protein ratio of approximately 2 (rosamines), respectively, 8 (coumarines) were used [10].

For the heterogenous immunoassay, antibodies were immobilized on quartz glass slides (suprasil) cut in 8 mm × 6 mm pieces. After the activation of the surface, primary amino groups were introduced by dipping the slides into a solution of 2% 3-aminopropyltriethoxy-silane in ethanol, followed by heat curing for 1 hr at 100°C. The silanized substrates were treated with a 2.5% solution of glutardialdehyde in PBS and subsequently washed three times. To immobilize the antibodies, the slides were dipped into aliquots containing 10 μg of the antibody in 1 ml PBS for 1 hr. After washing and blocking of the unoccupied positions with albumin bovine in TRIS buffer, the immobilized antibodies were incubated for 1 hr with antigens (in this case also antibodies in aliquots of 10 μg/ml PBS), linked with different fluorescent dyes. Excess of antigen was removed by repeated washing in PBS/Tween 20 [11,12].

All spectroscopic measurements were performed in borate buffer PBS pH 8. UV/VIS absorption spectra were recorded on a Lambda 7 UV/VIS spectrometer (Perkin Elmer) at room temperature. Corrected fluorescence spectra of the diluted solutions were measured with a LS 100 spectrometer (Photon Technology Instruments, PTI). The solutions were not degassed and optical densities for fluorescence measurements were kept strictly below 0.10 (1 cm cuvette). Radiative decay curves were registered by the time-correlated single photon counting technique using an instrument from Photon Technology International (PTI LS 100). All samples were excited in their respective long wavelength maximum. The excitation source was a hydrogen-filled flash lamp with a pulse of 1.7 ns FWMH. Fluorescence was observed at 20°C at the emission maxima. The goodness of the fit of the deconvoluted decay curves was controlled by the reduced chi-square statistical parameter. Most of the decays could be satisfactorily described by a mono- or biexponential model with $\chi < 1.2$.

RESULTS

To demonstrate the identification of biomolecules using multiplex dyes and time-resolved fluorescence spectroscopy, the antigen molecules were tagged directly with the fluorescent dyes. This approach differs from that in routine immunoassay techniques, where an additional tagged component is added to the analyte solution (competitive assay, sandwich assay).

The antigens were tagged with a fluorescent dye. In the work described here, the antigen mouse-anti-rabbit-IgG was labeled with 7-methoxycoumarin-4-acetic acid, whereas goat-anti-rat-IgG was labeled with 7-aminocoumarin-4-methyl-3-acetic acid. And the red dyes p-carboxyrhodamine and dicarboxyrhodamine, which consists of a mixture of different isomers. The structures of the coumarin and rhodamine dyes used are shown in Fig. 1.

As indicated in Table I, the coupling of 7-aminocoumarin-3-acetic acid (amca) to the antibody has no influence on the spectroscopic data of the dye. Independent of the environment for amca, a mono-exponential

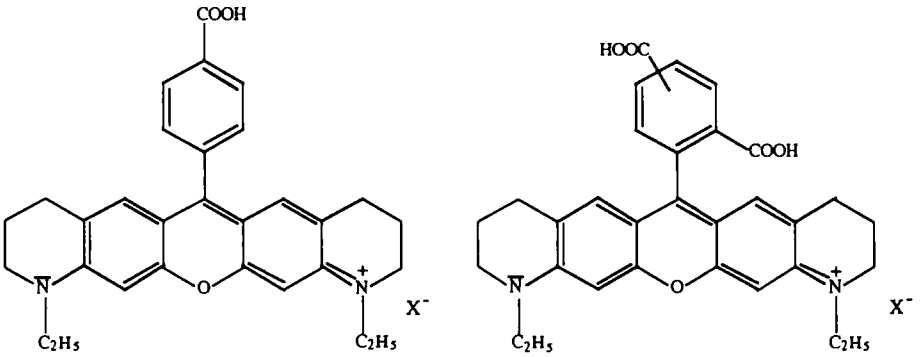

Fig. 1. Chemical structures of rosamine 5 (left) and rosamine 6 (right).

Table I. Spectral Data of the Used Coumarin Dyes 7-Amino-4-Methyl-Coumarin-3-Acetic-Acid (amca) and 7-Methoxycoumarin-3-Carboxylic Acid (mc) and the Coumarin-Antibody Conjugates

	λ_{abx} [nm]	λ_{em} [nm]	τ [ns]
amca	340	452	4,6
amca–antibody conjugate	340	449	4,6
mc	362	414	1,5
mc–antibody conjugate	348	408	1,9

Table II. Spectral Data of Rosamine 5 and 6 and the Rosamine-Antibody Conjugates

	λ_{abs} [nm]	λ_{em} [nm]	τ [ns]
Ros 5	564	597	2,91
Ros 5-ab	564	595	2,31
Ros 6	561	594	3,99
Ros 6-ab	565	599	4,00

fluorescence lifetime of 4.6 ns was determined. The absorption- and emission wavelength of the methoxy derivative 7-methoxycoumarin-3-acetic acid (mc) is slightly blue shifted due to linkage to the antibody. The fluorescence decay of the dye shows a second, longer component, which increases the observed average lifetime.

The spectroscopic data of the two coumarins in PBS pH 8.0 show that two dyes with nearly identical absorption wavelengths can easily be distinguished by their different fluorescence lifetimes. This example proves clearly the concept of "multiplex dyes" for application in biological analysis and demonstrates the feasibility of designing different multiplex dyes in each wavelength region, so that the concept of simultaneous detection of analytes in a mixture can be greatly extended.

In addition, two further dyes, rosamine 5 and rosamine 6 (see Fig. 1) were covalently linked via a succinimidyl ester to antibodies. As indicated in Table II nearly no influence, if any, on the fluorescence lifetime of rosamine 6 could be observed. Also, the spectral properties are more or less unaffected due to the covalent linkage. In comparison, rosamine 5 shows a decrease in fluorescence lifetime of 0.6 ns after coupling to the antibody. A small spectral shift could also be observed.

Multiplex Immunoassay

A heterogenous immunoassay is presented in Fig. 2 which shows that different antigens can be detected by one measurement at one wavelength by time-resolved fluorescence spectroscopy. To demonstrate how antigens can be identified simultaneously by fluorescence lifetime measurements, three experiments were performed: Donkey-anti-mouse-IgG was immobilized on a quartz surface; the added 7-methyl-coumarin-tagged mouse-anti-rabbit-IgG was recognized and linked at the binding sites of the immobilized antibodies. The fluorescence was detected by time-correlated single photon counting and the fluorescence lifetime was determined to be 1.8 ns; this is nearly equal to the fluorescence lifetime of the antibody-dye conjugate in solution (see Table I). The second immunoassay was performed in the same way, but the immobilized antibody was donkey-anti-goat-IgG-antibody and the identified antigen was 7-amino-coumarin-labeled goat-anti-rat-IgG. Here, the fluorescence lifetime increases to 5.0 ns on the surface compared with the fluorescence lifetime of the antigen-dye conjugate in solution.

Finally, both antibodies, donkey-anti-mouse-IgG and donkey-anti-goat-IgG were immobilized on the same

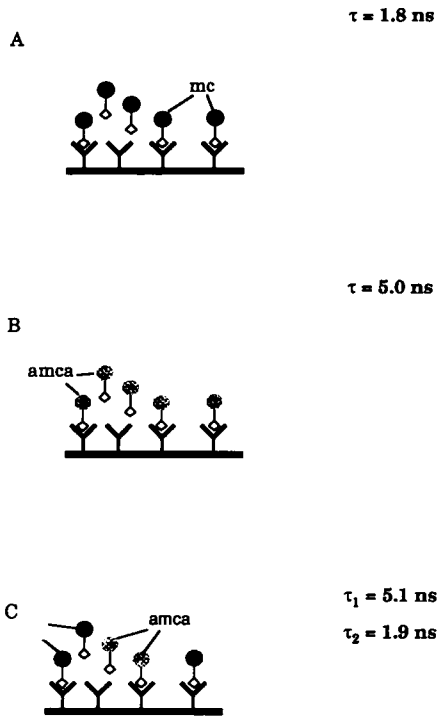

Fig. 2. Multiplex immunoassay: (A) mouse-anti-rabbit-IgG-7-mca-conjugate was linked to immobilized donkey-anti-mouse-IgG, (B) antigen–antibody reaction between goat-anti-rat-IgG-AMCA-conjugate and immobilized donkey-anti-goat-IgG-antibodies, (C) both antibodies were immobilized at the same surface and both tagged antigens were linked on the antibodies at the surface. Fluorescence lifetimes were detected at the surface using the lifetime spectrometer. Both antigens were identified by the fluorescence lifetime of the fluorescent tags.

surface. Subsequently both antigens, tagged with the two different coumarine dyes mentioned above were added to the solution and after incubation and washing, the slide was irradiated with the pulsed flash lamp. A biexponential decay with the components τ_1 = 5.1 ns and τ_2 = 1.9 ns was found. Hence, applying the multiplex dyes 7-methyl-coumarin and 7-amino-coumarin with different lifetimes but identical absorption wavelengths, the unequivocal identification, also in a mixture of two labeled antigens is realized. From the biexponential decay the two fluorescence lifetimes of the antibody-labeled coumarins amca and mc can be calculated and the antibody is identified.

DISCUSSION

It has been shown that the fluorescence lifetime of fluorescent dyes can be used as an intrinsic recognition parameter to identify dye molecules. The application of this principle has been successfully demonstrated with coumarin dyes as label molecules.

The present work points out two things: (a) The fluorescence lifetime is not changed so much that it cannot be recognized after binding to the antibodies used. The slight changes observed do not impair the multiplex immunoassay technique, if the differences in the fluorescence lifetime are large enough. This depends mainly on the time resolution of the spectrometer employed. In the experimental set-up described here, the dyes and therefore the antigen molecules could be identified easily. The use of a flashlamp was necessary, because no short pulse laser in this wavelength region was available in our laboratory. Recently, fluorescent dyes have been developed which can be excited above 600 nm, i.e., the fluorescence background of biological samples is reduced in this wavelength region. Furthermore, the fluorescence lifetime of these dyes are in the range from 1 to nearly 4 ns, so that they can easily be recognized by time-correlated single photon counting without an extremely expensive set-up. In addition, these dyes can be excited with diode lasers, also with pulsed ones, so that the system becomes even more compact. Presently, we are working on an all-solid-state fluorescence lifetime spectrometer, which includes a pulsed diode laser and a single-photon counting avalanche photodiode.

For application in routine diagnostics, further investigations are necessary to examine the effect of the matrix, e.g., a blood sample on the fluorescence lifetime of the multiplex dyes. Additionally, more multiplex dyes have to be designed, especially to work in the red wavelength region [13,14]. The combination of color information and fluorescence lifetime information allows a further increase of distinguishable tags, e.g., the use of three wavelength classes, each with three multiplex dyes with different fluorescence lifetimes, results in nine tags!

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