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Polyacrylonitrile Fiber as Matrix for Immunodiagnostics

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1. Introduction

Accurate assessment of various clinical, elemental, chemical antigenic substances from different sources is imperative for monitoring, preventive and treatment measures. Instrumental techniques, chromatographic analysis and immunological assays have progressed for the accurate measurement of various analytes over last decades [N. C. Van de Merbel, 2008; R. M. Lequin 2005; R. M. Twyman 2005; H. Richardson 1998; J. Garcia-de-Lomas 1997]. Immunoassays provide an easy, simple and sensitive route for the precise determination of analytical concentration. They utilize the concept of high specificity of antibodies to their analogues antigen forming a complex which can be detected using secondary antibody (Ab) coupled with certain labels. These markers or labeling agents can be radionuclides, chemiluminescent substrates, fluorophores or enzymes leading to measurable results. In the areas of safety regulations, instrumentation and convenience of protocol, enzyme immunoassays have easily surpassed others over the years. Enzyme catalyzed immunochemical test had caught the imagination of researchers leading to development of numerous immunoassays over the years. The future of enzyme immunoassays will bring more rapid test results with simplified procedures catering to wider audience for clinical applications. Extension of basic concept may also encompass a broader consumer-base consisting of increasing number of potential users which will transcend boundaries of technical disciplines [Maggio, E. T. 1979]. The following introduction descibes enzyme immunoassays in brief with emphasis on polymeric matrices as solid support in ELISA. This chapter describes the designing of solid phase immunoassay using surface functionalized polyacrylonitrile fibers for the sensitive and specific determination of various antibodies. Pendent nitrile groups on polyacrylonitrile fibres were successfully reduced to generate amino groups on the surface of the fibers. The newly formed amino groups of the fibers were activated by a bi-functional spacer-glutraldehyde for the covalent linking of antibodies. Sandwich immuno-complex was developed on these PAN fibers which provided high sensitivity, specificity and reproducibility for the detection of various small analytes.

1.1 Enzyme immunoassays

Enzyme immunoassays have become popular in clinical and medical fields. The concept first described by Landsteiner gained momentum in the late 1950s and 60s setting the stage for the

pioneering work for the rapid development of immunological assays. Utilization of enzymes as isotopic label has greatly overshadowed fluorophores and radioactive substances. The broad range of application of enzyme immunoassay to determine the concentration of serum proteins & hormone levels, illicit & therapeutic drugs, cardiovascular ligands, carcinofetal proteins, immune status, chemotherapeutics and pathogenic microbes will attest to this. Enzyme Immunoassay is a prominent methodology based on selective recognition and high affinity of antigen and antibody coupling along with the sensitivity of simple enzyme assays. They utilize antibodies or antigen coupled to an easily assayed enzyme that posses a high turnover number to enhance assay signal as some chromogenic substrate is converted to coloured product whose intensity is directly proportional to antigenic concentration [Lequin, 2005].

These immunoassays can be broadly categorized into two major types depending on the assay formats. The first one being homogenous assay in which the immunological reaction occurs in solution phase. Homogeneous immunoassays do not require a separation and washing step, but the enzyme label must function within the sample matrix. As a result, assay interference caused by the matrix may be problematic for samples of environmental origins (i.e., soil, water, etc.). For samples of clinical origin (human or veterinary applications), high target analyte concentrations and relatively consistent matrices are often present. Thus for clinical or field applications, the homogeneous immunoassay format is popular, whereas the heterogeneous format predominates for environmental matrices [Rubestein et al., 1972; Pulli, et al., 2005; Voller, 1979]. Heterogeneous assays such as Enzyme Linked Immuno-Sorbent Assays (ELISAs) are most widely used detection method which utilizes the concept of immobilization of biomolecules on solid support. These have atleast one separation step allowing the differentiation of reacted from un-reacted materials. The enzymatic activity is quantified either in bound state or free fraction by an enzyme catalyzed process of a relatively nonchromatic substrate to highly chromatic product.

1.1.1 Solid-phase immunoassays

Solid phase enzyme immunoassays, which include Enzyme Linked Immunosorbent Assay-ELISA and Western blot, have become popular as qualitative and semi-quantitative sample screening methods for the laboratory diagnosis of infectious diseases, auto-immune disorders, immune allergies and neoplastic diseases [Condorelli & Zeigler, 1993; Derer, et al., 1984; Gosling, 1990; Rordorf, et al., 1983; Voller, et al.,1976]. In ELISA, antibody immobilized on the solid support detects the specific antigen present in the sample and this immune complex is detected by a high turn-over enzyme conjugated antibody. The excess of reagents are washed off in each step and the subsequent substrate interaction yields a coloured product either for the direct visualization or for measuring the optical density. Thus, the ELISAs are among the most specific analytical techniques providing a low detection limit and are economical, versatile, robust, achieve easy separation of free and bound moieties and be automated on demand [Engvall 1977; Peruski A. H. & Peruski L.F., 2003; Wilson & Walker, 1994]. Within the past decade, immunochemical methods have proven to be an alternative or a supplement to the established chromatographic methods.

Sandwich ELISA is a dominant format where a “sandwich” type complex is formed with immobilized antibody, target molecule and secondary antibody labeled with enzyme. Immobilization anchors the first antibody which recognizes the specific antigen from the

sample which is detected by enzyme conjugate. Excess of reagents are washed off in each step and the subsequent substrate reaction yields coloured signal for direct visual or spectrometric assessment. The amount of enzyme activity is measured under standard conditions is directly proportional to the antigen present in sample. The immobilization however, should not lead to loss of activity of biomolecule due to change in the orientation and steric hindrance [Moulima, et al., 1998].

1.1.2 Immobilization techniques

1.1.2.1 Physical adsorption

Commonly used immobilization methods include physical absorption or adsorption of biomolecules on the solid support. This involves immobilization of biomolecules through weak forces such as vander waal, electrostatic, hydrophobic interaction and hydrogen bonding. However, non-specific interaction may lead to desorption of the biomolecule during the integral intensive washing steps of assay ensuing erroneous results [Honda, et al., 1995; Rejeb, et al., 1998; Palma, et al., 2004; Palmer, et al., 2004; Tedeschi, et al., 2003]. A controlled covalent attachment of Abs is more preferred to random adsorption so as to achieve better homogeneity in antibody coating.

1.1.2.2 Covalent attachment

Covalent attachment involves the chemical interaction of counter functionalities present on solid matrix and biological entity. The covalent bond induces flexibility to the bond relieving it from steric hindrance and crowding of biomolecules leading to conformational stability. Tethering analytical compound to solid support via functional groups leads to its reduced non-specific absorption, greater stability and better biological activity and enhanced signal output. Lehtonen and Vilijen [Lehtonen & Viljanen, 1980] have studied the antigen attachment in ELISA for the detection of chicken anti-bovine serum albumin antibodies and compared the non-covalent and covalent coupling of biomolecules. They have used polystyrene (PS), nylon and cynogen bromide (CNBr) activated paper and have reported the substantial leakage of antigen from both PS (30%) and nylon (60%) while less desorption was observed for the CNBr activated paper during washing steps. Covalent immobilization is difficult with the non-functionalized surfaces including PS. Eckert et al [Eckert, et al., 2000] have grafted glycidal methacrylate on PS microtiter plate for immobilizing proteins and have reported poor reproducibility of the results. Hence, modified and synthesized functional groups containing solid surfaces are being employed for ELISA.

1.1.3 Polymeric matrices as solid support for immobilization

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 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. Hence, surface and interface chemistry of lots of polymeric materials is currently manipulated to make them amenable for covalent immobilization. The conglomeration of material science and molecular biology has lead to the development of new technologies which benefit from the

exquisite specificity of biomolecules and controllable surface properties of polymeric materials. Polymeric materials are surface modified for the generation of an array of functional groups improving hydrophilicity, hydrophobicity, biocompatibility, conductivity apart from providing active groups for the covalent immobilization of biomolecules. Many polymeric materials such as polyethylene, nitrocellulose (NC), Dacron, polyvinyl chloride (PVC), nylon, polyacrylonitrile (PAN) etc. have been widely studied as bioassay's matrix over the years as a reliable interface between materials and biological moieties [Charles, et al., 2006; Jackeray, et al., 2010; Jain, et al., 2008; Venditti, et al.,2008].

1.1.4 Polyacrylonitrile fibers

Polyacrylonitrile in various forms like membranes, fibers and nano-fibers have been exploited in different fields of composites, protective clothing, pervoporation, water treatment, gas separation technology, nanosensors, enzyme immobilization, haemodialysis, biochemical product purification and other biomedical applications [Che, et al., 2005; Nouzaki, et al., 2002; Shinde, et al., 1995; Sreekumar, et al., 2009]. This wide popularity is due to their excellent thermal & mechanical properties, chemical stability, abrasion resistance, high tensile strength and tolerance to most solvents, bacteria & photo-irradiation [Frahn, et al., 2004; Iwata, et al., 2003; Kim, et al., 2001; Musale & Kulkarni, 1997]. Polyacrylonitrile (PAN) is the most important fiber and film/membrane forming polymer. PAN hollow fiber membranes such as AN 69 (produced by HOSPAL, fabricated from an acrylonitrile/methallyl sulphonate copolymer) have already been used as dialyzers and high flux dialysis therapy [Valette, et al., 1999; Thomas, et al., 2000]. PAN hollow fibers are already used as dialysers that remove low molecular weight compounds and proteins. PAN fibers have high surface area, very high mechanical strength, abrasion resistance & posses' insect resistance. Though PAN has many superior properties, it has few demerits of moderate hydrophilicity, low moisture absorption and lack of active functionality limiting its usages. However, the presence of nitrile groups along with the fiber backbone offers multidirectional approaches to modify fibers for specific applications unlike synthetic membranes which can be damaged during the modification [Wen & Shen, 2002, 41].

There is a lot of interest in modifying PAN by changing its surface structure by plasma and photo-induced graft co-polymerization [Deng et al., 2003; Hartwig, et al., 1994; Ulbricht, et al., 1995; Zhao, et al., 2005; Zhao, et al., 2004] enzymatic [E. Battistel, et al., 2001] and chemical modifications including hydrolysis and reduction of PAN fibers. Haiquing Liu *et al* [Liu & Hsieh, 2006 48] have hydrolysed PAN nanofibers to improve its water absorbing capacity. A PAN derivative of poly (acrylonitrile-maleic acid) containing reactive carboxy functionality were synthesized and fabricated to nanofiber and used to immobilize lipase by Sheng-Feng Li [Li et al., 2007]. Ezeo Battistel et al have used nitrile hydratase to enzymatically modify PAN fibers to introduce amide groups. Zhao Jia et al [Jia & Du, 2006] have hydrolyzed and chlorinated PAN fibers and then grafted natural polymer casein to improve moisture absorption and water retention properties. Fumihiro Ishimura [Ishimura & Seijo, 1991] 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.

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 [Li et al., 2007; Matsumoto, et al., 1980]. Zhao Jia et al

have grafted casein directly on PAN fibers to improve their antistatic and water retention properties [Leirião, et al., 2003]. Fumihiro 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 [Ishimura & Seijo, 1991].

2. PAN fibers-surface modification and their evaluation for the colorimetric detection of analytes

2.1 Reduction of PAN fibers

In a 250 mL RB flask equipped with water condenser, equivalent quantities of lithium aluminium hydride (LAH) (1.5 g) and polyacrylonitrile PAN fibers (1.5 g) were reacted in excess of pre-dried diethyl ether, AR grade (120 mL) [Matsumoto, et al., 1980]. The reaction mixture was stirred continuously in a moisture free environment under the nitrogen blanket at room temperature (27 ± 2^0C) for different time periods (0.5 h, 1 h, 6 h, 12 h and 24 h). The fibers were thoroughly washed to remove the excess of LAH and dried in vacuum oven for 4 h. They were then stored in the desiccator for further use.

2.2 Activation of the aminated fibers and immobilization of antibodies

10 mg aminated PAN fibers (PAN-NH_2) were activated using 12.5% glutaraldehyde/borate buffer (pH 8.5) in a micro-centrifuge tube at 4 °C for 3 h. The fibers were thoroughly washed with borate buffer (pH 8.5) and Tween/PBS (pH 7.2) to remove excess of glutaraldehyde [Leirião, et al., 2003; Matsumoto, et al., 1984]. Glutaraldehyde activated PAN fibers (PAN-NH_2-Glu) were used for the immobilization of enzyme conjugated antibodies. 10 mg of differently aminated PAN-NH_2-Glu were incubated with GAR-HRP (1 mL) of various dilutions ranging from 1:1000-1:64000 for 16 h at 4 °C with occasional shaking. The fibers were washed with Tween/PBS (pH 7.4). 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 3, 3′, 5, 5′ tetramethyl benzidine (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.

2.3 Evaluation of the modified fibers for the detection of analyte (RAG) by performing checkerboard ELISA

A checkerboard or 2-Dimensional serial dilution method was carried out to optimize the concentration and dilution of the analyte and enzyme-label respectively. A checkerboard titration is single experimental set in which the concentration of two components is varied that will result in a pattern. 10 mg of activated PAN fibers (PAN-NH_2-Glu) were immobilized with 1 mL of GAR-IgG antibody (1 µg/mL to 5 µg/mL) for 16 h at 4 °C. Unbound antibodies were removed and the fibers were washed with Tween/PBS. The unbound sites of the fibers were blocked with 12% skimmed milk (1 mL). These primary antibody immobilized fibers were incubated with a fixed concentration (1 mL) of complimentary antibody RAG-IgG (60 ng/mL) for 1.5 h at 37 °C. After washing with Tween/PBS, the fibers were again incubated with 1 mL of enzyme conjugate of the first antibody - GAR-HRP, conjugate dilutions ranging from 1:2000-1:32000 for 1.5 h at 37 °C. Subsequently, the conjugate was decanted and the fibers were washed with Tween/PBS

buffer. After the removal of unbound conjugate, the activity of HRP was evaluated, using its substrate TMB as mentioned earlier and the optical density was recorded. Non-specific binding (NSB) of the modified fibers was also evaluated. 10 mg of modified PAN fibers were immobilized with 1 mL of 60 ng/mL of the analyte RAG-IgG. They were blocked with 1 mL of 12% skimmed milk and were incubated with the subsequent GAR-HRP conjugate dilutions (1:2000- 1:32000) after the washing steps. A complimentary set of experiments were performed to determine the sensitivity of the assay i.e. to measure the minimal detectable concentration of the analyte RAG. The activated PAN fiber with optimized primary antibody GAR-IgG concentration, (determine by previous experiment) was incubated with serial dilutions (120 ng/mL-1 ng/mL) of RAG-IgG antibody taken as analyte for 1.5 h at 37 ^{0}C. After washing, fibers were incubated with GAR-HRP of 1:8000 dilutions (as optimized previously) for 1.5 h at 37 ^{0}C and then the fibers were washed again with Tween/PBS. The activity of peroxidase was measured using its substrate TMB and optical density was recorded with the ELISA plate reader.

The developed ELISA system was compared with conventional ELISA using polystyrene (PS) 96-well microtiter plates. Same experimental procedure was followed as that with the activated PAN fibers. The fibers were also compared with the ELISA system where the PS 96-well microtiter plates were pre-treated with 12.5% glutaraldehyde for 3 h at 4 ^{0}C.

2.4 Detection of human blood IgG's using the developed assay of modified PAN fibers

Human blood was taken and 10 µL was spotted on a wattman filter paper no. 1, the blood dots were air dried at 37 ^{0}C for 1 h and then stored at 4 ^{0}C for further use. When required, the filter paper with the dotted blood was punched from a standard puncing machine and discs of 5 mm diameter were obtained. All the blood spotted samples discs were eluted in 100 µL of PBS of pH 7.4 for 1 h at room temperature. After this, serial dilutions of the eluted samples were performed to obtain 1:10, 1:100, 1:1000 and 1:10000 dilutions. These were stored at 4 ^{0}C for further use.

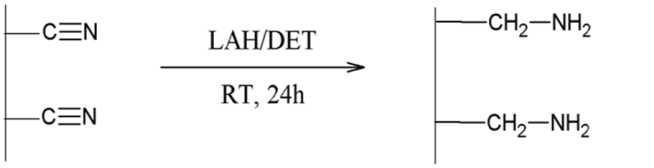
10 mg of the reduced PAN fibers were taken after the activation with glutaraldehyde in a microcentrifuge tube and incubated with 1 mL of 3 µg/mL of GAH-IgG for 16 h at 4 ^{0}C. Antibody immobilized fibers were washed with Tween/PBS and incubated with 1 mL of the human blood elute (undiluted) for 1 h at 37 ^{0}C. After washing, the non-specific binding sites were blocked with 12% skimmed milk at 4 ^{0}C. Blocking solution was removed and fibers were washed and incubated with 1:8000 enzyme conjugate of anti-species of human antibody RAH-HRP for 1 h at 4 ^{0}C. The fibers were washed and the activity of the peroxidase was measured by adding the substrate TMB. The OD was recorded in ELISA plate reader. To determine the sensitivity of the developed assay for human blood, the eluted and serially diluted human blood samples were incubated with the GAH-IgG antibody immobilized PAN fibers followed by the aforementioned ELISA steps. The specificity of the developed assay was further checked using rabbit blood.

3. Results and discussion

3.1 Amine content

The pendent nitrile groups present on the surface of polyacrylonitrile fibers were successfully reduced to primary amino groups with LAH as schematically diagrammed in

Scheme 1. The amine content determined by performing acid-base titrations revealed that with the increasing reduction time, the primary amine content increased and was found to be highest for 12 h reduction time after that amination decreased. As the reduction time increased, prolonged action of the reducing agent LAH ensures the conversion of more number of nitrile groups to amino groups. However, it was also observed that with very high reduction time e.g. 24 h the content of amino groups reduced. The explanation to the decreased amine value is not clear but similar pattern was also reported in US Patent No 4486549 [Matsumoto, et al., 1984]. Physical changes also indicated reduction of fibers such as change of colour from shinning white to pale yellow which increased with the advancement of reaction. Extent of the reduction also influenced and increased the brittleness (noted by ease of tearing of fibers) and roughness (gauzed by touching) in the fibers.



Polyacrylonitrile fiber

Reduced PAN fiber

Scheme 1. Reduction of pendent nitrile groups of polyacrylonitrile fibers to amino groups

SI No.	Time of reduction (h)	Content of Amino groups (μM/g)
1.	0.5	28.75
2.	1	36.29
3.	6	78.5
4.	12	126.1
5.	24	108.75

Table 1. Content of amino groups of PAN fibers as measured by acid–base titration method

3.2 ATR-FTIR spectroscopy

FTIR spectroscopic studies showed an appearance of broad band from 3400-3500 cm^{-1} after reduction, which is attributed to the N-H stretching vibration, demonstrating the formation of the primary amine groups. IR spectra were also used to monitor the relationship between surface amination and the reduction time. It was observed that as the reduction time increased from 0.5 h to 12 h, the band corresponding to amino groups increased, but decreased for fiber reduced for 24 h. Relatively, as the reduction time increased, the peak 2241 cm^{-1}, corresponding to the C N stretching vibration of nitrile group decreased in magnitude and

completely vanished in the spectra of the fiber reduced for higher time periods. Also as the reduction progresses, the peaks corresponding to C-N stretching and bending vibration diminished and vanished for the fibers reduced for higher time periods. A peak at 1730 cm^{-1} corresponding to C-O stretching of C=O is observed probably due to the addition of small percentage of methyl methacrylate/vinyl acetate added during the polymerization.

After glutaraldehyde treatment absorption peak of stretching vibration of imines group (N=C) comes at 1655 cm^{-1} (Fig. 2 A). However, the peak of free carbonyl group of glutaraldehyde at 1720 cm^{-1} was not visible as it is merged with the peak of methacrylate/acetate groups. The spectra of GAR-IgG antibody immobilized PAN fibers showed absorption band at 2506 cm^{-1} different from that of glutaraldehyde activated PAN fibers (Fig. 2 C), which also correspond to the spectrum of antibody (given in the Fig.2 B for comparison). The band around 2506 cm^{-1} may be attributed to the O-H stretching of carboxyl group present in the *Fc* region of Ab [Allmer, et al., 1989].

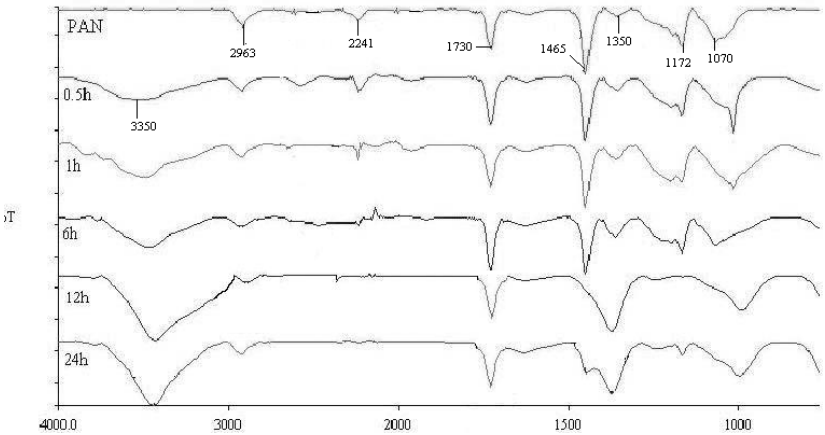


Fig. 1. ATR-FTIR spectra of unmodified and aminated PAN fibers

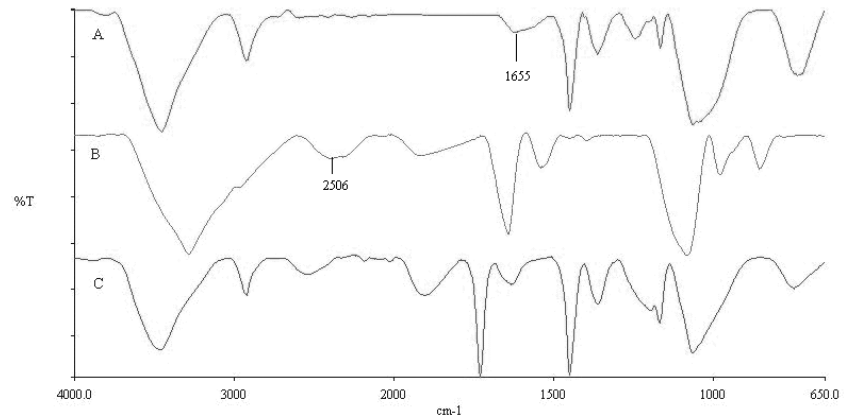


Fig. 2. ATR-FTIR spectra of (A) glutaraldehyde treated reduced PAN fibers (B) Antibody GAR-IgG (C) GAR-IgG immobilized PAN fiber

3.3 Differential scanning calorimetry

Thermal properties of unmodified and reduced PAN fibers were studied in DSC and are given in Fig. 3, 4 and Table 2. It was observed that two thermal transitions occurred for dry PAN fibers in the first cycle of heating and only one in the second cycle. On first cycle of heating a transition is observed at 96 ^{0}C. A sharp peak also appears at 150 ^{0}C which is attributed to the cyclization of PAN (Fig. 3a) involving the pendent nitrile groups present on its surface as given in Scheme II [S. Hajir, et al., 2003]. It was observed that for the fibers reduced for 0.5 h and 1 h, the peak cyclization temperature shifted to 162 ^{0}C and 166 ^{0}C respectively (Table 2). However, fibers reduced for 6 h, 12 h and 24 h (Fig. 3c) did not show any second transition. This altered cyclization behavior of modified PAN indicates that negligible nitrile groups were available to facilitate the cyclization process on heating as majority of them were converted to amino groups on reduction.

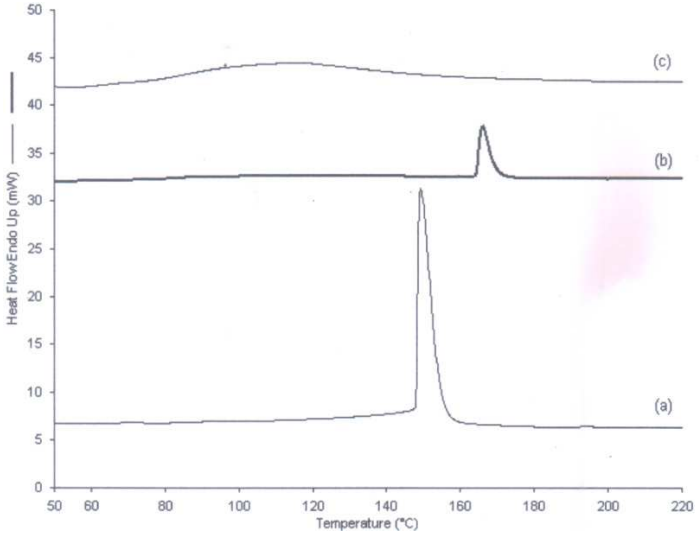
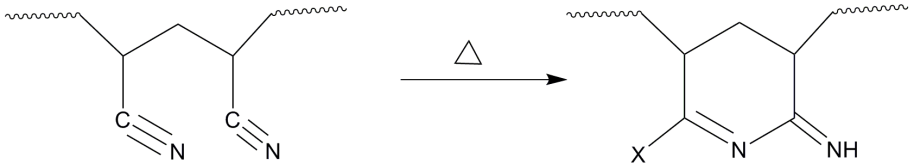


Fig. 3. Cyclization temperature of (a) unmodified PAN fibers and (b) 1 h reduced fibers (c) 6 h reduced fibers

Glass transition temperature (T_g) of PAN and reduced fibers as recorded during second cycle of heating is given in Table 2 and is graphically presented in Fig. 4. T_g of PAN was observed at 96 ^{0}C as a result of chain mobility caused by the weakening of vander waals forces in the amorphous region of the polymer [Zhang & Li, 2005]. However, no significant change in the glass transition temperature was observed in reduced fibers. No change in T_g indicate that polymeric backbone was not affected by the reduction of pendent nitrile groups.



Scheme 2. Cyclization of nitrile groups of PAN upon heating in DSC

Fiber type	Initial exothermic temp. (^{0}C)	End exothermic temp. (^{0}C)	Cyclization temp. (^{0}C)	Heat energy (mJg^{-1})	Glass transition temperature T_g (^{0}C)
PAN	146	160	150	0.374	96.351
PAN-NH_2 (0.5h)	157	180	162	0.348	110.7
PAN-NH_2 (1h)	164	174	166	0.352	98.294
PAN-NH_2 (6h)	-	-	-	0.356	101.327
PAN-NH_2 (12h)	-	-	-	0.388	98.921
PAN-NH_2 (24h)	-	-	-	0.604	102.350

Table 2. Thermal behavior of the unmodified and fibers reduced for different time periods

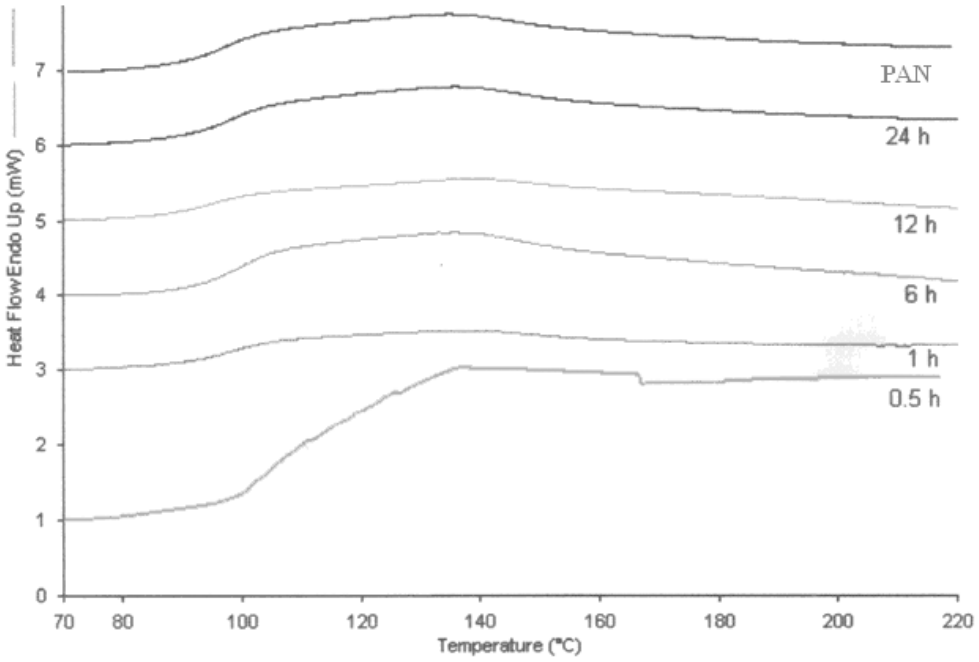


Fig. 4. Thermal studies of reduced fibers using DSC

3.4 Scanning electron microscopy

Scanning electron micrographs of unmodified and reduced PAN fibers are depicted in Fig 5 a-f. Unmodified fibers were found to be smooth and untangled with fiber diameter of 50-70 µm. No significant morphological changes were observed for 0.5 h and 1 h reduced fibers (Fig. 5 b-c). However, as the reduction time increased fibers gradually become rough, rugged and the extent of entanglement also increased, indicating conversion of a large number of nitrile to amino groups. Similar observations are also reported by other authors [Liu and Heish, 2006]. 24 h reduced fibers were further used for immobilization and development of the assay due to its consistent and reproducible immobilization results. Hence, its morphology was also studied after glutaraldehyde activation and antibody immobilization. The micrographs of activated and GAR-IgG antibody immobilized fibers are presented in Fig. 6. No major morphological changes were observed on activation of the fibers with glutaraldehyde. After immobilization of antibodies, topography of the fiber changed (Fig 6 b & c). Deposition of the antibody can be seen on the modified fibers after immobilization on higher magnification.

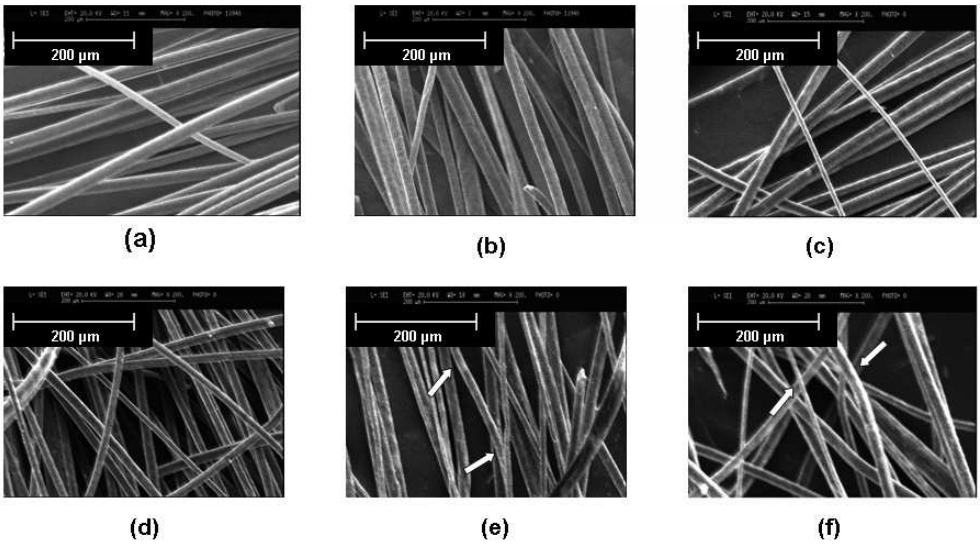


Fig. 5. Scanning electron micrographs of unmodified and reduced fibers (a) Unmodified PAN fibers (b) 0.5 h (c) 1 h (d) 6 h (e) 12 h and (f) 24 h reduced fibers

3.5 Antibody immobilization

Reduced PAN fibers were activated with excess of glutaraldehyde. The primary amine groups of the reduced fibers reacted with one of the aldehydic groups of the bi-functional glutaraldehyde to form the imine linkage (Scheme III). The free aldehyde group of glutaraldehyde covalently binds to the amino groups of residues/units (generally lysine) in the antibodies and the antibodies coupled with enzymes, providing a stable linkage. Thus, antibodies were covalently immobilized on the glutaraldehyde activated PAN fibers.

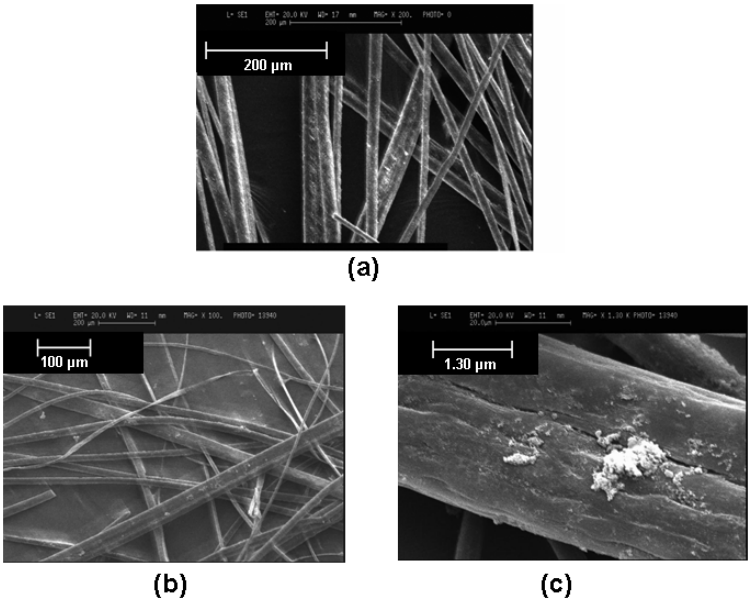
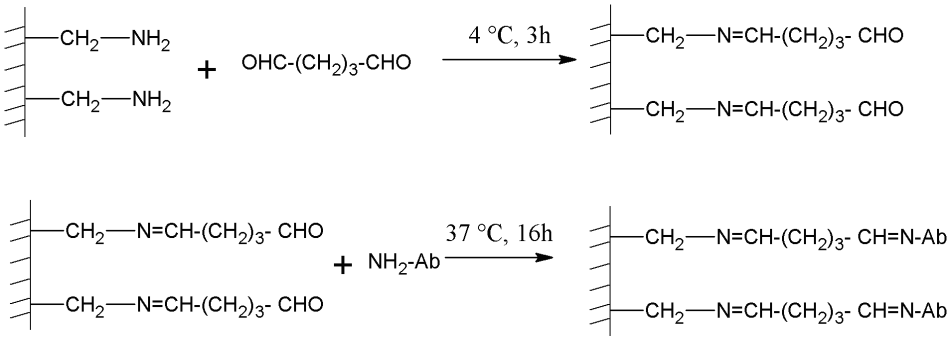


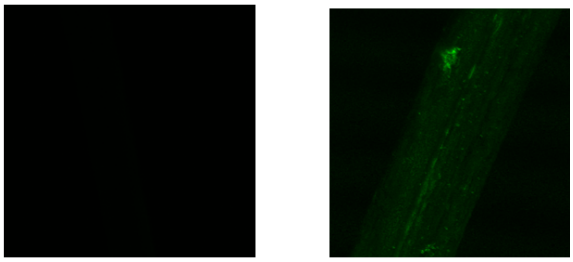
Fig. 6. Scanning electron micrographs of (a) glutaraldehyde activated fibers (b) antibody immobilized PAN fibers (c) Immobilized fibers at higher magnification



Scheme 3. Activation of the reduced fibers with glutaraldehyde and immobilization of antibody on the activated fibers

The attachment of the Ab on the fibers was visualized through confocal laser scanning microscope using fluorophore tagged antibody Goat anti-Rabbit-Fluorescien isothiocyanate (GAR-FITC) (Fig. 7 b). Glutaraldehyde activated fibers were coupled with 1:400 dilution of GAR-FITC for 16 h at 4 ◦C. They were washed with Tween/PBS and studied under the microscope in dark. The null method was applied to deduce the autofluorescence [Jang, et al., 2006] of the polyacrylonitrile (Fig. 7 a) from the fluorescence of dye FITC attached on fiber as GAR-FITC.

A range of HRP conjugated antibodies were prepared in 1X-PBS buffer to evaluate the efficiency of immobilization on aminated PAN fibers. Effect of conjugate dilution shows a



(a) (b)

Fig. 7. Confocal laser scanning electron microscope image of (a) unmodified PAN (b) modified PAN-NH_2-Glu-GAR-FITC at 512 HV voltage

typical sigmoidal curve (Fig. 8). With an increase in the conjugate GAR-HRP dilution from 1:2000 to 1:64000 in the immobilization system, there was a decrease in the optical density (O.D.) recorded. A sigmoidal pattern may be attributed to the saturation of PAN fibers with the Ab at the higher concentration/lower dilutions. Competitive Ab-Ab interaction of GAR-HRP molecule for binding to aldehydic groups and steric hindrance are the principal factor leading to the plateau of O.D. values for the immobilization at higher concentrations of conjugate. Very low optical density was observed at lower dilutions as negligible binding occurs, since less conjugate was present for binding. Similar pattern was observed for the time varied reduced fibers (0.5 h to 24 h) as given in Fig. 9. Efficacy of differently aminated PAN fibers for immobilization of Ab was studied. Various conjugate dilutions GAR-HRP, ranging from 1:2000-1:64000 were immobilized onto fibers reduced from 0.5 h to 24 h. The result showed that with increase in reduction time, the O.D. increased indicating greater immobilization of the antibody-HRP conjugate occurred on the modified fibers (Fig.9). It was also observed that the O.D. was highest for 12 h reduction time. However, the fibers, which were reduced for 24 h showed the most stabilized readings on repeated experimentation as against that of 12 h reduction time period. Therefore, 24 h reduced fibers were further used for the detection of the analyte.

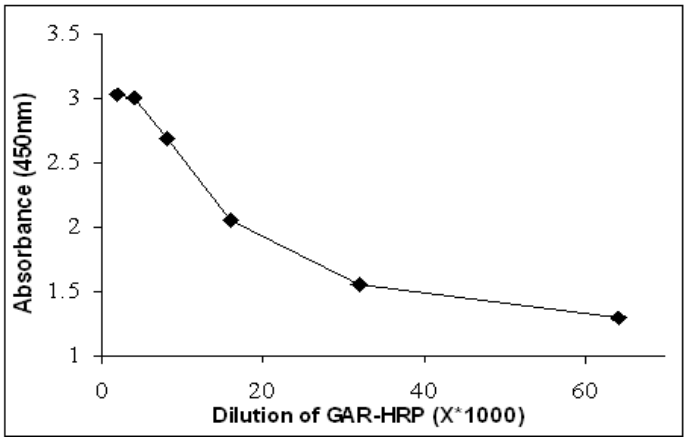


Fig. 8 Activity of GAR-HRP immobilized on modified PAN fiber

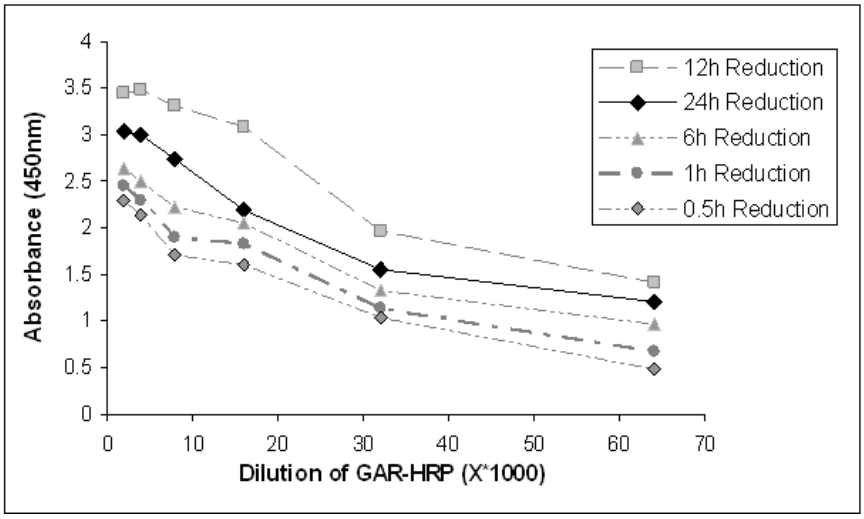


Fig. 9. Activity of GAR-HRP immobilized on PAN fibers reduced for different time periods (0.5 h to 24 h)

3.6 Evaluation of the modified fibers for the detection of analyte (RAG) by performing checkerboard ELISA

A checkerboard is a typical sandwich ELISA technique, where the antibody immobilized to the solid support binds with the complimentary Ab or antigen in the solution. This complex is incubated with the secondary Ab conjugated with an enzyme which provides a colorimetric reaction for the detection of antigen/analyte spectrophotometerically. Reduced PAN fibers were studied to develop a reproducible assay for the detection of analyte over a biologically relevant assay range. Therefore, optimum concentration of each assay reagent has to be standardized empirically. In the first experiment, a varied concentration of primary antibody GAR-IgG was immobilized on to reduced fibers activated by glutaraldehyde. Analyte RAG-IgG was immobilized with a fixed conc. of 60 ng/mL and then serially diluted secondary Ab conjugate GAR-HRP was incubated with the fibers. The assay was carried out in triplicate and the averages of the results are presented in Fig 10 and 11. The result showed that with the increase in primary antibody GAR-IgG concentration from 1 µg/mL to 5 µg/mL and secondary Ab dilution from 1:2000-1:32000, an antibody saturation pattern in optical density (Fig.10) was observed. This curve showed maximum O.D. for 3 µg/mLGAR-IgG conc. at 1:8000 conjugate dilutions, thus, these values were established as the optimized concentration and dilution. This indicated that at higher concentration saturation of primary Ab occurred due to Ab-Ab interactions and steric hindrance. The NSB of Ab on modified PAN fibers was observed from 0.85 to 0.23 for the dilutions 1:2000 to 1:32000 of the conjugate GAR-HRP.

Sensitivity is an important parameter while developing any immunogenic assay. The sensitivity of the assay was determined for the modified PAN fibers by varying the concentration of the analyte RAG-IgG. In the second experimental set up, primary antibody

GAR-IgG (3 µg/mL) and conjugate GAR-HRP (1:8000) were fixed for 10 mg of PAN-NH_2-Glu and the analyte RAG-IgG was varied over a range from 0.9 ng/mL to 120 ng/mL. Over this range of analyte concetration, it was observed that the lowest detectable concentration hence sensitivity of the assay was 3.75 ng/mL (Fig.11).

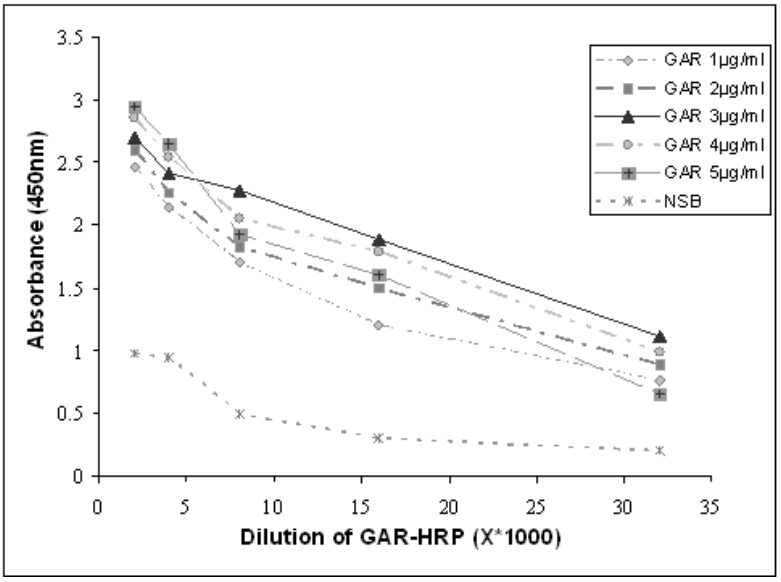


Fig. 10. PAN-NH_2-Glu fiber - ELISA at different primary antibody (GAR-IgG) and conjugate dilution (GAR-HRP)

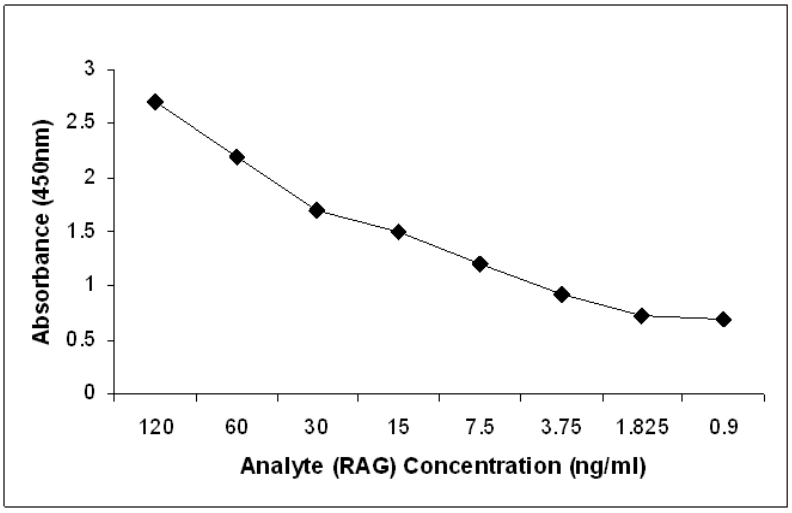


Fig. 11. PAN-NH_2-Glu fiber - ELISA at different analyte concentrations with the 3µg/ml primary antibody GAR concentration and 1:8000 antibody enzyme conjugate GAR-HRP

The results were compared with the conventional ELISA method where the assay was performed on 96-well PS microtiter plate as well as glutaraldehyde pretreated plate and are presented in Fig. 12. It was observed that with the increase in analyte concentration the O.D. increased for all the three solid supports. But the O.D. of modified PAN fibers was always higher than both of the plates. Glutaraldehyde pre-treated and non-treated plates showed decreased activity due to loss of reagents during extensive washing. This confirms, simply adsorbed antibodies bind to solid support get detached while washing, leading to less sensitive assays. Thus, covalent binding on modified PAN fibers leads to high sensitivity and specificity. The advantages of covalent binding are also reported by other authors [Palma, et al., 2004; Tedeschi, et al. 2003; Tyagi, et al., 2009]. From these studies it can be concluded that modified PAN fibers as a solid support are more sensitive, specific and cost effective as compared to PS 96-well microtiter plates.

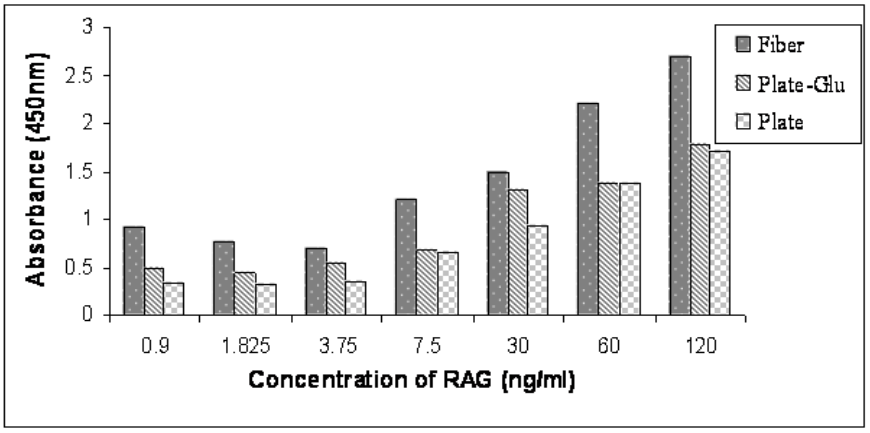


Fig. 12. Comparison of antibody immobilized PAN-NH_2-Glu fiber with the conventional PS microtiter plate and PS plate pre-treated with 12.5% glutaraldehyde

3.7 Detection of human blood IgG's

Modified PAN fibers were used for the detection of antibodies present in human blood. Antispecies of human blood IgG were covalently immobilized on the fibers, which specifically binds with the IgG's of the human blood. This complex was detected with the antispecies-HRP antibody conjugate as visualized by the development of the coloured product on addition of substrate. Sensitivity of the developed assay on modified PAN fibers coated for human blood was also checked and is given in Fig. 13. The human blood elute of 10 fold and 100 fold dilution gave high O.D. values. With further dilutions, the absorbance decreased and negligible intensity was recorded for 1:10000 dilutions. It was also observed that the O.D. of neat elute was lesser than that of 1:10 diluted sample, indicating lesser immobilization of human IgG's with the neat eluate. This can be attributed to the overpopulation of IgG, resulting in their deformed orientation and led to non-homogeneity during immobilization [Endo, et al., 1987, 60]. These results thus established that blood sample as low as 0.1 µL can be easily detected through the developed assay. GAH-IgG immobilized modified PAN fibers were also used for specificity test against human and rabbit blood elutes. Negligible readings were recorded for rabbit blood elute where the O.D.

of neat human blood elute was substantial. The GAH and RAH-HRP used in the assay specifically binds with the antispecies of human blood IgG's. This confirms the non-specificity of the assay with respect to blood of any other organism.

These results were compared with the conventional PS 96-well microtitre plates (Fig. 13). A slightly higher absorbance was recorded for modified PAN fibers in comparison to PS microtiter plate and the glutaraldehyde activated plate. This relates to better sensitivity of the assay due to covalent binding of Ab's to fibers. Modified fibers also showed lesser extent of non-specific binding caused due to physical adsorption and were more specific towards the detection of human blood.

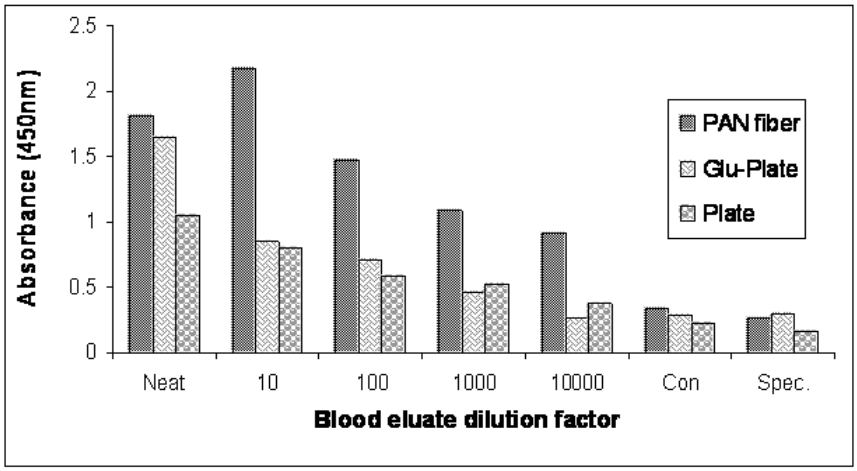


Fig. 13. Human antibody detection by modified PAN fibers-ELISA and comparison with conventional PS microtiter plate and PS plate pre-treated with 12.5% glutaraldehyde

4. Conclusions and prospects

In our work pendent nitrile groups of multifilamentous polyacrylonitrile (PAN) fibers were reduced to amino groups using lithium aluminum hydride for different time of reduction and amine content was estimated by performing acid-base titrations. Modified fibers were characterized by spectroscopic and analytical techniques for the generation of amino groups. The newly formed amino groups of the fibers were activated by using glutaraldehyde for the covalent immobilization of biomolecules. Modified PAN fibers were evaluated as a matrix for sandwich ELISA by using Goat anti-Rabbit antibody (GAR-IgG), Rabbit anti-Goat (RAG-IgG) as analyte and enzyme conjugate GAR-HRP. The fibers reduced for 24 h were able to detect the analyte RAG-IgG at a concentration as low as 3.75 ng/mL. PAN-ELISA gave more promising results when compared with the conventional polystyrene (PS) 96-well microtitre plate-ELISA. The sensitivity, specificity and reproducibility of the developed immunoassay was further established with antibodies present in human blood using Goat anti-Human (GAH-IgG) antibody and the corresponding anti-species HRP enzyme conjugate. These standardized modified PAN fibers when applied for human blood antibody identification showed that 0.1 μL blood elute was sufficient for ELISA. These immunoassays

demonstrate that Modified PAN-ELISA can be exploited as a solid matrix for the detection of variety of biomolecules. Immunoassay developed on modified PAN fibers provides a low detection limit, is versatile, robust and achieve easy separation of free and bound moieties. The sensitivity, specificity and the reproducibility of the developed immunoassay indicate the potential application of modified PAN fibers in the field of immunodiagnostics.

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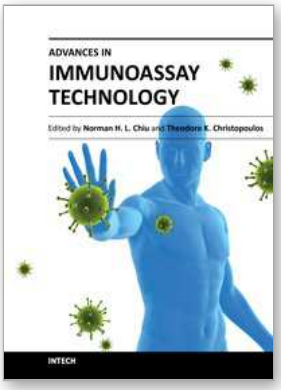

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From the basic in vitro study of a specific biomolecule to the diagnosis or prognosis of a specific disease, one of the most widely used technology is immunoassays. By using a specific antibody to recognize the biomolecule of interest, relatively high specificity can be achieved by immunoassays, such that complex biofluids (e.g. serum, urine, etc.) can be analyzed directly. In addition to the binding specificity, the other key features of immunoassays include relatively high sensitivity for the detection of antibody-antigen complexes, and a wide dynamic range for quantitation. Over the past decade, the development and applications of immunoassays have continued to grow exponentially. This book focuses on some of the latest technologies for the development of new immunoassays.

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