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Studies on visual detection and surface modification testing of glass microfiber filter paper based biosensor

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ABSTRACT

Glass microfibers are commonly used as biomolecule adsorption media, as structural or disposable components of the optical biosensors. While any improvement in these components are appreciated, utilizing basic tools of traditional approaches may lead to original sensor opportunities as simple, functional designs that can be easily disseminated. Following this pursuit, surface modification of glass microfiber paper surface was performed by 3-aminopropyltriethoxysilane (APTES) and resulting improvement in the cell entrapment capacity could be observed visually, only after Gram staining. Gram staining offered rapid validation of enhanced binding on the glass surface. The same APTES-modified samples were also tested for binding of complementary DNA sequences and the results were less straightforward due to the necessity of DNA visualization by using a fluorescent stain, YOYO-1. Accordingly, when there were no surface modification, DNA and YOYO-1 adsorbed readily on the glass microfiber filter paper, and prolonged the interaction between DNA and YOYO-1. YOYO-1 adsorption on glass could be recognized from the color profile of YOYO-1 emission. This phenomenon can be used to examine suitability of APTES coverage on glass surfaces since YOYO-1 emission can be distinguished by its glass adsorbed *versus* DNA-bound forms. Aptness of surface coverage is vital to biosensor studies in the sense that it is preceding the forthcoming surface modifications and its precision is imperative for attaining the anticipated interaction kinetics of the surface-immobilized species. The proposed testing scheme offered in this study secures the work, which is aimed to be carried out utilizing such sensing systems and device components.

1. Introduction

Glass is a substantial medium for optical measurements and sensor applications for being a transparent medium. It can be implemented in discrete grounds, together with a number of its morphological sorts like beads, porous membranes, cover slides, and filters. Likewise, one of these sorts, the glass fiber paper, is employed in diverse fields, including electro-blotting (Aebersold et al., 1986) as an example. Besides, it is engaged in sampling or filtration of the media in gas analysis applications (Bartman et al., 1995). It is even reported as an appropriate material for separating plasma from blood (Milunic and Russell, 2006), in the form of composites. Its surface properties, modification, activation and analysis are valuable, with this regard.

The 3-aminopropyltriethoxysilane (APTES) treatment is a common method for glass surface modification, which dates back to 1969 (Messing et al., 1969; Weetal, 1969a, 1969b). The protocol results in amine ending surfaces that can further be adapted for biomolecule attachment on a variety of glass media such as porous glass (Messing et al., 1969; Weetal, 1969a, 1969b; Robinson et al., 1971), glass beads (Wachter et al., 1973) and cover slides (Qın et al., 2007). Within the frame of the presented work, glass microfiber paper was chemically modified by using APTES, for increasing the efficiency of yeast cells' (*Saccharomyces cerevisiae*) entrapment within the meshwork of glass microfibers.

The use of glass fiber paper as cell immobilization media in biosensor studies was recognized earlier. Kumar et al. (2006)) reported to use the *Flavobacterium* ssp. whole cells-adsorbed on unmodified glass fiber samples as disposable elements. The set-up was established as an optical microbial sensor that was utilizing a miniature optical fiber spectrophotometer, as the transducer element (Kumar et al., 2006). Yet, the contribution of surface modification is indispensable. It is normally performed exclusively for the molecule of interest that needs to be captured. No such surface modification was considered in the mentioned work. Conversely, in this work, a costly transducer was not used to prove the amendment, which relied on the boosted cell entrapment. Instead, detection of improved cell entrapment was achieved merely by applying conventional Gram staining (Basu and Biswas, 1969) on the paper samples. Conventional staining techniques which are

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generally applied in microbiology have not been used for visual detection and/or identification of the microbial cells on glass microfibers, to the best of our knowledge.

APTES modification can be chosen as a medium for DNA adsorption as well (Kim et al., 2007; Musayev et al., submitted for publication), which makes the approach suitable for using glass as a substrate for the APTES modification. Glass is already a common substrate for assembling microarrays (Malainou et al., 2012), which mostly utilize optical detection methods of the small biomolecules through fluorescence signals. Jang and Liu (2009) studied the fabrication of protein microchips based on APTES monolayer construction on glass slides. Inherent tendency of the fluorescein isothiocyanate (FITC) to bind to the primary amine group of APTES was utilized and fluorescence intensities after the immobilization of FITC, protein A-FITC, antimouse IgG-FITC, and anti-bovine albumin-FITC were all measured (Jang and Liu, 2009). However, they (Jang and Liu, 2009) analyzed only the green component of the images, because the FITC fluorescence yields an emission peak in the frequency that corresponds to the color green. Yet, red, green, and blue (RGB) analysis has the potential to provide further, valuable information. Previously, Riccio et al. (2011) highlighted the importance of colors in life sciences and demonstrated the use of RGB analysis in immunocytochemistry. Also, Lou et al. (2012) utilized the chlorophyll fluorescence and RGB intensity to correlate the internal chemical parameters of the mulberry fruits.

Emission of the fluorescent dyes can shift to longer wavelengths upon adsorption onto surfaces with higher polarities (Albani, 2007). A method can get use of this phenomenon, in order to test the success of surface coverage during modifications that are mainly applied to block direct interactions with the surface and to create an attractive surface specific for some biomolecules. For this purpose, applicability of a cationic cyanine based fluorescent stain, YOYO-1, is realized in this work. The fluorescence of YOYO-1 yields an emission peak in the frequency that corresponds to the color green, when bound to DNA (Rye et al., 1992). However, direct adsorption of YOYO-1 onto the glass surface yielded extra emission at longer wavelength. So, green component of the emission frequency was not the only color component that was analyzed in the images, which were obtained through this study. The RGB channels were inspected simultaneously and yielded unique results for APTES-modified *versus* unmodified glass microfiber filter paper surfaces. This is offered as a simple testing strategy of proper APTES coverage of the glass surface. This test can be a key step during sensor fabrication. Moreover, significance of performing such tests lurks behind the immense influence of surface chemistry on the interaction tendencies, kinetics, and mechanisms of surface-immobilized *versus* glass-adsorbed species.

Eventually, this study shows the use of APTES-modified glass microfiber filter paper as a biosensor, through surface adsorption of yeast cells and DNA, separately. Detection was performed by Gram staining and YOYO-1 staining, respectively. Therefore, this work covers two distinct uses of the glass microfiber filter paper, as a contribution to biosensors research. The latter usage renders the possibility of utilizing direct adsorption of YOYO-1 onto glass as a testing method to check the appropriateness of surface coverage, by means of the additional fluorescence emission of glass-adsorbed YOYO-1.

2. Material and methods

Glass microfiber filter paper was purchased from Filter-Lab (Ref: MFV1). APTES was purchased from Sigma-Aldrich. Yeast cells and Gram staining kit were purchased respectively from the market and a local dealer, which was named Kimsan Kimyasal Maddeler. DNA oligomeres were synthesized by Alpha DNA, Canada. YOYO-1 was purchased from Invitrogen. Olympus SZX12 model microscope, which was illuminated with a mercury arc lamb and equipped with a GFP filter, was used for the fluorescence imaging.

2.1. APTES modification

The 90 mm diameter samples of glass microfiber filter paper were cut into small pieces and transferred into petri dishes, to work with fewer volumes of sample solutions. The glass microfiber paper was modified with APTES to obtain positively charged, amine ending surface, by incubating them in APTES solution (5% by volume in an anhydrous toluene solution), for 4 h, at 25 °C, in a nitrogen chamber, before rinsing in ethanol and acetone, subsequently, and drying on a heater at 110 °C, for 1 h. Modification was also performed by the use of another protocol. This alternative protocol was modified from an earlier work on the modification methods of glass surfaces for protein immobilization (Qin et al., 2007). It basically involves dipping the samples inside H_2SO_4/HNO_3 (3:2 v/v) for 5 min, followed by rinsing in ethanol and acetone, and drying on a heater at 110 °C, for 1 h. The samples are eventually treated with APTES, as described above, and turned out to be ready for use.

2.2. Yeast cell binding

Yeast cells were prepared by mixing in deionized water and incubated on APTES-modified or unmodified glass microfiber filter papers for 1 h, at room temperature. Afterwards, samples were rinsed with deionized water several times and observed under the light microscope. The data is saved as image files. Then, Gram staining of the samples was performed according to the manufacturer's instructions, with Gram staining kit. The kit included Crystal Violet, Gram's Iodine, alcohol, and Basic Fuchsin. The glass microfiber paper samples were kept in a petri dish during staining, so as not to damage the samples and to handle them with ease. Brief explanation of the staining procedure is given in the supporting document. After staining, samples were photographed and observed under the light microscope. The data is saved as image files. Results are presented in the first part of the *Results and Discussions* section.

2.3. DNA binding

At first, DNA hybridization tests were carried out with probe (polyC 50mers) and target (polyG 50mers) oligonucleotides. The oligonucleotides, which were obtained in lyophilized form, were dissolved in sterile, deionized water and separated into aliquots. 125 μl of 200 nM polyC oligomers were added onto each sample, except for the controls, and incubated for 1 h, at room temperature. Afterwards, 125 μl of 200 nM polyG oligomers were added onto each sample, except for the controls, and incubated overnight, at room temperature, in a closed petri dish. For visualization, DNA was stained with the cationic cyanine dye, YOYO-1. 1 μl of 1 mM YOYO-1 iodide (491/509) in DMSO was diluted to 1 μM YOYO-1 in deionized water. 125 μl of 1 μM YOYO-1 was incubated for 20–25 min on the samples and observed immediately after incubation. Then, samples were rinsed again with deionized water and observed both 2.5 h and 15 h after. The molar ratio of YOYO-1 and (hybridized) DNA was 10:1, which was lower in effect, considering that DNA was concentrated on 2-dimensional surface of the glass microfibers, rather than being freely floating in solution. For control samples, only 125 μl of 1 μM YOYO-1 was incubated on the samples for 20–25 min and observed immediately after this incubation and also 2.5 h later. Observations were

performed under the fluorescence microscope, through the GFP filter. Data was saved as image files.

2.3.1. Image processing and analysis

Fluorescence microscope images were captured at 200 ISO. Brightness of the images was increased 64%, for clarity during inspection. Resulting brightness of the images after this much enhancement corresponded well to the brightness of the images that were captured at 1600 ISO. This was tested manually by increasing the brightness of a 200 ISO image until the brightness matches with that of the same image, which was captured at 1600 ISO.

In some images, there were invalid regions such as surfaces of the underlying petri dishes. Before analysis, those invalid regions were removed from the original images and the same amount of cropping was performed for all images, in order to end up with the same image sizes of all the data to be analyzed. The analyzed sections of the images are delineated in the figures.

As an additional processing, brightness of the images for the control samples were reduced 66%, to bring the YOYO-1 fluorescence intensities down to the equivalent level to that of a sample with 3 folds less YOYO-1 concentration. This is because the concentration of the YOYO-1 in the samples with DNA was diluted 3 folds, as a result of the preliminary presence of DNA solution on the samples. Preprocessing of the images that are described herein was performed for improving clarity of the color changes, and to equilibrate the control and test sample images that were having 3 folds difference in final YOYO-1 concentrations on the surface, during imaging. Besides, images of the control samples without any reduction in brightness were also analyzed.

Color histograms were obtained for the processed images, after drawing a rightwards descending diagonal line on the selected area of the images. This analysis was performed by using the color histogram plugin of ImageJ 1.47v. 2-D column plotting of the RGB mean values of the color histograms were performed with the Microsoft Office Excell 2010. Results are presented in the second part of the *Results and Discussions* section.

3. Results and discussion

3.1. Yeast cell binding on glass microfiber filter paper

The glass microfiber paper was modified with APTES, to obtain a positively charged amine-ending surface. This was aiming to improve the affinity of the surface to the yeast cells in water, which are negatively charged. As mentioned in Section 2, APTES modification was performed by either incubating the samples directly in the APTES solution, or by a preliminary H_2SO_4/HNO_3 (3:2 v/v) treatment. The results were similar for both, with no significant change in the outcomes of the forthcoming steps. Fig. 1b shows the photo of two samples that were modified with APTES through different approaches, in comparison to a sample with no modification. *Sample* 1 is unmodified, while *Sample* 2 and *Sample* 3 are the one with no pretreatment before APTES modification and the one with pretreatment, correspondingly (Fig. 1b). Indistinctive results of the two -slightly- different modification strategies could be due to the relatively long duration of incubation inside the APTES solution. Prolongation of incubation leads to thickening of the APTES layer, but it also eliminates the risk of ending up with unmodified regions on the surface to be modified. This long incubation was preferred because increased layer thickness was not expected to be having any adverse effects on the current tests. Further information on the microstructure of APTES-modified glass microfiber filter paper samples and high magnification images that were obtained with the scanning electron microscopy are provided in the supporting document (Fig. S1).

As it is clear from the images in Fig. 1a, paper samples that were incubated with cells were not readily distinguishable by their appearance, without any staining. Additionally, cell immobilization alone was leading to a faint change in the microscopy images of the samples (Fig. 2a and b). When Gram staining was applied, color changes of the samples were indicative of different levels of dye loading due to diverse amounts of cell entrapment (Fig. 1b). The microscopy images of the Gram stained samples revealed improvement of cell entrapment better. This is observed through comparing both Fig. 2c and d images with Fig. 2e and f, one-to-one, respectively, for unmodified and APTES-modified samples. A discussion about the relationship between immobilization efficiency and yeast cell concentration of the incubation solution is provided in the supporting document, along with the data for non-biofouling layer usage for diminishing binding affinity to the surface-modified samples (Fig. S9). The latter proves the applicability of some control on the cell binding affinity, for developing this approach as a micro-array prototype, or so.

Microscopy images of Gram stained samples with immobilized cells reveal augmented cell-binding capacity of the surface-modified sample by more than a factor of four (Fig. 2e and f, in comparison to the respective images in Fig. 2c and d). Therefore, this approach can be benefited for designing a simple dip-stick type or a filter-mode sensor, with improved binding capacities and

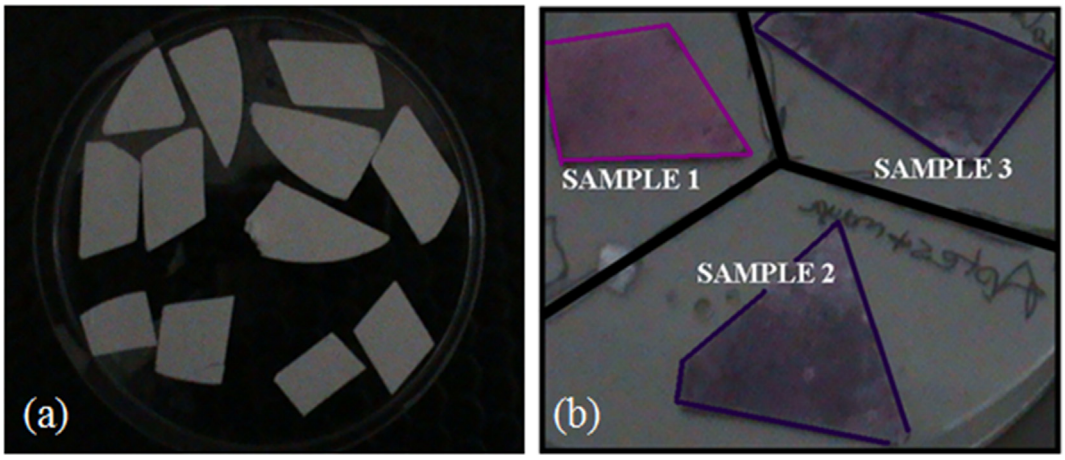

Fig. 1. The appearances of unmodified or APTES-modified glass microfiber paper samples that were incubated with cells, before Gram staining (a) and after Gram staining (b). In the Gram stained samples' image (b), *Sample* 1 was not treated with APTES before incubation with the cells. *Sample* 2 and *Sample* 3 were treated with APTES. *Sample* 2 encountered no pretreatment before incubation in APTES solution, and *Sample* 3 was initially treated chemically, then with APTES.

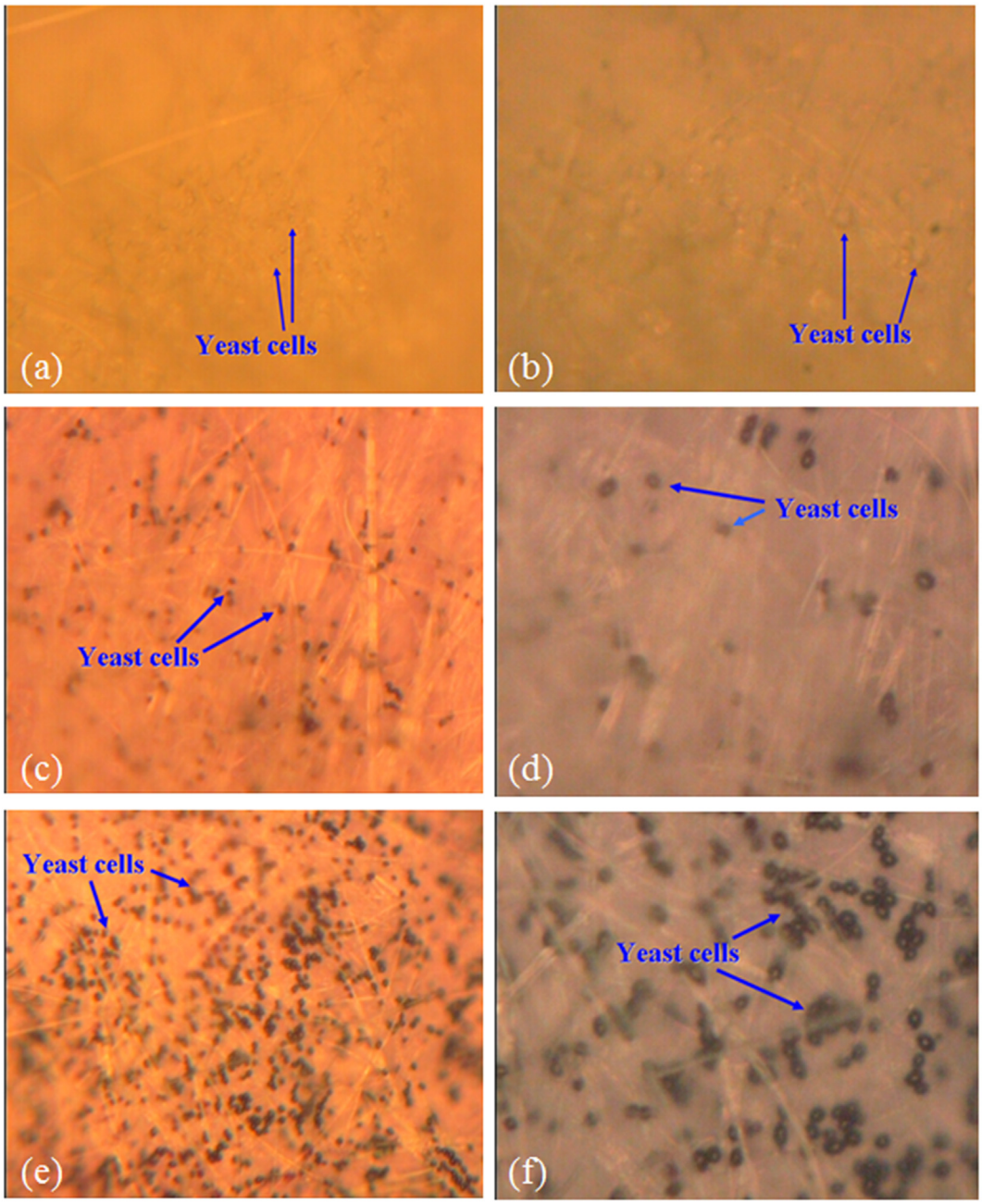

Fig. 2. Enhanced view of APTES-modified glass microfiber paper sample that was incubated with cells ((a) and (b)), in comparison to unmodified ((c) and (d)) and APTES-modified glass microfiber paper samples ((e) and (f)) that were incubated with cells and colorized by Gram staining. Zoom out view of the images were obtained by using 20× ((a), (c), and (e)) and 50× ((b), (d), and (f)) objectives, with further magnification sourced by the video-camera of the microscope. Yeast cell diameter is about 3–5 μm.

using a basic visual detection scheme. It also allows the application of similar staining techniques that are widely used in microbiology, to the optical biosensors studies. Moreover, it can be regarded as a signal enhancement tool.

3.2. DNA binding on glass microfiber filter paper

As the second part of this work, APTES-modified glass microfiber paper was tested for surface binding affinity of negatively charged DNA oligomers. YOYO-1 staining was used to visualize surface-adsorbed 50mers in double stranded form. YOYO-1 leads to a characteristic green fluorescence, when intercalated into the double-stranded DNA. This was not observed when DNA was not present in the media. Yet, green color of the DNA–YOYO-1 complex was recognized when YOYO-1 was incubated with the DNA solution, on the APTES-modified samples. Corresponding images are Fig. 3a and b, respectively, for the medium without, and with DNA. The fluorescence microscope images are on top, along with their analysis results at the bottom, within the 2-D column plots of the RGB mean values of the color histograms. In Fig. 3c, the image and its analysis results revealed that YOYO-1 sourced emission remained with little intensity loss, after rinsing the sample and incubating it for 2.5 h more. This intensity loss was remediated till 15 h. It can be seen in Fig. 3d, through the image and its analysis results. The data presented in Fig. 3 is in coherence with the fact that green channel values are dominating in the images of the samples with DNA–YOYO-1 complex. Relationship between the concentration of target DNA and fluorescence intensity of YOYO-1 was also studied and the

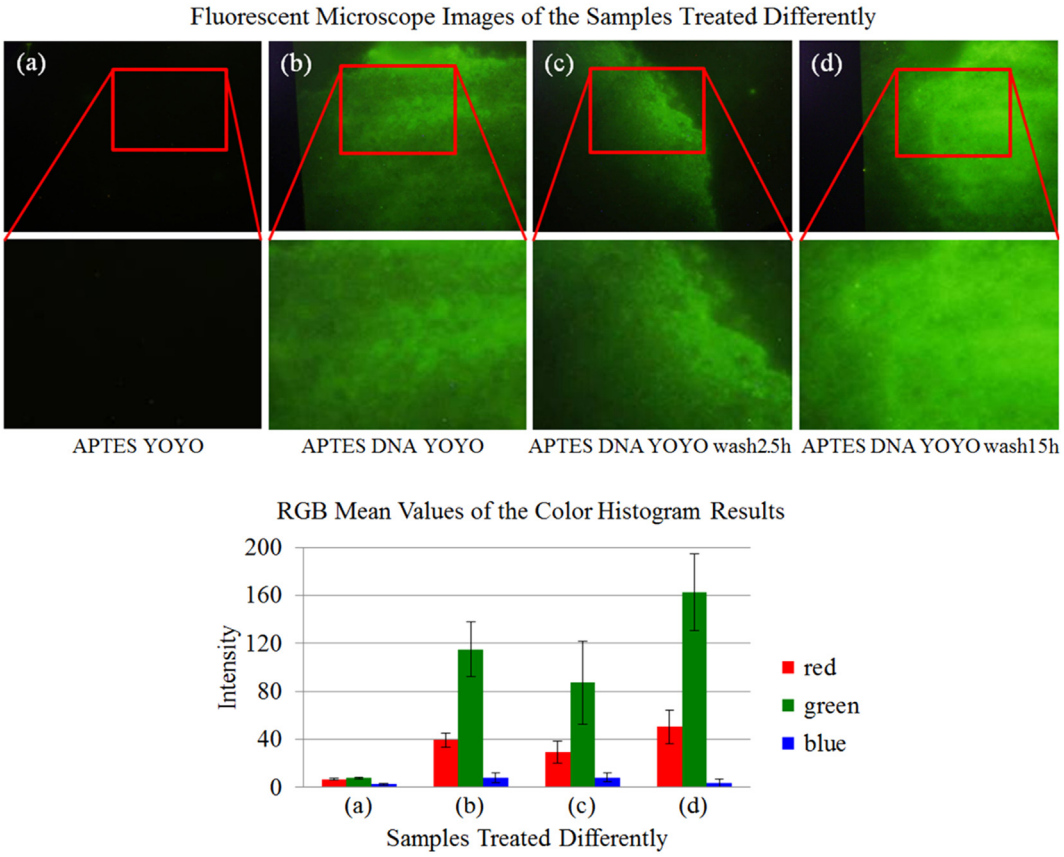

Fig. 3. Fluorescence microscope images of surface-modified samples that were treated differently (on top), together with 2-D column plot of their RGB mean values of the color histograms (at the bottom). On top, fluorescence microscope images depict YOYO-1 on APTES-modified sample, either without DNA or with DNA, but under varying conditions as follows: The image was captured after incubating YOYO-1 on APTES-modified sample without DNA, for 20–25 min (a); and the image captured after incubating YOYO-1 on APTES-modified sample with DNA, for 20–25 min (b), followed by rinsing, and incubating the sample 2.5 h more (c), 15 h more (d). The images (on top) are presented with the same denotation as 2-D column plot of their RGB mean values of the color histograms (at the bottom).

results are provided in the supporting document (Figs. S4–S6), along with a discussion on the relevant changes in the fluorescence intensities (Table S1).

Test results for the unmodified samples were revealing additional emission frequency of YOYO-1, which was adsorbed on glass. When DNA was excluded, image of YOYO-1 on the unmodified sample revealed a pale fluorescence signal, distinct from the characteristic green color of the DNA–YOYO-1 complex (Fig. 4a, on top). When DNA was present, appearance of the fluorescence microscope image was still different from the characteristic green color of the DNA–YOYO-1 complex (Fig. 4b, on top). So, green color was not noteworthy at first, meaning that it was not attained immediately after incubating YOYO-1 with the DNA on the surface. It was barely noticeable after rinsing the sample and incubating it 2.5 h more (Fig. 4c, on top), and rather visible after prolongation of the incubation to 15 h (Fig. 4d, on top). However, mere visual inspection is insufficient and thus can be misleading. This is revealed by RGB analysis of the images. Besides, more information can be extracted than the single color analysis, due to overlapping fluorescence signals. According to the 2-D column graph of the color histograms, green channel values are distinctive, along with the red channel values (Fig. 4a, image analysis results). In case of the sample with DNA, green channel value increases after 20–25 min treatment with YOYO-1, but not as much as that observed in case of the sample with APTES-modification (image analysis results of Fig. 4b, in comparison to that of Fig. 3b). Additionally, red channel value is still higher (Fig. 4b, image analysis results). After rinsing the sample and incubating further, green channel values increase to those observed for the image analysis results of the APTES-modified sample (image analysis results of Fig. 4c and d, in comparison to those of Fig. 3). Besides, there is an accompanying increase in the red channel values. So, after rinsing the sample and incubating further, green channel values increase up to those that were observed for the APTES-modified sample, along with elevated red channel values.

As informed through the Invitrogen's web-site, cationic cyanine dyes, including YOYO-1, are readily adsorbed out of aqueous solutions onto surfaces, particularly glass. This was observed here, through the results of 3 folds concentrated YOYO-1 fluorescence on samples without DNA (Fig. 5). Concentrated YOYO-1 on unmodified sample led to a distinctive color, enabling it to be differentiated from the characteristic green color of the DNA–YOYO-1 complex (Fig. 5b, on top). Adsorption onto glass was probably causing a disregarded preference for employment of this dye in detection of surface-adsorbed DNA. However, YOYO-1's having no affinity for the positively charged surface should be enabling it to move and interact freely, and maybe better, with DNA. In relevance, prolonged duration for DNA–-YOYO-1 complex formation was leading to the observations about the unmodified surface. Such an influence on the interaction kinetics can be problematic for designing and developing biosensors comprising similar reaction schemes.

Proper APTES coverage of the glass surfaces is important for surface modifications with small biomolecules and for the kinetics and mechanisms of the molecular interactions. This is critical in biosensor studies. Here, immediate adsorption of the cationic cyanine dyes, particularly YOYO-1, onto the glass surfaces is offered as a simple testing strategy of proper surface modification. Imaging of the surface under fluorescence microscope would be

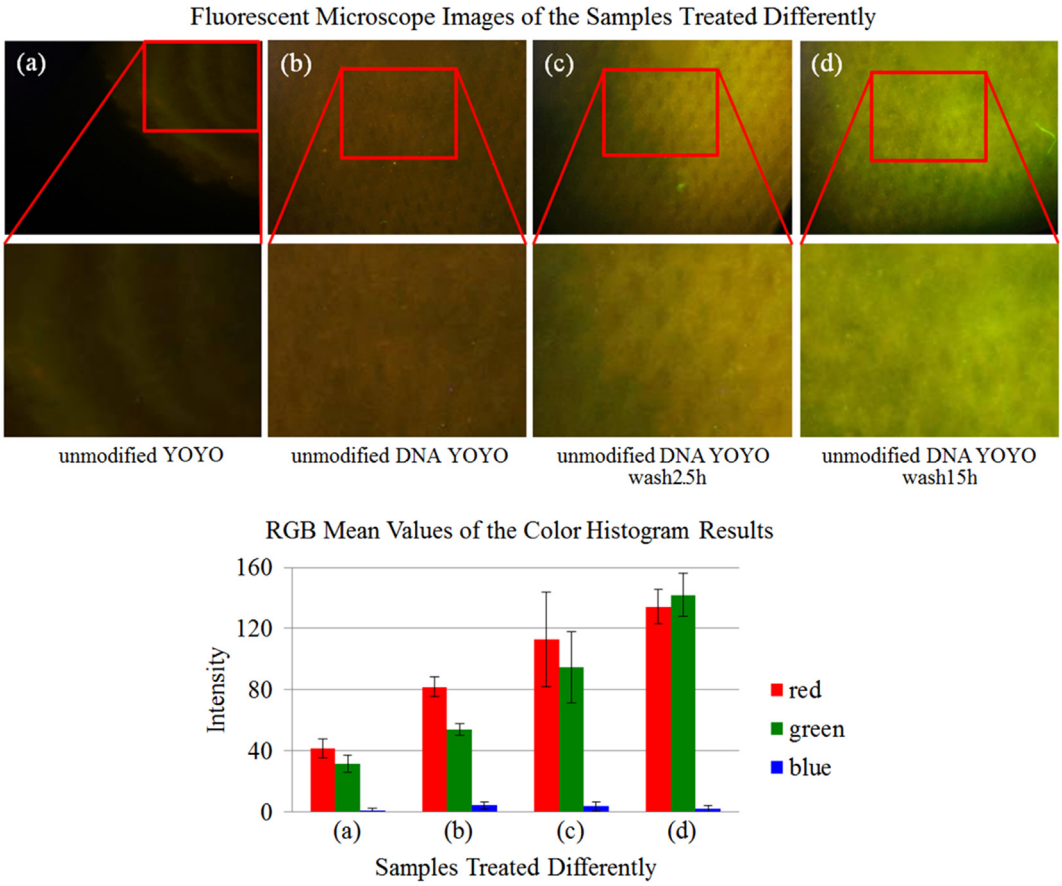

Fig. 4. Fluorescence microscope images of the unmodified samples that were treated differently (on top), together with 2-D column plot of their RGB mean values of the color histograms (at the bottom). On top, fluorescence microscope images depict YOYO-1 on unmodified sample, either without DNA or with DNA, but under varying conditions as follows: The image was captured after incubating YOYO-1 on unmodified sample without DNA, for 20–25 min (a); and the image captured after incubating YOYO-1 on unmodified sample with DNA, for 20–25 min (b), followed by rinsing, and incubating the sample 2.5 h more (c), 15 h more (d). The images (on top) are presented with the same denotation as 2-D column plot of their RGB mean values of the color histograms (at the bottom).

revealing the success of modification, thorough RGB analysis of the YOYO-1 fluorescence emission. Data on the relationship between the duration of sample incubation in APTES solution, in relation to the surface coverage with APTES, and fluorescence intensity of YOYO-1 is provided in the supporting document (Fig. S3).

4. Summary and outlook

This work highlights following a simplistic approach for quantitative improvement and visualization of the entrapped yeast cells on surface-modified glass microfiber paper samples. In accordance, Gram staining was utilized in an effective and easy manner. Gram staining is very common and affordable. It is applied in most microbiology laboratories. Such advantages facilitate the transmission of the technique successfully over rural districts. Additional strength of the method is that it can be generalized and the other bacterial and cell staining procedures can possibly be implemented. It can also be used to develop novel improvement strategies for lab-on-a-chip devices and components. It can even be used in designing filtering systems with new functionalities.

This work contributes also to the optical biosensors research in the sense that it provides means to decipher the appropriateness of glass surface modification, by getting use of varying emission properties and DNA-binding affinities of glass-adsorbed *versus* free YOYO-1. To the best of our knowledge, using YOYO-1 for detection of DNA on APTES-modified or unmodified glass microfiber filter paper surfaces were realized for the first time. Moreover, adsorption of YOYO-1 on glass, with a unique color content of the emission wavelength, was claimed to be a testing scheme for checking the suitability of glass surface modification. RGB analysis provides ease in estimation of each color channel's intensity for an image. Computer monitors, scanners, cameras and vast number of scientific instruments reproduce colored images by assigning varying intensities among the red, green and blue pixels that are evenly distributed on the screen or the generated image. This universal feature enables a very nice analyzing scheme for different experimental designs. For instance, possibility of using single color led light as an excitation source and simpler equipments than a microscope for imaging can be investigated for diminishing the equipment needs of the current approach and presenting it as an even simpler tool.

Testing of glass surface modification by staining can have potential uses in biosensors studies, together with the capability of being widespread. Alternatively, dye-adsorbed glass microfiber filter may be realized as a ready-to-use dip-stick type sensor. Also, it can be used for watching the DNA presence in a flow through system, through remote monitoring of the emission wavelength. Such designs may not be accounted as fast-response sensors, but are likely to outrage their competitors by bearing such benefits like being cheap, simple, effective, and robust, all at once.

Finally, staining techniques can also be employed as signal enhancement tools for the optical sensors, once detection schemes are designed accordingly. Determining detection limits of the proposed glass microfiber based sensor is left as a future work,

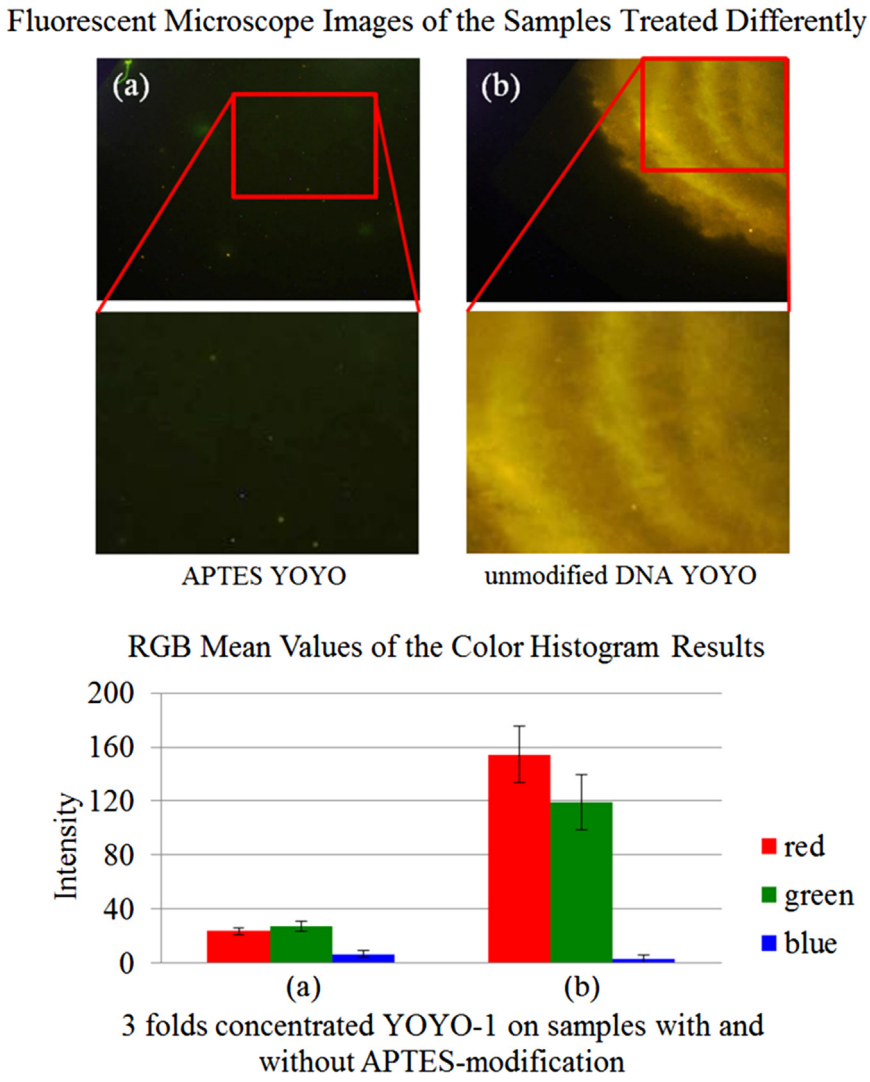

Fig. 5. Fluorescence microscope images of 3 folds concentrated YOYO-1 on samples with and without APTES-modification (on top, (a) and (b), respectively), along with 2-D column plot of their RGB mean values of the color histograms (at the bottom). The images (on top) are presented with the same denotation as 2-D column plot of their RGB mean values of the color histograms (at the bottom).

along with testing robustness, durability, and storage time of the surface modified samples; and tests with single base mismatch DNA oligomers, cells, and other biological media, like proteins.

5. Conclusion

Several simplistic approaches that can be combined for benefiting in biosensors research is presented through the novel use of surface-modified glass microfiber filter paper as a biosensor. This was achieved through visual detection of surface-immobilized yeast cells by Gram staining. DNA detection was additionally tested on these surfaces with complementary DNA sequences, and presence of DNA was visualized by using YOYO-1. Results allowed us to conclude that adsorption tendency of YOYO-1 onto glass can be generalized for testing proper surface coverage, for sensors bearing glass-based active surfaces, by using RGB analysis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.10.042.

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