



## Ratiometric Alcohol Sensor based on a Polymeric Nile Blue

Nirmala Chandrasekharan, Sherif Ibrahim, Yordan Kostov\*,  
Govind Rao

Center for Advanced Sensor Technology, Department of Chemical and Biochemical  
Engineering University of Maryland, Baltimore County, 1000 Hilltop Circle,  
Baltimore, MD

E-mail: [kostov@umbc.edu](mailto:kostov@umbc.edu), [grao@umbc.edu](mailto:grao@umbc.edu)

\* Corresponding author

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**Abstract:** We present a sterilizable ratiometric fluorescent ethanol sensor with sensitivity over a wide range (0-100%) of ethanol concentration v/v. The sensor is composed of a near infra red fluorescent solvatochromic dye, Nile Blue methacrylamide polymerized into a polyethylene (glycol) dimethacrylate matrix. The dye can typically exhibit two or more wavelength dependent shifts in the fluorescence intensities based on its different micropolar environments. Two different concentrations of the Nile Blue methacrylamide dye were prepared and polymerized into homogenous films. The fluorescence properties of the two different films were investigated with a view to determining their ethanol sensing capabilities. The sensor was immersed in a water-ethanol solvent mixture. Excitation of the dye was performed at 470 nm. The range of emission wavelengths was 480-800 nm. The ratio of the fluorescence intensities at 620 nm and 554 nm was obtained for ethanol concentrations varying from 0-100% and the calibration curve of the ratiometric fluorescence intensities over the entire concentration range of ethanol was plotted. A ratiometric intensity change of over 33% has been obtained for pure ethanol compared to that obtained for pure water. The sensor response was rapid ( $\leq 10$  minutes). The sterilizable ethanol sensor exhibits good potential for on-line monitoring of the ethanol generated in an LB fermentation chamber.

**Keywords:** Alcohol sensing, Optical measurements, Fluorescence, Ratiometric sensor.

### Introduction

The rising cost of the crude oil sparked again research in the alternative energy sources. As a result, the use of ethanol as a fuel, fuel additive or in fuel cells [1] has received renewed interest. Ethanol is also a useful solvent and is utilized in the production of perfumes, paints, lacquers and explosives. It is also produced via fermentation in the manufacturing of alcoholic beverages such as beer and wine. It is also of great importance in clinical, industrial and biochemical areas. With numerous industries that utilize or produce ethanol, it is apparent that reliable methods are needed for its measurement and control.

Many analytical methods have been developed during the years for the measurement of ethanol and other alcohols. Primarily, these methods include chromatographic, enzymatic or/and optical. The chromatographic method is the most accurate and sensitive [6, 8] with a lower limit of ethanol detection in the order of 0.005% v/v [17]. The downside of this method is the necessity for sample pretreatment, long operation time and high costs. Somewhat less precise but more rapid measurements are achieved by the use of enzymes. Determination of ethanol concentration is based on either of two enzymes, alcohol oxidase (AOX) or alcohol dehydrogenase (ADH), by monitoring  $O_2$  consumption or  $H_2O_2$  formation [2, 5]. The specificity of the enzyme binding sites provides highly selective and accurate sensors. The

disadvantage of the sensor lies in the instability due to protein denaturing when exposed to high temperature, low or high pH, pressure and other external factors. In recent years, attempts have been made to place the “sensing” enzymes in close proximity to the transducer, an electrode [7] or to be immobilized in a matrix [3, 16] in order to increase the stability of the enzymes and to allow for continuous monitoring. Another approach has been the use of screen-printing technology for the mass-production of low cost disposable sensors [5]. However, the most simple and wide spread approach to the determination of alcohols is an optical method. It has the advantages of low-cost manufacturability, safety and miniaturization. Optical sensors are intended to be used for real-time, in-situ monitoring of alcohols. Recently, life-time based [10] and fluorescence-based [4, 11, 12, 14] alcohol sensors have been introduced utilizing different alcohol sensitive dyes. Though extremely promising, these sensors suffer from dye-leaching, cross-sensitivity to pH and low specificity. They also lack high temperature stability and are subject to interference due to autofluorescence.

This paper presents an autoclavable ratiometric optical alcohol sensor that operates in the red-infrared region of the visible spectrum. It was synthesized by using Nile Blue methacrylamide (NBA) a dye with an absorption maximum at ~663 nm in aqueous medium. The absorption maximum of the dye is well situated to prevent any possible interference from most organic solvents and water which show absorption bands in the region between 200 to 400 nm. Leaching behavior exhibited by the pristine dye was circumvented by immobilization of the derivatized, Nile Blue methacrylamide in a polymer matrix of polymethyl di methacrylate. Immobilization of the dye has the combined advantage of high temperature and pressure resistance, as well as high hydrophilicity. The sensor can thus withstand autoclaving and has relatively short response time in the range of minutes. The ratiometric approach was chosen for its advantages over the conventional steady-state methods based on fluorescence intensity [9, 13].

## Experimental section

### *Materials*

Nile Blue chloride, methacrylic acid, DP, polyethylene (glycol) dimethacrylate (average molecular weight 1000) (PEG), 1,3-diisopropylcarbodiimide and N-methylmorpholine were purchased from Aldrich and used without further purification. Anhydrous methanol was purchased from Fisher and Darocur was purchased from Polyinc.

### *Synthesis of Nile Blue N-Methacrylamide*

Nile Blue methacrylamide (NBA) was prepared by employing a procedure reported in the literature [15]. The compound was obtained by reacting NB with MAA in methanol using 1,3-diisopropylcarbodiimide under nitrogen atmosphere and in the presence of a small amount of N-methylmorpholine as catalyst. The sample was characterized using  $^1\text{H}$  NMR and FTIR.

### *Synthesis of the Nile Blue methacrylamide anchored sensor film*

**Preparation of a film containing  $0.0197 \text{ gmL}^{-1}$  of the sensor dye (HC film)** 8.2 mg of Nile Blue methacrylamide was added to 320 mg of polyethylene glycol dimethacrylate and the mixture dissolved in 280  $\mu\text{L}$  of 70% ethanol and 120  $\mu\text{L}$  of water. 16  $\mu\text{L}$  of the photoinitiator, Darocur was then added and the solution was vortexed for 15 minutes in order to blend the mixture completely. The solution was further deaerated by bubbling nitrogen gas for 20 minutes. The solution was then spread over a nylon mesh cut to size and placed on a glass slide 2” wide and 0.004” thick. The thickness of the slide was controlled using aluminium spacer tape. Photopolymerization of the methacrylate groups was carried out by exposure to UV light for

20 minutes. The polymer along with the nylon mesh was then peeled off from the glass plate and washed first in water to hydrate and later immersed in 200 proof absolute ethanol. Unreacted Nile blue leached out of the film into the surrounding solvent. The film was soaked in ethanol until no further leaching was observed.

**Preparation of a film containing  $0.0049 \text{ gmL}^{-1}$  of the sensor dye(LC film)** 5.1 mg of Nile blue methacrylamide was added to 960 mg of poly (ethylene glycol) dimethacrylate and the mixture dissolved in 1000  $\mu\text{l}$  of 70% ethanol. 48  $\mu\text{l}$  of the photoinitiator, darocur was then added and the solution was vortexed for 15 minutes in order to blend the mixture completely. The solution was then deaerated using flowing nitrogen and the sensor film was prepared, as mentioned in detail in the foregoing paragraph.

The two different HC and LC films were subsequently used for fluorescence measurements.

### Absorbance measurements

Absorbance measurements were performed using a commercial spectrometer from Agilent Technologies.

### Fluorescence measurements

In order to perform ratiometric fluorescence measurements, a conventional commercial fluorimeter was used. The excitation source was a xenon lamp with a power output of 180 watts. The scan rate was 600 nm/min with signal averaging time of 0.1 second. Excitation and emission slits were both set to 5 nm. The sensor film was cut to dimensions of 2 cm length and 0.8 cm width and placed in a glass cuvette containing an alcohol-water mixture. Following the excitation of the sensor film at 470 nm, the fluorescence signal intensity was collected over a wavelength range of 480-800 nm, for various percentages of alcohol, ranging from 0-100% v/v.

## Results and discussions

The sensor employs a solvatochromic dye, Nile blue methacrylamide that is immobilized in a hydrogel, poly(ethylene glycol) dimethacrylate with a molecular weight of 1000. The structure of the monomeric dye is shown in Fig. 1.

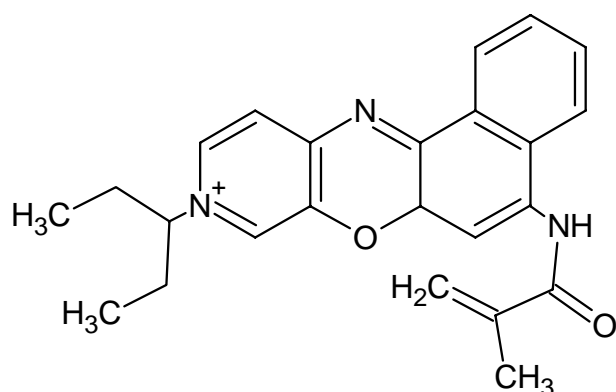


Fig. 1 Nile blue methacrylamide

On immersing the sensor in a solution containing alcohol as one of the solvents, and irradiating it with the appropriate excitation wavelength in the visible, the resulting absorption event causes the molecules of the dye to be distributed among various excited states. The excited states vary in energy due to the variation in the polar environment around the dye molecules. Subsequent relaxation by the excited molecules from the different excited energy states to the ground state via fluorescence can be easily detected as shifts in the emission wavelength of the emission

intensity. The ratio of the emission intensities at two wavelengths that characterize two different polar microenvironments around the dye molecule is directly proportional to the concentration of alcohol in the solution.

Fig. 2 is a snapshot of two different HC films in water and absolute alcohol respectively. The blue colored film is an HC film immersed in water and the pink colored film is an HC film immersed in absolute alcohol. It is immediately obvious from the color of the sensor in the two different media that the absorbance of the film in water is redshifted as compared to that of the film in absolute ethanol. It is important to note that Nile blue methacrylamide does not exhibit such a color change when not polymerized.

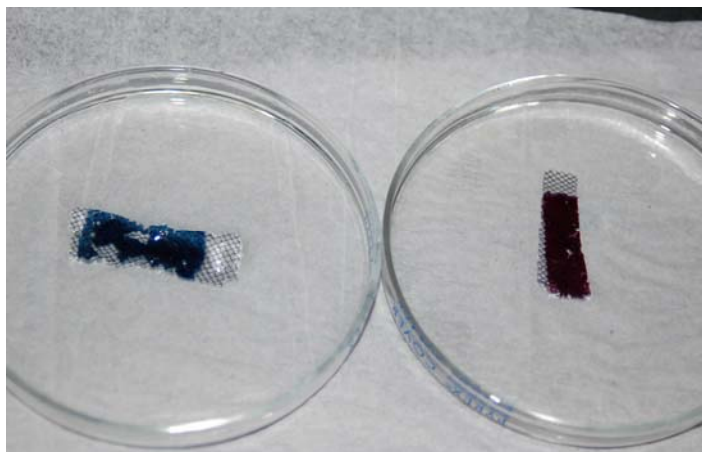


Fig. 2 Snapshot of the sensor film with a concentration of 0.0049 g/ml of the dye immersed. The film is colored blue in water and it is pink in absolute alcohol.

at 663.6 nm for the LC film is 1.5 times more intense than the corresponding peak of the HC film. It also exhibits a narrower spectral distribution and correspondingly less scatter in the near range. The corresponding absorbance spectrum of the LC film in pure ethanol is shown as a grey solid line. It has a peak at 517.1 nm and a shoulder at 654.3 nm. It is clear from the absorbance spectra that a sensor based on absorbance is a practical consideration for detection of 0% and 100% ethanol. However, it was found that for intermediate ranges the sensitivity was poor since the resolution was within the instrumental short noise.

Fig. 3 shows the absorbance spectra of an HC and an LC film, each film measured in two different media, water and absolute ethanol. The dashed black line is the absorbance spectrum of the HC Nile blue polymethacrylamide film in water. It exhibits a peak at 662.3 nm and a broad shoulder at 614.4 nm. The grey dashed line is the absorbance spectrum of the HC film in absolute ethanol. It exhibits a broad peak with a maximum at 523.8 nm. The solid black line is the absorbance spectrum of the LC film in water. It has a peak at 663.6 nm and a shoulder at 614.4 nm. The intensity of the peak at 663.6 nm for the LC film is 1.5 times more intense than the corresponding peak of the HC film.

Fig. 4 shows the fluorescence spectra of HC film over a range of ethanol concentration. The film was first immersed in water. The excitation wavelength was fixed at 470 nm. The fluorescence emission was scanned over a wavelength range of 500-800 nm. The fluorescence spectrum in water is shown as a solid black line. The spectrum exhibits three distinct peaks in water, at 688 nm, 630 nm and at 554 nm. As Nile blue polymethacrylamide is a solvatochromic dye, it is reasonable to assume that the polymerized dye has essentially three distinct and differing micro polar environments within the film. The most polar being the region which exhibits emission at 688 nm in water and the least polar environment being the region which exhibits an emission peak at 554 nm in water. As the percentage concentration of ethanol in water is increased, the fluorescence peak at 554 nm remains essentially unperturbed whereas, the peaks at 630 nm and 688 nm display a gradual increase in intensity. It is also interesting to observe that the peak at 630 nm remains unshifted for concentrations of ethanol  $\leq 20\%$  v/v. In 25% and 50% v/v ethanol the peak is shifted to  $\sim 628$  nm and in 75% ethanol, it is 625.9 nm. The peak is blue shifted greatly to a value of 618.7 nm in absolute alcohol, a difference of 11.3 nm as compared to that of water. In pure ethanol the peak at 554 nm disappears and the peak at 688 nm is barely visible as a shoulder. The film exhibits a 5.6 fold increase in fluorescence intensity at 620 nm when compared to the same film when immersed in water. It is also immediately obvious that the intensity of the emission at 620 nm increases exponentially as the concentration of the percent ethanol increases from 25-100%.

Studies in this laboratory have shown that pristine Nile blue chloride when embedded in PEG exhibits fluorescence peaks at 700 nm and 630 nm, similar to that observed in NBA (at 630 nm and 688 nm). Therefore, it would be reasonable to conclude that the origin of these two peaks is similar in the two different dyes. Studies have also shown that for concentrated solutions of NB in PEG the emission peak at 700 nm was dominant. For diluted NB the peak at 630 nm was dominant [15]. Since, NB and NBA are sparingly soluble in water but easily dissolve in ethanol, the peak at 700 nm has been assigned to dye aggregates which predominate in the more polar aqueous medium. Increasing concentrations of ethanol in water, allows for more of the dye monomer to form. It is possible that the fluorescence peak at 554 nm is also a contribution from aggregates but with a different orientation. Since the Nile blue methacrylamide dye has a dipole moment, it can orient either head-to-tail (J aggregates) or in a parallel form (H aggregates).

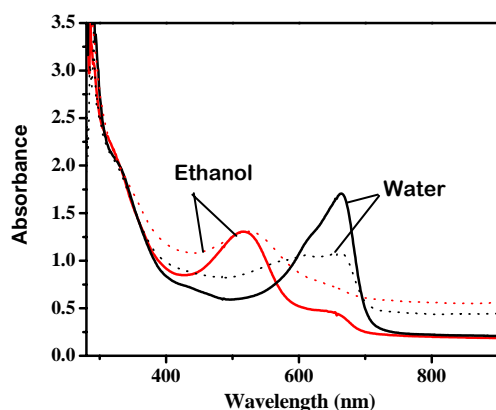


Fig. 3 Absorbance spectra of polymethacrylamide films with different dye concentrations. Solid lines – LC film, dashed lines – HC film.

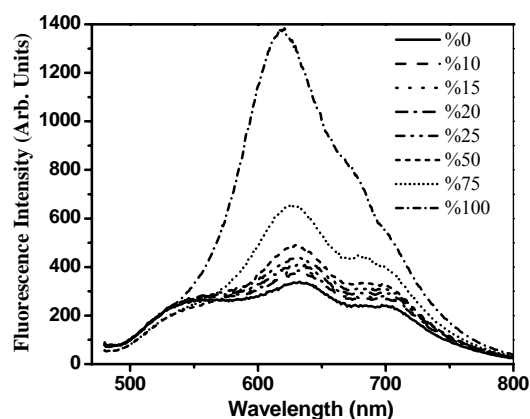


Fig. 4 Fluorescence spectra of Nile blue polymethacrylamide HC film at different ethanol concentrations. Excitation – 470 nm.

Fig. 5 shows the wavelength dependent fluorescence intensity of the LC film in varying concentrations of ethanol. The LC film has an emission spectral profile quite different from that of the HC film. The solid black line shows the fluorescence spectrum of the film in water. It exhibits an intense broad peak at 554 nm and a less intense peak at 710 nm. The fluorescence peak at 630 nm exhibited by the HC film is absent in the water immersed LC film. Therefore, it is reasonable to conclude that the LC film essentially has two different micropolar chemical environments that display wavelength dependent shifts in their spectral profile when immersed in water. However, as the percentage of ethanol in the water medium is increased, there is a gradual evolution of a new band at ~620 nm which is seen as an increase in the fluorescence intensity between 575-675 nm as the concentration the volume percent of ethanol is increased from 0-20%. As the concentration of ethanol is increased from 20% to 100%, there is the evolution of a well defined peak at blue shifted to 591 nm. In absolute ethanol, the peak maximum is at 614.4 nm, and the LC film has emission intensity at 620 nm which is ~6.3 times that of the LC film in pure water. In the case of the LC film, it is interesting to note that in water, the aggregates predominate and the peak at 630 nm is virtually absent. On increasing the concentration of ethanol the monomers of the dye gradually increase, until in absolute ethanol the monomers of the dye predominate with a small concentration of the J aggregates represented by the shoulder at 710 nm. The emission spectral profile of both the HC and LC films are very similar in absolute ethanol.

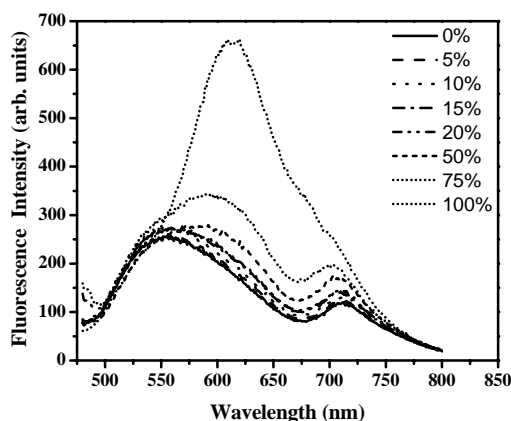


Fig. 5 Fluorescence spectra of Nile blue polymethacrylamide LC film at different ethanol concentrations. Excitation – 470 nm.

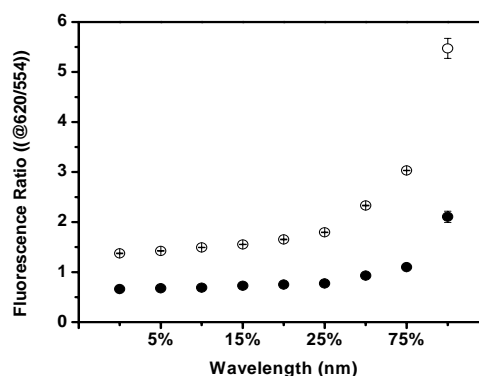


Fig. 6 Film calibration (emission ratio 620/554 nm) for different concentration of the immobilized dye. Solid circles – LC film, open circles – HC film.

Fig. 6 shows the ratiometric fluorescence intensities of the HC and LC films at 620 nm and 554 nm for the range of ethanol concentration from 0-100%. The ratio of the emission intensities at the two wavelengths is directly proportional to the concentration of alcohol in the solution. The ratiometric fluorescence sensing method circumvents the drawbacks that are associated with conventional intensity based detection; that is, it is independent of sensor orientation and position, coloring of the solution medium, concentration and thickness of the dye in the sensor film, background fluorescence signal and fluctuations in excitation source intensity or temperature. The ratiometric intensities are linear in the concentration range of 0-25% of ethanol v/v and exponential in the concentration range of 25-100% ethanol v/v for both the HC and the LC films. In order to determine the efficacy of the films as sensors for quantitative determination of ethanol in fermentation processes, the sensitivity of the ratiometric fluorescence intensities of the films were tested for a concentration range of 0-5%.

Fig. 7 shows the ratiometric fluorescence intensities at 620 nm and 554 nm for the HC film for a concentration range of ethanol, 0-5%. The square symbols represent the sensitivity plot for a pristine HC sample whereas, the triangles represent the same HC sample, after being autoclaved. The autoclaved sample does not differ significantly in sensitivity as compared to that of the pristine sample. Hence it can be concluded that the sensor films in general can be autoclaved (an important prerequisite before being introduced in the fermentation vat) without any significant loss in sensitivity. In order to observe the effect of aging, the sensitivity of the ratiometric fluorescence intensity of a representative LC film was studied as a function of ethanol concentration.

Fig. 8 shows the sensitivity plot of an LC film, 3 days after synthesis (seen as solid circles). The triangles represent the sensitivity of the same film after 20 days (subsequent to the initial measurement). As can be seen, the sensitivity of the film is considerably enhanced after 20 days of aging (numbers). The fluorescence plot of the LC after 20 days of aging is seen in Fig. 9. The general emissive characteristics of the LC film (described above) are preserved after aging. However, the fluorescence intensity of the peak exhibited at 620 nm in water, has increased several folds for e.g. for a percentage increase of ethanol from 15% to 20%, the emission at 620 nm increases 5 folds after 20 days as compared to the initial value.

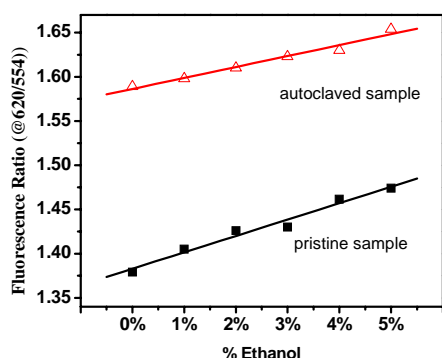


Fig. 7 Ratiometric fluorescence intensities ratioed at 620 nm and 554 nm as a function of ethanol concentration v/v for a HC sensor film

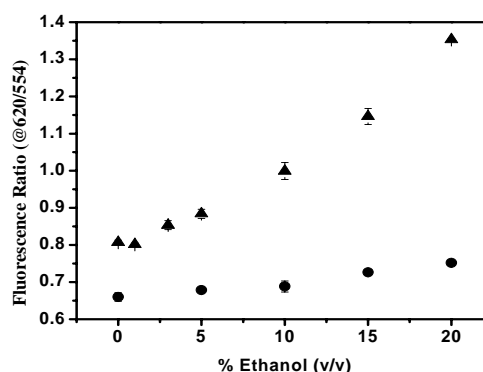


Fig. 8 Ratiometric fluorescence intensities ratioed at 620 nm and 554 nm as a function of ethanol concentration v/v for LC sensor film. Circles: film 3 days after synthesis, triangles – same film after 20 days of aging.

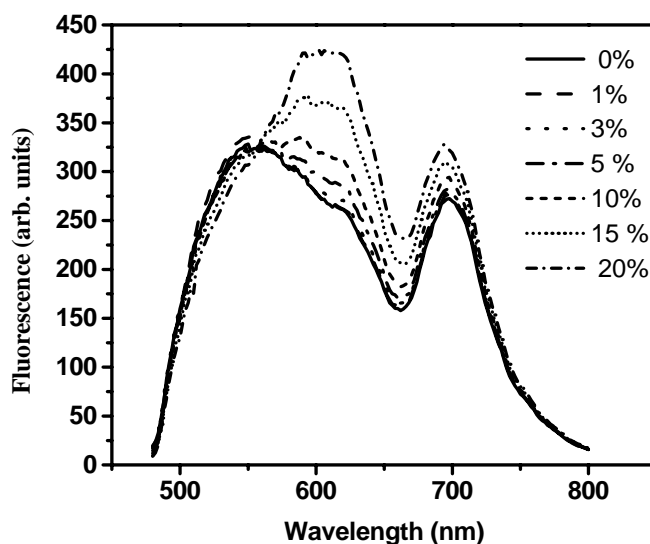


Fig. 9 The fluorescence intensities of the sensor film with a concentration of 0.0049 g/ml of the dye for a range of ethanol concentrations v/v (0-20%) after 23 days of aging subsequent to synthesis

### Conclusion

An ethanol ratiometric sensor has been developed by immobilization of the red region dye Nile Blue methacrylamide in poly(ethylene glycol) dimethacrylate. It is sensitive to a broad range of ethanol concentration, from 0-100% v/v. Two different concentrations of the dye have been investigated for sensitivity to ethanol concentrations. The film with dye concentration of 0.0049 g/ml, exhibits good sensitivity in the concentration range 0-20% ethanol v/v. It is thus a good potential candidate as a sensor in fermentation processes.

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**Yordan Kostov, Res. Assoc. Prof., Ph.D.**E-mail: [kostov@umbc.edu](mailto:kostov@umbc.edu)

Dr. Yordan Kostov received his B.Sc. and M.Sc. with honors in 1987 from Department of Electrical Engineering, Odessa Polytechnic Institute, Ukraine. He received a Ph.D. Degree in Engineering from Bulgarian Academy of Sciences for his work in the area of optical chemical sensors and biosensors. After that, Dr. Kostov was post-doctoral fellow at the Institute for Technical Chemistry, University of Hannover, Germany. In 1994 he accepted a position as an Assistant Professor in the Department of Biotechnics, Sofia Technical University, Bulgaria. In 1999 he became a post-doctoral fellow at University of Maryland Biotechnology Institute, at the Medical Biotechnology Center. Since 2000, he is a Research Assistant Professor, and since 2006 – a Research Associate Professor at the Department of Chemical and Biochemical Engineering, University of Maryland, Baltimore County (UMBC). Dr. Kostov also holds a position as an Assistant Director of the Center for Advanced Sensor Technology at the same university. He is involved in the development of chemical and biochemical sensors for biotechnology and biomedical applications.

**Govind Rao, Prof., Ph.D.**E-mail: [grao@umbc.edu](mailto:grao@umbc.edu)

Dr. Govind Rao received B.Sc. from Indian institute of Technology in Madras, India and Ph.D. degree from Drexel University in the field of Biotechnology. Since 1987 he is a professor at the University of Maryland, Baltimore County. In 1991, he was recipient of the prestigious Presidential Young Investigator Award. In 1993, he was visiting professor at Facultes Universitaires Notre-Dame De La Paix, Namur, Belgium. In 2000-2006 Dr. Rao was Chair of the Department of Chemical and Biochemical Engineering at UMBC. He has been also in the editorial boards of Biotechnology Letters, Biotechnology Techniques, Journal of Industrial Microbiology and Biotechnology and Bioengineering. Currently, he is the Director of the Center for Advanced Sensor Technology at UMBC. His interests are in the field of high-throughput bioprocessing as well as bioprocessing methods and devices.