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Detection of anti-tetanus toxoid antibody on modified polyacrylonitrile fibers

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ABSTRACT

Accurate determination of concentration of immunoglobulin (IgG) to tetanus toxoid is important in order to evaluate the immunogenicity of tetanus toxoid vaccines, immune competence in individual patients and to measure the prevalence of immunity in populations. Surface modified polyacrylonitrile (PAN) fibers were evaluated as a matrix to develop highly sensitive method for the detection of anti-tetanus antibody in a sandwich ELISA format. In the proposed method tetanus toxoid immobilized on modified PAN fibers was used to detect anti-tetanus antibody (raised in horse hence represented as horse anti-tetanus toxoid or HAT-Ab) with horse raddish peroxidase enzyme conjugated with Rabbit anti-Horse IgG (RAH-HRP) as the label within 2.5 h. A sigmoidal pattern for the detection of different concentration of antibody ranging from 1.0 to 0.0001 IU mL^{-1} was validated. The immunoassay recorded a very high sensitivity as concentration as low as 0.0005 IU mL^{-1} of HAT-Ab was detected. The intra- and inter-assay precision for 3 parallel measurements of 0.01 and for 0.001 IU mL^{-1} of antibody varied from 5.4% to 11% and 5.7% to 20% respectively. PAN fibers were also used to qualitatively access the presence of different level of anti-tetanus antibody spiked in human blood. Seroepidemiological studies to measure the immunity against tetanus were conducted with twenty-five human beings belonging to various age groups using modified PAN-ELISA. The sensitivity, specificity and the reproducibility of the developed immunoassay indicate the potential application of modified PAN fibers in the field of immunodiagnostics.

1. Introduction

Determining the level of antibodies against toxins is clinically used as an indicator of immunization of either an individual or a population through vaccines [1,2]. Tetanus or lockjaw is caused by the action of highly potent toxin released by an anaerobic bacteria *Clostridium tetani* and belong to family of metaloproteases called clostridium neurotoxins. The tetanus toxin consist of two di-sulphide linked polypeptide chains of 150 kDa: Heavy chain involved in nerve-specific binding & cell penetration and one light chain which is zinc-endopeptidase involved in neuroexocytosis. The spores of the pathogen that get through injured skin into tissues are transformed into bacilli, which synthesize the toxin into the surrounding necrotized tissue. It is one of the most toxic substances with estimated human lethal dose of less than 2.5 ng per kg of body weight. The toxin directly affects the central nervous system of the body causing spastic paralysis [3,4]. The neurotoxin binds with cholinergic nerve terminals and internalizes in the CNS neurons affecting the physiological processes of synaptic vesicles, which leads to the proteolysis, and cleavage of protein substrate. The spinal spastic paralysis results from the intoxication of neurotoxin arising from loss of spinal inhibitory control of motorneuron activity [5–7]. There exists a direct correlation between proteolysis and effect of toxin on neuroexocytosis. The toxin can be inactivated by formaldehyde or heat to yield toxoid that is used to immunize. Immunity to tetanus is solely determined by the antibodies produced against the toxins [8–10]. The most popular way of measuring the specific immunoglobulin to toxoid is the in-vivo toxin neutralization test, which is time consuming, expensive and raises ethical issues regarding the use of live mammals. In addition, the accuracy of the neutralization assay depends on the nature of toxin, toxin test and weight of mice. Dr. Sesardic and collaborators have conducted in-vivo neutralization test using two different set of 20 serum pools and have performed the validation of serological methods for potency testing of tetanus toxoid vaccines for human use [11,12]. The study results may be used in support of the replacement of the multi-dilution direct challenge procedures in different animal models by a single dilution serology test, where appropriate, and to use sera from the same animals for potency testing of several components in combined vaccines. *In-vitro* tests like ELISAs

Abbreviations: PAN, polyacrylonitrile; IgG/Ab, antibody; Ag, antigen; HAT-Ab, horse anti-tetanus toxoid antibody.

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are developed for the detection of anti-tetanus antibodies, which are accurate, ethical, clinical, economic and can be automated as per requirement [13]. Lot of polymeric materials have widely been used as a solid support in ELISAs or enzyme linked immunosorbent assays for the laboratory diagnosis of infectious diseases, disorders and allergies [14,15].

Efficient tailoring of physico-chemical properties of polymers like molecular weight, shape, size, and easy functionalization render them amenable for the covalent attachment of biomolecules in ELISA. A controlled covalent linkage of antibodies to solid support is preferred which gives more sensitive assays as negligible desorption occurs during extensive washing steps and imparts very low extent of non-specific interaction of biomolecules. Nylon, polystyrene, Dacron, polyacrylonitrile are some of polymers used as interface for the biological entities [16–21].

Polyacrylonitrile in various forms like membranes, fibers and nano-fibers have been exploited in different fields of pervoporation, water treatment, enzyme immobilization and haemodialysis. This wide popularity is due to their excellent thermal & mechanical properties, chemical stability and tolerance to most solvents, bacteria & photo-irradiation [22–24]. PAN fibers have high surface area, very high mechanical strength, abrasion resistance & insect resistance and the presence of nitrile groups along with fiber backbone offer multidirectional approaches for their modification unlike synthetic membranes which can be damaged during the modification [25]. PAN hollow fibers are already used as dialysers that remove low molecular weight compounds and proteins. These fibers show potential application in field of composite materials, protective clothing, biomedical materials and nanosensors. Nitrile groups of PAN fibers have been partially & completely hydrolyzed and reduced to generate amide, carboxy and amine functionality respectively by researchers using chemical, irradiation and enzymatic techniques [26–29]. Jia et al. have grafted casein directly on PAN fibers to improve their antistatic and water retention properties [30]. Ishimura has reduced the PAN fiber and immobilized penicillin acylase to study the activity of the enzyme after the attachment on the fibers in terms of specific activity and immobilization yields [31].

In our previous paper, we have reduced the pendent nitrile groups of polyacrylonitrile fibers to generate amino functionality and evaluated them as a matrix for the detection of antibody in a triple antibody sandwich assay [32]. The goal of this contribution is to evaluate the reduced and activated PAN fibers for the immobilization of tetanus toxoid for the effective, sensitive and specific detection of anti-tetanus antibodies.

2. Experimental

2.1. Materials and reagents

Polyacrylonitrile fibers (PAN) (wet spun multifilamentous-containing approx. 500 filaments) were received from Department of Textile Technology, IIT-Delhi (New Delhi, India). Diethyl ether, AR grade was obtained from Merck (Darmstadt, Germany) while boric acid and skimmed milk were from CDH (Mumbai, India). Lithium aluminum hydride (LAH), glutaraldehyde and Tween-20 were purchased from Sigma–Aldrich (St. Louis, USA). Rabbit anti-Goat (RAG-IgG), Goat anti-Rabbit (GAR-IgG), Rabbit anti-Horse-Horse Raddish Peroxidase (RAH-HRP), Rabbit anti-Human-HRP (RAHu-HRP) and 3,3′,5,5′ tetramethyl benzidine (TMB) were purchased from Bangalore Genie (Bangalore, India). Ampoules of tetanus toxoid of 500 lf mL^{-1} concentration were received from Serum Industries of India Ltd. (Pune, India). It was reconstituted as 50 lf mL^{-1} in 1X-PBS to yield a working concentration. Ampoule of the polyclonal tetanus toxoid immunoglobulins (Ab against toxoid) of equine origin represented as HAT, horse anti-tetanus antibody was also obtained from Serum Industries. HAT-Ab was supplied as a 1 mL solution with 1500 International Units and was reconstituted in 1X-PBS to yield a working dilution of 150 IU mL^{-1}. All further dilutions were prepared from these concentrations.

Serum and blood samples directly taken from humans were provided by IIT-Delhi hospital (New Delhi, India). All buffers – borate and phosphate buffer saline (PBS) were prepared in milliQ water (Millipore Ltd.). All solvents and reagents were used as received unless noted otherwise.

2.2. Reduction of PAN fibers

In a 250 mL RB flask equipped with water condenser, equivalent quantities of lithium aluminum hydride, LAH (1.0 g) and polyacrylonitrile PAN fibers (1.0 g) were reacted in excess of pre-dried diethyl ether (120 mL). The reaction mixture was stirred continuously in a moisture free environment under the nitrogen blanket at room temperature (27 ± 2 °C) for 24 h. The fibers were thoroughly washed with distilled water to remove the excess of LAH and dried in vacuum oven for 4 h. They were then stored in the desiccator for further use.

2.3. Characterization

2.3.1. Instruments

Perkin Elmer UV–vis spectrophotometer LS-55 was used to measure amine content and BioRad ELISA plate reader to determine the activity of peroxidase enzyme conjugate in the immunoassay procedure.

2.3.2. Evaluation of amine content

Primary amine content of the surface aminated PAN fibers was evaluated by calculating the uptake of acidic dye acid orange II [33,34]. 4 mg of the aminated fibers were immersed in a solution of 5 mL of 500 μM acid orange II prepared in 50 mL of 0.1 M HCl at pH 2–3. The fibers were kept for shaking for 24 h at room temperature and were washed thoroughly using excess of 0.1 M HCl. Treated fibers were re-immersed in 2 mL of 0.1 M sodium hydroxide solution (pH 12) for 20 min and the optical density of the colored solution was recorded at 454 nm in UV–vis spectrometer.

2.3.3. Scanning Electron Microscope

Surface morphology of unmodified, reduced and toxoid immobilized PAN fibers were studied through ZEISS EVO Series Scanning Electron Microscope EVO 50 (Cambridge, U.K.). Fibers were mounted on a metallic stub using a double sided tape and silver paint was applied on fibers to make them conducting for better resolution. Gold sputtering was done on the sample for conduction using the sputtering instrument (BioRad Polaran Sputter, Model 50X).

2.4. Attachment of tetanus toxoid onto modified PAN fibers

2.4.1. Activation of the aminated fibers

4 mg of aminated PAN fibers (PAN-NH_2) were activated using 12.5% glutaraldehyde solution prepared in borate buffer (pH 8.5) in a micro-centrifuge tube at 4 °C for 3 h. After that, the fibers were thoroughly washed with borate buffer (pH 8.5) and Tween/PBS (pH 7.2) to remove excess of glutaraldehyde. The activated fibers were stored at 4 °C till further use.

2.4.2. Immobilization of tetanus toxoid

4 mg of glutaraldehyde activated PAN fibers (PAN-NH_2-Glu) were incubated with 500 μL of 2.5 lf mL^{-1} tetanus toxoid for 16 h at 4 °C with occasional shaking. Unbound toxoid was removed and

the fibers were washed with Tween-20 and 1X-PBS (pH 7.4) thrice. The immobilized fibers were stored at 4 °C till further use.

2.5. Detection of tetanus toxoid antibody using modified PAN fibers

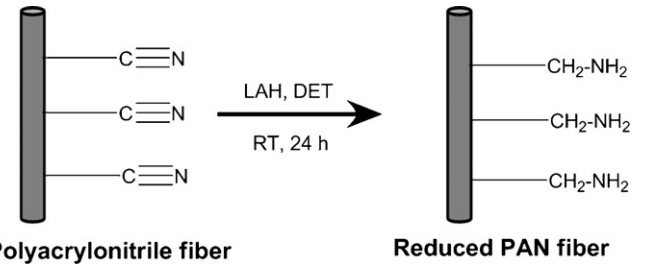
A two dimensional serial dilution method was performed to determine the optimum concentration of analyte and dilution of enzyme conjugate [30]. The first set of experiment was carried out to optimize the dilution factor of peroxidase conjugate RAH-HRP. 4 mg of tetanus toxoid immobilized PAN fibers (PAN-NH_2-Glu-Tox) were incubated with 12% skimmed milk for 1 h at 37 °C to block the unbound sites to avoid non-specific binding. They were then washed three times with PBS and incubated with 500 μL of fixed concentration of 0.1 IU mL^{-1} of the HAT antibody taken as analyte. After washing with Tween/PBS, the fibers were again incubated with 500 μL of different dilutions of enzyme conjugate of anti-horse antibody RAH-HRP (1:2000–1:16,000) for 1 h at 37 °C with occasional shaking. The fibers were removed from the excess of conjugate and were finally washed with Tween/PBS. After the removal of unbound antibodies, peroxidase activity of the bound antibody on the fiber was measured by the means of conversion of colorless substrate TMB to a colored product immediately after 10 min. 100 μL of this solution was transferred to the 96-well microtiter plate and the color development was quenched by adding equal volume of conc. sulphuric acid (0.5 M). The optical density was measured at 450 nm with BioRad ELISA plate reader. Constant amount (4 mg) of modified PAN fibers was used for each conjugate dilution separately and data was collected in triplicate. Tetanus toxoid from couple of ampoules was used for immobilization on modified PAN fibers and the subsequent assay was performed.

In the complimentary set of analysis, the sensitivity or the lowest detection limit of the assay was determined for the PAN-ELISA method for the detection of HAT-Ab. The activated PAN fibers immobilized with 2.5 lf mL^{-1} of tetanus toxoid were incubated with different concentrations of the antibody ranging from 1.0 to 0.0001 IU mL^{-1} for 1 h at 37 °C. After thorough washing, the fibers were incubated with the standardized dilution of RAH-HRP determined from the first experiment. Negative control for the experiment was also performed where 1X-PBS was taken instead of analyte or HAT-Ab. 4 mg of the tetanus toxoid immobilized fiber PAN-NH_2-Glu-Tox was blocked with 12% skimmed milk. They were again incubated with 500 μL of different dilutions of RAH-HRP (1:2000–1:16,000) for 1 h at 37 °C.

Specificity of the assay was measured with different antibodies like GAR-IgG, RAG-IgG and RAHu-IgG in place of antibody against tetanus toxoid (HAT-Ab) in the detection protocol. All experiments were carried out in triplicates. The intra- and inter-assay precision (coefficient of variation) were determined with three parallel measurements for two different antibody concentrations – 0.01 and 0.001 IU mL^{-1} on same day and on different days respectively using the formula:

$$\text{Coefficient of variation} = \left(\frac{\text{Standard deviation of replicates}}{\text{Mean of replicates}}\right) \times 100$$

The developed ELISA system was compared with the conventional ELISA using polystyrene (PS) 96-well microtiter plates. Same experimental procedure was followed where tetanus toxoid (2.5 lf mL^{-1}) was physically coated on the wells of the plate. They were blocked with 12% skimmed milk, incubated with different concentrations of HAT-Ab followed by incubation with 1:8000 dilution of RAH-HRP. Washings were done with Tween/PBS before each incubation step which were done for 1 h at 37 °C. The absorbance was recorded following the above-mentioned steps.



Scheme 1. Reduction of pendent nitrile groups of polyacrylonitrile fibers to amino groups using lithium aluminum hydride (LAH) in solvent diethyl ether (DET) at room temperature (RT).

2.6. Detection of HAT antibody spiked in human blood

Modified PAN fibers immobilized with tetanus toxoid were employed to detect HAT antibody spiked in human blood. 4 mg of toxoid immobilized fibers (PAN-NH_2-Glu-Tox) were incubated with 500 μL of blood spiked with different concentrations of HAT-Ab for 1 h at 37 °C after blocking with skimmed milk. HAT-Ab spiked blood was obtained by mixing equal volumes of human blood and different concentrations of HAT-Ab 1.0, 0.1, 0.01, 0.0015, 0.001 IU mL^{-1}. After washing, the fibers were incubated with 500 μL of RAH-HRP conjugate at a dilution of 1:8000 for 1 h at 37 °C. Final washings were done and absorbance at 450 nm was recorded as mentioned in Section 2.5.

2.7. Evaluation of the immunity of patients against tetanus

Twenty-five different human blood samples were collected from the IIT-Delhi hospital and were used for the detection of antibodies of tetanus toxoid present in humans by modified PAN-ELISA. Modified PAN fibers immobilized with tetanus toxoid were blocked with 12% skimmed milk for 1 h at 37 °C. After washing with Tween/PBS, fibers were incubated with 500 μL of different human sera for 1 h at 37 °C. PAN fibers were washed and were further incubated with 1:8000 dilution of RAHu-HRP for 1 h at 37 °C. Optical density was recorded for the peroxidase conjugate immobilized on modified PAN fibers after washing as reported in Section 2.5.

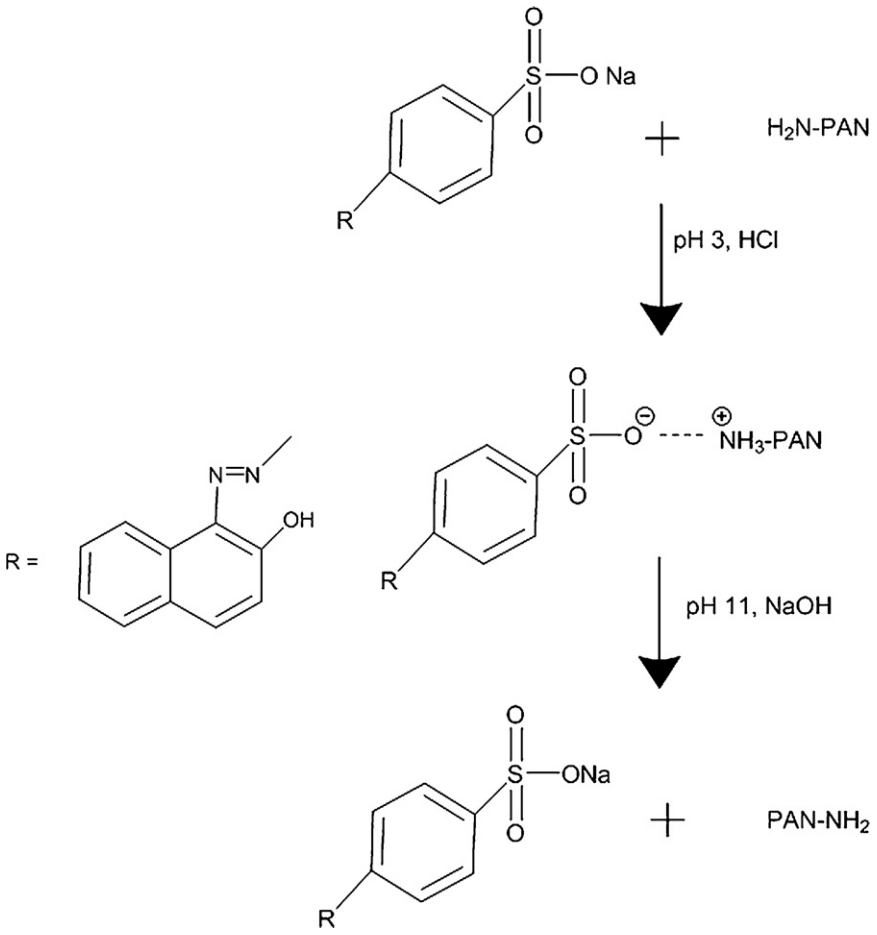
3. Result and discussion

3.1. Reduction of PAN fibers

The pendent nitrile groups of the PAN fibers were successfully reduced to generate primary amino groups on the surface as depicted in Scheme 1. As reported earlier in our paper [32], the reduction results in slight increase in yellowness, brittleness, and roughness of the aminated fibers.

3.1.1. Estimation of amine content

The reaction of amino groups of aminated PAN fibers with acid orange II dye is depicted in Scheme 2. Colorimetric estimation of amine content of the aminated PAN fibers was done by the uptake of acidic dye–acid orange II. In the acidic solution of the dye at pH 2–3, the amino groups of the modified PAN fibers are protonated and are ionically linked with the negatively charged dye molecule. The original configuration of the dye molecules (uncharged state) was achieved at high pH of 12 when dye is detached from the amino groups of the PAN fiber in alkaline NaOH solution. The amine content was calculated with the help of a calibration curve and the assumption that one amino group binds with one dye molecule. Amine value was determined as 234 nmol/g of PAN fibers by this method.



Scheme 2. Colorimetric estimation of amino groups generated on PAN fibers using acid orange II dye.

3.1.2. Scanning Electron Microscope

Fig. 1 represents the scanning electron micrographs of unmodified and modified PAN fibers. It can be observed that unmodified PAN fibers (Fig. 1a) were smooth and untangled with a mean diameter of 50–70 μm. Morphology of 24 h reduced fibers remained intact with a slight increase in roughness and entanglement of fibers (Fig. 1b). This can be attributed to the conversion of pendent nitrile to the amino groups on the surface of the fiber. On immobilization of tetanus toxoid, a homogenous deposition of toxoid on the surface of immobilized fiber can be visualized from the micrograph (Fig. 1c). Other authors also report similar observations [29].

3.2. Immobilization of tetanus toxoid

The representation of the activation of reduced PAN fibers with excess of glutaraldehyde is depicted in Scheme 3. Bifunctional glutaraldehyde reacts with amino groups of the fiber to produce aldimine units which have free aldehyde groups, these further react with the amino groups of the toxoid. Thus, the tetanus toxoid anchors onto modified PAN fibers through stable covalent linkages. The optimized concentration of tetanus toxoid was also evaluated where activated PAN fibers were incubated with 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 lf mL^{-1} of tetanus toxoid. Since, the maximum optical density in the immunoassay was obtained for the concentration 2.5 lf mL^{-1} of the toxoid, it was chosen as ideal for the immunoassay.

3.3. Detection of HAT tetanus toxoid antibody

The schematic representation of the immune complex formed on modified PAN fibers is presented in Scheme 4. Modified PAN fibers are able to detect HAT antibody in the sandwich ELISA format developed using the toxoid, antibody, and the corresponding peroxidase conjugate. It was observed that on addition of TMB substrate, a blue color was developed on the surface of the fibers, confirming the covalent attachment of above-mentioned biomolecules. Optimum dilution of RAH-HRP conjugate was standardized empirically from the checkerboard ELISA. Modified PAN fibers were immobilized with fixed concentration of toxoid and HAT-Ab and the serially diluted peroxidase conjugate were incubated with the fibers. As shown in Fig. 2, an Ab/conjugate saturation pattern in optical density can be observed. With the increase in conjugate dilution from 1:2000 to 1:16,000 in the assay, there was a subsequent decrease in the O.D. was recorded. Same trend was also observed for the negative control for each dilution. Since the O.D. in the negative control for the lower dilutions (1:2000 and 1:4000) was too high and absorbance for the test of 1:16,000 dilution was low, the working dilution for the assay was optimized to be 1:8000.

Different ampoules of tetanus toxoid were utilized for the immunoassay for the detection of antibodies. Similar results were obtained for different ampoules of tetanus toxoid and no variation was observed in the immunoassay.

3.4. Evaluation of analytical sensitivity, specificity and reproducibility of the modified PAN fiber ELISA system

The dose response curve for the detection of tetanus toxoid antibody for concentration ranging from 0.01, 0.05, 0.005, 0.00075, and 0.0005 is depicted in Fig. 3. Results show a typical sigmoidal pattern where very high optical density was recorded for antibody

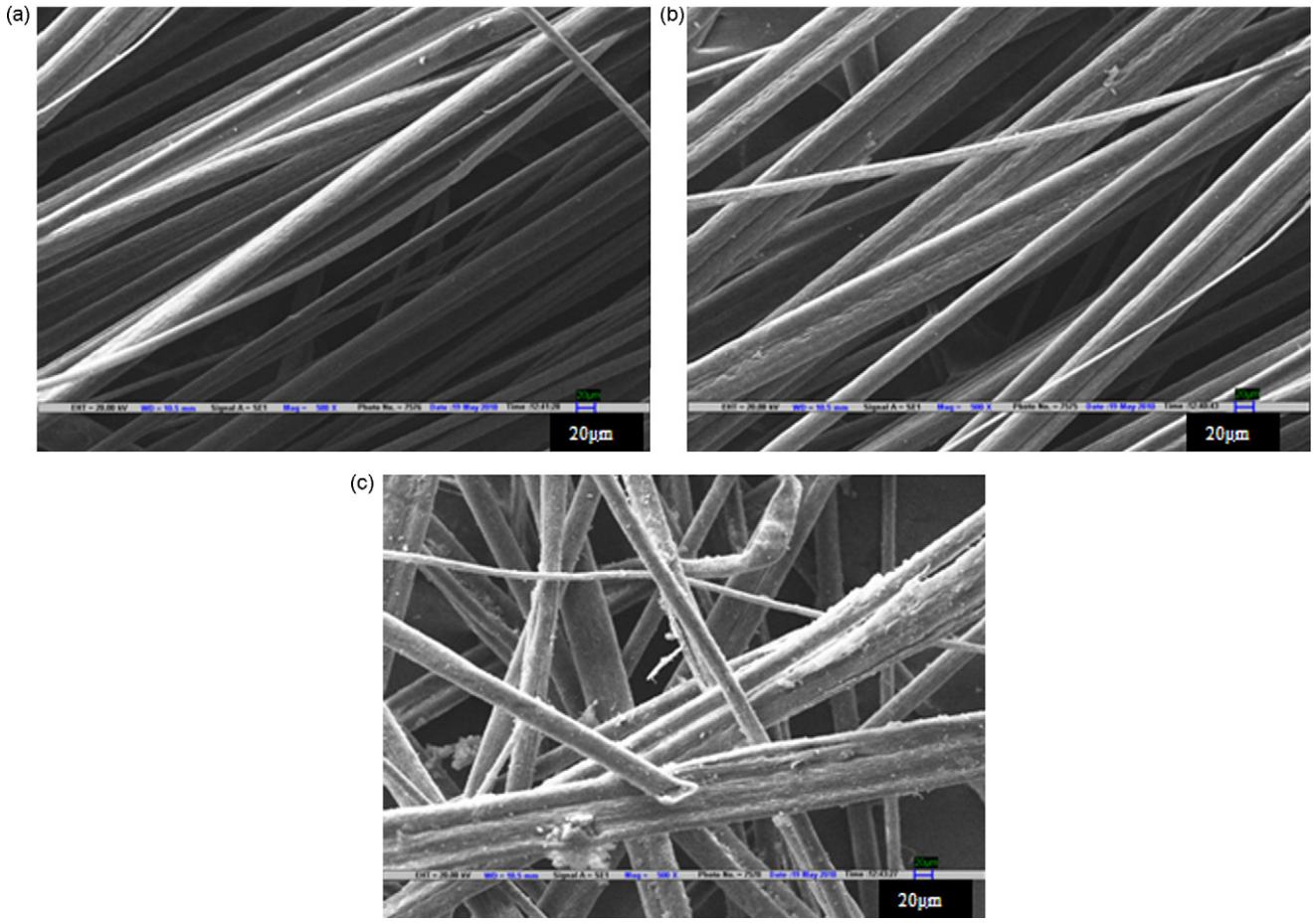
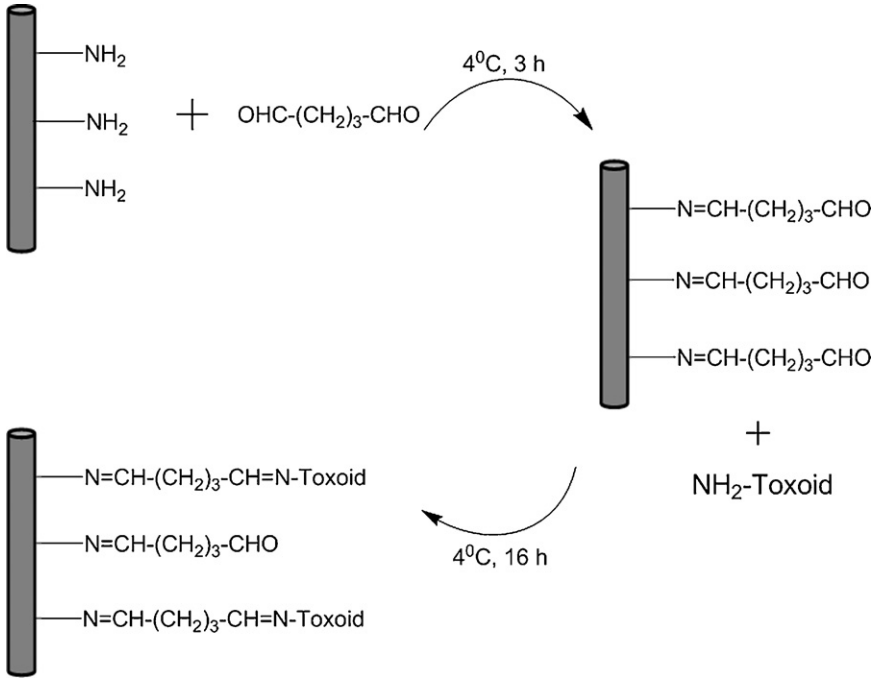
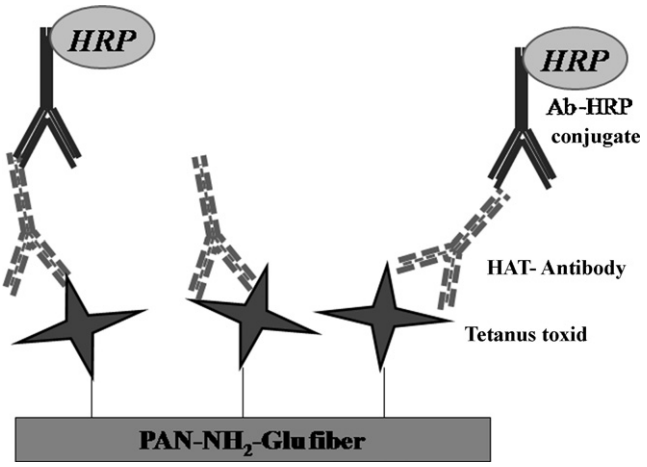


Fig. 1. Scanning electron micrographs of (a) unmodified PAN fiber (b) 24 h reduced and (c) Immobilized PAN fibers.



Scheme 3. Activation of the reduced fibers with glutaraldehyde and covalent immobilization of tetanus toxoid.



Scheme 4. Schematic representation of the sandwich immunoassay developed on modified PAN fibers for the detection of anti-tetanus antibody.

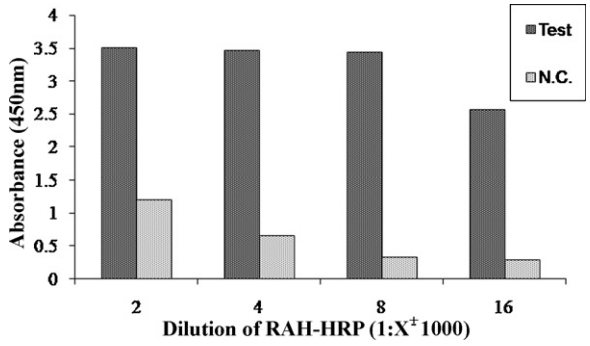


Fig. 2. Activity of various dilutions of RAH-HRP in the assay for test and control experiment.

concentration greater than 0.1 IU mL^{-1} indicating the saturation of Ab concentration at level higher than 0.1 IU mL^{-1}. The optical density decreased in a linear fashion with the decrease in the HAT-Ab concentration. The sensitivity or the lowest detection limit is a very important parameter of any immunogenic assay [35–38]. Sensitivity of assay for the detection of HAT-Ab on modified PAN fibers was found to be 0.0005 IU mL^{-1} (which corresponds to 8.5 ng mL^{-1} since 1 IU mL^{-1} of HAT is equivalent to 17 mg L^{-1}) [2] as statistically significant difference between the negative control and the lowest detection level of Ab was observed. The absorbance value at concentration 0.0001 IU mL^{-1} of HAT-Ab was significantly low and close to the negative control absorbance value of 0.328.

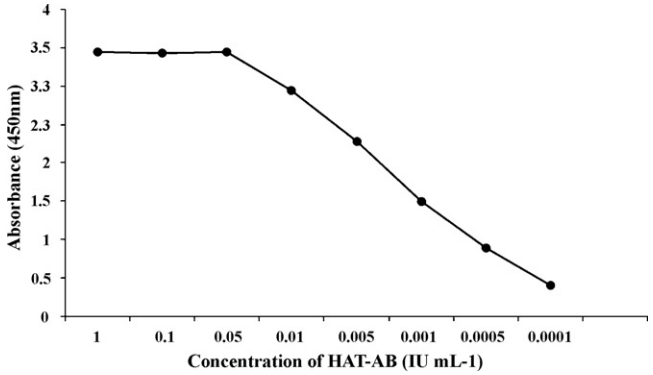


Fig. 3. Evaluation of analytical sensitivity of modified PAN-ELISA with 2.5 lf mL^{-1} of toxoid and 1:8000 dilution of RAH-HRP for the detection of HAT-Ab.

Table 1
Inter- and Intra-assay coefficient of variation for 0.01 IU mL^{-1} and 0.001 mL^{-1} of HAT-Ab.

Concentration of HAT-Ab (IU mL^{-1})	Coefficient of variation (CV %)	
	Intra-day	Inter-day
0.01	11	5.7
0.001	5.4	20

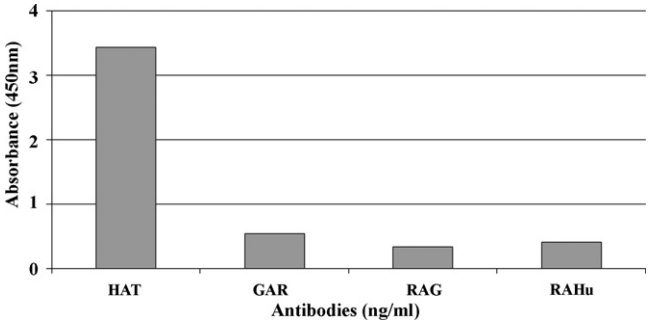


Fig. 4. Specificity of modified tetanus toxoid immobilized PAN fibers in comparison with other IgG's – GAR (Goat anti-Rabbit), RAG (Rabbit anti-Goat), RAHu (Rabbit anti-Human) IgG.

We evaluated intra-assay coefficient of variation by analyzing the same concentration sample three times with multiple replicates ($n = 5$) and inter-assay ($N = 4$) coefficient of variation of same concentration samples on different days respectively. Good reproducibility of the developed immunoassay can be observed from the data given in Table 1. Specificity of the immunoassay depends on the interaction of antigen and antibody involved. This method of Ab detection has the advantage of specificity since the interaction of antibody and antigen i.e. toxoid and its antibody is specific intrinsically. This was proved when a very high O.D. was obtained when HAT-Ab taken as analyte for the modified PAN fibers immobilized with tetanus toxoid as compared to other antibodies like GAR-IgG, RAH-IgG and RAHu-IgG in the assay protocol (Fig. 4).

Comparative analytical studies of modified PAN-ELISA with conventional ELISA method where the assay was performed on 96-well PS microtiter plate and presented in Fig. 5. It was observed that with increase in the dilution of antibody, the absorbance value decreased for both the matrices but the O.D. of modified PAN fibers was always higher that that of microtiter plates. The sensitivity or the lowest detection level of HAT-Ab detected by the Plate-ELSIA was 0.001 IU mL^{-1} while PAN-ELISA has detection limit of 0.0005 IU mL^{-1}. This may be attributed to the large surface area of the fiber as well as covalent binding of biomolecules which are not lost during extensive washing steps in modified PAN-ELISA. More-

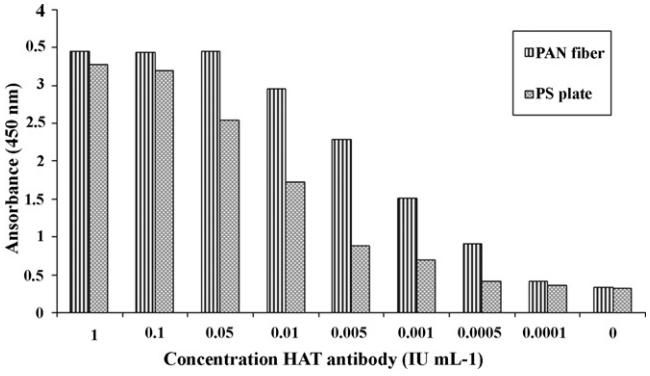


Fig. 5. Comparison of assay performance of PAN-ELISA and conventional 96-well microtiter plate ELISA.

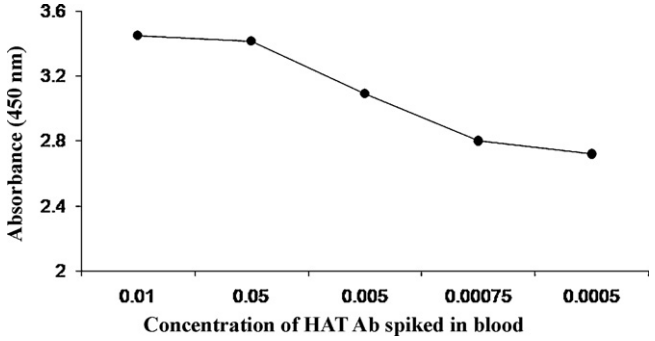


Fig. 6. Detection of different concentration of HAT-Ab spiked in human blood.

over, the PAN-ELISA gave substantial absorbance for the HAT-Ab at a concentration as low as 0.0005 IU mL^{-1} where the plate ELISA produced absorbance value very near to the control (no analyte) at low Ab concentrations up to 0.001 IU mL^{-1} [10]. These data implicated that analytical sensitivity of PAN-ELISA was higher than the conventional plate method.

3.5. Detection of HAT antibody in human blood sera

The potential of immunoassay developed on modified PAN fibers was elucidated for the detection of toxoid antibodies in human blood. Modified fibers immobilized with tetanus toxoid were able to detect the corresponding HAT antibodies spiked in human blood. Tetanus toxoid covalently immobilized on modified PAN-NH_2 fibers binds specifically to the toxoid antibody. This complex was detected with the antispecies-HRP conjugate as visualized by the development of the colored product on addition of TMB substrate. It was observed that with increase in the antibody concentration from 0.0001 IU mL^{-1} to 0.1 IU mL^{-1}, the absorbance linearly increased (Fig. 6).

Table 2
Immunization level of twenty-five individuals.

Serum sample	Anti-tetanus toxoid antibody titer value (IU mL^{-1})	Level of immunity
1.	0.00922	Non-protective
2.	0.0095	–
3.	0.01	–
4.	0.00058	–
5.	0.00047	–
6.	0.0043	–
7.	0.0006	–
8.	0.124	Protective
9.	0.0485	Non-protective
10.	0.0005	–
11.	0.0043	–
12.	0.0064	–
13.	0.005	–
14.	0.0987	Protective
15.	0.125	–
16.	0.2	–
17.	0.145	–
18.	0.063	Non-protective
19.	0.098	Protective
20.	0.057	Non-protective
21.	0.112	Protective
22.	0.062	Non-protective
23.	0.084	Protective
24.	0.24	–
25.	0.157	–

3.6. Evaluating the immunity of patients against tetanus

Blood samples of twenty-five different patients were analyzed to evaluate the immunization condition of the individual using the modified PAN fibers by estimating the antibodies against tetanus toxoid. Quantitative estimation of the antibodies in each patient was extracted from their respective absorbance values from the reference curve (Fig. 3), where the concentration of HAT-Ab is plotted against absorbance. The modified PAN fibers immobilized with tetanus toxoid access the presence of tetanus antibody in human sera up to a very low concentration. The status of immunity to tetanus toxoid is described according to the serum level of specific immunoglobulin against the toxoid. The diagnostically relevant cut-off of tetanus toxin antibody has been recommended by World Health Organization (WHO) to be 0.1 IU mL^{-1} with any ELISA testing [4,13]. 0.1 IU mL^{-1} concentration of antibody has been termed as the protective level of immunity and concentration lower than this level are interpreted as non-protective and vaccination is recommended (see Table 2).

4. Conclusions

Surface modified polyacrylonitrile fibers were successfully evaluated as a matrix to develop an immunoassay for the detection of tetanus toxoid antibody. Sandwich ELISA developed with PAN fibers was able to measure accurately, precisely very low amounts of tetanus toxoid antibodies required for the clinical investigation of various pathological conditions. The sensitivity of the assay was found to be 0.0005 IU mL^{-1} of HAT antibody which was much higher than the conventional plate method. The immunoassay was validated and assay parameters (inter, intra-run coefficient of variation, sensitivity, specificity, etc.) were established. The method was also applied for the determination of HAT antibodies spiked in human blood. It was found that toxoid immobilized PAN fibers were able to empirically detect the HAT antibodies even from a complex biological system like human blood. Antibody levels of humans belonging to different age groups were measured, and the protective and non-protective level of immunization was established by calculating the Ab titer values of 25 patients. Immunoassay developed on modified PAN fibers provides a low detection limit, is versatile, robust and achieve easy separation of free and bound moieties. Hence, aminated polyacrylonitrile fibers have the potential to be exploited as a matrix in the field of immunodiagnostics.

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