Surface engineering of solid supports; Cellulose, Nitrocellulose and Nylon to increase the efficiency of antibody immobilization in diagnostic systems

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Abstract_ Immobilization of antibodies is a critical stage for design and manufacturing of sensitive biological sensors. In this study, in order to increase the capacity of antibody capturing by the supports, different materials and polymer layers such as Cyanogen bromide, Acetonitrile, PEI and MAMEC were used for processing the surface of Cellulose, Nitrocellulose and Nylon, respectively. Increasing the surface charges in nitrocellulose and creating new active groups on the surfaces of activated nylon and cellulose were the most important chemical modifications on the mentioned solid supports. Assessment of immobilized antibody showed that the chemical modifications applied on the surface of supports have had significant effects on increasing their capacity to accept antibody, compared to control samples. Efficient immobilization of antibody on the supports can increase the sensitivity of immunochromatographic-based diagnostic systems. Immobilization processes can alter the physiochemical features of supports such as their capillarity, flexibility, surface charges, delicacy etc. which may intensively influence the quality of immobilization.

Index Terms— Activation, Antibody, Cellulose, Immobilization Nitrocellulose, Nylon, Support.

1 INTRODUCTION

POINT-of-care diagnostic assays, the rapid, simple to use and inexpensive devices have become the common bioassay systems. Lateral Flow Immunoassay (LFIA), designed based on the immuno-chromatographic processes, is the standard format in such settings. This system has found many applications in diagnosis of a wide variety of biological and chemical agents. Sensitivity, specificity, precision and speed are of the important parameters in designing such systems. Diagnostic systems designed based on immunochromatographic processes and focusing on antibodies, generally present a limited sensitivity. This means that, in comparison with other diagnostic methods, these systems require bigger amounts of analyte and are not able to recognize analytes with concentrations lower than the threshold level. Among techniques, which can increase the sensitivity of system and improve the quality of diagnosis, are the use of inherent physicochemical characteristics of supports such as capillary flow rate, porosity, chemical composition and application of chemical methods as a useful means for changing the structure of supports and altering the format and structure of such systems. For instance, Fu and colleagues developed a paper network platform that extends the LFA conventional test to two dimensions. The two-dimensional structures allow the incorporation of multistep processes for improved sensitivity [1]. Increasing the binding rate of the biocatalyst to analytical supports used in such systems leads to increase their sensitivity. The chemical modification of functional groups of the supports is also helpful in increasing their capacity to accept the biocatalyst. One of the factors which impress the sensitivity, specificity, precision and speed measures is how the biocatalysts are immobilized on the support surface.

Antibodies and enzymes are usually stabilized through immobilization process, and remain attached to the support surface with stronger bonds, though incorrect immobilization can reduce the activity of enzymes and antibodies [2-3]. Among several important factors involved in the immobilization engineering, support plays a significant role in the efficiency of this procedure. Cellulose is one of the appropriate supports for antibody immobilization in diagnostic systems. Cellulose is a polysaccharide with hydrophilic properties and chemical formula $(C_6H_{10}O_5)_n$ composed of hundreds to thousands of D-glucose molecules [4-5]. Because of the –OH functional group of cellulose, various compounds like antibody molecules can be bound to it [6]. However, at first, the support should be activated and its capacity must significantly be improved. Many methods have been

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developed for the activation of cellulose support [7]. Some activator agents containing Ethylene chloroformate, Carbo-di-imidazol, Nitrobenzoate, HNS, Cyanogen bromide (CNBr) etc. are used for modification of support surface in chemical immobilization of antibodies [8-10]. Among these agents CNBr is widely used to activate the cellulose support [7]. Reaction between CNBr with the –OH group forms the highly active cyanate ester, which can form a stable neutral compound called carbamate, or convert to an unstable isomer called cyclic imidocarbonate, or promote the production of iso-urea which is the best model for immobilization of the antibody [11]. The reaction between hydroxyl groups of cellulose with some acids and anhydrides produces the cellulose ester. Cellulose nitrate is produced through the reaction of cellulose with nitric acid in the presence of sulfuric acid [12]. The negative charge of nitrocellulose, created by resonance structure of nitrate oxygen atoms, is one of its properties which can be used in antibody immobilization. Nitrocellulose binds to protein through the electrostatic bonds between nitrate esters and peptide bonds [12]. The capacity of nitrocellulose for antibody capturing can be increased several times by using activators like acetonitrile. Another appropriate support for antibody immobilization is nylon 6, 6. Nylon 6, 6, a type of nylon materials, is composed of adipyl chloride and hexa-methylene di-amide monomers. Nylon 6, 6 is a polyamide composed of repetitive (-CO-NH-) units, and has a high similarity to nylon 6, in structure [8]. Nylon supports can be used to immobilize the antibodies; however, to reach the maximum capacity for antibody capturing it should firstly be treated using several activators and polymers like PEI, DCC and MAMEC [13]. In this paper, we report a process in which the antibody is immobilized on the surface of cellulose, nitrocellulose and nylon supports. This study aims to compare three different frequently applied supports and explore their advantages through surface process engineering and investigation of the supports.

2 MATERIALS AND METHODS

Different activators and polymer layers used to process the surface of supports include Cyanogen bromide, sodium hydroxide, dimethyl formamide, ethanol, polyethyleneimine (PEI), N,N'-dicyclohexylcarbodiimide (DCC), maleic anhydride methylvinyl ether copolymer (MAMEC) purchased from Sigma company and acetonitrile and formic acid purchased from Merck Co. Different kinds of support including nylon 6, 6 were purchased from Sigma and nitrocellulose sheets, Hi-Flow Plus HF180 and Hi-Flow Plus HF135, cellulose tapes and glass fiber sheets were purchased from Millipore Co. BSA used as a blocker, and mouse anti-Beta HCG monoclonal antibody as an immobilizing antibody were purchased from DCN Co. Beta HCG protein was used as the antigen purchased from Abcam company, and Rabbit anti-Beta HCG polyclonal antibody HRP enzyme conjugated as a detector antibody was purchased from Razi Teb Co.

2.1 Immobilization of antibody on cellulose support

Activation of cellulose support

To prepare the thin cellulose bands, its tape was cut in 5mm×20mm dimensions with the cutter. The bands were placed into 5 ml of dimethyl formide for 10 min in a plate with diagonal of 60 mm. Then, they were placed into 5ml of 1N sodium hydroxide for 10 min in 4°C and were dried after washing with 0.5X PBS. In the next stage, the bands were put in 5ml of 25 mg/ml CNBr for 10 min in a plate. Then, they were removed and washed three times with 0.5X PBS and dried between two glass slides in the room temperature [11, 14].

2.2 Immobilization of antibody on the activated cellulose support

Activated cellulose bands were submerged into 5 ml of 0.1 mg/ml mouse anti-Beta HCG monoclonal antibody in 1X PBS for 3 hours in 4°C in a plate. The bands were then washed with 0.5X PBST three times each time for 5 min. In order to occlude remainder active sites, the bands were blocked with 5ml of 0.1 mg/ml BSA in 1X PBS for 1 hr and washed twice with 0.5X PBST each time for 10 min and dried between two glass slides. To compare the activated and non-activated supports, a raw cellulose band was just faced with antibody without being activated as mentioned above. The remaining active sites on this support were occluded with the blocker and the band was tested in the evaluation step.

2.3 Immobilization on nitrocellulose strips

Two different models were applied for this purpose.

2.3.1 First Model; Activation of nitrocellulose support and immobilization of antibody on it

Acetonitrile, as one of the activators of nitrocellulose supports was used to activate the nitrocellulose support. Since acetonitrile solvates the nitrocellulose, the critical concentration of acetonitrile, should firstly be determined. In order to obtain this concentration, the bands cut in 5mm×20mm dimensions, were submerged in 5 ml of each dilution series of acetonitrile including; 5, 10, 15, 20, 25, 30% in 1X PBS for 1 hr in a plate. After starting the process, the bands in the 30% solution began to decompose and the bands in the 25% solution were selected to be used in the next stages. The bands were then washed and dried between two glass slides in the room temperature. Activated bands were submerged into 5 ml of 0.1 mg/ml mouse anti-Beta HCG monoclonal antibody in 1X PBS for 3 hours in 4°C in a plate. The bands were then washed with

0.5X PBST twice each time for 10 min. The remaining activated sites on the support were blocked and washed as described above and then dried between two glass slides.

2.3.2 Second model; Simultaneous activation and antibody immobilization on the nitrocellulose support

In the second method which includes the activation accompanied with immobilization, the bands are prepared in 5mm×20mm dimensions and submerged in the 5 ml of 0.1 mg/ml mouse anti-Beta HCG monoclonal antibody solution and 25% acetonitrile solution with 1X PBS buffer in 4°C for 3 hr in a plate [15]. After washing the bands with 0.5X PBST, they were blocked in the way which mentioned earlier. In order to compare the activated and non-activated states of the support, a raw nitrocellulose band was faced with the antibody without being activated with acetonitrile and the remaining activated sites were occluded with blockers. The band was evaluated in the testing stage.

2.4 Use of nylon support for Immobilization of antibody

Activation of nylon support

The first stage is preparing a thin layer of nylon. For this purpose, the paper band made from glass fiber was cut in 5mm×20mm dimensions. 100 mg of nylon 6, 6 was solved in 1 ml formic acid and smeared on the glass fiber band and dried in the room temperature (25°C) for 1 hr. The nylon layer was activated using 5 ml of 2.5 N HCl in 30°C for 30 min in a plate. To prepare the second polymer, 5 ml of 1.25% solution of polyethyleneimine (PEI) and DCC in 87.5% methanol was prepared and the nylon layer was submerged in it for 2.5 hr in room temperature and dried after washing. DCC was used to accelerate the binding of PEI to nylon; it serves as a condensing agent to start and catalyze the reaction. The supports were placed in 5 ml of 2% MAMEC copolymer prepared in net acetone for 12 hr in room temperature [13]. The bands were then removed and washed twice with 1X PBS, and dried between two glass slides in room temperature for 2 hr.

2.5 Immobilization of antibody on the activated nylon support

To immobilize the antibody, the activated supports were submerged in 5 ml of 0.1 mg/ml mouse anti-Beta HCG monoclonal antibody in 1X PBS for 3 hours in 4°C in a plate and were dried between two glass slides in room temperature for 3 hr [13].

In order to occlude the active sites on the third polymer remained from the previous stage, dried supports were put into the 5 ml of 0.1 mg/ml BSA solution in 1X PBS for 1 hr and after washing with 0.5X PBST, were dried between two glass slides. To compare the treated samples with non-treated ones, a nylon support without being activated was used as the negative control. The raw nylon support was faced with the antibody and the remaining activated sites were occluded with the same blocker and were evaluated in the testing stage.

2.6 Testing the strips

All types of bands (including cellulose, nitrocellulose, nylon and the control bands) were incubated in 5 ml of 10 µg/ml of Beta HCG protein solution in 1X PBS as the antigen for 1 h at RT (25°C) then were washed three times with 0.5X PBST. After preparing a 1:5000 solution of Rabbit anti-Beta HCG polyclonal antibody HRP conjugated, the bands were submerged in 5 ml of it for 3 hr in 4°C in a plate. The bands were removed and washed with 0.5X PBST buffer and were dried between two glass slides. Dried bands are ready to be used in testing stage. The bands were placed into tubes containing 100 µl of the enzyme substrate (TMB). The reaction time, i.e. since the substrate is added until the color is appeared, was measured and compared. In addition, the optical density (OD) of colored solution was measured at OD_{450nm} in 1-min time intervals using a Nano-Drop spectrophotometer.

2.7 Statistical analysis

In order to confirm the experiments and to ensure the correct immobilization process, each experiment was repeated three times under similar conditions and the average values were reported. The control samples of different supports were also experimented three times under similar conditions.

3. RESULTS

Immobilizing the antibody on the activated cellulose support

Reaction of the –OH group of cellulose with CNBr forms the cyanate ester as an intermediate which can enter three different pathways. It can form a stable neutral compound called carbamate which blocks the considered process. In the second pathway, it converts to an unstable isomer called cyclic imidocarbonate, which can react with amine group of the antibody and produce N-substituted imidocarbonate, leading to production of N-substituted carbamate through a hydrolytic process. The third pathway promotes the production of iso-urea which is the best model for immobilization of the antibody [11] (Fig. 1).

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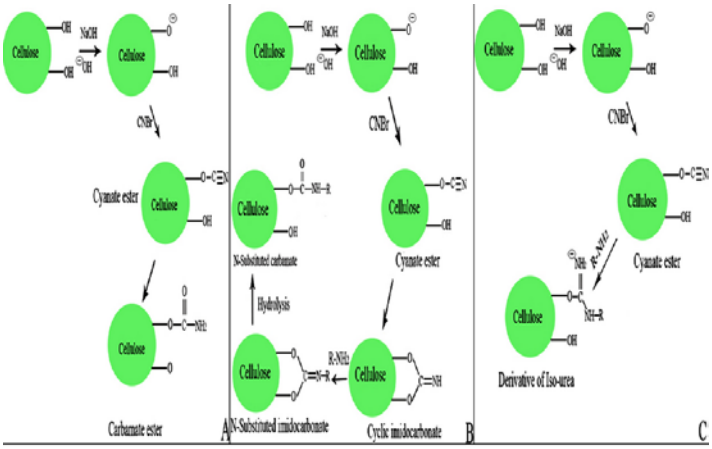

Fig. 1. Reaction of the hydroxyl group of cellulose with CNBr enters three different pathways. A) Formation of carbamate ester, a stable neutral compound, B) Production of cyclic imidocarbonate, an unstable isomer, C) Production of iso-urea, as the best model for immobilization.

As shown in Fig. 2, the color saturation in the tube containing cellulose band increases with time. Increasing the optical absorption of colored solution on the bands in different time intervals results from the action of the enzyme attached to the secondary antibody. After three times repeating the experiment, the best response for cellulose test bands was obtained in 8th minute, which showed the maximum enzymatic reaction with average optical density (OD_{450nm})=1.07. On the other hand, the best response for the control samples was also obtained in 8th minute, which showed the maximum enzymatic activity with average OD_{450nm}= 0.32. Data on the Fig. 2 show the significant difference between the maximum OD_{450nm} of the test sample and that of the control sample. These results demonstrate the efficiency of performed stages on the cellulose support.

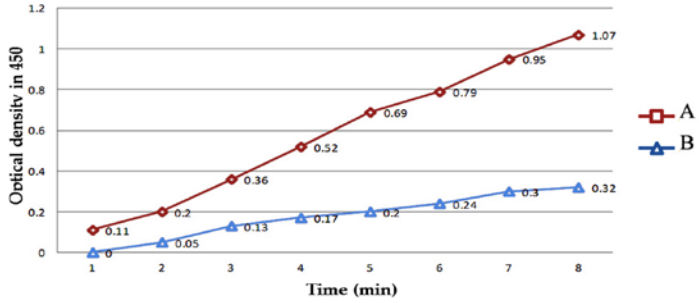

Fig. 2. Average results of three times testing the activated and control cellulose bands in different time intervals. Data were obtained based on the average optical density at 450nm as the enzymatic reaction progresses. A) Average optical absorption by the colored substrate on the activated cellulose band in different times. B) Average optical absorption by the colored substrate on the raw cellulose band in different times.

3.1 Immobilizing the antibody on the activated nitrocellulose support

Results of this stage show that the activated nitrocellulose support has immobilized much more of mouse anti-Beta HCG monoclonal antibody on their surface, in comparison with non-activated control bands. Qualitative and quantitative evaluation of the antibody immobilization was performed in different time intervals for both immobilization models and it was repeated for three times. The averages of obtained results confirm the greater number of antibody molecules immobilized on the activated part of the support.

As shown in Fig. 3, the color saturation has increased with time, which leads to the increase in the optical absorption in different time intervals. The absorption was measured since the first minute of enzymatic reaction until the 8th minute, for the test models and control samples. In the 8th minute, the average OD_{450nm} of the first model of test sample was 0.53 and for the second model of test sample was 0.46. In the 8th minute, OD_{450nm} of the control sample was 0.24 which showed a significant difference.

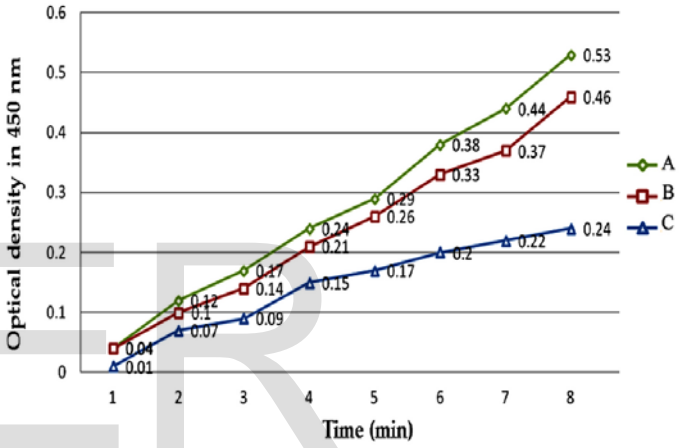

Fig. 3. Average optical density at 450nm resulted from the enzymatic reaction progresses after three times testing the nitrocellulose bands in different times. A and B) Average optical absorption by the colored substrate on the activated nitrocellulose band in different times obtained from first (A) and second model (B). C) Average optical absorption by the colored substrate on the raw nitrocellulose band in different times.

3.2 Immobilizing the antibody on the activated nylon support

In order to use nylon as the support, a band made from glass fiber sheet in 0.5×2cm dimensions was prepared for antibody immobilization. As shown in Fig. 4, homogenous functional groups capable to attach the antibody were generated as a result of a 4-step process. In this study, the efficiency of antibody immobilization was increased using different treatments which have been shown in Fig. 4.

The immobilization process was also performed three times for the nylon support, and average of the obtained values has been summarized in Fig. 5. The absorption was measured since the first minute of enzymatic reaction until the 8th minute, for the test and control samples using the spectrophotometer. In the 8th minute, the average OD_{450nm} of the test sample was 1.02 and that of the control sample was 0.32. Results obtained from the above experiments prove

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the efficiency of activating processes in increasing the immobilization rate of the antibodies.

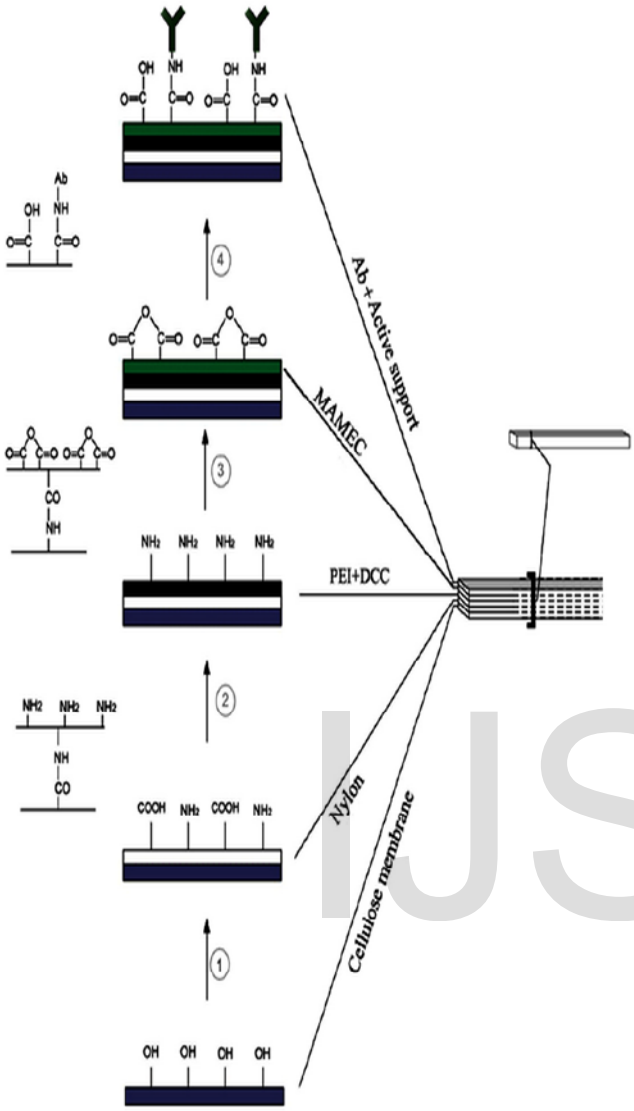

Fig. 4. A schema of surface engineering of the support, through mounting multiple polymers on each other. Several steps were taken to activate the Nylon to achieve an appropriate support for antibody immobilization. 1) Nylon 6,6 granules were solved in formic acid and then poured on glass fiber as the base support. 2) Nylon support was treated with 2.5% HCl, leading to increasing its active groups. The NH_2 groups of PEI polymer were laid on carboxyl groups of the nylon. 3) Third polymer (MAMEC) reacted with NH_2 groups of PEI. The anhydride groups of this polymer formed the ends for capturing the antibody. 4) Antibodies were covalently immobilized through hydrolyzing of anhydride groups.

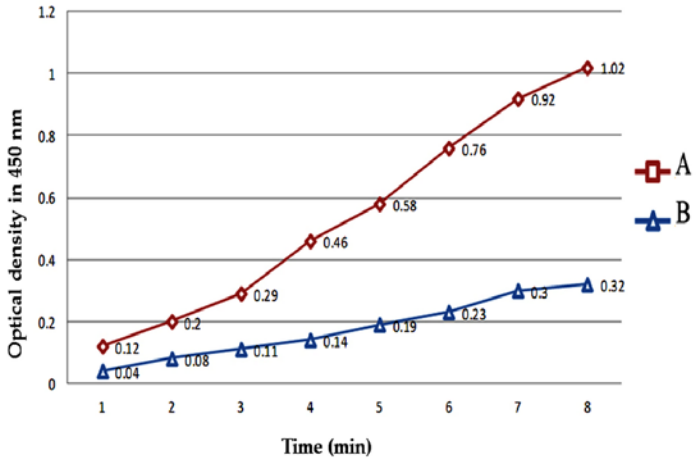

Fig. 5. Data are based on the average optical density of colored substrate at 450nm after the enzymatic reaction progresses on nylon bands in different times, obtained from three times repetition of the experiment. A) Average optical absorption by the colored substrate on the activated nylon band in different times. B) Average optical absorption by the colored substrate on the raw nylon band in different times.

4 DISCUSSION

Nowadays, application of strip diagnostic systems is being developed as an efficient tool to diagnose the biological agents. One of the important parameters affecting the qualitative and quantitative properties of these systems is the correct immobilization of biocatalysts on the support. On the other hand, the type and quality of the applied support plays an important role in immobilization engineering and the efficiency of the diagnostic system. Therefore, surface engineering of the supports is of a great importance. This paper has investigated three different common supports and presented a straight picture of each, by activating them and comparing their capacities. On the other hand, since the antibody plays an essential role in diagnostic systems, the attachment of antibodies and its efficiency focused in this study.

In the first experiment, the antibody was attached to the cellulose support through a covalent band by using a support activator (CNBr). In a similar work by Haider and colleagues, β-galactosidase enzyme obtained from *Aspergillus oryzae* was immobilized on cellulose beads [14]. They first immobilized the antibody on cellulose beads and using this immuno-affinity support, β-galactosidase enzyme was attached to the support by surface absorption method, and the properties and performance changes were then evaluated. In addition, Yang and colleagues immobilized the peroxidase enzyme directly on cellulose nanocrystals in order to remove the phenol compounds together with Cl [11]. The nanocrystal and bead formats of this powerful support cannot be used in strip diagnostic systems. In practice, the strip format of this support is used. Therefore, in this study, cellulose characteristics in its strip format were investigated.

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Another support investigated in this paper was nitrocellulose. As can be realized from its structure, the resonance between two oxygen atoms connected to the nitrogen atom generates a partial negative charge, which makes the nitrocellulose a support with negative charge. When faced with low concentrations of acetonitrile, the nitrocellulose support is changed so that its surface charges increase and provides appropriate conditions for further and stronger binding of the antibodies.

Antibody binds to the nitrocellulose support by the electrostatic bonds between nitrate esters and peptide bonds through the surface absorption mechanism. Sun and colleagues have reported the simultaneous activation and immobilization of the antibody on the nitrocellulose support. They used activated nitrocellulose bands for affinity-directed mass spectrometry for the analysis of interactions between antibody and antigen [11]. In this research, in addition to simultaneous activation and immobilization of antibody on the support, we evaluated another model of antibody immobilization on nitrocellulose support which included separate activation and immobilization steps. Significant results obtained from this experiment revealed that the antibodies immobilized in the step-by-step immobilization are of a higher quality. Such difference could be resulted from the effect of the activator (acetonitrile) on the molecular structure of the antibody, or it may be due to other mechanisms which require more investigation. The corrosive concentrations of acetonitrile were also investigated in this study.

Another support used in this study was nylon 6,6. Nylon, with its few amine and carboxyl groups, cannot immobilize the antibody. Thus, in order to convert it to an ideal support, its surface charges are needed to be modified so that the antibody can bind correctly with the highest capacity. This can be done using activation treatments. As seen in Fig. 4, after different stages and using different treatments on the nylon support, the generated amine group is an appropriate group for immobilizing the antibody. Honda and colleagues were pioneers of establishing a similar technique, but with some differences with the present study [13]. In their investigation, how the nylon is applied for making its layered structure has not been mentioned and appropriate solvents and primary support have not been investigated. In addition, the support has not been blocked specifically, which leads to many non-specific reactions. The activated and non-activated states of the nylon support have not been compared. They measured the amount of immobilized antibody by using anti-rabbit immunoglobulin labeled with alkaline phosphatase. They reported that the nylon optical density at 405 nm (OD_{405nm}) in the ELISA was ≥ 2.0, which is different from results of our investigation. As a result, the critical aspects of their support have not been evaluated and therefore it lacks the required criteria to be applied in diagnostic systems. In addition to describing many hidden aspects of immobilization on cellulose, nitrocellulose and nylon supports, this investigation could demonstrate the strengths and weaknesses of each method and present a real picture of the mechanisms behind the increase of immobilization efficiency.

5 CONCLUSION:

Efficiency of antibody immobilization process can be improved by activating the supports through processing their surfaces, and applying them to make diagnostic systems.

In general, from the obtained results, we can conclude that, A- The capacity to accept the antibody in activated cellulose support is greater than that of activated nylon support, and this capacity in the nylon support is greater than that of nitrocellulose support. B- The liquid motion on the cellulose strip is very fast and irrestrainable, which could generate false responses or errors in methods based on chromatography and liquid phase motion on the support. On the other hand, dealing with such supports is difficult, because of the difficulties in the use of such irrestrainable supports in strip processing stages which take a high precision in measurements. C- The capillary motion decreases drastically in the nylon support. Therefore, the use of nylon in designing the systems based on capillary motion is not recommended, while the nitrocellulose is an appropriate support for capillary motions of liquids. D- From viewpoint of elegance and delicacy of supports, the nitrocellulose bands show a higher delicacy in comparison with the nylon and cellulose supports. E- Working with nitrocellulose support takes less time and materials, compared to the nylon and cellulose supports.

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