Nanocellulose as functional filler in starch/polyvinyl alcohol film for preparation of urea biosensor

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Urea analysis is of considerable interest in clinical, agricultural and environmental chemistry. Urea biosensor helps in on-site monitoring of urea. The aim of this study was to evaluate the potential use of nanocellulose reinforced composite film as substrate for urea biosensor. Nanocellulose was prepared from non-spinnable short staple cotton fibres by controlled microbial hydrolysis process. This was used as a substrate to immobilize urease enzyme and, subsequently used to reinforce the starch/polyvinyl alcohol biocomposite film. Nanocellulose acted as both carrier for the enzyme and reinforcing agent in the film. Use of 1.5% nanocellulose increased the tensile strength of the resultant film to the tune of 2.5-fold. About 68% immobilization efficiency of urease onto nanocellulose was observed in the reported process. The biosensor could detect the presence of urea linearly in the range of 10 to 1000 ppm concentration in water with a response time between 30 sec and 1 min. The storage lifetime was 2 months when stored in 4°C with the activity more than 90%. The study revealed that the nanocomposite film could significantly improve the performance and storability of urea biosensor.

Keywords: Functional filler, nanocellulose, starch film, urea biosensor, urease immobilization.

BIOSENSORS find applications in medicine, pharma, food and process control, environmental monitoring, defence and security[1]. Compared to other analytical methods, enzyme-based sensors have several advantages like high sensitivity and specificity, cost-effectiveness and mass production[2]. Among various kinds of substrates for attaching enzyme, cellulose and its derivatives are one of the ideal matrices because they are low cost, non-toxic, renewable, biodegradable and biocompatible[3]. Two enzymes (trypsin and α-chymotrypsin) were immobilized on a microcrystalline cellulose by adsorption but the process is reversible[4] and hence, not scalable. One of the challenges in making robust enzyme-based biosensors is ensuring that the immobilized enzyme remains functional over a period of time[5]. Recombinant urease immobilized via entrapment in photopolymer onto the surface of the transducer served as an effective biosensor for a wide range of urea determination (0.5–15 mM) and quick response time (1–2 min)[6].

The large surface area-to-volume ratio of nanocelluloses and their chemical properties favour their application in biosensors. Enzymes can be linked with nanocellulose through covalent linkages, salt bridges and with physical inclusion complexes. The surface properties of nanocellulose provide a biocompatible matrix for such a selective activity to occur. The enzyme glucose oxidase immobilized on nanocrystalline cellulose with gold nanoparticles attached through thiols of polyethylenimine acted as a colorimetric biosensor derivative giving varying levels of activity as a function of the thiol linker[7]. Urease covalently immobilized onto the modified cotton fibres after activation through glutaraldehyde treatment, remarkably improved temperature and operational stability, which made it more attractive in the application aspect for urease enzyme[8]. Bacterial cellulose nanofibres derivatized with reactive green 5 dye ligand showed very high adsorption capacity towards urease from its aqueous solution due to the nanosize of bacterial cellulose[9].

In the present study, we report the preparation of urease immobilized nanocellulose and its use in functional fillers in starch/polyvinyl alcohol biocomposite film (urea biosensor). Nanocellulose, in addition to acting as substrate for urease immobilization, improved the mechanical properties of the biosensor film due to its inherent crystalline nature.

The biosensor film was prepared by a solution casting method as reported earlier[10]. Briefly 5% (w/v) solution of starch (potato starch, HiMedia® Laboratories) and 5% (w/v) solution of polyvinyl alcohol (Fisher Scientific DP ~200,000) were prepared separately and blended in a mechanical stirrer at 1500 rpm for 10 min along with 5% (w/v) citric acid as a cross linking agent. This blend solution was casted in square-shaped plastic moulds (12 cm × 12 cm) and dried to obtain the film with a thickness of 180 ± 4 μm.

To prepare the nanocellulose, the microcrystalline cellulose (MCC) prepared from non-spinnable short staple cotton fibres (variety: *Bengal Desi*, India; sourced from local market) by conventional 4 N hydrochloric acid hydrolysis process was used as the raw material. This MCC was further size reduced to nanocellulose by controlled hydrolysis using the fungus *Trichoderma reesei* (ATCC culture no. 13631) as reported in our previous study[11]. Briefly, the 24 h inoculum of the fungus *T. reesei* was prepared in potato dextrose broth by inoculation of spore suspension ($\sim 3 \times 10^6$ spores/ml). The optimized concentration (5.0%) of inoculum was added in Mandel's medium having MCC as the sole carbon source in 250 ml conical flask at 25°C under shaking condition (150 rpm). After fermentation, the broth was subjected to differential relative centrifugal forces to sediment the particles of size more than 1 μm. The resultant supernatant was filtered through 100 kDa ultrafiltration membrane by vacuum

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suction and the nanocellulose trapped on the surface of the membrane was removed with a jet of ultrapure water. The particle size distribution and zeta potential of nanocellulose were measured using Nicomp™ 380 ZLS size analyser by dynamic light scattering (DLS) principle. This instrument employs a design that permits both multi-angle particle size analysis by DLS and low-angle zeta potential analysis by electrophoretic light scattering. Size calibration was carried out using 90 nm size polystyrene latex spheres and zeta potential calibration using 491 nm polystyrene latex spheres. The hydrodynamic diameter was obtained based on the dynamic light scattering and autocorrelation principle. The mean diameter of the particles was calculated from their Brownian motion via the Stokes–Einstein equation. For this, He–Ne laser (632.8 nm) was used and the scattering intensity was analysed by Avalanche photodiode detector at 90° orientation. Nanocellulose thus prepared was loaded in four different concentrations, viz. 0.5 wt%, 1 wt%, 1.5 wt% and 2 wt% into the starch film.

The urea biosensor was prepared in a three-step process. The first step was amine functionalization of nanocellulose. This was achieved by using amino propyl triethoxy silane (APTES) as a linker molecule and the reaction was carried out in pH 8.0 adjusted using NaOH at 0.5 mM concentration of APTES at 37°C (ref. 12). In the second step, the amine functionalized nanocellulose was conjugated with urease enzyme using 0.5 M citric acid as a linking agent at pH 4.8 (ref. 13). Finally the third step consisted of coating the backside of the urease immobilized biocomposite film with an ammonia sensitive layer prepared by using a mixture of sodium nitroprusside, sodium hypochloride and sodium salicylate in toluene[14]. The ammonia sensitive layer was bound onto the film using silicone binders that were solublized in toluene at 5% (w/v) concentration to form a complete solid state disposable biosensor that is sensitive to urea. When the film is in contact with solutions containing urea, the immobilized urease enzyme catalyses the decomposition of urea to form ammonia, which reacts with the ammonia-sensitive layer in accordance with Berthelot's reaction. Blue colour (indophenol dyes) is formed, resulting in a colour change of the film from very light green to blue formed due to reaction with ammonia. The urease used for immobilization was commercially available jack bean urease (HiMedia® Laboratories). All other chemicals were of AR grade procured from HiMedia® Laboratories and toluene AR grade was procured from Sd Fine Chemicals®. The silicone binder was procured from Dow Corning®.

The urease activity of the urease immobilized films was measured based on Berthelot's reaction using 100 mM urea as substrate in a phosphate buffer of pH 7.0 as per a previously reported procedure wherein the activity was reported in mM of ammonia[13]. The standard curve was generated using ammonium chloride. The efficiency of immobilization was reported as per cent urease activity of immobilized film as compared to that of free urease activity. The response of the fabricated biosensor, as also the linearity range, was measured by adding 10 µl of urea in the biosensor film. The absorbance was read at 660 nm in a UV spectrophotometer (Shimadzu UV 700) using the film holder accessory after incubation for 2 min at room temperature.

The tensile strength and per cent elongation of films were determined using Universal Testing Machine (LR-50 K, LLOYD instrument, UK) with a 500 N load cell, in accordance with the standard ASTM D 882. Samples were cut to 200 mm × 15 mm in dimension, and conditioned at 50 ± 5% RH, 23 ± 2°C for 24 h. The gauge length and crosshead speed were 100 mm and 10 mm/min respectively. The tests were carried out at 50 ± 5% RH and 23 ± 2°C. Each determination was taken from an average of five specimens. To test the stability, the prepared biosensor was stored at 4°C and their urease activities were measured up to 2 months according to the urease assay procedure mentioned previously.

The average size (hydrodynamic diameter) of nanocellulose as analysed by DLS particle size analyser was 91.8 ± 8.4 nm and the zeta potential was –14.46 mV. The size distribution graph is given in Figure 1. The effects of various loading concentrations of nanocellulose on the mechanical properties of the starch/PVA films are given in Table 1. It was observed that the addition of nanocellulose as a filler improved the mechanical properties of the films. There was a 2.5-fold increase of the Young's modulus of films with 1.5% loading of nanocellulose as compared to that of control. Further addition of nanocellulose (2%) reduced the mechanical property of the film that can be attributed to probable aggregation of nanocellulose. Similar reduction in mechanical property at higher loading of nanocellulose was reported earlier[15]. Thus the composite film loaded with 1.5% nanocellulose as filler that showed better mechanical property was chosen as the substrate for preparation of urease biosensor films. The increased Young's modulus may be related to the increased stiffness and brittleness of composite films due to addition of nanocellulose[16]. Hence, the elongation percentage reduced simultaneously.

After immobilization of urease, the protein content on the starch/PVA film was determined to check the immobilization efficiency. Protein was estimated based on Lowry's method[17]. The immobilization efficiency was found to be 68% based on protein content. The surface morphology of the control and urease immobilized biosensor was analysed by AFM as given in Figure 2 *a* and *b*. The surface roughness of the urease immobilized film increased indicating the binding of urease. The response of the biosensor to the presence of urea in the solution was evaluated by adding a solution of 700 ppm urea dissolved in distilled water and the films were scanned from 400 to 850 nm in a UV-Vis spectrophotometer.

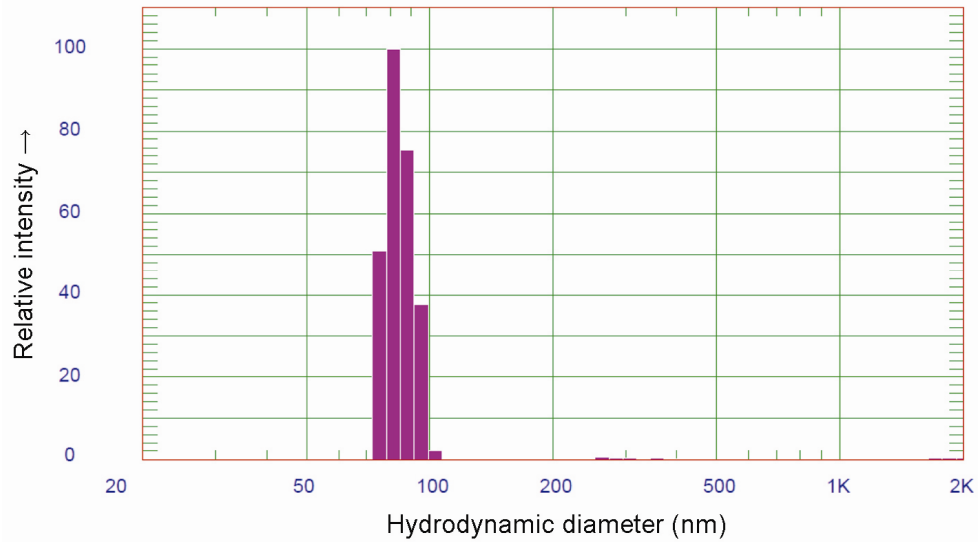

Figure 1. Particle size distribution of nanocellulose analysed by DLS particle size analyser.

Table 1. Mechanical properties of starch/polyvinyl alcohol biocomposite film reinforced with nanocellulose*

Samples	Stress at maximum load (MPa)	Strain at maximum load (%)	Young's modulus (MPa)
Control	7.62 ± 0.89	9.48 ± 1.85	286.55 ± 28.7
0.5% Nanocellulose	5.89 ± 0.68	7.51 ± 1.55	223.49 ± 21.30
1.0% Nanocellulose	8.92 ± 0.93	6.19 ± 1.23	419.48 ± 31.22
1.5% Nanocellulose	12.68 ± 1.02	3.67 ± 0.95	1065.89 ± 38.21
2.0% Nanocellulose	9.08 ± 0.96	6.37 ± 1.15	458.88 ± 32.90

*Values are average of five replications.

Maximum absorbance was obtained at 660 nm as given in Figure 2 *c*. To check the response characteristics of the biosensor to different concentrations of urea, it was tested using different concentrations of urea dissolved in distilled water and the absorbance was measured at 660 nm. The linearity curve was plotted with respect to the absorbance against the concentration of urea as shown in Figure 2 *d*. The curve was linear with the R^2 value more than 0.99 in the range of 10 to 1000 ppm (0.167 to 16.7 mM). Above 1000 ppm, there was saturation and a plateau was observed. The response time was measured as the time between the addition of the sample and the appearance of blue colour and it was between 30 sec and 1 min.

The stability of the biosensor films was estimated by storing the sensor strips in a refrigerator and at room temperature and analysing the films for their urease activity. The activities were assayed at an interval of 7 days and the values are given in Table 2. The reported values are the average of three replications for each treatment. It was observed that the biosensor films retained 90% of their initial activity when stored at 4°C for 2 months. When stored at room temperature, there was a rapid loss in activity of the films and at the end of 2 months, only 3% of the initial activity was retained. Earlier studies reported that the development of urea enzyme electrodes prepared with urease enzymes obtained from *Helycobacter pylori* and Jack bean based on poly (vinyl chloride) membrane ammonium-selective electrode, had stability over 2 months with less decrease in sensitivity and response time of 1–2 min (ref. 18). However, the present study reported a response time of 30 sec to 1 min with a stability of 2 months.

The nanocellulose prepared by controlled microbial hydrolysis was used as a substrate for immobilization of urease enzyme, in addition to imparting strength to the matrix of the biosensor – starch/PVA. Nanocellulose in addition to acting as a carrier for immobilization of urease enzyme was also responsible for improvement in mechanical properties of the biocomposite film. The 2.5-fold increase in tensile strength of the starch/PVA biocomposite film could help sustain high tensile loads during the handling of urease sensors. The biosensor could detect the presence of urea down to 10 ppm concentration in water and up to 1000 ppm. The developed biosensor can be stored at 4°C for 2 months without any significant loss in activity. Also, it showed a better response time between 30 sec and 1 min when compared to the previous reported value of 1–2 min (ref. 18). The reported process

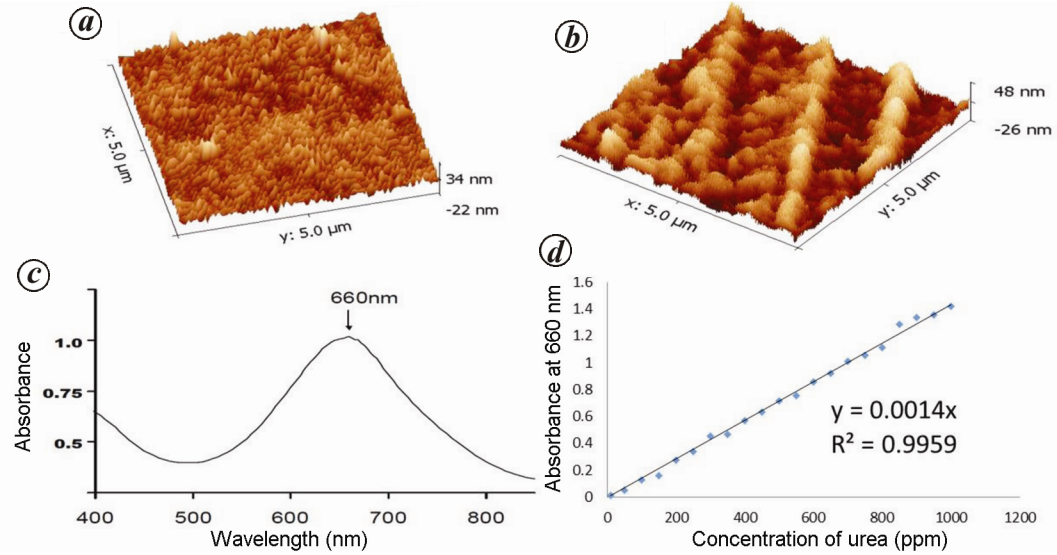

Figure 2. AFM images of nanocellulose reinforced starch/polyvinyl alcohol film before (***a***) and after (***b***) urease immobilization. The spectrum (***c***) shows the response of biosensor to urea with lambda max at 660 nm and (***d***) shows the linearity curve of biosensor.

Table 2. The urease activity of biosensor on storage

	Urease activity*		% Activity with respect to initial activity	
Sampling interval (days)	After storing at 4°C	After storing at room temperature	After storing at 4°C	After storing at room temperature
0	15.01 ± 0.17	14.95 ± 0.15	100	100
7	14.94 ± 0.13	14.44 ± 0.28	99.53	96.59
14	14.86 ± 0.28	12.43 ± 0.35	99	83.14
21	14.55 ± 0.35	8.65 ± 0.21	96.94	57.86
28	14.54 ± 0.28	4.11 ± 0.23	96.87	27.49
35	14.24 ± 0.42	3.81 ± 0.13	94.87	25.48
42	14.14 ± 0.17	3.11 ± 0.11	94.2	20.8
49	14.04 ± 0.35	2.11 ± 0.32	93.54	14.11
56	13.94 ± 0.21	1.11 ± 0.11	92.87	7.42
60	13.54 ± 0.28	0.56 ± 0.05	90.21	3.75

*Values are average of three replications.

for preparation of urea biosensor remarkably improved the response time and tensile strength without compromising its storage stability.

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Identification of unique characteristics of deception from facial expression

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Facial asymmetry provides important information for detecting deception. The present study aims at deciphering deception in facial expression unique to Indian culture and to detect differences between parameters of expression of 'felt' emotion and 'deceived' expression. Facial expressions are analysed based on Facial Action Coding System. Results reveal that participants deamplify happiness whereas they neutralize and mask negative emotions.

Keywords: Deception, facial asymmetry, facial expression.

MOST people rely on facial expressions to understand emotion. However, not all emotional facial expressions of the partners reflect actual emotional experience. When these expressions intend to transmit misleading information or to suppress information, it may be termed as deception. The fine line of distinction between posed and deceived emotions in facial expressions has rarely been examined. This is important as facial expressions of emotion are culturally constructed and there is a great degree of variation between cultures, especially of Western and Eastern cultures.

Several studies have been conducted on posed facial expressions using Facial Action Coding System (FACS)[1]. These studies however, did not distinguish between posed and deceived emotions. According to Ekman[2], posed emotion is defined as an expression of the facial configuration without felt emotional experience. However, in the case of deceived emotion the facial configuration provides two types of information: false but convincing emotional expression and concealed felt emotional expression. The present study aims at deciphering the structural composition of the face during felt and deceived emotions, using FACS. The study however does not aim at examining cross-cultural differences in these expressions. Therefore the study is conducted with Indian encoders of facial expressions only.

The sample consisted of normal, healthy, 20 female young adults in the age range 18 to 25 years. Their average educational age was 13 years and all of them were right-handed. Participants were chosen randomly for portraying their facial expression. Skin texture of participants was normal and without makeup. Participants were also asked to uncover their forehead to fully show their eyebrows. For photographs, participants with rough skin texture, eye glasses and anatomical facial asymmetry were not selected. Participants who were not naïve for the purpose of the experiment were not considered.

Informed written consent was obtained from each subject. The purpose of the research was explained to the participants and they were assured of confidentiality of their expressions. They were also given the right to withdraw from the study at any stage.

FACS coding manual has been used for facial action coding of the facial expressions of portrayers. FACS[3], as informed by the pioneering work of Hjortsjo[4], is a comprehensive tool[5] for coders to manually code all possible facial displays, which are decomposed into 30 action units (AUs) and 14 miscellaneous actions. The fundamental actions of individual muscles or groups of muscles

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