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COMPARISON OF ALEXA 488, DR110 AND FITC CONJUGATED TO ANTIBODY FOR MICROSCOPIC ASSAYS

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Abstract: *The fluorescent dyes DR110 and Alexa 488 were obtained. Synthetic fluorescent dyes that are conjugated to antibodies are useful tools in microscopic imaging. Alexa 488, DR110 and fluorescein 5(6)-isothiocyanate (FITC) were compared in applications using various conjugates with anti-sheep IgG antibody. Antibody-fluorescent dye conjugates with variety degree of labelling were obtained. Their fluorescence characteristics were observed by fluorescence spectrophotometer and fluorescence microscope. Brightness, photobleaching and background of the fluorescent conjugates were examined. Alexa 488 labeled antibody has brighter fluorescence and negligible photobleaching and background in microscopic assays, then DR110 and last FITC dye.*

Keywords: *Alexa 488, DR110, FITC, anti-sheep IgG antibody, fluorescent conjugates, microscopy.*

INTRODUCTION

During the last decades, fluorescent dyes and their conjugates are greatly applicable in histochemical and cytochemical research. Brightness and photostability are main characteristics in photostaining techniques (Mahmoudian et al., 2011). Unfortunately, the fluorescence emission even of dyes with high quantum yield, such as fluorescein and sulforhodamine, is considerably quenched on conjugation to biological molecules, particularly to proteins (Panchuk-Voloshina et al., 1999). Based on the chemical structure of dyes, their photostability and photobleaching profiles are very different. The cyanine dyes have brighter fluorescence and more photostability than fluorescein isothiocyanate (FITC) (Panchuk-Voloshina et al., 1999; Mahmoudin et al., 2011). It was interesting to obtain conjugates between these dyes with protein and to study which ones had brighter fluorescence and greater photostability. In the present study fluorescent dyes DR110 and Alexa 488 were obtained. Then the conjugates between Alexa 488, DR110 and FITC with immunoglobulin G (IgG) were prepared, compared and visualized by a fluorescence microscope.

EXPOSITION

Preparation of DR110 dye

A mixture of 3-aminophenol (0.25 g, 2.3 mM), mellitic anhydride (0.33 g, 1.3 mM) and sulfuric acid (1 mL) was homogenized and heated to 190°C for 12 h (Fig. 1). Distilled water (10 mL) was added to the reaction mixture and the obtained dark solid material was separated by

filtration. The solid material was extracted by acetonitrile (10 mL). The obtained orange solution was concentrated to a dry product. Then the dried product was dissolved in carbonate/bicarbonate buffer (250 mM, pH 8, 10 mL). The solution was acidified with hydrochloric acid. Red sediment was obtained and collected by centrifugation, then dried in a vacuum centrifuge. The red solid compound - DR110 was 5% yield (32 mg). The emission maximum of the DR110 solution in 40% acetonitrile in ammonium acetate buffer is 516 nm.

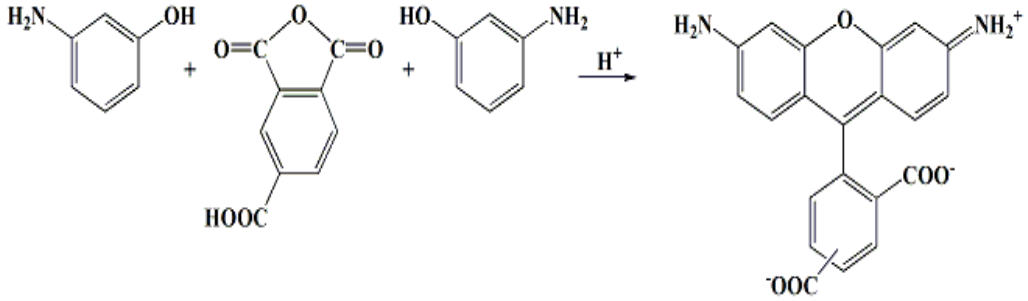

Fig. 1. Fluorescent dye DR110 preparation scheme

Preparation of Alexa 488 dye

The obtained DR110 (8.14 g, 19.8 mM) was added slowly to 30% oleum (50 mL) placed in an ice bath and was incubated 12 h at 0°C. Then the solution was poured in cold dioxane (600 mL) and diethyl ether (1 200 mL) was added. The suspension was filtered through diatomite. The filtrated cake over the diatomite was resuspended in methyl alcohol (1 200 mL) and pH was corrected to 10 with triethylamine. The mixture was filtered and the filtrate was evaporated. The concentrated filtrate was purified by Sephadex LH-20. The elution was performed with distilled water. Finally, the colored fractions were collected and dried. An orange solid compound - Alexa 488 (9 g) was obtained (Fig. 2).

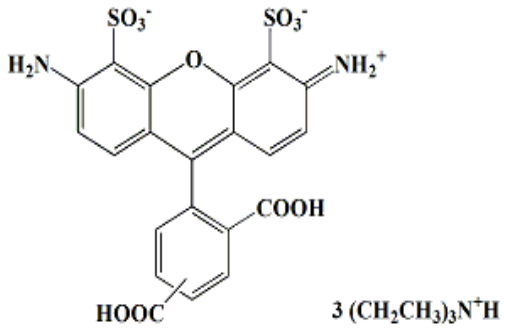

Fig. 2. Chemical formula of fluorescent dye Alexa 488

Esterification of Alexa 488 and DR110 dyes

First, the obtained dyes were dissolved in dimethylformamide (DMF). Then the carbodiimide activation method was performed by N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), purchased from Sigma Aldrich (Germany). Variety ratios of dye:NHS:EDC were used - 1.0:1.2:0.7; 1.0:1.8:1.0; 1.0:2.8:1.5; 1.0:5.5:3.0. The incubation time was 1 h, at room temperature (RT), on a shaker. The activation buffer was 10 mM phosphate buffer saline (PBS) pH 6.

Fluorescence conjugates - obtaining, purification, proving

All Alexa 488 and DR110 dyes were used in succinimidyl ester form for conjugation with the *primary amines of biomolecules. Anti-sheep IgG antibody* (*1 mg*) *was added to the* esterificated form of dyes (2 mg for both dyes). The conjugation buffer was 100 mM PBS pH 8. Incubation of the activated dye with antibody was made for 2 h at RT, or overnight at 4°C.

Conjugates of anti-sheep IgG antibody with FITC were made as described in our previous paper (Becheva et al., 2017). Different dye:antibody ratios were obtained by addition of variety concentrations of FITC (from 50 μg to 160 μg) to 1 mg of the antibody. The incubation for conjugation was performed for 2 h at RT, or overnight at 4°C.

Finally, all of the obtained conjugates were purified from unreacted compounds by size-exclusion chromatography using Sephadex G25 Medium column (1 x 22 cm), flow rate 0.5 mL/min, fraction volume 2 mL. All of the steps in conjugation procedures were performed protected from light.

The obtained conjugates were proved by UV-Vis and fluorescence spectrophotometric measurements (6900 UV/Vis spectrophotometer Jenway and Perkin Elmer LS45 fluorescence spectrophotometer). Conjugate fractions were concentrated by VivaSpin 500 (5 000 MWCO).

Conjugate emission characterisctics and microscopic assay of conjugate stained cells

Protein concentrations of the obtained and concentrated fluorescent conjugates were determined by Bradford method (Bradford, 1976). Emission of the conjugates (30 μg/mL in 10 mM PBS pH 7.4) was compared by Perkin Elmer LS45 fluorescence spectrophotometer, 750 V, 491 nm excitation, 518 nm emission.

Microscopic assay was performed with bovine blood neutrophil cells and anti-neutrophil antibody obtained as described in our previous paper (Becheva et al., 2017). Neutrophils were suspended in balanced salt solution to concentration 7 x 10^6 cell/mL. Then, 50 μg of anti-neutrophil antibody was added and the suspension was gently stirred and incubated for 30 min at 37°C in a shaker. The samples were centrifuged at 3 000 rpm for 10 min, for antibody excess removal. Six samples with different conjugates and different degree of labeling (DL) were prepared: Anti-sheep IgG antibody - Alexa 488, DL 1.4; Anti-sheep IgG antibody - Alexa 488, DL 3.0; Anti-sheep IgG antibody - DR110, DL 1.65; Anti-sheep IgG antibody - DR110, DL 2.75; Anti-sheep IgG antibody - FITC, DL 3.35; Anti-sheep IgG antibody - FITC, DL 7.65. Conjugates (50 μL) were added to the cell suspensions with bound primary antibody. The conjugate concentration was 30 μg/mL in 10 mM PBS pH 7.4, and the incubation was 30 min at 37°C in a shaker.

The images were viewed with a fluorescence microscope Olympus BX51, 470 nm laser, magnification x 100 and QImaging Retiga 2000R camera. The main fluorescent conjugate characteristics for microscopic assays were examined. Brightness and background of the conjugates was compared at same gain and exposition. Photobleaching of the conjugated fluorescent dyes was observed in 1st min, 4th min and 9th min. All of the experiments were performed by fluorescence microscopic images and comparison of the obtained photos.

Results and discussions

The cyanine dye DR110 was prepared as described above. Then, the Alexa 488 dye was prepared by sulfonation of DR110. Sulfonation decreases the inherent tendency of molecules to form aggregates, presumably due to the increased polarity imparted by the sulfonic acid moiety (Mujumdar et al., 1993). The synthezised dyes were esterificated to obtain active dye forms for conjugation to antibodies IgG type. For comparison, the commercial dye - FITC was used for preparation of conjugates with antibodies IgG type. Fluorescent conjugates with different DL were obtained by variation of activation reagents (dye**:**NHS:EDC**)**, as well as variation of conjugation time (2h, overnight) and temperature (RT, 4°C**),** (Table 1). Degree of labeling (DL) was determined as the ratio of moles of fluorophore to moles of protein as described by Haugland (1995). Different DL values of the conjugates suggested different fluorescence characteristics. A graphic presenting correlation between fluorescence intensity and DL of the obtained conjugates is shown in Figure 3. The optimal DL for Alexa 488 conjugates was 1.4, for DR110 conjugates was 1.65, and for FITC conjugate was 3.35.

Table 1. Variation of activation reagents, as well as conjugation time and temperature, for preparation of conjugates with different DL

Dye:NHS:EDC	DL of Alexa 488 conjugates (2h, RT)	DL of DR110 conjugates (2h, RT)	DL of Alexa 488 conjugates (overnight, 4°C)	DL of DR110 conjugates (overnight, 4°C)
1.0:1.2:0.7	0.01	0.09	0.40	0.70
1.0:1.8:1.0	0.46	1.20	1.30	1.55
1.0:2.8:1.5	1.20	1.30	2.30	2.00
1.0:5.5:3.0	1.40	1.65	3.00	2.75

The curves of fluorescence intensity vs DL typically show a peak and then quenchering of the signal after reaching the optimal DL. However, DR110 and Alexa 488 conjugates retain their high emission even at higher moles of dye per moles of antibody (after reaching the optimal DL). Where as the fluorescence of FITC conjugates quenched after optimal DL. Consequently, the preferred conjugates for microscopic assay are that with the optimal and the highest DL.

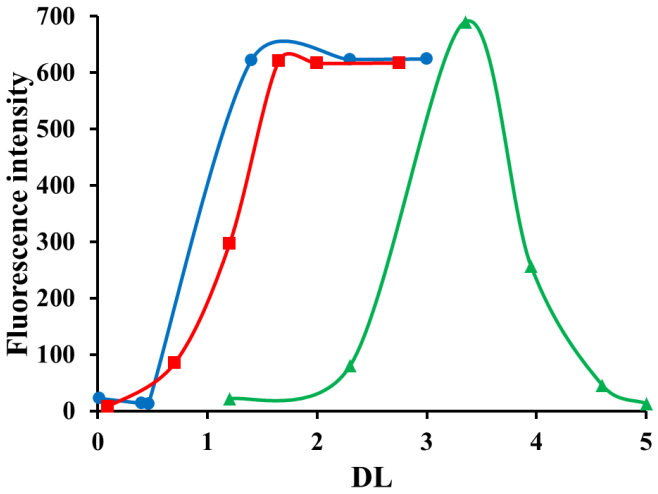

Fig. 3. The relationship between fluorescence intensity and DL of anti-sheep IgG antibody - Alexa 488 (●), anti-sheep IgG antibody - DR110 (■) and anti-sheep IgG antibody - FITC (▲).

Microscopic assays of the neutrophil cells stained with these conjugates were performed in same conditions, for obtaining of comparable results. Indirect cell staining is an often used technique in the laboratories. In our study, anti-neutrophil antibody, produced in a sheep, was used as a primary antibody for bovine neutrophils.

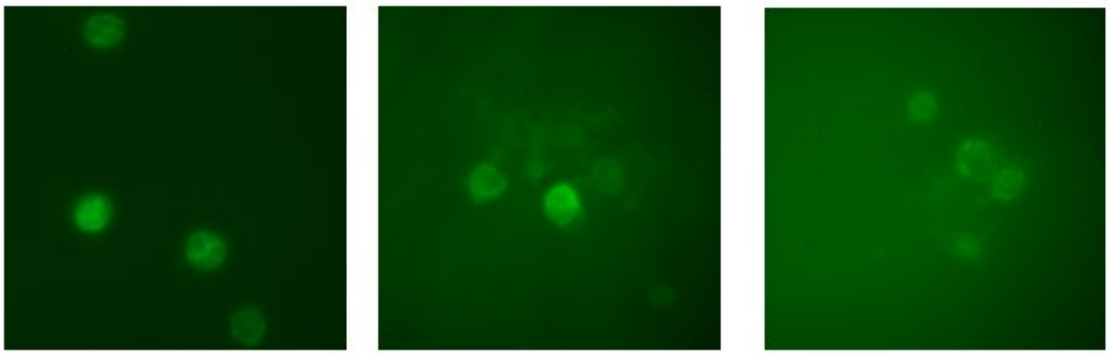

Fig. 4. Fluorescence microscopic assay of bovine neutrophils with anti-sheep IgG antibody - fluorescent dye conjugates, at optimal DL.

After that, the obtained fluorophore labeled secondary antibody was added (conjugates of anti-sheep IgG antibody with Alexa 488, DR110 and FITC with different DL). Images of the stained cells with the obtained anti-sheep IgG antibody conjugates with Alexa 488, DR110 and FITC with optimal DL, are shown in Figure 4. Table 2 presents the results from characteristics of the obtained fluorescent conjugates IgG-fluorescent dye. It was evident that the conjugate IgG-Alexa 488 has the highes brightness and has not background. The photobleaching of obtained fluorescent conjugates was investigated for 10 min. Anti-sheep IgG antibody - Alexa 488 and anti-sheep IgG antibody - DR110 are shown slightly photobleaching in 9 min, but anti-sheep IgG antibody - FITC has a visible photobleaching in 4 min (Table 2).

Table 2. Comparison of brightness, photobleaching and background of fluorescent conjugates of anti-sheep IgG antibody conjugated with Alexa 488, DR110 and FITC

Conjugate type	DL	Brightness	Photobleaching	Background
Anti-sheep IgG antibody - Alexa 488	1.4	high	Slightly in 9 min	No
Anti-sheep IgG antibody - Alexa 488	3.0	high	Slightly in 9 min	No
Anti-sheep IgG antibody - DR110	1.65	medium	Slightly in 9 min	No
Anti-sheep IgG antibody - DR110	2.75	high	Slightly in 9 min	No
Anti-sheep IgG antibody - FITC	3.35	medium	Visible in 4 min	Visible
Anti-sheep IgG antibody - FITC	7.65	medium	Visible in 4 min	Visible

CONCLUSION

Comparison of fluorescence characteristics of conjugates obtained with Alexa 488, DR110 and FITC was made. Alexa labeled antibody has brighter fluorescence and negligible photobleaching and background in microscopic assays than the other two dyes. Furthermore, Alexa and DR110 offers constant emission signal even after reaching the optimal DL, unlike FITC. Consequently, Alexa 488 is a good choice of fluorescent conjugate obtaining intended for microscopic analyses.

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