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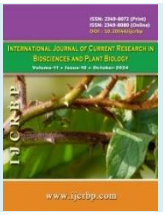

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Bismuth oxide nanoparticles - synthesis and functionalization for the detection of proteins

S. Anitha, Pradeepa Jeeva, N. Anandhi, D. Thamizhini, D. Yugashni, S. Loheshwari, Bharathi Purushothaman, Ajuy Sundar Vijayanandan*

Department of Biotechnology, Karpaga Vinayaga College Of Engineering Technology, GST Road, Chinna Kolambakkam, Palayanoor (Po),Chengalpattu (Dt) 603 308Madhuranthagam(Tk), Tamil Nadu, India.

*Corresponding author; e-mail: ajsdr08@gmail.com

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Abstract

Polymer membranes remain a popular preference as solid support for biomolecular analyses, especially for the discovery of specific proteins or peptides and nucleic acid sequences. But low abundance biomarker proteins detection is frequently based on various types of membrane-based devices. Decreasing of the protein detection limits is vital in commercial applications such as lateral flow assays and in western blots extensively used in proteomics. These currently suffer from inadequate detection sensitivity and low retention for low molecular weight proteins. Normally metal nanoparticles have high binding capacity towards protein and exhibit unique properties in terms of particle aggregation, photoemission, electrical and heat conductivity, catalytic activity and high fluorescence capacity. Bi_2O_3 nanoparticles blended membrane offers the advantage of being able to prepare artificial membranes with improved detection sensitivity of lowering of the protein in Blotting application. In this work, we focused on the role of nano composite membrane exhibit high protein binding activity and provide increased sensitivity for detection of low molecular weight protein in Western Blot. The effects of Bi_2O_3 nanoparticles as additive on the preparation of nitrocellulose blend membranes were investigated in terms of detecting low molecular weight protein. Bi_2O_3 nanoparticles was synthesized by precipitation method using sodium hydroxide solution as an reducing agent. A nitrocellulose membrane was modified by dispersing nanoparticles of bismuth oxide in a nitrocellulose polymer solution with varying concentration of nanoparticles. The membranes were prepared with 30 wt.% of polymer nitrocellulose by phase inversion method. Acetone and deionized water were employed as solvent and coagulant, respectively. The membrane modified by nanoparticles and synthesized bismuth oxide nanoparticles were characterized by UV-Vis Spectroscopy, SEM with EDAX, PSD, TEM and XRD. The detection sensitivity of normal and modified nitrocellulose membrane was assessed statistically via densitometry analysis. Overall, the detection sensitivity was 25 times higher for blended membrane than the normal as they effectively capture trace proteins by keeping small molecules on the metal membrane.

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Introduction

Nitrocellulose membrane is widely used in molecular biology to hold nucleic acids, and it has become effective in binding proteins too. This membrane was among the first used for western blotting and remains a favored choice due to its strong binding ability and low background staining. A key use is for transferring proteins from gels to nitrocellulose during "immunoblotting"(Towbin et al., 1979). The proteins can then be analyzed by binding specific antibodies or using detection stains(Jahn et al., 1984). The binding of proteins to nitrocellulose is rapid, nearly irreversible, and can effectively capture up to 80 to 100µg/cm2. Nitrocellulose is easily wetted and works with many protein detection systems, but detecting small proteins (below 10-15 kDa) can be challenging because they might pass through the membrane during transfer. Improvements to the membrane surface have been recommended to enhance its properties(Xie et al., 2011).

Nanotechnology is an emerging field that allows scientists to create new materials and products at the molecular level. The term "Nanotechnology" was first used by Japanese researcher Professor Norio Taniguchi in 1974, describing it as involving the manipulation of materials at the atomic or molecular level. Nanotechnology deals with materials that are sized between 1 to 100 nanometers, which display unique physical characteristics different from larger materials. It is expected to significantly impact the economy and society in the early 21st century, similar to developments in semiconductor and information technologies. Research into nanoscale materials is growing due to their applications in areas such as microelectronics, sensors, drug delivery, and catalysis. Nanotechnology has multiple benefits and applications across various sectors. In cosmetics, nanomaterials offer improved clarity, cleansing, and skin treatment properties. In electronics, nanoscale transistors lead to more efficient memory storage. In energy, nanotechnology enhances fuel efficiency and production. In food, nanocomposites in packaging prevent spoilage and maintain freshness. The textile industry benefits from treatments that protect fabrics. In catalysis, nanoparticles reduce the need for more expensive catalytic materials. In the environment, nanotechnology aids in affordable clean water solutions. In medicine, research is focusing on using nanotechnology to stimulate nerve cell growth and improve medical imaging. Nanomaterials have unique properties, including magnetic properties, which allow their use in various applications like imaging and biological treatment. They also display interesting optical properties, where size reduction alters the energy states, affecting color and light emission. Electronic properties change based on system size, where smaller systems can exhibit insulator properties. Additionally, nanoparticles made of transition metal oxides show unique catalytic properties due to their large surface area.

Bismuth (III) oxide, or Bi_2O_3, is a significant compound known for its various properties and uses. It is thermally stable and has good optical and electrical characteristics. Bismuth oxide can be obtained from natural sources or as a by-product of smelting. Bi2O3 is employed in ceramics, electronics, and fuel cells due to its ionic conductivity (Oudghiri-Hassani et al., 2015). It can be found in different forms such as powders and pellets, and bismuth oxide nanoparticles exhibit nontoxicity and high fluorescence, making them ideal for applications in sensing and as catalysts. Bismuth oxide nanoparticles (Bi_2O_3NPs) can be synthesized through simple methods like gel conversion. These nanoparticles have a band gap that enhances their catalytic properties and applications in biomedical fields, including biosensing and imaging. The importance of low molecular weight proteins, which typically range from 2-5 kDa, is also highlighted due to their crucial roles in regulating biological processes. Examples include insulin, progesterone, oxytocin, and estrogens, each contributing uniquely to body functions. Nanocomposite membranes are being developed to combine natural molecular properties with synthetic materials. Coating membranes with noble metal nanoparticles boosts their ability to trap proteins, especially small molecular weight proteins during electro-transfer in western blotting. This is achieved through a phase-inversion process, where a polymer transitions from liquid to solid, allowing for customizable membrane characteristics (Ding *et al*.,2010). Adding bismuth oxide nanoparticles to the membrane synthesis process aims to improve protein retention and enhance detection sensitivity, making it possible to capture small proteins effectively alongside larger ones. The aim of the study was therefore the investigation of modified nitrocellulose membrane (Bi_2O_3NPs incorporated membrane) for efficient electro-transfer of low molecular weight protein of >5kDa through western blot experiment.

Materials and methods

Materials

Bismuth sub nitrate, sodium hydroxide and nitric acid are needed for the synthesis of Bi_2O_3NPs. Nitrocellulose as the base polymer and acetone as the polymer solvent for the manufacture of nitrocellulose membrane. Low molecular weight protein of β-insulin was purchased from Sigma-Aldrich.

Preparation of 0.05M Bismuth Subnitrate

Bismuth sub nitrate $(Bi)_5O(OH)_9(NO_3)_4$ is the precursor for the synthesis of Bi_2O_3NPs by precipitation method. 7.3095 g of bismuth sub nitrate will be dissolved in 100 ml of 0.05M HNO_3. This would give 0.05M concentration of bismuth sub nitrate solution (Fig. 1).

Fig. 1: Bismuth Sub Nitrate Powder.

Preparation of 4M Sodium Hydroxide Solution

Weigh 26.5 g of NaOH and dissolve it in 166.66mlofdistilledwater to get4M NaOH solution which would act as a reducing agent (Fig. 2).

Fig. 2: Sodium hydroxide pellets.

Formation of Bismuth Oxide Nanoparticles

Take 100 ml of 0.05M Bismuth subnitrate solution dissolved in 0.05M of HNO_3. Add 166.66ml of 4M NaOH solution drop by drop in above with continue stirring. Keep stirring the mixture at 500rpm until yellow color appears. By increasing the temperature and stirring, the solution becomes dry. Incubate the solution at 90ºC for 2hours under constant stirring of 500 rpm (Fig. 3). Filter the precipitate with Whatmann filter paper No.1. Wash the precipitate with ethanol and distilled water. Dry the precipitate at 60ºC until fine powder forms.

Characterization of bismuth oxide nanoparticles

UV-Visible Spectroscopy

The Bi_2O_3NPs solution will be analyzed in Shimadzu double beam spectrophotometer for its maximum Surface Plasmon Resonance (SPR) between the wavelength of 400 to 700 nm to find out the maximum absorbance as the indication ofthe presence ofBi_2O_3NPs in the solution. They are further characterized by SEM (Philips XL30), EDAX, Particle Size Distribution (PSD), TEM (HRTEM, JEOL JEM-2000 CX2) and XRD.

Metal Nano composite Membrane

Blending the nitrocellulose membrane with Bi_2O_3NPs can be prepared using phase inversion induced by immersion precipitation technique. Nitrocellulose polymer dissolving in acetone solvent to prepare neat Nitrocellulose membrane. Bi_2O_3NPs blended membrane will be manufactured by dissolving with six different concentration of nanoparticles such as 0.02g, 0.04g, 0.06g, 0.08g, 0.10g and 0.125 g in 78 ml acetone solvent for three hours by mechanical stirring at 200 rpm and room temperature. Subsequently, adding 30 g polymer to the nanoparticle/solvent solution and stirred for 24 h at 500 rpm and 40°C of temperature.

After formation of homogenous solution, cast the films. Immerse the membrane films in non-solvent bath (distilled water at 20°C) for precipitation. The membrane afterwards repeatedly washed with distilled water to remove the remaining solvent and wet store (Lin et al., 2013). To reveal the structure of the nitro cellulose membrane blended with Bi_2O_3 NPs, the morphology of membrane surface and cross-section observed by SEM. And also carried out SEM imaging of the uncoated nitrocellulose membrane for the comparison study.

Western Blot Experiment

The western blot experiment is carried out, in order to assess the membrane performance of normal nitrocellulose membrane and Bi_2O_3 blended nitrocellulose membrane on low molecular weight protein detection. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide. The separated proteins are then transferred to a membrane (typically nitrocellulose) using electric current; where they are probed (detected) by using antibodies specific to the target protein or else they are stained (Fig. 4, 5, 6).

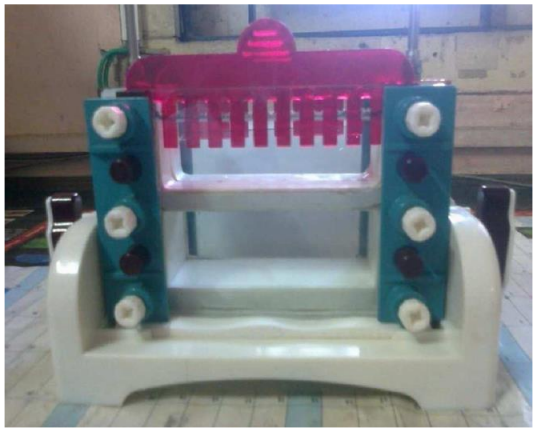

Fig. 4: SDS-PAGE gel setup.

Fig. 5: Blotting assembly.

Densitometry Analysis

Densitometry is the quantitative measurement of optical density in light-sensitive materials, such as photographic paper or photographic film, due to exposure to light. Image J Software offers free densitometric analysis of Western blots and the following steps are to be followed.

Synthesis of Bismuth Oxide Nanoparticles

Separation of 0.05M Bismuth Subnitrate

Bismuth sub nitrate $(Bi)_5O(OH)_9$ $(NO_3)_4$was the precursor for the synthesis of Bi_2O_3NPs by precipitation method. 7.3095 g of bismuth sub nitrate was dissolved in 100 ml of 0.05M HNO_3. Thus 0.05M concentration of bismuth sub nitrate solution was prepared as shown in Fig.:7.

Fig. 7: 0.05M Bismuth subnitrate.

Preparation of 4M NaOH

26.5 g of NaOH was weighed and dissolved in 166.66 ml of distilled water to get 4M NaOH solution which act as a reducing agent as shown in Fig.:8.

Fig. 8: 4M NaOH solution.

Formation of Bi_2O_3 nanoparticles

100 ml of 0.05M Bismuth sub nitrate was dissolved in 0.05M of HNO_3. 166.66 ml of 4M NaOH solution was added to the above under vigorous stirring. The color of the solution turned yellow color after sometime. The solution was incubated at 90ºC for 2 hours under constant stirring of 500 rpm. The precipitate was filtered with Whatmann filter paper No.1 and washed with ethanol and distilled water. The precipitate was driedat 60ºC until fine powder forms as shown in Fig.:9, 10. Arun Prakash Periasamy*et al*. (2011) obtained Bi_2O_3NPs as the formation of faint yellowish precipitate.

Fig. 9: Yellow color indicates nanoparticles..

Fig. 10: Synthesized Bi_2O_3NPs.

Characterization of bismuthoxide nanoparticles

UV-Visible Spectroscopy

Spectrum analysis for the development of Bi_2O_3NPs was observed by UV – vis spectroscopy using a Shimadzu UV-1800 double beam spectrophotometer to know its maximum surface plasmon resonance (SPR). Luan et al. (2015) observed a strong absorption at the wavelength range from 230 to 400 nm and confirmed the formation of Bi_2O_3NPs in the solution. In addition,theyobserved that the absorption band of the Bi_2O_3sample is red-shifted, which is a well-known largely studied material for photodegradation, and the Bi_2O_3sample has obvious absorption in the visible region. The absorbance was noted for every 20nm of wavelength from 360nm to 480nm. The wavelength and absorbance value are plotted in Table 1. The maximum peak value for the synthesized Bi_2O_3NPs was found to be 460±3nm in the Fig. 11.

Table 1. UV-Vis spectral data of Bi_2O_3 NPs.

Wavelength(nm)	Absorbance(A)
360	0.021
380	0.052
400	0.062
420	0.096
440	0.125
460	0.857
480	0.078

SEM

Scanning electron microscope (SEM) was employed to visualize the morphological characterization of the Bi_2O_3NPs. A Philips XL30 SEM was used. (Periasamy et al., 2011) obtained 50 nm for Bi_2O_3NPs as confirmed by the SEM analysis. A dried sample of Bi_2O_3NPs was used to determine the size and the shape of Bi_2O_3NPs. The size of Bi_2O_3NPs was found to be 20 to 50 nm as found in Fig.12.

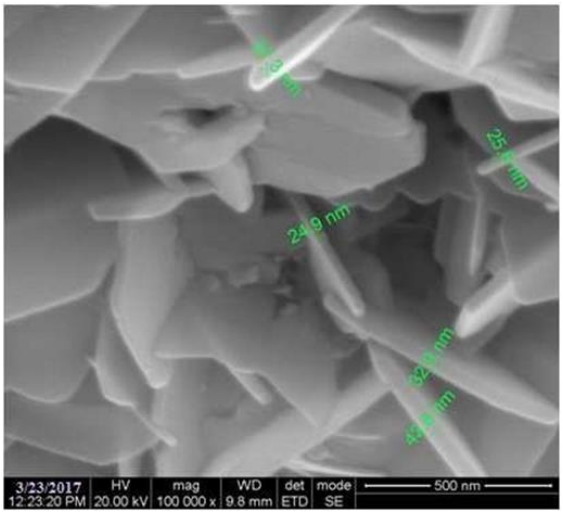

Fig. 12. SEM image of Bi_2O_3NPs.

EDAX

EDAX was a technique used for identifying the elemental composition of the specimen, or an area of interest therefore. The EDAX spectrum was just a plot of how frequently an X-ray isreceived for each energy level. An EDAX spectrum normally displayed peaks corresponding to the energy levels for which the most X-rays had been received. Each of these peaks was unique to an atom, and therefore corresponds to a single element. The higher a peak in a spectrum, themore concentrated the element is in the specimen. An EDAX spectrum plot not only identifies the element corresponding to each of its peaks, but the type of X-ray to which it corresponds as well.The percentage of bismuth percent in the synthesized nanoparticle was found to be 92.66wt% (Fig.13) (Table 2).

Table 2. EDAX for Bi_2O_3 NPs.

Element	Wt%	At%
OK	07.34	50.84
BiL	92.66	49.16

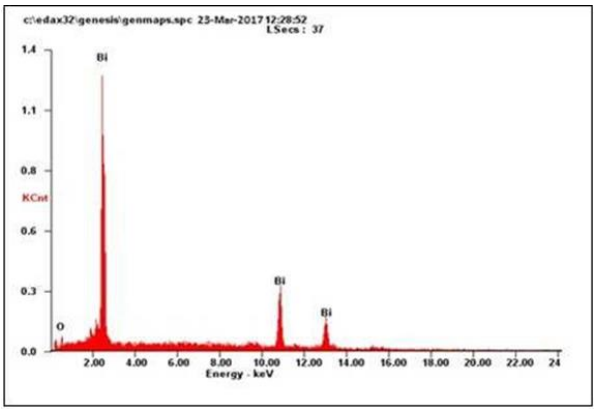

Fig. 13: EDAX spectrum data of Bi_2O_3 NPs.

Particle Size Distribution

In order to study the size distribution of the synthesized samples, scanning electron microscopy (SEM) image was used. (Luan et al., 2015) reported the particle sizes of them are in the range of 50-90 nm. In this work, particle size distribution of the synthesized Bi_2O_3NPs was assessed using Image J Software and particles were found maximum in the range of 20-50 nm in Fig. 14.

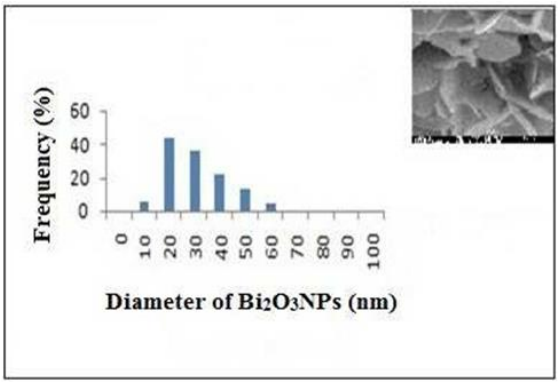

Fig.14: Particle size distribution of Bi_2O_3 NPs.

TEM

The TEM picture was recorded with JEOL model 1200 EX instrument at the accelerating voltage of 100 kV. (Patil et al., 2005)found the average particle size of powders by TEM study is found to be 50 nm. The fine powders were dispersed in amyl acetate on a carbon coated TEM copper grid. The samples were analyzed for the presence of carbon by microanalysis technique. TEM analysis was performed for verification of particle size and crystallite structure of bismuth oxides powders. The morphology of these powders was shown in Fig. 15. The figure shows that the powder was agglomerates of fine particles. The powder consists of mostly in the range 30-40 nm in size.

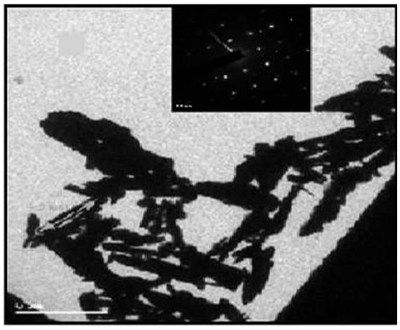

Fig. 15: TEM image of Bi_2O_3 NPs.

XRD

The values for Bi_2O_3phase was obtained from (JCPDS no. 27-50).The calculated lattice parameters by least square fit are a = 7.736 A° and c = 5.614 A° from (JCPDS no. 27-50).D = k λ /β cosθ, where D is the crystallite size, k is a constant (k=0.9 assuming that the particles are spherical), l is the wavelength of the X ray radiation, β is the line width and θ is the angle of diffraction. The particle size obtained from XRD data is 50nm was shown in Fig.:16. (Patil et al., 2005)confirmed the particle size obtained from XRD data was 75 nm.

Membrane Preparation

Nitrocellulose Membrane

Nitrocellulose membrane was prepared as shown in Fig.:17 using phase inversion induced by immersion precipitation technique. Nitrocellulose polymer was used and dissolved in acetone solvent to prepare neat nitrocellulose membrane. The film wascasted on nonwoven polyester as support layer. The membrane film was immersed in non-solvent bath (distilled water at 20°C) for precipitation. The membrane was afterwards repeatedly washed with distilled water to remove the remaining solvent and wet stored.

Fig. 17: Normal nitro cellulose membrane.

Bi_2O_3 nanoparticle blended nitrocellulose membrane

Bi_2O_3NPs blended nitrocellulose membrane was shown in Fig.:18 was also prepared with six different concentration of nanoparticles such as 0.02g, 0.04g, 0.06g, 0.08 g, 0.10 g and 0.125 g using phase inversion induced by immersion precipitation technique. Bi_2O_3 blended membrane was manufactured by dissolving 0.125 g of Bi_2O_3NPs in 78 ml of acetone solvent for three hours by mechanical stirring at 200 rpm and room temperature. Subsequently added 30 g of polymer to the nanoparticle/solvent solution and stirred for 24 h at 500 rpm and 40°C of temperature. After the formation of homogenous solution, the films were casted on nonwoven polyester as support layer. The membrane films was immersed in non-solvent bath (distilled water at 20°C) for precipitation. The membrane was afterwards repeatedly washed with distilled water to remove the remaining solvent and wet stored. Similarly other five concentrations of nanoparticles such as 0.02 g, 0.04 g, 0.06 g, 0.08 g and 0.10 g were also prepared.

Fig. 18: Bi_2O_3 nanoparticles blended nitrocellulose membrane

Characterization of membrane

SEM

The SEM image clearly shows that difference between normal as shown in Fig. 19 and cross-sectional observations of synthesized nanoparticle blended membrane as shown in Fig.20 due to deposition of Bi_2O_3 taking place in blended membrane. (Xie et al., 2011) imaged the PVDF membrane before and after incubation with gold nanoparticles by SEM. Stefan (Sotto et al., 2011) observed SEM images of the polyethersulfone membrane surface before addition of zinc oxide nanoparticles was similar to these of the same membranes after embedding zinc oxide. (Sotto et al., 2011) studied SEM micrographs of the neat and blended-titanium oxide membranes exhibit a higher porosity for the modified membrane.

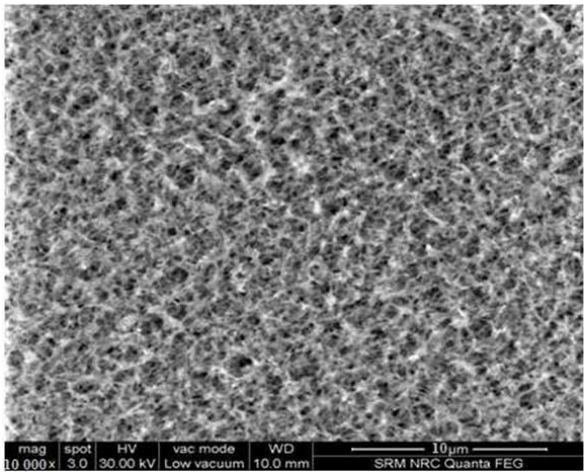

Fig. 19: SEM image of normal nitro cellulose membrane

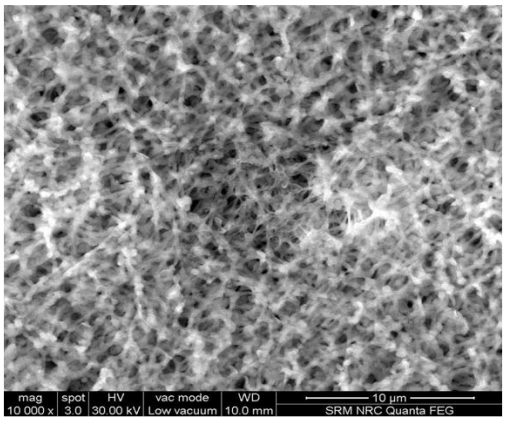

Fig. 20: SEM image of Bi_2O_3 NPs blended nitrocellulose membrane

Western Blot Experiment

The Bi_2O_3 covered the nitrocellulose membrane with a homogenous layer of nanoparticles. The membrane performance of normal and nanoparticle blended membrane were studied by western blot experiment using low molecular weight protein of βchain of Insulin (3.3kDa). In this study, the commercially availableβ-Insulin protein from porcine pancreas was used. The SDS-PAGE analysis shows the band with respect to broad range marker which confirmed that molecular weight of β chain of Insulin as >5 kDa as shown in Fig.:22. During Western blotting, the functional groups of the peptides bound to the Bi_2O_3NPs and they were kept in the membrane. The presence of the Bi_2O_3NPs prevented diffusion of the peptides from the membrane after applying the 200mA. After the blotting, both the normal and nanoparticle blended membrane containing

β chain of Insulin were stained with Ponceau S Staining solution and the band of the reaction was visualized with this technique (Table 3).

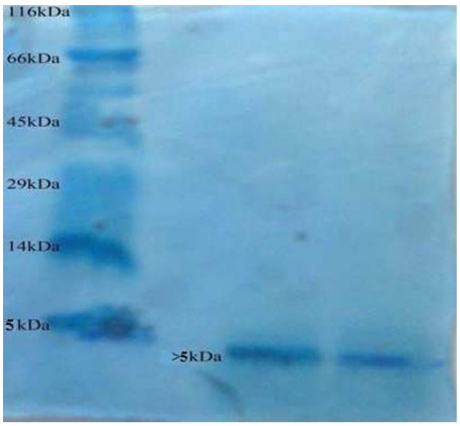

Fig. 21: SDS-PAGE gel (15%): Broad range marker and βchain of Insulin (>5kDa).

Table 3. Densitometric analysis data of western blot images.

Conce tration of Bi_2O_3NPs (g)	Band Area (a.u.)
0	1287.4
0.02	12388
0.04	15946
0.06	16558
0.08	18458
0.10	25984
0.125	26295

The thiol group (Cys) and amino groups (Arg, Lys, His, Asn, Gln) of the proteins and peptides have high affinity in binding to some metals, such as gold and silver. These metals were used previously in staining proteins. (Duchesne and Fernig, 2007) and (Emami et al., 2012) proposed using a silver and gold nanoparticle-coated membrane for femtomole detection of small proteins and peptides by dot and Western blot, which used a 2.28kDa and 1.4kDa peptides respectively to transfer from SDS-PAGE gel to nanoparticle-coated membrane and prevent diffusion of the peptides and small proteins. In this study, a low molecular weight protein of insulin (3.3kDa) was used.

During electro-blotting, the proteins were loaded onto the SDS-PAGE gel (15%), which is commonly used to separate proteins in the mass range of 3 to 100kDa. The normal nitrocellulose membrane showed low detection sensitivity for the low molecular weight protein. The protein was transferred to the Bi_2O_3NPs blended membrane by the Western blot technique. The β chain of Insulin with a molecular weight of 3.3 kDa was kept in the blended membrane, and the Fig.:23 showed that detection sensitivity of low molecular weight protein has been improved compared to that normal nitrocellulose membrane. The detection sensitivity has improved with increasing concentration of the Bi_2O_3NPs in the blended membrane from 0, 0.02, 0.04, 0.06, 0.08, 0.10, 0.125 g. But 0.10 g and 0.125 g Bi_2O_3NPs blended membrane showed almost equal band intensity. Hence, no further concentration of nanoparticles was prepared and blotted. Taken 0.125 g of Bi_2O_3NPs blended membrane showed maximum detection sensitivity than the normal nitrocellulose membrane.

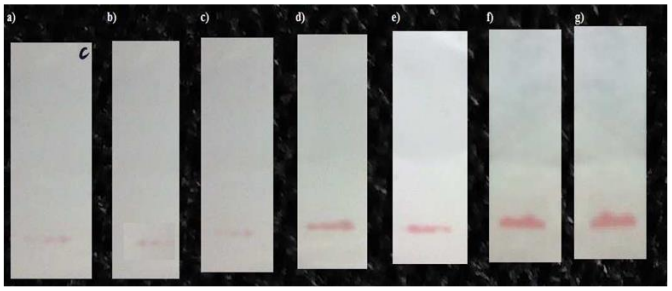

Fig. 22: Western blot analysis of βchain of Insulin (3.3kDa) with increasing concentration of nanoparticles [a)0, b)0.02, c)0.04, d)0.06, e)0.08, f)0.10, g)0.125 g of Bi_2O_3 NPs] and constant nitrocellulose polymer concentration

Densitometry Analysis

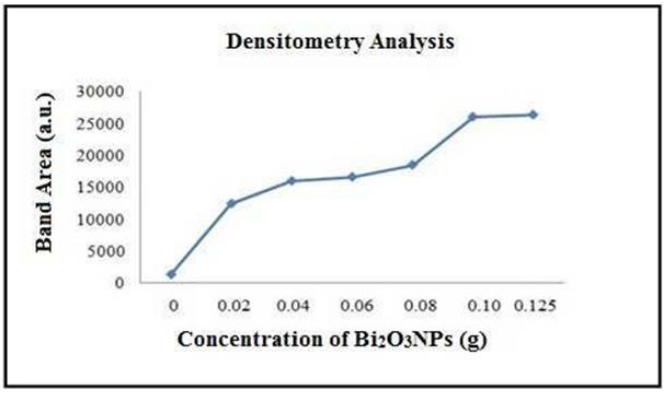

Fig. 23: Densitometry analysis of western blot images.

From the above plot, we conclude that the maximum concentration of both 0.1g and 0.125g of Bi_2O_3NPs blended nitrocellulose membrane showed the band intensity much closer and brighter. So further concentration was not taken in account for membrane modification.

Thus, the manufactured Bi_2O_3NPs blended

nitrocellulose membrane with a better performance than the normal nitrocellulose membrane, in terms of 25 times improved detection sensitivity of low molecular weight protein was explored.

Hence that metal nanoparticles embedded in the membrane show other highly desirable properties, such as the ability to capture low molecular weight proteins, this modification can facilitate more sensitive fluorescence-based bioassays and other biochemical procedures.

Conclusions

Here the Bi_2O_3 nanoparticles were synthesized by precipitation method below 100ºC. The optimum temperature for the production of Bi_2O_3 nanoparticles was found to be 90ºC. From the UV plot the maximum absorbance were absorbed at 460 nm for the Bi_2O_3 nanoparticle solution. The SEM image proves that the particle size ranges between 20nm to 50 nm. The EDAX spectrum reveals that 92.66 wt % of bismuth was present in the synthesized Bi_2O_3NPs. From the particle size distribution graph Bi_2O_3NPs was found to be in the range of 20-50nm. TEM study shows that the powders was agglomerated and found in the range of 30-40 nm in size. From the XRD plot the size of the nanoparticle was found to be 50 nm.

Membrane modification has been done by metal nanoparticles and their detection performance was estimated by western blot experiment. For this, the surface of nitrocellulose membrane can be modified by phase inversion induced by immersion precipitation technique with a bismuth oxide nanoparticles. A low molecular weight protein and a protein stain was used for western blot experiment. During Western blotting, the functional groups of the peptides bound to the Bi_2O_3NPs and they were kept in the membrane. The presence of the Bi_2O_3NPs prevented diffusion of the peptides from the membrane afterapplyingthe200mA. Given that metal nanoparticles embedded in the membrane show other highly desirable properties, such as the ability to capture low molecular weight proteins, this modification can facilitate more detection sensitive-based bioassays and other biochemical procedures. The normal nitrocellulose membrane showed low detection sensitivity for the low molecular weight protein.

These results showed that detection sensitivity of the Bi_2O_3NPs blended nitrocellulose membrane was increased than the normal nitrocellulose membrane. The concentration of 0.125 g of bismuth oxide nanoparticles blended nitrocellulose membrane showed maximum detection sensitivity among the other five concentration of the Bi_2O_3NPs (0.02, 0.04, 0.06, 0.08 and 0.10).This preparation method of Bi_2O_3NPs blended nitrocellulose membrane could easily be applied to bioassays and proteomic applications.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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