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Original Article

Utilizing Different Supports and Comparing their Performances in the Construction of Morphine Rapid Detection System Based on Lateral Flow Assay

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Abstract

Lateral flow assay, a simple and rapid test to detect various agents; have found many applications in different fields. One of the most important issues in the construction of a Lateral Flow assay is the increasing of its sensitivity and efficiency by processing of analytical layer. This study, aimed to investigate the performance of, Nellulose, Nitrocellulose, Nylon and PVD as different analytical layer in morphine Lateral flow strip as a model. Cyanogens bromide, acetonitrile, methanol and specific polymers were used to activate cellulose, nitrocellulose, PVDF and nylon respectively. BSA-morphine and anti morphine polyclonal antibodies were immobilized orderly in test and control bands different concentrations of morphine were prepared as sample solution. Results showed that activated supports have better detection level in comparison with control strips. Among activated supports nitrocellulose showed more reliable results rather than others and introduced as a suitable support for construction of analytical layer and antibody immobilization. Nylon and PVDF supports due to its hydrophobic nature and cellulose due to high capillary property and non-uniform texture showed inappropriate results. Using proper supports for design of lateral flow strips can improve the sensitivity and detection level of this system.

Keywords: LFA System, Immobilization, Cellulose, Nitrocellulose, Nylon, PVDF

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Introduction

Nowadays rapid diagnostic systems such as Lateral Flow Assay (LFA) have found widespread applications in medicine, industry, agriculture and etc. These systems are frequently used to accurately determine the presence or absence of a particular target in different samples. Lateral flow assay is very cheap and easy to useand do not need to special training for application [1, 2]. Levels of detection, sensitivity and specificity of LFA are the most important factors for the construction of these systems LFA-based diagnostic systems have been composed of different pads including, sample pad, conjugated pad, analytical pad and absorbent pad [3].

The main part of this system is analytical layer and what has a direct impact in the optimal performance of this layer is selectionofappropriate support and activation procedure for the most immobilization of antibody. Immobilization defines as limiting biomolecule in a specific spaceto enhance stability and performance efficacy without any change in its biological activity. Of course it is very important how to perform the immobilization process which does not reduce the efficiency of the fixed substance and its biological activity [4-6]. To obtain the appropriate method and the best conditions for immobilization process a proper method should be selected according to the type of biomolecule, their reaction and applications and physical and chemical characteristics of the substrate. This study aimed to investigate the effect of different supports in efficacy of rapid detection system designed for morphine. Competitive supports include: Nitrocellulose, Cellulose, Nylon and PVDF. Nitrocellulose substrate is made of cellulose nitrate and is the product of a reaction between cellulose and nitric acid in the presence of sulfuric acid as a catalyst. Nitrocelluloseis insoluble in waterand it looks like white cotton fibers [7]. Negatively charged nitrate groups are the most important functional groups in nitrocellulose support biomacromolecules such as antibodies, which are typical positively charged proteins due to the amino acids in its structure, can be connected simply to nitrate ester by electrostatic bonds. Of course activation of supports before immobilization of antibodies, increase supports capacity to capture antibodies.Acetonitrile is an importantactivator for nitrocellulose supports [8].

Another support used in this study is Nylon. Nylon is a generic name for a family of synthetic polymers known as Polyamides. These materials have considerable heat, electrical and chemical resistance. Polyamide 6 and 6.6 is the most consuming types of polyamides that despitethe similarityproperties aredifferent together. In this research, Nylon 6.6 is used [9]. To achieve maximum capacity of nylon to immobilized biomolecules, it should be treated. It means that the groups placed on nylon surface should be organized and if necessary, should be changed, so activation of nylon support with the proper activator may be a helpful.HCl increases amine andcarboxylgroups ofthe support surface so that these groups, form the primary basis for making next substrates or polymers [10, 11].

Celluloseis another support thatused in this study cellulose is a polysaccharide which composed of many glucose molecules connected together with β (1 → 4) glycoside bond and created a strong linear structure [12]. Activation of a large number of OH groups that are in cellulose structure will facilitate antibodies immobilization on its surface [13]. In this study, Cyanogens bromide is used to activate the cellulose.This agentreactswithOH groups and provides veryunstablegroupthat easily brake down and linked to the amine group of antibody with covalent bond [5]. The fourth support which hasbeen studied in this research was PVDF that is as emi-crystalline polymer and is made of crinkle chains, as including CF_2-CH_2 groups. Thermodynamic stability and chemical resistance of this support is among the factors that makes it appropriate for using in a variety of fields such as construction of filters [14]. But, due to its hydrophobic nature and have not active reactant group for direct reaction with the other materials such as antibodies, at first it should be activated. Ethanol and Methanolare the most common activators of PVDFsupport [5] that make it hydrophilic. Thus, content of aqueous solutions can be penetrating the support and connected it. Therefore, the transmission capacity increased. Chemical structures of presented supports are shownin figure 1. In this study how to activation of these supports and immobilization of anti-morphine antibody on their surfaces was evaluated. Also their performance as analysis layer in rapid detection system of morphine was compared.

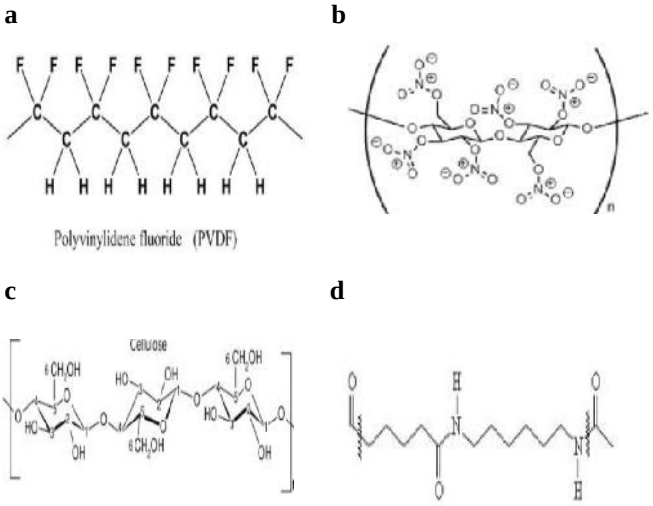

Figure 1. Chemical structure of the investigated substrates.
A: PVDF, b: Nitrocellulose, c: cellulose, : Nylon 6.6.

Materials and Methods

Necessary materials are as follows: PVDF, cellulose and nitrocellulose purchased from Millipore Corporation and Nylon 6.6 from Sigma. Monoclonal mouseanti-morphine antibody and polyclonal goatanti-mouse antibody were purchased from Fitzgerald. Polymers including PEI (Polyethylene imine), DDC (Dicyclohexyl-carbodiimid), MEMAC (Maleic Anhydride Methyl vinyl Ether Copolymer), Cyanogen Bromide (CNBr), sodiumhydroxide (NaOH), Dimethylformamide (DMF), Methanol from Sigma and BSA from Roche, Acetonitrile, Potassium carbonate, Formicacid and Twin-20 from Merck, colloidal gold nanoparticle from Kestrel, PVP, Morphine Sulfate and $NaN_3$from Sigma. First the different parts of a LFA system, apart from the analysis layer including sample pad, conjugated pad and absorbent pad were prepared for mounting on the baking [1, 15]. Nitrocellulose paper was cut in two pieces with dimensions 30 × 50 mm. One piece was immersed in 25% acetonitrile solution for 5 minutes and then was washed with distilled water and was placed between two glass slides to dry. Other piece was kept inactive as control. Activated nitrocellulose piece, were mounted in their place on the backing. The same work was done also for not activated nitrocellulose. Then morphine-BSA complex and polyclonal antibody were immobilized in test and control bands over the nitrocellulose substrate. To try the strips and determine their detection level, morphine sulfate solution with gradient series concentrations including (0.5, 0.05, 0.005, 0.0005, 0.00005, 0.000025, 0.000005 mg/ml) was prepared and applied to strips.

In order to test nylon support, 600 mg of nylon 6/6 was dissolved in 1 ml of formic acid then two pieces of glass fiber with 30 × 50 mm in dimensions were immersed in nylon solution and placed at room temperature for 1 hour. For activation process, one of them was immersed in hydrochloric acid 2.5 normal for 30 minutes. Next polymer that was encountered with the nylon platform was the solution of PEI polymer (1.25%) and DCC as a catalyst. Maleic anhydride methylvinyl ether copolymer (MAMEC) polymer (2%) in pure acetone was the next polymer that added to nylon for 12 hour. Finally the strips were washed twice with 1X PBS and were dried between two slides at room temperature. Then the activated and not activated nylon supports were placed on a previously described baking card. After creating the test and control bands and mounting other mentioned pads the strips were tested as before with different concentrations of morphine sulfate solution.

PVDF was cut in two pieces in 30 × 50 mm dimensions and was washed with distilled water until possible dust remove on it. Then one of them was immersed in 100% methanol for 10 minute and after washing with distilled water was dried between two glass slides. Activated and non-activated PVDF were placed on the baking card then the test and control bands were created on the strips similar to what was done about nitrocellulose and nylon. One of two cellulose strips with 30 × 50 mm in size was placed in Dimethylformamide (DMF) for 10 minutes and then was immersed in 1 normal sodium hydroxide for 10 minutes at 4°C. Then were washed with PBS 0.5 X and were dried between two glass slides. The resulting pieces were placed in Cyanogens Bromide with 25 mg/ml concentration for 10 minutes and after washing with PBS 0.5 X were dried between two glass slides. Two cellulose layers, that one of

them was active and another was inactive, were placed on a baking card and after creating test and control bands, were tested with morphine sulfate solution as before. To obtain more accuracy in results all steps were repeated three times and finally the average was reported.

Results

The effect of the activation process on the efficiency of stripsas it was expected was positive. The detection level of the valid strips prepared with activated supports was better than of strips whit inactive supports. In the strips that their analysis layer was made from cellulose, detection level of morphine was 50 ng/ml. Therefore, when the morphine sulfat solution with a concentration of 0.00005 mg/ml was applied to these strips, the test bands began to be colored in red. Detection level of strips with inactive cellulosein analysis layer, were obtained in the same range, but the color of the test band in this strips was less highlighted than the test band in strips with activated cellulose. The same results as cellulose were obtained for nitrocellulose strips. But, after applying the morphine sulfate solution with concentration of 0.00005 mg/ml to these strips, the color of the test band was more highlighted than the cellulosic strips (Fig. 2). In the strips that theiranalysis layer was made from nylon and PVDF because of their natural properties specific result was not obtained.

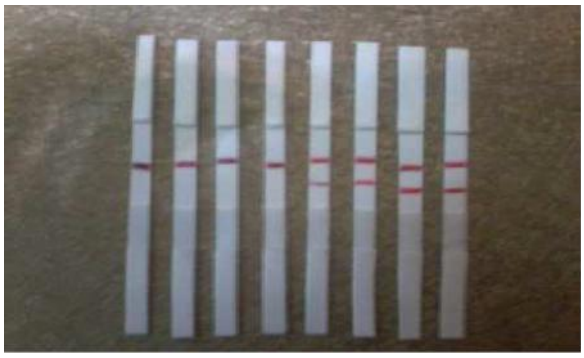

Figure 2. The picture of performance of nitrocellulose bed as analysis layer. Morphine sulfate solution with gradient series concentrations including (0.5, 0.05, 0.005, 0.0005, 0.00005, 0.000025, 0.000005 mg/ml (left to right) was applied to strips.

Discussion

Evaluation of the detection level of valid strips that are made of nitrocellulose and cellulose supports showes a sensible improvement in detection sensitivity. In thestrips which their analysis layer were made of nylon and PVDF because of these supports were highly hydrophobic [16, 17] and have a little capillary properties, movement of the conjugated substance were not donein a way that will leadtoa certain result and movement of the conjugated substance was stopped before reaching to the analytical bands. Activation of supports used for creation of the analytical layerhas a great influence on their capacity to immobilize the antibodies. These results obtained by modifying of the properties of each of the supports such as their hydrophilic or hydrophobic nature, type of functional groups presented on their surface and how to increase the number of these groups, the structure of support in terms of being rough or uniform and subsequently selecting appropriate materials for activation of each of the substrates. Results also confirmed that utilizing of chemical activatorsas a tool tomodify the structure of the supports and organization of their functional groups can increase supports capturing capacity. Extensive study on the effect of the activation processon the supports binding capacity to capture of antibody in the study of Heiat *et al*. are reported [4, 5]. in spite of their research, the present study is specifically about morphine detection system through different strategy .

In their study the effect of the activation process was evaluated by immersing the activated supports in a monoclonal antibody and then encountering with HRP conjugated polyclonal antibody and then adding the enzyme substrate and measuring the dye concentration of the enzymatic reaction by Nano-drop Spectrophotometers, while in the present study antibody and morphine–BSA, were immobilized onto the activated supports in a delicate bond and conjugated material interacted with test and control band in a capillary lateral flow procedure [18]. By investigating the performance of different supports it can be concluded that although activated cellulosic support shows a high capacity to immobilized antibody and BSA-morphine and this matter causesto improve the level detection of strips to 50 ng/ml, but its high capillary velocity and non-uniform tissue causes conjugated material trapped in the pores of its structure and create colored smear. In fact cellulosic support such a filter separates particles from suspension solution and obviously it is not pleasant in order to manufacture of LFA strips (Fig. 3). Capillary rise of cellulose is too high that after the release of conjugated material from the conjugated layer, it is traversing quickly the length of support and does not have the enough time to encounter with the BSA-morphine and antibody in test and control band and this can lead to false results, because the sample moving at high speed reduces the sensitivity of the strip. Therefore, using this supportas the analytical layerin the morphine detection stripis not recommended. This observation suggests that activation of cellulosic support improved the detection level of strip.

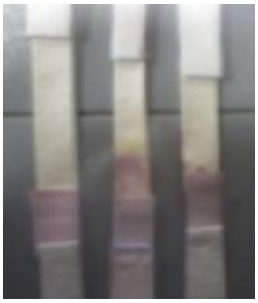

Figure 3. The picture of performance of cellulose bed as analysis layer. It is clear that this substrate is not suitable in the field of diagnostic kits.

It was the results of elimination of background in nitrocellulose strips. However,in general, this study confirmed that using nitrocellulose supports compared with others supports are preferred for LFA strip construction PVDF and nylon, due to their natural properties including high hydrophobisity and low capillary velocity, are not recommended directly for applications in LFA diagnostic strip. But perhaps those are effective and suitable for some applications such as western and dot blotting in which, entire support is immersed in the antibody or antigen.

Conclusions

In general, it can be concluded that a supports is useful for construction of analytical layer of LFA that has the appropriate capacity for antibody capturing, capillary and hydrophilic properties and the uniform structure. Results in this study revealed that nitrocellulose support is more qualified rather than others. Because its surface is negatively charged and due to confronting appropriate concentrations of acetonitrile, altered in such a way that its surface charges increased and provided a better conditions for antibody and morphine-BSA capturing. However, the nitrocellulose support has disadvantages such as high fragility and flammability, possibility of changes in its properties due to changes in environmental such as temperature and humidity and ability to react with organic solvents.

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