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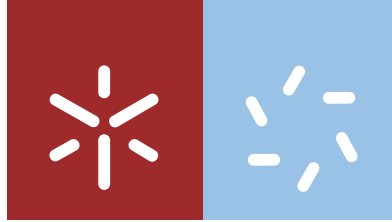Escola de Ciências

Ana Raquel Carvalho Bertão

**On-site and rapid optical assay
to test biological samples**

outubro de 2017

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On-site and rapid optical assay to test biological samples

Dissertação de Mestrado
Mestrado em Técnicas de Caracterização e Análise Química

Trabalho realizado sob orientação da
Professora Doutora Maria Isabel Pontes Correia Neves
e do
Professor Doutor Tao Dong

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ABSTRACT

Currently there is a need for diagnosis processes, which uses urine samples and offer quick, easy, and accurate results, since most of the traditional systems require time, material, facilities, and specialized personnel, which can affect the efficiency of the therapy chosen for the patient.

In this context, this work reports two different strategies to improve the stability and latency of the results provided by a device specifically developed for the analysis of urine samples on diapers and early medical condition assessment. The main goal was to obtain stable, rapid, and easily interpretable results, by employing colorimetric detection, without the need of electricity to analyze several urine biomarkers.

The device design already comprises components to provide the conditions for the results stability, such as a self-locking system and a cover layer. These same conditions were tested, and the outcomes suggest that the device has great potential to keep valid results for long periods. The encapsulation of the dye molecules, normally used in the bioassays, into hosts like zeolites nanostructures, which were used to enhance color stability, was also studied in order to have a chemical approach to this issue. These obtained dye nanomaterials were characterized by employing several techniques (Room Temperature Fourier Transform Infrared (FTIR), N_2 adsorption isotherms, scanning electron microscopy (SEM/EDX), thermogravimetric analysis (TGA) and powder X-ray diffraction (XRD)) and, the results show that the encapsulation of the dyes was successfully achieved, preserving both the guest and host structures. Regarding the colorimetric results, a long-time stability of the dye colors was achieved due to their encapsulation into the zeolite nanostructures. The choice of the type of paper for the μPAD and the zeolite nanostructures properties influences the colorimetric response. The results obtained in this work were promising for the studied assays, and the strategy could also be extended to other biomarkers.

KEYWORDS: Urinalysis; Zeolites; Dyes molecules; Encapsulation; Paper-based analytical devices; Colorimetric studies.

RESUMO

Atualmente, existe uma necessidade de processos de diagnóstico que utilizem amostras de urina e ofereçam resultados rápidos, fáceis e precisos, uma vez que a maioria dos métodos tradicionais requerem tempo, material, instalações e pessoas especializadas, o que pode afetar a eficiência da terapia escolhida para o paciente.

Neste contexto, este trabalho explora duas estratégias diferentes para melhorar a estabilidade e latência dos resultados obtidos por um dispositivo especificamente desenvolvido para a análise de urina em fraldas e avaliação médica prévia (monitorização da saúde). O objetivo principal foi obter resultados estáveis, rápidos e fáceis de interpretar, ao utilizar a deteção colorimétrica, sem necessidade de eletricidade para analisar vários biomarcadores da urina.

O design do dispositivo já contém componentes para fornecer as condições para a estabilidade dos resultados, tal como um sistema de fecho automático e uma camada de cobertura. Estas mesmas condições foram testadas e os resultados sugerem que o dispositivo tem grande potencial para manter a validade dos resultados por longos períodos de tempo. O encapsulamento de moléculas de corantes, normalmente utilizadas em ensaios biológicos, em hospedeiros como zeólitos, no sentido de aumentar a estabilidade das cores das moléculas, foi também estudado com o objetivo de explorar uma abordagem química ao problema. Os nanomateriais obtidos foram caracterizados por várias técnicas (Espetroscopia de infravermelhos (FTIR), isotérmicas de adsorção de N_2, microscopia eletrónica de varrimento (SEM/EDX), análise termogravimétrica (TGA) e difração de raio-X (XRD)) e os resultados confirmam que o encapsulamento das moléculas, com a preservação das estruturas dos corantes e dos respetivos hospedeiros. Em relação aos resultados colorimétricos, foi alcançada uma estabilidade das cores dos corantes por um longo período de tempo devido ao seu encapsulamento nas estruturas dos zeólitos. A escolha do tipo de papel para os µPADs e as propriedades dos zeólitos demostraram ter influência na resposta colorimétrica. Os resultados obtidos neste trabalho são promissores para os ensaios estudados, e o método pode também ser estendido a outros biomarcadores.

PALAVRAS-CHAVE: Análise de urina; Zeólitos; Corantes; Encapsulação; Dispositivos de análise de microfluídica em papel; Estudos colorimétricos.

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ABBREVIATIONS

BPB – Bromophenol blue dye;

BSA – Bovine serum albumin;

BTB – Bromothymol blue dye;

EDX – Energy-dispersive X-ray spectroscopy;

FAU – Three letter code for a faujasite structure;

FTIR – Fourier transform infrared spectroscopy;

MFI – Three letter code for the *pentasil* structure;

MR – Methyl red dye;

NED - N-naphthylethylenediamine dihydrochloride;

POC – Point-of-care;

PTFE – Polytetrafluoroethylene;

SBU – Secondary building units;

SEM - Scanning electron microscopy;

TGA – Thermogravimetric analysis;

USY – Ultrastable Y zeolite;

UTI – Urinary tract infections;

XRD - X-ray diffraction;

ZSM5 – Zeolite Socony Mobil-five;

µPADs - Microfluidic paper-based devices.

CHAPTER 1 |

Thesis overview and General Introduction

CONTEXT AND OUTLINE

Urine analysis is a fundamental process for diagnosis, screening, or monitoring purposes. Since it can be used to evaluate body homeostasis and several diseases, examination of the urine is part of the initial patient assessment [1-3]. Together with physical examination and clinic history, it can elucidate the patient health status, and therefore is one of the most required tests in a clinical laboratory [4, 5].

This process includes biochemical, physical and microscopic examinations [6]. The chemical analysis covers a wide range of parameters that, in certain situations, are correlated with several pathologies. Their measurement is most of the times related with a chemical reaction that leads to a color change, allowing an easy, less expensive and visual readout of the results [5, 7]. Normally, a plastic strip with several reagents pads attached to it, called dipstick, is used for urine analysis. It is an easy, fast and cheap solution, which reduces the need for laboratory tests [1, 2]. However, each pad must be read at a specific time, which can be the same or not between pads. Since the results vary with time, the user must be able to quickly interpret the colorimetric results [6]. Other Point-of-Care (POC) testing platforms, like microfluidic paper-based devices (µPADs), have been used to analyze biological fluids. One of the advantages of µPADs relatively to dipsticks is the possibility of designing the microfluidic channels to separate the sample into several regions, allowing the determination of several parameters simultaneously [8], and reducing the risk of cross-contamination between the zones with reagents.

Besides the test method, the sampling procedure for urine analysis can also be a problem, especially for elderly, children, or patients with incontinence, to whom a catherization is normally done to obtain a urine sample, which is an invasive and painful approach [3, 9, 10].

This main objective of this work was to develop a system that could rapidly analyze a urine sample, by using a diaper as sampling platform, for a more comfortable alternative, but also to enhance the results stability for longer periods. Firstly, a novel design for the device was analyzed: the provided conditions and their role in the stability of the results for glucose and nitrite were studied. Afterwards, a new approach was also considered: the use of zeolites in µPADs for keeping the colors for longer periods. The zeolites nanostructures were used as a solid support for the dye molecules normally used in pH and protein assays. Several characterization techniques as N_2 adsorption isotherms, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM), energy-dispersive X-ray

spectroscopy (EDX) and thermogravimetric analysis (TGA) were used for analyzing the obtained dyes nanomaterials. The colorimetric results were studied over time to investigate their stability.

This work is divided into four chapters: in chapter I, the fundamental theory for a better understanding of the work is presented; chapter II is related to the influence of the conditions provided by the novel device design for urine analysis in diapers; chapter III presents the work developed with the zeolites for color stability; and finally, chapter IV concludes this thesis by summarizing the main ideas and results.

This MsC thesis was carried out at the Department of Microsystems of the University College of Southeast Norway, and at the Department of Chemistry of the University of Minho.

1.1 Urinalysis

Since ancient times the urine is investigated and used as a source of information for diagnosis [11]. Conversely to the analysis of other biological matrices, like blood, the urinalysis offers the advantages of being non-invasive (when catheterization is not needed), easy (without the need of pretreatment) and not expensive [5, 6, 12].

The information about patient's health can be obtained through physical, chemical, and microscopic examinations of the urine sample. Physical observations include the study of color, clarity and solutes concentration of the urine sample. Other characteristics, like odor or taste, are no longer interpreted [2]. On the other hand, microscopic analysis of urine allows the detection of cells, crystals, casts, and bacteria in the samples, and is an essential part of urinalysis [13]. However, and even though the urine culturing is accurate, it is also time-consuming [14] therefore, the chemical analysis of urine offers a faster, easier, and cheaper solution. Currently, this kind of analysis enables tests for pH, specific gravity, protein, glucose, nitrite, ketones, urobilinogen, bilirubin and occult blood [15].

These tests are normally performed with reagent strips, called dipsticks, which contain several reagent-impregnated pads specific for each parameter. After being in contact with the sample, a color change will occur in each pad, and when compared with a reference chart, it will give qualitative results for each reaction [2]. To achieve accurate results, one of the requirements is the time of the readout specified by the dipsticks manufacturer. Other factors like the storage of the strips, which should be handled in close containers away from heat, direct sun light, moisture can influence the colorimetric results [1, 15].

Dipsticks are one the most traditional POC tests [8, 16], mainly because of their simplicity and portability, but have also some drawbacks, like the possibility of cross-contamination between reagent pads. Other POC testing devices, like µPADs, have received more attention, since they can perform several biochemical assays simultaneously and without cross-contaminations [17].

1.2 Microfluidics on paper

The concept of µPADs was introduced by Martinez et al. [18] in 2007. Since then, a lot of interest was devoted for this kind of devices [19 -22], mostly due to the aforementioned advantages.

There are several methods to define the fluid path in the µPADs, which can create chemical or physical barriers [23]. These patterns allow the multiplicity of the reactions and the diffusion of the sample only to desired zones of the µPADs [24]. The fabrication methods include photolithography, ink jet printing,

screen printing, cutting, wax printing and others [16]. The wax printing is one of the most used due to the simple and, fast fabrication process, which enables mass production [25], but it requires a heating step and an expensive printer. On the other hand, the cutting is an inexpensive process, but it is associated with less precision in the microchannel size [24].

The use of paper as substrate platform make them affordable [23], biocompatible [7], disposable and easy to transport, store and manipulate [19, 26, 27]. The modification of cellulose materials allows the improvement of μPADs performance and functionality. Functionalization of μPADs with nanomaterials were already described [27-29]. The employment of paper in microfluidic devices also allows the system to be used without the need of an external power source: the fluid moves within the hydrophilic matrix toward the reaction zones through capillarity [23, 30]. In addition, the versatility of μPADs can be associated to the wide range of paper properties (e.g. thickness, pore size, etc) [8, 26], and the possibility of employing several kinds of detection methods (colorimetric, electrochemical, fluorescence, chemiluminescence, etc.) [30, 31].

The most used is the colorimetric detection due to its low-cost and simplicity for getting and interpret the results, which are attained with a chemical or enzymatic interaction between the target analyte and the reagents spotted in the detection zones [31]. This method normally uses a calibration curve, which can be easily obtained with an electronic reader such as a scanner or phone camera, to have semi-quantitative and more accurate results in comparison to visual interpretation [28]. The latter has some drawbacks when compared with electronic readers, due to different visual interpretations of people or to different light conditions [7].

1.3 *TOUCHSENSOR* PROJECT

Specially focused on facilitating the urinalysis process for patients suffering from urinary incontinence or reduced mobility, the main objective of the *TouchSensor* project is the development of a device that combines disposable diapers, as sampling platform, with part of microfluidics in paper and the dipstick method, for the chemical analysis of the urine sample. Designed to be portable and easy-to-use, it allows a less uncomfortable and stressful process for the patient, while it monitors the patient health.

The device (Figure 1.1) should to be placed on the inside surface of the diaper. When the diaper is removed, after a urine sample being collected, and the device is detached from it, the colorimetric results can be read directly through the transparent bottom of the device or analyzed with an electronic reader.

As idealized by A. Couto et al. [32], this design considered the necessity of collecting and retaining one single urine sample and the need to extend the validity of the colorimetric results of the reagents pads, normally used in dipsticks, which have determined reading times. It was possible to retain for more time the data by having a self-locking system, which avoids the entrance of more body fluid when there is enough sample to perform the test, and by isolating the testing area from the external environment, to avoid sample evaporation or system contamination. The design and the influence of the device conditions on the results obtained for the reagent pads of nitrite and glucose will be further disclosed in chapter 2. Since this work makes use of microfluidics in paper, other possibilities to improve the validity of the colorimetric results related with the adopted reagents, were also explored. The use of nanomaterials in the modification of the µPADs surface was already well reported in literature [29]. Following this line of thought, a new approach exploiting zeolite nanostructures to stabilize the color results for longer periods of time was investigated.

Figure 1.1 - TouchSensor device for analysis of several biomarkers in urine samples [33].

1.3.1 Zeolites applied to µPADs

Zeolites are solid crystalline and microporous materials built from a 3-dimmensional arrangement of SiO_4 and AlO_4^- tetrahedra, which are linked by oxygen atoms, to form secondary building units (SBU) [34]. Other elements can be part of the structure, rather than Si and Al, like Ti, Ge or P, in the case of synthetic zeolites [35]. The use of zeolites in industrial, medical and environmental applications, like catalysis [36-

38] and adsorbent [39] processes or even anti-bacterial studies [40, 41], is increasing due to their unique properties like compositional and structural flexibility, non-toxicity, high surface area, high thermal and physical stability, among others [42].

The building units of the zeolites are arranged in a periodic way, forming micropores that connect the surrounding medium with their interior, allowing the access of molecules from the exterior to the internal spaces of the zeolites. These materials can be classified based on their structure but also by their pore size: small, medium or large [37].

In this work, different zeolite structures are proposed as hosts [43] for encapsulated dyes molecules, normally used for urine biomarkers detection and analysis. The objective is to obtain stable materials suitable for the µPADs fabrication, in order to maintain the colorimetric results for longer periods. Two types of zeolites were employed: ultrastable Y zeolite (USY), and Zeolite Socony Mobil five (ZSM5) (Figure 1.2).

The large-pore USY zeolite presents a faujasite structural framework type (FAU), with two types of SBUs: sodalite cages connected via hexagonal prisms, forming a pore system with spherical cages, known as supercages, with a diameter of 1.3 nm (13 Å) and a pore aperture of 0.74 nm (7.4 Å). Conversely, the ZSM5, a medium-pore zeolite, belongs to the *pentasil* family (MFI), with two types of 10-ring channels system: straight 10-ring 0.55 x 0.51 nm channels connected by sinusoidal 0.56 x 0.53 nm channels with the intersection cavities (approximately 9 Å) [36].
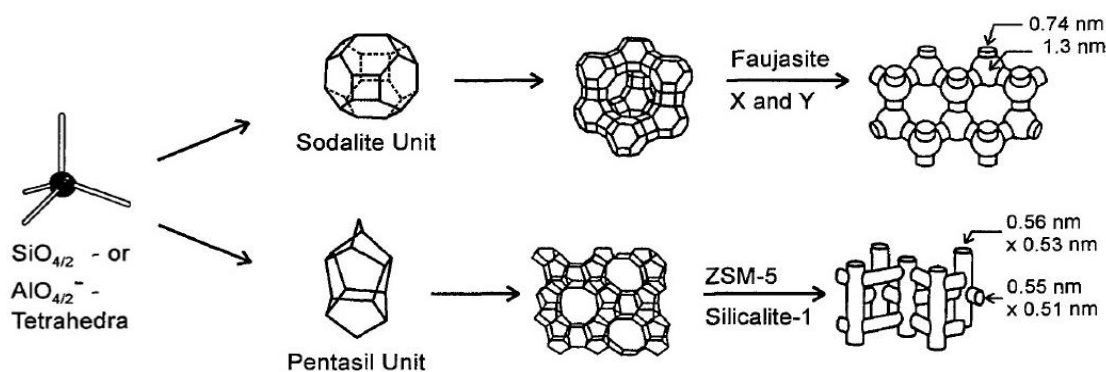
Figure 1.2 - Structure of zeolites Y and ZSM5, with their respective micropores systems and dimensions [36].

These structural features of USY and ZSM5 were taken into consideration for the choice of the right host-guest system, especially because of the pore sizes and the tested molecules, as explored in chapter 3.

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CHAPTER 2|

Stability of Colorimetric Results in the Detection of Urine Biomarkers Using a Paper-based Analytical Device[1]

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ABSTRACT

Urinalysis is used to obtain information about many of the body functions. Normally, urine is a easily collected specimen, but in some cases, it can be an uncomfortable process. Moreover, standard methods such as microscopic examination or urine culturing are time-consuming procedures. In this regard, this work explores the design of a device that combines disposable diapers, as a platform for sample collection, with the dipsticks method. Even though this is a desirable combination for a faster and comfortable approach, it is also convenient to extend the prescribed time to read the results, provided by the dipstick manufacturer, preferably until the moment of the diaper changing, and this requirement was also considered in the design. The desired conditions were reproduced for the reagent pads of glucose and nitrite. The results show that the RGB profile, obtained in glucose detection, varied after approximately 60 min. However, when the developed colors are visually compared with the reference colors, the results suggest that there were not significant variations. In the nitrite assay, by analyzing the RGB profile or by visual comparison with the reference colors, the color does not appear to change over time, even though no nitrite was detected, contrary to what was expected. It seems that the proposed design is promising for the results stability, but more experiments need to be done to check if the variations are constant for more biomarkers in the conditions provided by the device.

2.1 INTRODUCTION

Urinalysis comprises a series of tests frequently requested by physicians for diagnostic purposes since the substances or cellular material that can be detected in the urine, in certain quantities, are associated with several pathologies, drug abuse or exposure to harmful chemicals [1]. Even though this process is also done as a preventive measure, it is common to perform it only after the detection of some visible symptoms which, sometimes, are found when the disease is already in an advanced state. Furthermore, the most usual procedures are a complete routine analysis which includes macroscopic and microscopic exams and urine culturing. These standard methods are accurate but also time-consuming [2, 3], from the moment of the sample collection until the results are obtained, which may be crucial to the success of the treatment chosen for the patient.

Dipsticks have gained popularity as a faster and cheaper instrument to obtain information about urine status. The dipstick is made in plastic strips with several attached reagent pads that change color after contact with the sample. Each reagent pad detects a certain analyte and the reaction color is correlated with the analyte concentration in the sample. This kind of procedure is normally used for screening or treatment monitoring, without the need of complex protocols, serving as the basis for additional and more accurate urine examinations [4].

Another issue associated to urinalysis is the sample collection, especially for the elderly, patients with urinary incontinence or even children. In these cases, the urine sample is taken through catheterization, but it can be painful and it can lead to contaminations [2, 5]. A more comfortable alternative is the use of disposable diapers as a platform for urine sample collection. However, the extraction of urine substances from absorbent media can be complex and it is influenced by factors like temperature and urine pH, among others [6]. Therefore, it would be more practical to perform sample collection and analysis in the diaper itself.

2.2 DESIGN AND STRATEGY

The device was designed based on the need for collecting a single urine sample and detecting several of its health parameters, for early medical condition assessment, without disregarding the well-being of the patient, and the simplicity of the process to the analyst, with or without medical training.

The patient can wear the diaper with the device placed on its inside surface. The sample is collected when the urine reaches the diaper, and through capillary flow it will reach the reagent pads present on the filter paper (Figure 2.1). In contact with the urine sample, each reagent pad will change its color depending on the detected biomarker and the relative concentration, giving information about possible abnormalities in the urine composition. When the diaper is removed from the patient, the device is detached and the color change can be observed through the transparent bottom layer. It is possible to analyze the change by using a reference color chart that displays the full colorimetric reaction divided into several distinguishable color steps for each reagent pad, as is usually done with dipsticks.
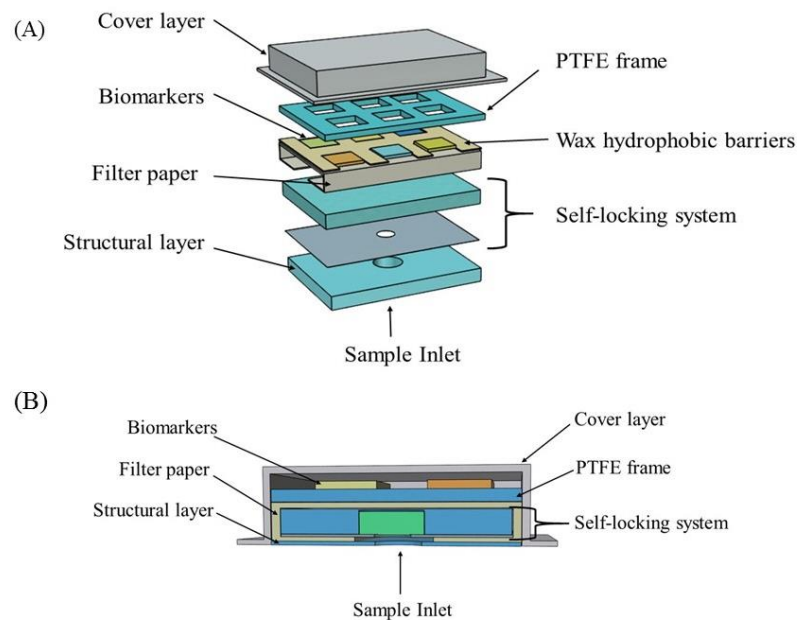
Figure 2.1 - The design concept of the purposed device for urinalysis in a diaper. (A) The exploded view of the device. The wax hydrophobic barriers and the polytetrafluoroethylene (PTFE) frame were included to prevent the runover of reagents between pads. (B) Cross section of the device. The urine sample will reach the biomarkers on the filter paper through flow capillarity. The self-locking system was designed to prevent the entry of more body fluids, and it is described by A. Couto et al. [8].

The use of dipsticks implies the reading of the results after a certain time, determined by the strips manufacturer. Considering that diapers are changed at regular intervals, and these are longer than the suggested reading time of dipsticks, it is important to ensure the validity of the results until the moment

of the analysis. This issue was considered in the design of the device with the implementation of a self-locking system and the cover layer, to prevent the fluid evaporation, the system contamination, or the deactivation of reagents [7]. In this design, described by A. Couto et al. [8], the self-locking system consists of a set of layers that, when combined, and after the collection of a sample, prevent the entry of more body fluids. Furthermore, to avoid the leakage of the reagents between pads, and to improve the possibility of accurate results, the design also comprises wax hydrophobic barriers and a polytetrafluoroethylene (PTFE) frame (Figure 2.1 (A)).

One of the most common substance monitored in the urine is glucose because the tests are usually used to confirm a diagnosis of diabetes mellitus or to monitor it [9]. The colorimetric detection of glucose is based on its reaction with oxygen by the action of the glucose oxidase to form gluconic acid and hydrogen peroxide. The latter oxidizes the indicator (e.g. Tetramethylbenzidine) in the presence of peroxidase leading to a color change in the pad. The reactions that allow the detection of glucose are summarized in figure 2.2. These can lead to different color results, depending on the glucose concentration, as shown in Figure 2.4.

$$\text{Glucose} + O_2 \longrightarrow \text{Gluconic acid} + H_2O_2, \text{catalyzed by glucose oxidase} \quad (1)$$

$$H_2O_2 + \text{Chromogen} \longrightarrow \text{Oxidized chromogen} + H_2O, \text{catalyzed by peroxidase} \quad (2)$$
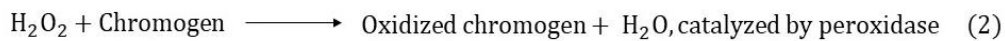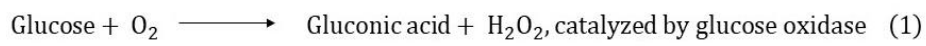
Figure 2.2 - Reactions that lead to the colorimetric detection of glucose [4,10].

On the other hand, the routine screening for nitrite is useful for urinary tract infections (UTIs) detection. The nitrate consumed through diet can be converted to detectable levels of nitrite, by the nitrate-reducing bacteria causing the UTI, if the urine is retained for enough time in the bladder. Even though this approach is commonly used for identifying patients with asymptomatic bacteriuria [10], it can also be applied to the cases of patients with symptoms, but who may be unable to communicate the presence of this signs [5], like the elderly or children.

The detection of nitrite is based on the principle of Griess test: in the presence of an acid buffer, the nitrite reacts with an aromatic amine (e.g. *p*-arsalinic acid or sulfanilamide) to form a diazonium compound, which couples with an indicator (e.g., 3-hydroxy-1,2,3,4-tetrahydrobenzo-(h)-quinoline or N-naphthylethylenediamine dihydrochloride (NED)) to produce a pink color. The reaction chain is summarized in Figure 2.3. Depending on the nitrite quantities, there are 3 possible colors, as shown in Figure 2.4.

$$\text{Aromatic amine} + \text{Nitrite} \longrightarrow \text{Diazonium salt} \quad (3)$$

$$\text{Diazonium salt} + \text{Indicator} \longrightarrow \text{Azo dye} \quad (4)$$

Figure 2.3 - Reactions that lead to the colorimetric detection of nitrite [10].

In this work, the reagent pads for glucose and nitrite detection were tested with a coating of plastic film, to avoid evaporation or contamination problems, as mentioned before. These issues were also considered in the device design, so the purpose of the experiment is to reproduce these same conditions and study the results variations over a period of 8 hours.
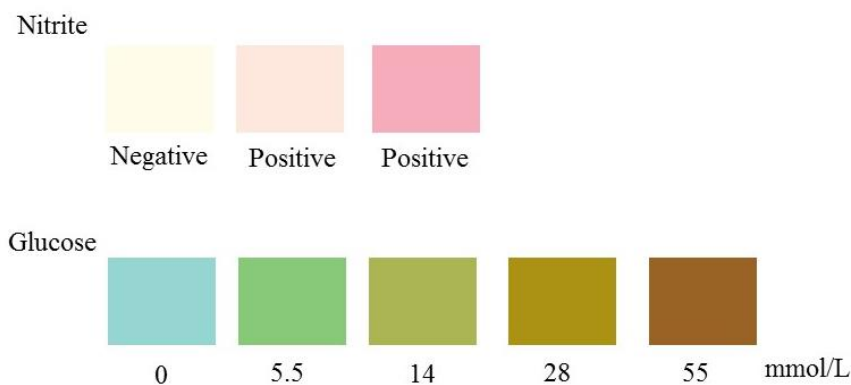
Figure 2.4 - Reference colors of the reagent pads for nitrite and glucose detection. Each color is associated with a negative or positive result or even to a concentration value.

2.3 MATERIALS AND METHODS

The PTFE sheet with a thickness of 1 mm was acquired from Yangzhong Fuda Insulated Electrical Co., Ltd., China. A multifunction printer (Aficio™MP C300SR) was purchased from Nashuatec. The dipsticks were purchased from Jilin Shenhua Medical Equipment Co., Ltd, China, and the artificial urine sample (qUAntify® Control) was acquired from Bio-Rad Laboratories, Inc.

The developed colors were recorded using the scanner mode of a multifunction printer (Nashuatec, Aficio™MP C300SR) with a 600-dpi resolution. The captured images were acquired in JPEG format and imported to an imaging process program to get the mean intensity values of the pixels in RGB scale (Red, Blue, and Green channels), within a selected image area.

2.4 COLOR RETENTION ASSAYS

2.4.1 Light Conditions

A square with a reference color was printed (RGB values: R: 128; G: 128; B: 128) and scanned several times. The RGB values were not as expected, since none of the channels presented a value of 128, but that does not necessarily mean that the scanner measured the wrong color. It can be, for example, the printer that prints the incorrect color. Nevertheless, and for this assay, from the results significant differences between replicas (Figure 2.5) could not be inferred, suggesting that the use of the scanner, to record the results, would keep the light conditions, which is known as an important factor in the color detection [11,12].

2.4.1 Test Procedure

The main purpose of the conducted experiment was to check, after the reaction, the stability of the resulting color of the reagent pad for a period of, at least, 4 hours. To simulate the desired conditions of the device, 4 reagent pads for each biomarker (nitrite and glucose) were placed separately in a PTFE sheet to have a hydrophobic surface between the pads.

For each set of pads: a volume of 3 mL of artificial urine was added to 3 of the pads, and other 3 mL of distilled water to the other pad, to be used as a reference. The assembly was covered with a plastic film to prevent the pads from drying out.

The recording of the developed colors started 1 minute (the waiting time referred by the manufacturer) after the beginning of the reaction and it was performed over a period of 8 hours. The images were analyzed with an imaging process software to get the RGB values of each color.

The values of the 3 assays obtained with the use of artificial urine sample were compared with those obtained as a reference, and their absolute difference was plotted against the time of reaction, to have a background-corrected response [12] For statistical analysis, the Tukey test was applied.
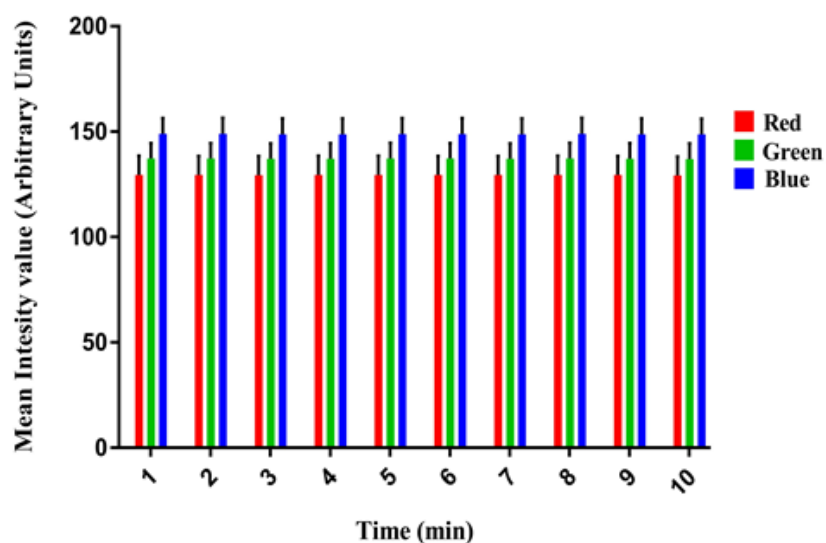
Figure 2.5 - Experimental colorimetric results of a printed square painted with a reference color (RGB values: R: 128; G: 128; B: 128) obtained with a scanner.

2.4.1.1   Glucose assay

After measuring the color at the recommended time, the results indicate that there were significant changes in the values of the red channel after 120 min. For the green and blue channels, the values displayed a transient change before reaching a steady state after 1 hour. (Figure 2.6 (B)). However, when the colors are interpreted by eye and compared with the reference (Figure 2.4), as it is done for the dipstick diagnostic, the results suggest that the color was stable for the whole experiment (Figure 2.6 (A)). According to the manufacturer, it is possible to have the colors correlated with a concentration between 5.5 and 55 mmol/L. So, besides of being apparently stable during the experiment, the visual interpretation is also in agreement with the expected colors.
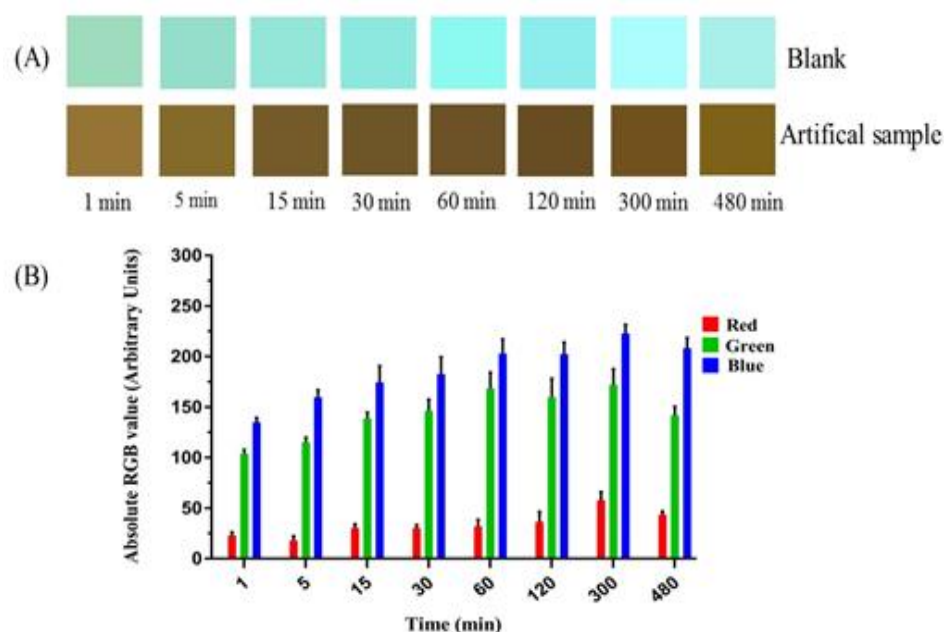
Figure 2.6 - Monitoring of the colorimetric response of the reagent pads for glucose detection over 8 hours. (A) Experimental colorimetric results of the reagent pads spotted with distilled water (blank) and artificial urine sample. (B) The absolute difference between the intensity values of the pixels obtained from the images of the reagent pads spotted with the artificial urine sample and distilled water, in RGB scale. The values were recorded over a period of 8 hours. Each datum is the mean of 3 assays.

2.4.1.2 Nitrite assay

For the nitrite pad, the results suggest that there is not a significant difference of the RGB profile of the recorded images since the beginning of the reaction until the end of the experiment. When the obtained colors (Figure 2.7) are visually compared with the reference colors (Figure 2.4) and with the outcomes of the reagent pads spotted with distilled water, the results suggest that the artificial sample does not have nitrite in its composition. However, according to the information provided by the sample manufacturer, it should have given a positive result.
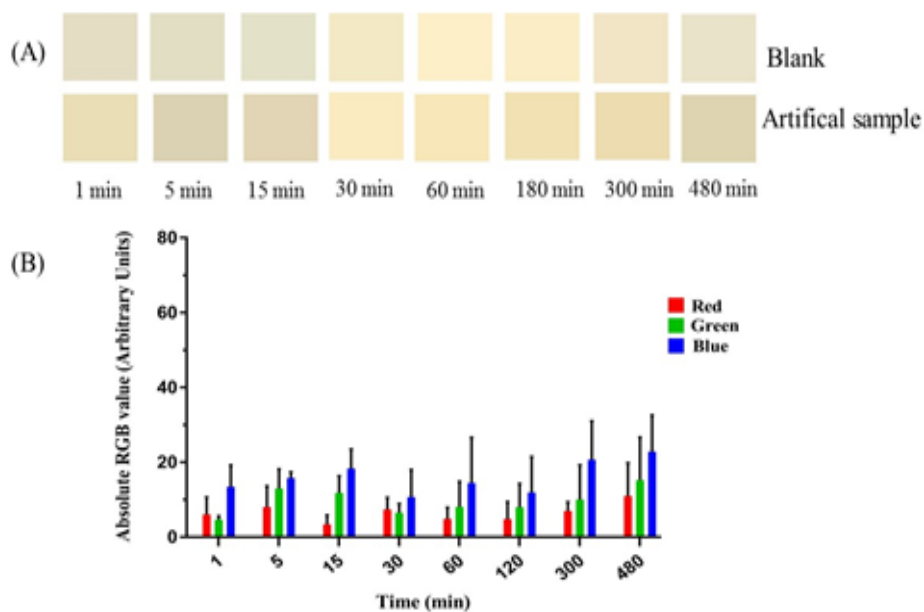
Figure 2.7 - Monitoring of the colorimetric response of the reagent pads for nitrite detection over 8 hours. (A) Experimental colorimetric results of the reagent pads spotted with distilled water (blank) and artificial urine sample. (B) The absolute difference between intensity values of the pixels obtained from the images of the reagent pads spotted with the artificial urine sample and distilled water, in RGB scale. The values were recorded over a period of 8 hours. Each datum is the mean of 3 assays.

2.5 CONCLUSION & FUTURE WORK

By using diapers as an instrument of simultaneous sampling and urinalysis, it is possible to improve the patient life quality as well as the efficiency of the work of the personnel responsible for their treatment. The device presented in this work allows the collection of one single urine sample, preventing contamination and entrance of more body fluids, and at the same time is capable of detect and analyze the sample in a short period of time [8].

The extended color retention, from the moment of sample collection until color analysis, allows a more efficient medical care since there is no need of additional and long procedures, but rather it simply requires the analysis of the developed colors when the diaper is changed.

The color changes are easily visible, and they may indicate problems pertaining the patients. It is also important to remember that this kind of procedure it's mostly applied to discard the presence of illness, rather than diagnose. If there is any signal of abnormalities, it is important to make more specific analysis to support the initial suspicion.

The device purposed in this work provides the necessary conditions to hold the results longer than the required time recommendations indicated by the dipstick manufacturer. In the case of glucose detection, the colors showed variations in their RGB profile, but visually it appears as they are associated with the same reference color. For the nitrite assay, even though the results were not the expected (negative instead of positive), the RGB profile it is constant during the experiment. This observation is also supported when the obtained colors are visually compared with the reference colors. Another method should be used to verify the nitrite presence in the sample. However, this manual procedure of color comparison is often associated with lower accuracy because each individual can have a different color interpretation. It is preferable to use an electronic reader, such as a camera or a scanner because it allows a more precise record of the results, analogously to the procedure performed in this work. The use of a smartphone with a built-in camera and a proper software application to record, analyze and send the data is considered as fast and reliable solution [11].

The device holds great potential for keeping the results stability, so the future work will focus on testing this color changes in the fabricated device, using an appropriate reader and software to analyze the developed colors, and check how much the variations can influence the achievement of the results for each biomarker. It would be important to test different standard concentrations of the analyte of interest and study the color response during the time, in the intended environment, to investigate, for each substance, if such variations are constant.

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CHAPTER 3 |

Modification of Microfluidic Paper-Based Devices with Dyes Nanomaterials Obtained by Encapsulation of Compounds in Y and ZSM5 Zeolites[2]

ABSTRACT

Zeolite nanostructures were used as hosts for dyes normally used in some bioassays (pH and protein), and they were applied on microfluidic paper-based analytical devices (µPADs). The obtained dye nanomaterials were characterized by several techniques (structural (FTIR and XRD), surface (SEM/EDX), textural (N2 adsorption) analyses and dye loading determination by TGA analysis), to confirm the stability of both host and guest. After their deposition on the paper surface, the color change was studied by analyzing the RGB values. The resulting colors were stable for a period of one week, and it was observed that the choice of the paper for the µPAD as well as the zeolite nanostructures properties also influences the colorimetric response. This encapsulation of the dyes into zeolites solve current challenges in colorimetric sensors where, long-term stability of the colorimetric results is an issue. The results obtained were promising for the studied assays, and the strategy could also be extended to other bioassays.

3.1 INTRODUCTION

The interest in paper as a platform for analytical processes has being growing in the last years, especially with the development of microfluidic paper-based devices (μPADs) [1, 2]. The main reasons for μPADs popularity are their low-cost, safe disposal, simplicity, the use of small quantities of reagents and samples, portability and the fact that there is no need of an external source of power [2-5].

The μPADs are associated with several types of detection methods, among them the colorimetric detection is one of the most usual, because it offers an easier readout of the results [6]. For this method, a colored reagent must be added to the detection zone on the surface of the μPAD to allow the detection of the respective analytes by a color change [7]. The analytical performance of μPADs was extensively studied and several solutions based on treatment and modification of the cellulose fibers with nanoparticles [8-10] or biopolymers [4] were reported in the literature as a procedure to improve the detection step. However, these modifications were only made regarding the color uniformity without consider its stability during long periods of time. Keeping the validity of the results for more time it is also important to keep the analysis independent on the short and specific interval of time needed for reading the results, as it happens with dipsticks [11], improving the interpretation and precision of the data.

In order to preserve the colorimetric results, the encapsulation of the dyes into zeolite nanostructures appears to be interesting since, the solid support as host preserves the molecular integrity of the components for the assays during time. In the host-guest chemistry, several works have shown that the zeolite nanostructures are interesting as host [12,13]. Zeolites are solid aluminosilicates normally used as catalysts or sorbents [12, 14, 15].

However, from our research in literature, there were no studies with zeolites for color stability purposes and applications on μPADs. Nevertheless, it has already been reported zeolite deposition on cellulose fibers through different methods [16].

Inspired in the host-guest chemistry, three dyes were encapsulated into two different zeolite structures. Due to their clinical relevance, the tests described in this work were focused on pH and protein analysis. So, bromophenol blue dye (BPB) and bromothymol blue dye (BTB) were encapsulated into USY zeolite and methyl red dye (MR) into ZSM5. The obtained dye nanomaterials were characterized by Room Temperature Fourier Transform Infrared (FTIR), N_2 adsorption isotherms, scanning electron microscopy (SEM/EDX), thermogravimetric analysis (TGA) and powder X-ray diffraction (XRD). A simple process was performed for deposition of the dyes nanomaterials in the surface of μPADs, and solutions with differences

of pH values and protein concentrations were tested. The color change was analyzed by RGB measurements. The stability of the μPADs after deposition was analyzed *via* scanning electron microscopy (SEM). These results are relevant and promising for optimization of these dye nanomaterials for colorimetric clinical diagnosis processes.

3.2 EXPERIMENTAL SECTION

3.2.1 Materials and Reagents

Two zeolite nanostructures, commercially available in white powder form, $(NH_4)USY$ (ultrastabilized Y zeolite, CBV500) and $(NH_4)ZSM5$ (CBV3024E), were obtained from Zeolyst International. Both zeolites were used after a thermal treatment at 120 °C for 12 h in an oven. The two types of filter paper (Whatman No. 1 and Whatman No. 542) used in this work were acquired from Merck. The dyes, bromophenol blue dye (BPB), bromothymol blue dye (BTB) and methyl red dye (MR) as well as the nujol, citric acid, sodium citrate, and the bovine serum albumin (BSA) were purchased from Sigma Aldrich. Other chemicals used in experiments were of reagent grade. The stock solution of BSA (60 μM) was prepared by dissolving the proper amount of BSA in water. The same stock solution was further diluted in different ratios to obtain the desired concentration range used in this work. Solutions with different pH values were prepared by combination of different volumes of sodium hydroxide (0.5 M) and hydrochloric acid (0.1 M) solutions and analyzed by a pH sensor acquired from Hanna instruments.

3.2.2 Encapsulation Method

The dyes were successfully encapsulated inside the zeolite nanostructures using the method described by Neves et al. [13]. Briefly, each dye (0.50 mmol) was dissolved in 50 mL of ethanol and these solutions were added to 1 g of the zeolites. The resulting mixtures were stirred for 48 h at room temperature. The suspensions were filtered off, washed with ethanol, and dried in an oven at 60°C for 12 h. Finally, the samples were subjected to Soxhlet extraction for 12 h in ethanol (150 mL) to remove the remaining dye molecules and the species adsorbed on the external surface of the zeolite. After that, the resulting dye nanomaterials were dried in an oven at 60°C for 24 h. Bromophenol blue (BPB) and bromothymol blue (BTB) dyes were encapsulated into USY and methyl red (MR) dye into ZSM5. The dye nanomaterials obtained were denoted as BPB@USY, BTB@USY and MR@ZSM5.

3.2.3 Characterization

Philips Analytical X-ray model PW1710 BASED diffractometer system was used for obtaining the powder X-ray diffraction patterns (XRD) of the samples. The powder patterns were performed at room temperature, using Cu Kα radiation in the 2θ range between 5° and 60°. ASTM D 3906-80 method was used to determine the crystallinity of USY, BTB@USY and BPB@USY, while ASTM D 5758 method was used for ZSM5 and MR@ZSM5. SEM micrographs were collected on a JOEL JSM-6010LV/OXFORD scanning microscope equipped with an energy-dispersive x-ray spectroscopy (EDX) system, INCAx-Act-PentaFET Precision. To avoid surface charging, samples were coated with gold in a vacuum environment prior to analysis, by used of a Fisons Instruments SC502 sputter coater. The textural characterization of the samples was based on the N_2 adsorption isotherms, determined at -196 $^{\circ}$C with a Quantachrome NOVA 4200e apparatus. The samples were previously outgassed at 150 $^{\circ}$C under vacuum. The micropore volumes (V_{micro}) were calculated by using the t-method. Surface areas were calculated by applying the BET equation. Room-temperature Fourier Transform Infrared (FTIR) spectra of the samples in KBr pellets were measured using a Bomem MB104 spectrometer in the range 4000-500 cm^{-1} by averaging 20 scans at a maximum resolution of 4 cm^{-1}. The thermogravimetric analysis (TGA) was performed with a STA 409 PC/4/H Luxx Netzsch thermal analyzer to determine the thermal stability and the amount of the dyes in the samples. The atmosphere used was high purity air (99.99 % minimum purity) with a flow rate of 50 cm^3/min. The sample holders used were crucibles of alumina oxide, supplied by Netzsch. The samples have been heated between 50 and 700 $^{\circ}$C at 10 $^{\circ}$C/min to evaluate their thermal stability.

3.2.3 Bioassays

Both filter papers were cut based on a device design with four branches (3 x 5 mm) and testing areas (4 x 7 mm), and a central zone (10 x 10 mm) for sample inlet (Figure 3.1). For the protein assay, each testing zone was firstly spotted with 4 µL of 250 mM citric buffer solution (pH 1.8), and allowed to dry for 15 min at room temperature. After that, 4 µL of a mixture containing 5 mg of BPB@USY and 3 drops (approximately 0.1 mL) of nujol was spotted in each testing area, and the paper dried for additional 60 min at room temperature. For the pH assay, 4 µL of a mixture with BTB@USY and 3 drops (approximately 0.1 mL) of nujol was added to two testing areas and 4 µL of a mixture with MR@ZSM5 and 3 drops (approximately 0.1 mL) of nujol in the other two remaining testing zones. The paper was dried for 60 min in the same conditions as before. Finally, the test solutions (60 µL) for protein or pH were added to the central zone and allowed to reach all the testing areas of the device. The paper was allowed to be air-

dried during 90 min before an image was acquired using an office scanner (Toshiba e-STUDIO 2050c) with a 600-dpi resolution. The recorded images were converted to a RGB scale and analyzed with Adobe Photoshop CS4 software, by recording the mean of the pixel intensity, for the red, blue, and green channels, within each detection zone.
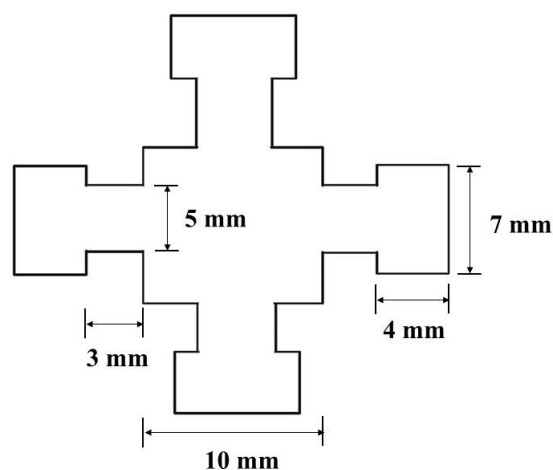
Figure 3.1 - Schematic representation of the μPAD design used in this study. The proposed μPAD has four branches (3 x 5 mm) and testing areas (4 x 7 mm), and a central zone (10 x 10 mm) for sample deposition.

3.3 RESULTS AND DISCUSSION

3.3.1 Preparation and Characterization of the dye nanomaterials

Three different dye molecules usually used as pH indicators were encapsulated in void space of two zeolites nanostructures, USY, a large pore and ZSM5, a medium pore. The choice of the zeolite was associated to the molecular structure of the dyes (Figure 3.2). The pH indicators, BTB dye, 4,4′-(1,1-Dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis(2-bromo-6-isopropyl-3-methylphenol)) and BPB dye, 4,4′-(1,1-dioxido-3H-2,1-benzoxathiole-3,3-diyl)bis(2,6-dibromophenol) show comparable molecular structures. For these guest molecules, the zeolite USY was selected since its structure has a 3D network with large void spaces, with a diameter of 1.3 nm known as supercages [14]. However, for the linear molecule of the methyl red (MR, 2-(N,N-dimethyl-4-aminophenyl) azobenzenecarboxylic acid) dye, the zeolite ZSM5, 3D network with pore diameters around 0.56 nm, was used as host [14].
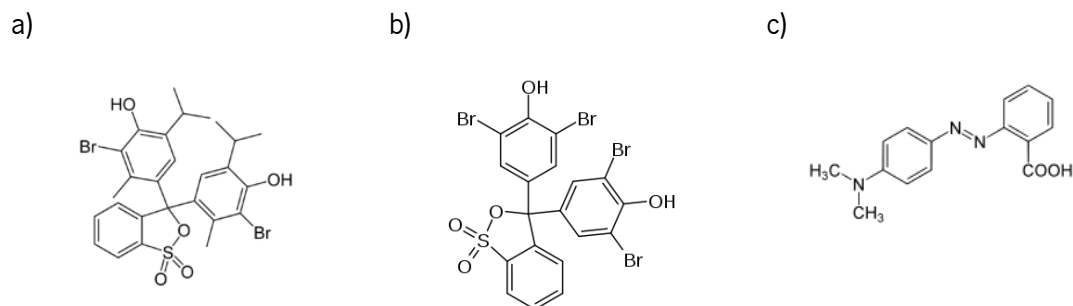
Figure 3.2 - Molecular structure of the dyes: a) bromothymol blue (BTB), b) bromophenol blue (BPB) and c) methyl red (MR).

These dyes were successfully encapsulated into the zeolite nanostructures and the method used in this work does not affect the molecular structure of the dyes (guest) and the respective zeolite (host). Figure 3.3 shows that the hosts assimilated the same color as the dyes used in this work, indicating that these compounds are effectively encapsulated inside the host after the encapsulation process.
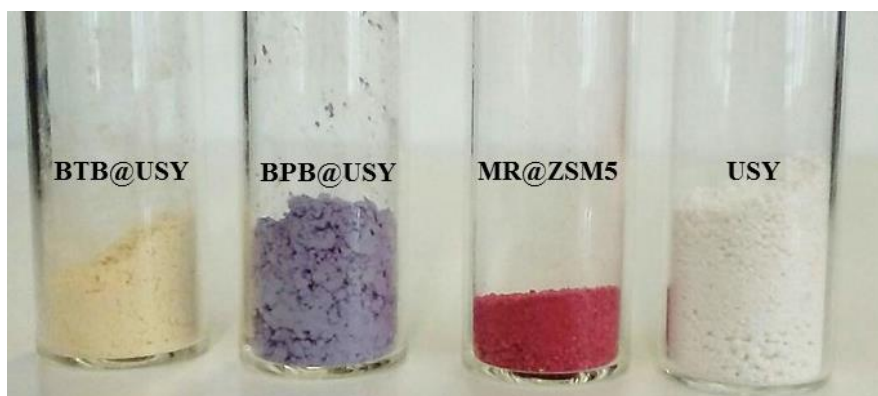
Figure 3.3 - Photograph of BTB@USY, BPB@USY, MR@ZSM5 and USY in powder form. The zeolite ZSM5 is a white powder similar to USY.

The structural characterization of the dye nanomaterials was performed by XRD and FTIR analysis. The results of X-ray powder diffraction (data not shown) confirm that both zeolite nanostructures, after encapsulation, exhibited the typical and similar patterns of a crystalline faujasite and MFI zeolite structures [18]. The powder XRD diffraction patterns of zeolites and dye nanomaterials were recorded at 2θ values between 5 and 60°. All XRD patterns present the characteristic peaks of the host zeolites, even after the dyes encapsulation, with high crystallinity: 90% for BPB@USY, 95% for BTB@USY and 95% for

MR@ZSM5.FTIR analysis confirm the preservation of the molecular integrity of the dye molecules and the zeolites. FTIR spectrum display the characteristic bands of the dye molecules and zeolite nanostructures (Figure 3.4).

Figure 3.4 - FTIR spectrum of a) bromothymol blue dye (BTB), b) zeolite USY and c) BTB@USY.

All dye nanomaterials were dominated by the typical strong bands of the host structures: the ν(O–H) stretching vibration at 3500 cm^{-1} and the δ(O–H) deformation band at 1650 cm^{-1} from the T-OH groups and the physiosorbed water were identified and the bands corresponding to the lattice vibrations were observed in the spectral region between 1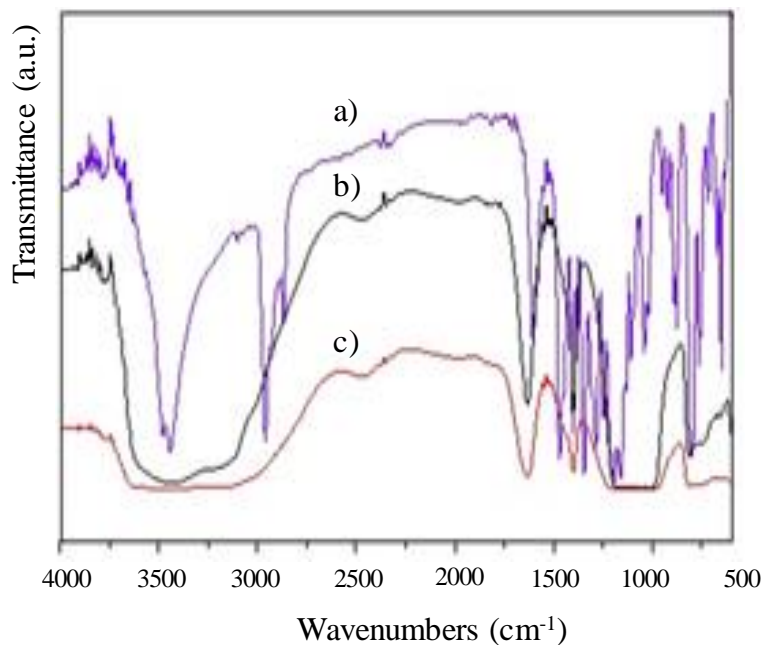250 and 500 cm^{-1}[19,20]. In the FTIR spectrum of BTB@USY and BPB@USY (data not show), the characteristic vibration bands of dyes at 1195 and 1025 cm^{-1} attributed to SO$_3$ group and the ν(C–Br) stretching vibration at 650 cm^{-1}, were detected and corresponded to the typical bands of these dyes [21]. In the case of the FTIR spectra of MR@ZSM5, the presence of the dye was detected by the vibration bands at 1550 and 1530 cm^{-1} attributed to azo chromophore [21]. SEM analysis confirmed that the morphology of the zeolite nanostructures was preserved after the dyes encapsulation. The typical morphology of the zeolites hosts is present in all dye nanomaterials and no significant differences were found after the preparation. For the dyes prepared with USY, the EDX analysis confirmed the presence of the dye molecule in the zeolite by the quantification of bromine in both dye nanomaterials. Figure 3.5 shows the SEM micrographs of USY and BTB@USY and the EDX analysis of BTB@USY and BPB@USY, as example.
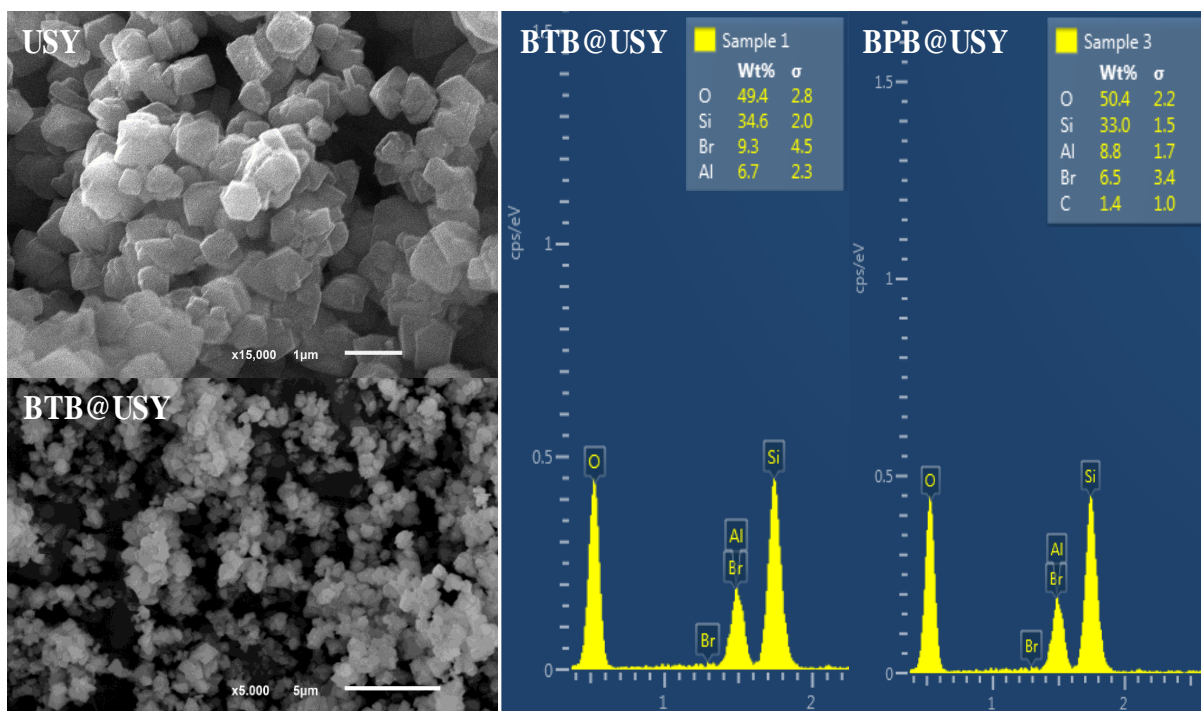
Figure 3.5 - SEM images of USY and BTB@USY with different resolutions and EDX spectrum of BTB@USY and BPB@USY.

Thermogravimetric analysis (TGA) was carried out to quantify the amount of the dyes encapsulated into zeolite nanostructures. TGA curves of the dye nanomaterials are similar and they present weight changes between, 120 °C and 500 °C, in the studied temperature range from 50 up to 700 °C. As expected, all dye nanomaterials showed a small weight loss at 150 °C, attributed to the removal of physiosorbed water from the zeolite structures [23]. The weight loss observed in the extended range of temperature was attributed to the decomposition of the dye molecules. The amount of the dyes into the hosts are displayed in Table 3.1. As expected, MR@ZSM5 has a higher amount of the encapsulated dye due to the small size of the molecule followed by the large BTB and BPB molecules in the USY host. So, the encapsulation efficiency was higher for ZSM5 with 60% followed by USY, with 48% for BTB@USY and 40% for BPB@USY.

Table 3.1 - Initial amount of the dye molecules and TGA results.

Dye nanomaterials	Dye$_0$ (mmol/g$_{host}$) [a]	Dye$_f$ (mmol/g$_{host}$) [b]	Yield (%) [c]
BPB@USY	0.50	0.20	40.0
BTB@USY	0.50	0.24	48.0
MR@ZSM5	0.50	0.30	60.0

[a]Initial dye amount in the solution; [b]dye encapsulated into zeolite host determined by TGA; [c]Encapsulation efficiency of dye into zeolite hosts.

A type-I isotherm, typical solids with a microporous structure, was identified for all dye nanomaterials by the N_2 adsorption isotherms, with slight hysteresis loops which suggests some degree of mesoporosity, typical of ZSM5 and USY [20, 24]. Similarly, the encapsulation method does not significantly modify the original porosity, since the shapes of both adsorption and desorption isotherms of the dye nanomaterials were similar to those of the hosts. The micropore volumes (V_{micro}) were calculated by the t-method, and the total surface areas were calculated by applying the BET equation (S_{BET}), as displayed in Table 3.2. The encapsulation of the dye molecules led to a decrease of 35% for both BTB and BPB, and 41% for MR in the adsorption capacity of the host zeolites. These results are consistent to the reduction of the microporosity as a consequence of the encapsulation of the dye molecules.

Table 3.2 - Textural characterization of the hosts and dye nanomaterials.

Sample	S_{BET} (m^2/g)	V_{micro} (cm^3/g) [a]	$V_{P/Po=0.95}$ (cm^3/g) [b]
USY [23]	750	0.269	0.355
BTB@USY	485	0.198	0.289
BPB@USY	490	0.207	0.289
ZSM5 [19]	395	0.096	0.231
MR@ZSM5	235	0.080	0.154

S_{BET} – Surface area calculated from BET equation; [a]Micropore volume obtained by the t-method; [b]Total pore volume for P/P$_0$=0.95

3.3.2 Protein and pH assays

Before starting the colorimetric assays, the office scanner, used to record the images, was tested. The light conditions and the focus of the images are important factors to obtain reproducible results[24]. Therefore, a square of a reference color (RGB values: Red channel - 128; Green channel - 128; Blue channel - 128) was printed and scanned several times. Since there were not significant differences between the replicas, the same scanner was used to record the images of the bioassays.

Two of the selected dyes, BTB and MR, are normally used to determine pH values [26]. With BTB, a yellow color is obtained at pH values below 6 and a blue color at values above 7.6 [28]. For the MR, a red color is observed at pH values below 4.4 and a yellow color at values higher than 6.2 [29]. After the encapsulation process, the BTB@USY and MR@ZSM5 were spotted in separated testing areas of the μPAD, and several solutions with different pH values were analyzed. The color results are displayed in Figure 3.6. For both dye nanomaterials, the color change occurred at pH values higher than expected. This could be explained by the acidity behavior of the zeolites [27], which contribute to a more acidic environment and therefore the color change is only observed at higher pH values.

In order to analyze the effect of the dyes encapsulation in the color changes, the stability of the colorimetric results was also studied (Figures 3.7 and 3.8). The results of both dye nanomaterials did not show a significant difference over the time, for all the RGB channels, with acid or basic pH values Comparing with parent μPADs, the use of these dye nanomaterials successfully improved the color stability and intensity. The RGB values vary between values of 0 (the darkest colors) and 255 (the lightest colors). As shown in Figure 3.6, lighter colors were observed with parent μPADs, since the RGB values are closer to 250 when compared with the results obtained with dye nanomaterials.

The pH factor was selected because of its importance in several areas of science, especially the human physiology. Urine is one of the body fluids that can be analyzed and give several information about the patient health. The pH of the urine is normally between 4.5 and 8.0, and values bellow or above this range are physiologically impossible. Unless, for example, a highly alkaline substance, like a therapeutic agent, has been given to the patient, which can result in pH values above 8.0 [26]. The colorimetric results (Figure 3.6) showed that the dye nanomaterials can be used in the clinically relevant ranges, despite the need of higher pH values for observing color changes.
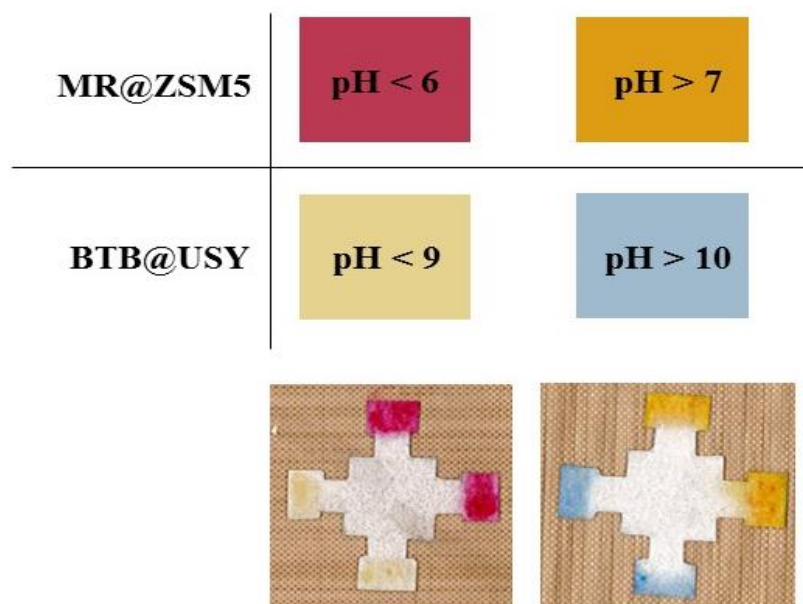
Figure 3.6 - Experimental colorimetric results of the pH assay obtained with the dyes nanomaterials and respective pH values.

The pH of the urine can also vary due to several reasons, from pathologies to the kind of diet that is being made, or the medication that the patient use [2]. Consequently, despite the utility of studying the pH values of urine, this parameter should be related with to other parameters in urine, like protein. It is essential to compare these two parameters since alkaline pH can lead to false-positive results in the protein assay that is used to get colorimetric results [30, 31].

The protein assay uses the principle known as protein error indicator. The pH value is held constant by the buffer (pH 3), which in the absence of urine protein allows the yellow color of the reagent area. The development of any green to blue color indicates the presence of urine proteins, due to the release of hydrogen ions by the indicator dye (e.g., BPB dye) to the protein (acts as a hydrogen receptor) [25, 30]. Proteinuria is the name given when there is an increased amount of protein in the urine, and it can be associated, in the majority, to renal diseases. There are other conditions such as fever and exercise that can also lead to abnormal quantities of protein in the urine [30]. Healthy adults present a urine excretion of total protein less than 150 mg/day, and a concentration less than 4 μM [32].

Figure 3.7 - Stability of the RGB values obtained for a solution with a pH value of 4.5: a) Results obtained with the dye nanomaterials; b) Values recorded with parent μPADs. The images were recorded until one week, and each value is the mean of three assays.
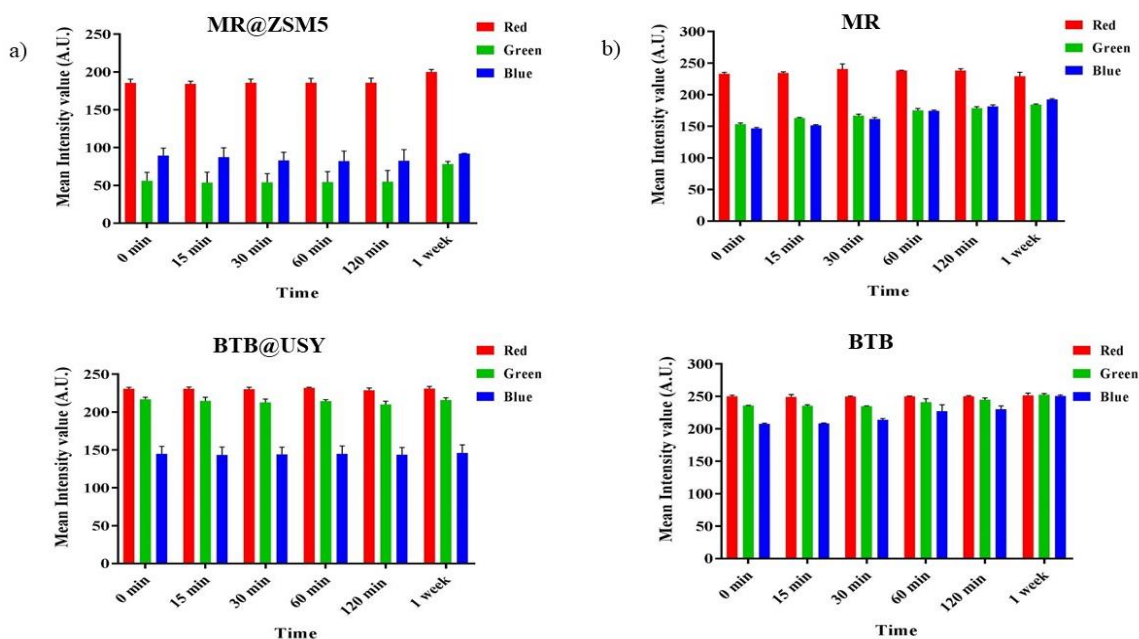

Figure 3.8 - Stability of the RGB values obtained for a solution with a pH value of 13: a) Results obtained with the dyes nanomaterials; b) Values recorded with parent μPADs. The images were recorded until one week, and each value is the mean of three assays.
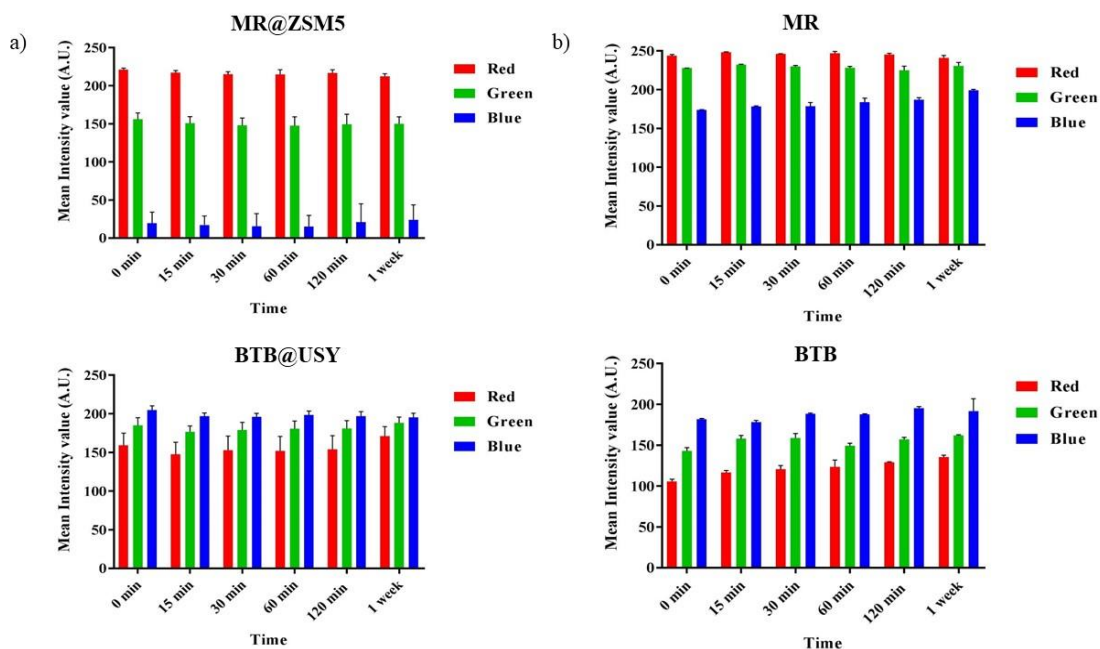
The protein assay was performed with two types of Whatman papers: No. 1 and No. 542. The results displayed in the Figure 3.7 have suggested that, even though the three RGB channels had a similar behavior, the paper type influences the obtained colors. To have a background-corrected response, the values obtained for three assays were compared with those obtained as a reference (0 μM) and they are plotted over BSA concentrations. Comparing the values obtained with both types of paper, it was observed that for Whatman No. 1 there is less color variation (the values are smaller) when compared with the results detected with Whatman No. 542. The visual interpretation of the colors also asserts this assumption (Figure 3.9 c)).
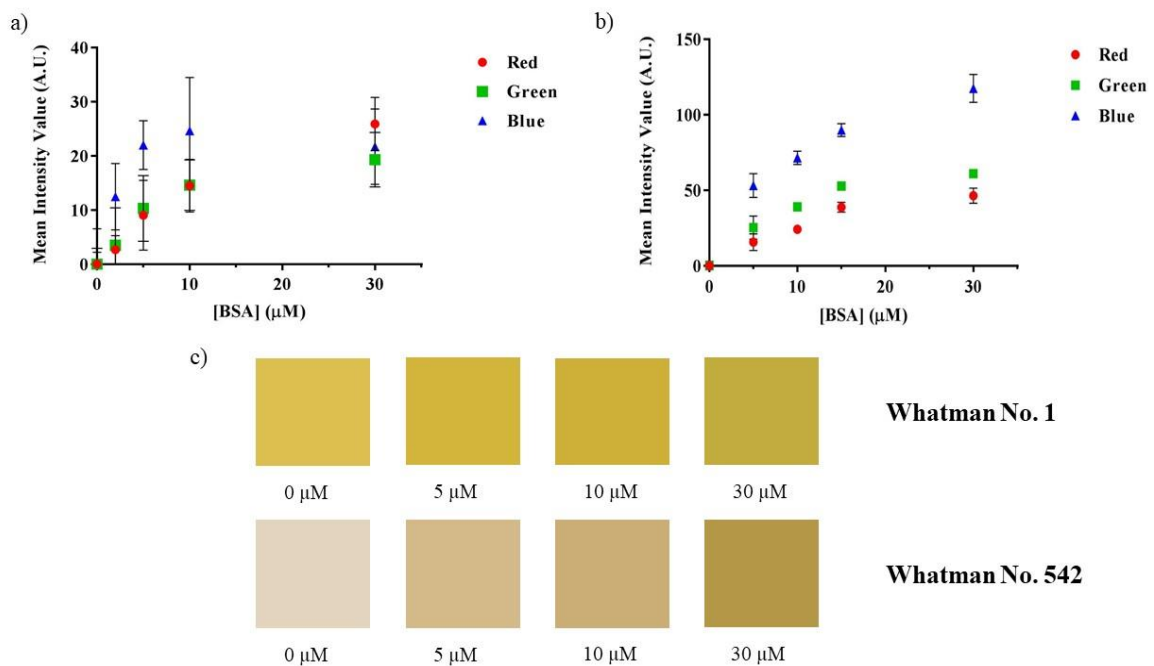
Figure 3.9 - Results of the protein assay with BPB@USY. a) Results obtained with Whatman No. 1 and b) Whatman No. 542. For colorimetric background-corrected response, the RGB values were compared with reference values (0 μM) and plotted over BSA concentrations. Each value is the mean of three assays. c) Experimental colorimetric results and respective BSA concentration.

Comparing visually the obtained colors for both papers, the results also suggested that Whatman No. 542 allowed less intense colors. This can be a consequence inherent to the paper properties. According to the manufacturer, Whatman No. 1 has a typical thickness of 180 μm, while the Whatman No. 542 has a thickness of 150 μm. This difference in the thickness values can influence the spread of the dye nanomaterials in the testing zones of the μPAD. In a thinner paper, the spread of the dye nanomaterials

will be greater than on thicker paper because of their limited downward penetration [33]. When the sample is loaded in the µPAD, it has the same behavior of the reagents deposited in the testing zones, depending on the used paper and, therefore if the paper allows larger diffusion of the analytes the colors will be less intense.

SEM analysis also confirmed this difference, where it was possible to observe that the BPB@USY was present in larger areas of testing zones when Whatman No. 542 was used (Figure 3.8), which can also explain why less color variation was observed with Whatman No.1 for different BSA concentrations. As the thickness of the paper is higher, the absorption of the sample is also greater and, therefore, the mass of the analyte which reaches the testing zones can be different, when compared with thinner papers [33]. Nonetheless, both types of paper can be used to detect proteins: Whatman No. 542 allows visual interpretation, although both types of paper can be analyzed with an electronic reader, with a scanner or a camera, to obtain results with higher accuracy [12]. Furthermore, the results for all RGB channels seems to have a similar behavior, which can be used for analysis of real samples.

Similarly, to the pH assay, the color stability feature for the protein assay with BPB@USY was evaluated. The results showed (Figures 3.10, 3.11 and 3.12) that for both types of paper and for all concentrations, the values did not exhibit significant differences for a period of one week, as it happened for the other dye nanomaterials.
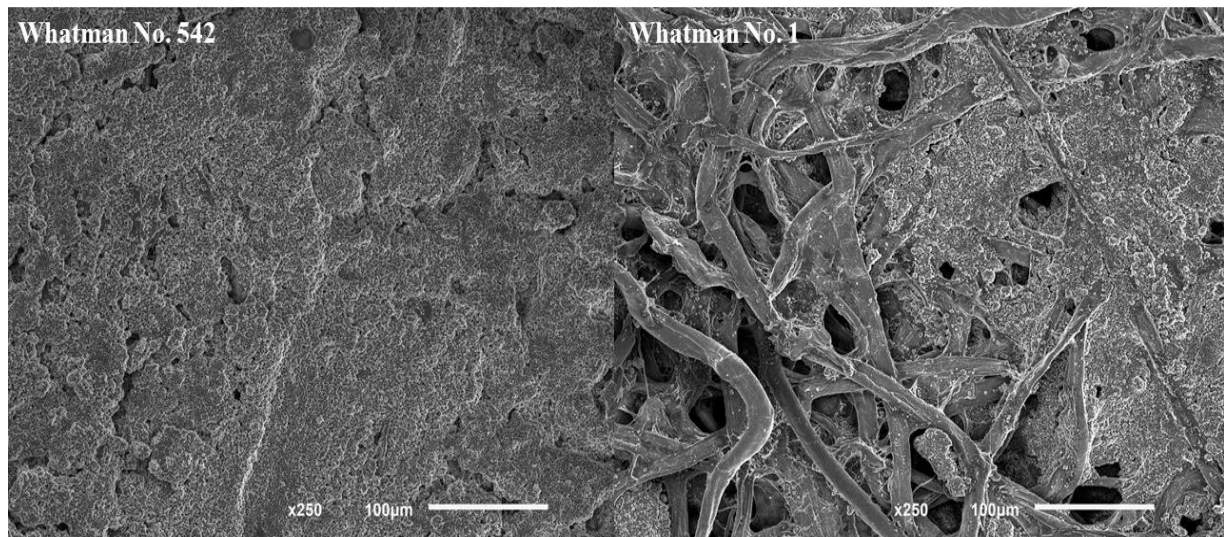
Figure 3.10 - SEM micrographs of Whatman No. 542 with BPB@USY and Whatman No. 1 with BTB@USY, with the same resolution.
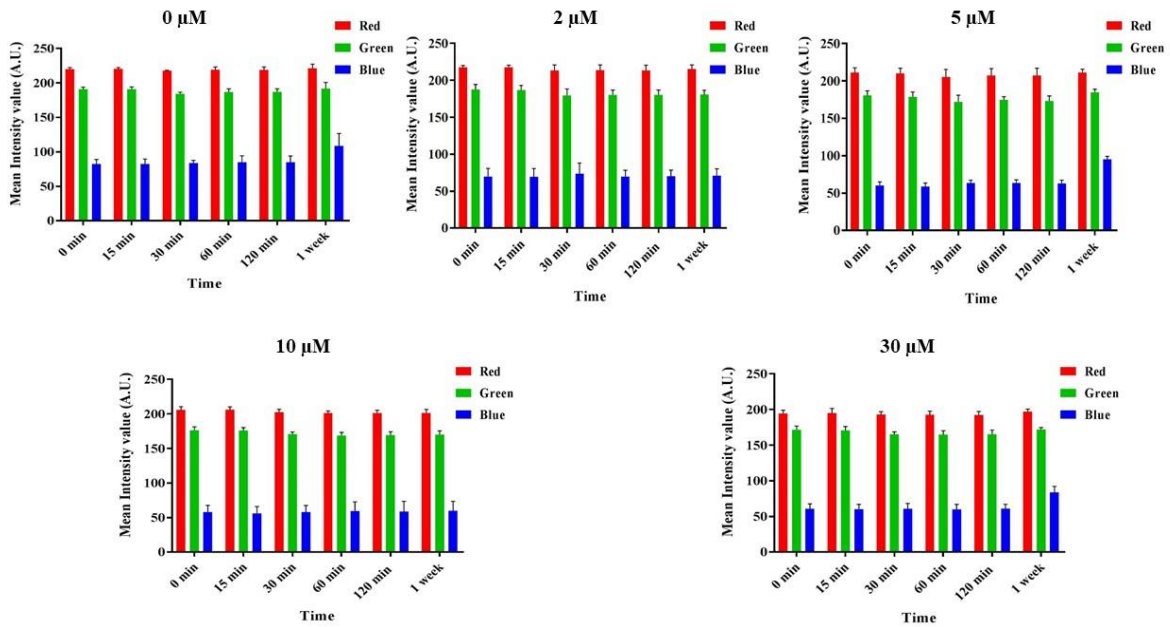
Figure 3.11 - RGB values obtained for the protein assay with BPB@USY and paper Whatman No.1. The images were recorded for one week, and each value is the mean of three assays.

Figure 3.12 - RGB values obtained for the protein assay with BPB@USY and paper Whatman No.542. The images were recorded for one week, and each value is the mean of three assays.

3.4 CONCLUSION

The successful encapsulation of three dyes, for protein and pH assays, on zeolite nanostructures enhances color stability when compared to original µPADs with no previous preparation. The encapsulation process was chosen to provide a solid support for the dyes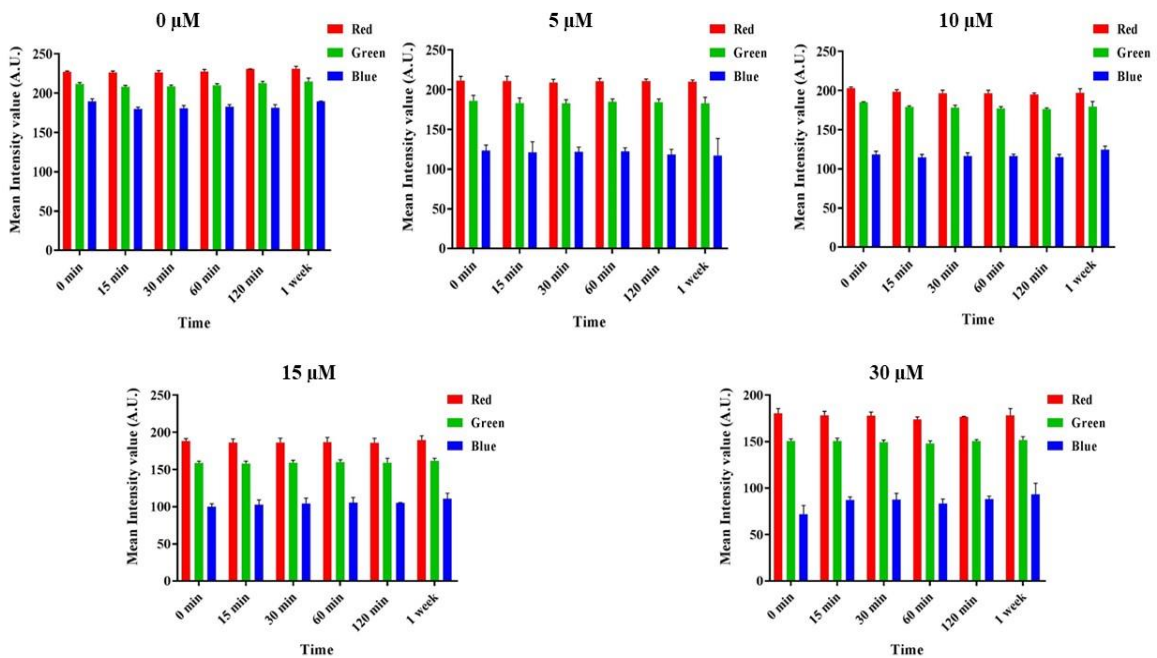, ensuring their molecular integrity and the preservation of the host structure. However, it is important to reckon the zeolites properties, for the correct choice of the hosts due to their influence on the colorimetric results. The type of paper used for producing the intended µPAD is also a factor to consider since it can influence the spreading of reagents and analytes. Nevertheless, both Whatman No.1 and No.542 can be used if the correct detection method is applied. The results are encouraging, and further work should be done for testing real samples and determination of the analytes based on the calibration data. The association of dye nanomaterials and µPADs a seems promising solution, which could be applied in other kind of assays, in addition to pH and protein, for clinical diagnosis.

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CHAPTER 4 |

Conclusions & Future work

Easy and real-time diagnosis or monitoring processes are a current demand in clinical environments, and therefore it is essential to improve the features of devices used for these objectives. In this work, a novel device design for urinalysis on diapers was studied, regarding the long-term stability of colorimetric results, which can be an issue, especially because the current solutions make the user dependent of short and specific intervals of time for data analysis. This device employs both microfluidics on paper and colorimetric detection, allowing a lower sample volume, when compared with other existing urinalysis techniques, and easier analysis and recording of the results.

First, the conditions provided by the device and their role in the results stability were analyzed, since the design comprises a self-locking system and a cover layer to prevent fluid evaporation or deactivation of reagents. The results showed that the device has great potential to improve color stability, as shown by the visual interpretation of the developed colors.

On the other hand, another strategy, which was never applied before, was considered: the encapsulation of dyes, normally used for biomarkers analysis (in this case, pH, and protein), in zeolite nanostructures, and their application on microfluidic paper-based analytical devices (µPADs). Several characterization techniques employed for the analysis of the obtained dyes nanomaterials showed that the encapsulation process was successful, since maintained the dye molecule integrity and the preservation of the host's structure. It was also observed that zeolite nanostructures properties and choice of paper for the device influence the colorimetric response and the results showed an improvement of the stability (for a period of one week).

Both approaches are promising, and future work will be conducted for testing real samples, and other environmental variations, like temperature, since this device is designed to be constantly exposed to body temperature. Furthermore, these strategies should be employed and tested for other bioassays, considering that both the device and use of nanomaterials can be combined to have stable colorimetric results for longer periods, for several bioassays, and in the future, it will be tested in real situations.

More improvements can be attained with the utilization of supplementary devices, like smartphones with a built-in camera and a software application to record, analyze and send the obtained data, allowing a more precise, rapid, easy, and portable diagnostic/monitoring clinical system.