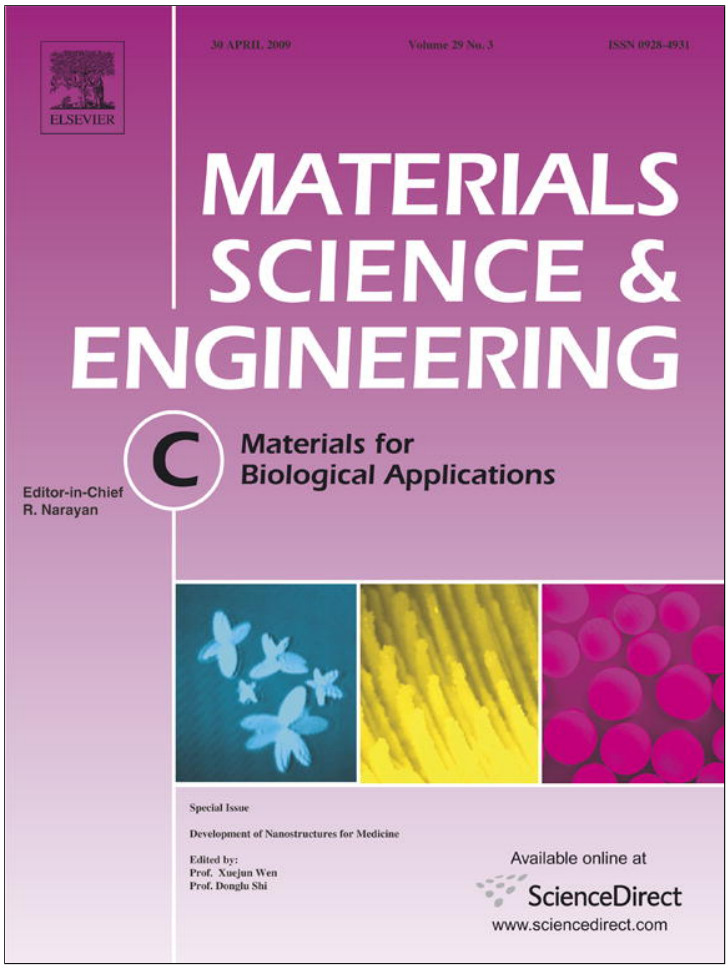

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Fabrication of nanocoated fibers for self-diagnosis of bacterial vaginosis

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ABSTRACT

Bacterial vaginosis (BV) is the most common disease which causes preterm birth and other adverse pregnancy outcomes linked with reproductive tract infection. Here, we report development of active fiber-based materials for early self-detection detection of BV. The proposed approach relies on the visual detection and measurement of the enzyme sialidase, which is present in elevated concentrations in vaginal fluid of BV patients. The developed colorimetric biosensor can detect sialidase in small droplets of vaginal fluid. Our experiments show that colorimetric sialidase substrate can be readily and with high yield immobilized on polymeric fibers coated by a nanolayer of a cationic polymer. Thus prepared fibers change color from white to bright blue in the presence of sialidase. The existing tests for BV are performed either in a lab setting or in a physician's office. In the proposed method, nanofiber colorimetric sensors can be incorporated into female panty liners so to enable the patient to monitor her BV status without visiting physician's office. It is expected that the proposed approach will lead to a new generation of fiber-based biosensors incorporated into everyday use household items.

1. Introduction

Bacterial vaginosis (BV) is an abnormal vaginal condition caused by changes in the vaginal ecosystem, characterized by the overgrowth of *Gardnerella vaginalis*, *Prevotella* species, *Mobiluncus* spp., and *Mycoplasma hominis*, and decreased numbers of *Lactobacillus* species [1,2]. BV is the most common vaginal condition in women of childbearing age. In the United States, 29% of women between the ages of 14 and 49 years [3] and as many as 16% of pregnant women have BV. BV is associated with spontaneous abortion, post-gynecologic surgery infection, and pelvic inflammatory disease that can lead to infertility. In pregnant women, the presence of BV is strongly associated with preterm birth and prematurity [4,5], a risk that is mitigated by early diagnosis and treatment with oral clindamycin [6].

Vaginal fluid of both pregnant and nonpregnant women with BV is characterized by elevated levels of sialidase activity [7]. Sialidases (neuraminidases) are a group of enzymes that catalyze the hydrolysis of terminal sialic acid residues from oligosaccharides, glycolipids, and glycoproteins by cleaving alpha-ketosidic bonds. Sialidases are produced by several pathogenic organisms, including *Streptococcus pneumoniae* [8], *Corynebacterium diphtheriae, Vibrio cholerae* [9], and group B streptococci [10], and are recognized as their virulence factors [7].

In BV patients, increased sialidase activity is believed to result in the removal of sialic acid residues from cervical mucins and diminish the viscosity of cervical mucus. As a result, mucus organization may be lost, leading to the decrease of its effectiveness as a mechanical and bacteriostatic barrier. In addition, removal of sialic acid residues results in exposure of cryptic structures in the oligosaccharide layer of vaginal epithelial cells, which can promote bacterial adhesion to the secreted mucus and the underlying epithelial glycocalyx. The combination of these two factors may create conditions for bacterial invasion of the upper reproductive tract. In pregnant women, such invasion can result in the release of inflammatory mediators that initiate labor [11,12]. In fact, a significant risk of preterm birth in has been found to be independently associated with sialidase activity in the vaginal secretions [13].

The diagnosis of BV is generally made using the Amsel criteria [14]. This test is considered positive if three out of the following four criteria are satisfied: (i) presence of abnormal vaginal discharge, (ii) elevated vaginal pH (>4.5), (iii) positive amine odor, and (iv) presence of clue cells on Gram stain or saline prep of vaginal secretions. This test is laborious and requires extensive lab skills, often leading to poor sensitivity for BV. Alternatively, a number of colorimetric tests have been proposed for detection of sialidase [15], most of which are based on the use of a chromogen-modified D-*N*-acetylneuraminic acid, a natural substrate for sialidase. One of these tests named BVBlue® has recently been approved by FDA [16]. This test utilizes a proprietary colorimetric substrate, which changes color from yellow to blue upon

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enzymatic cleavage by sialidases. It enables rapid detection of BV in a physician's office and generally shows better performance than the Amsel test.

Early detection and treatment of BV is important because this condition is associated with a number of pregnancy complications, including endometritis and preterm labor. Since BV is a very common disorder, and its clinical manifestations may often remain cryptic, availability of a self-diagnostic test embedded into an everyday use feminine hygiene item would greatly benefit a large number of patients, especially in high risk populations, such as those from lower socioeconomic groups. Here, we address this problem by developing fiber-based sensors for BV embedded into female panty liners that would not require any action from the patient [17]. Some controversy exists regarding whether or not early detection of BV is beneficial for pregnant patients. A recent meta-analysis concluded that screening of asymptomatic pregnant patients with low risk for BV followed by their treatment did not reduce the risk of preterm birth [18]. This led to U.S. Preventive Services Task Force recommendation against performing BV screening for pregnant women at low risk for BV [19]. However, this meta-analysis did not include results of a large randomized trial involving 494 women [20], which showed that early diagnosis and treatment of asymptomatic pregnant patients with oral clindamycin significantly reduces the risk of preterm birth. Since changes in BV are fairly common, availability of a test for frequent assessment of BV throughout the course of pregnancy may help to resolve this controversy [21]. Thus, it is highly desirable to have a simple non-invasive self-diagnostic test that would enable a patient to monitor her BV status on a regular day-to-day basis, without visiting a physician's office. Such a test would be especially valuable because in ~50% cases BV patients do not develop abnormal vaginal discharge and thus the condition can stay unnoticed and untreated, resulting in severe consequences later, especially for pregnant patients.

Here, we report fabrication and in vitro testing of surface-modified nanocoated fibers suitable for early colorimetric detection of BV. Such fibers can be incorporated into female panty liners or used for point-of-care analyses. Since most of colorimetric substrates for sialidase contain a negatively-charged D-*N*-acetylneuraminic acid moiety, we chemically modified the surface of the fibers by covalent attachment of a few nanometer-thick layer of a polycationic polymer, which serves as the anchor for electrostatic attachment of the negatively-charged sialidase substrate (Fig. 1). Because of higher sialidase concentrations in BV discharge and increased amounts of discharge in most of the patients, such fibers are expected to change their color if BV is present. Since panty liners are used by women on a day-to-day basis, availability of such an affordable test would enable early detection and treatment of BV. In another embodiment, the fibers can be incorporated into swabs, which can be used in physician's office to perform quick BV test.

2. Experimental

2.1. Materials and methods

Nylon yarn was obtained from Middleburg Yarn Company. Thickness of the yarn was about 0.3 mm. The yarn (containing ~170 filaments with diameter of ~25 μm), had estimated surface area of 1385 cm^2/g. Ethanol (99%), methyl ethyl ketone (MEK) (99%) and polyethylenimine (Mn = 25,000 g/mol) were purchased from VWR International and used as received. Glycidyl methacrylate (min 95%) was purchased from TCI America. Glycidyl methacrylate was radically polymerized to give poly(glycidyl methacrylate), PGMA, as described elsewhere [22].

Atomic force microscopy (AFM) studies were performed using a Dimension 3100 (Digital Instruments, Veeco, Inc.) microscope operated in tapping mode. Silicon tips with a spring constant of ~50 N/m were used to obtain the morphology of the model films in air at ambient conditions. Thicknesses of the layers deposited on model nylon films were obtained from ellipsometry. Ellipsometry was performed using a COMPEL automatic ellipsometer (InOmTech, Inc.) at an angle of incidence of 70°.

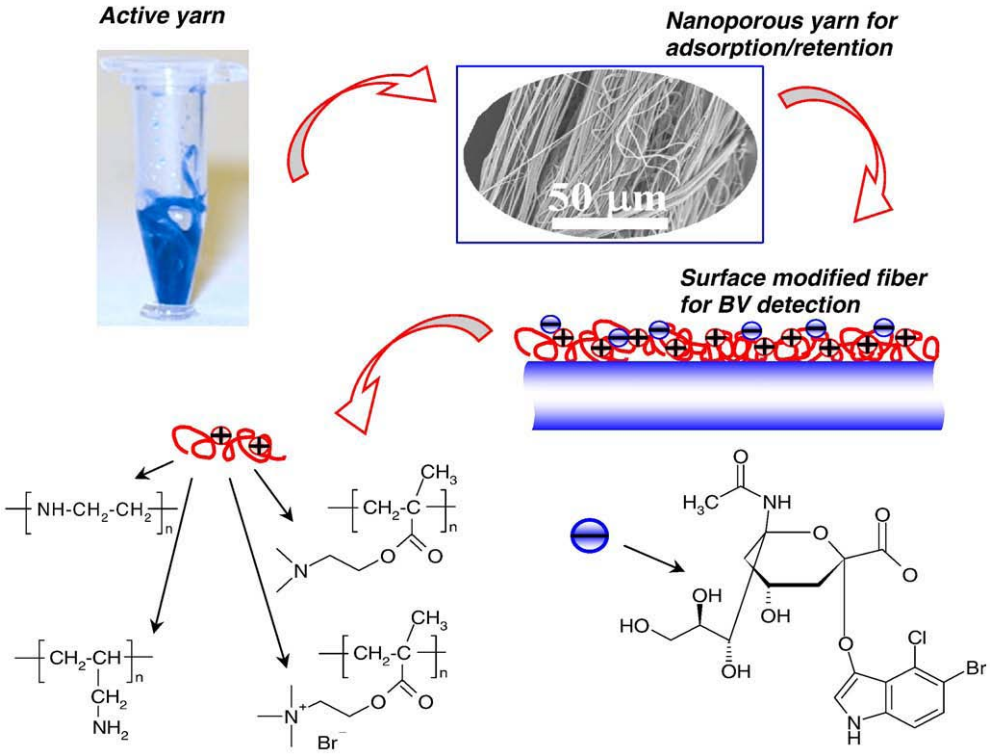


Fig. 1. Schematic illustrating the approach for fibers modification.

2.2. Surface modification of nylon fibers by positively charged polymer

The procedure for surface modification employing PGMA is described elsewhere in detail [23,24]. In brief, to achieve fiber modification, the nylon yarn was first treated by radiofrequency plasma to form surface reactive groups (such as –OH and –COOH). To modify the nylon fibers with PGMA, the fiber was dipped in a 1 wt/vol.% PGMA solution in MEK. Then, the fiber was withdrawn from the polymer solution and air-dried. Next, the fiber was placed in a vacuum oven at 105 °C for 10 min. After the annealing, the fiber was washed by MEK to remove any non-reacted PGMA from the surface. This procedure results in the formation of ultrathin anchoring layer containing epoxide functionalities on the fiber surface. At the next stage of the surface modification, the fiber was dipped in a 1 wt/vol.% PEI solution in ethanol. After treatment by PEI, the fiber was air dried and annealed in a vacuum oven at 100 °C for 1 h. Next, the fiber was washed by excess of ethanol to remove non-reacted PEI from the surface. The procedure resulted in the formation of ultrathin layer of positively charged polyelectrolyte on the fiber surface. To observe surface morphology and evaluate thickness of the coating model surfaces comprising of nylon thin films deposited on silicon wafers by spin-coating were also used in this work. Surface modification of these model surfaces was performed using the procedure similar to that described above for nylon fibers.

2.3. Immobilization of sialidase substrates on the fibers

We studied electrostatic immobilization on positively charged fibers for two colorimetric sialidase substrates: a commercially available BVBlue® detection reagent (Gryphus Therapeutics), and cyclohexylammonium salt of 5-bromo-4-chloro-3-indolyl-a-D-*N*-acetylneuraminic acid, BCIN (Rose Scientific, Edmonton, Alberta, Canada). BVBlue® contains a proprietary colorimetric substrate to sialidase. We assumed that it exists in the form of an anion because of the necessity for the presence of a neuraminic acid fragment in its structure.

For immobilization, approximately 1 cm of surface-modified fiber (190–220 mg) was incubated overnight with 0.5 mL of a 0.5 mg/mL aqueous solution of BVBlue® reagent or 0.58 mg/mL BCIN solution in 0.5 M potassium acetate buffer (pH 5.5) followed by washing with copious amount of HPLC-grade water and drying in a nitrogen flow.

The amount of deposited BVBlue® reagent has been determined by UV–VIS spectroscopy using Synergy HT microplate reader (Bio-Tek). Solutions of BVBlue® reagent before and after its incubation with the fiber were placed into a UV-transparent 96-Microwell plate, and optical density measured at 280 nm. Similar approach has been used to determine amount of deposited BCIN; optical density in this case was measured for its peak at 290 nm.

2.4. Sialidase activity assay

Sialidase from *Arthrobacter ureafaciensi* (SigmaAldrich, Cat. No. N8271) was dissolved in 50 mM phosphate buffer saline (pH 5.5) at 10 U/mL. The fiber was placed into a 0.5 mL test tube and treated with 6 μL of sialidase solution. In the case of fibers with immobilized BVBlue® reagent, 50 μl of 0.1 M NaOH solution was added to the test tube after 10 min incubation at 37 °C. Treatment by NaOH is necessary because BVBlue® requires basic medium (pH > 11) for color development. In the

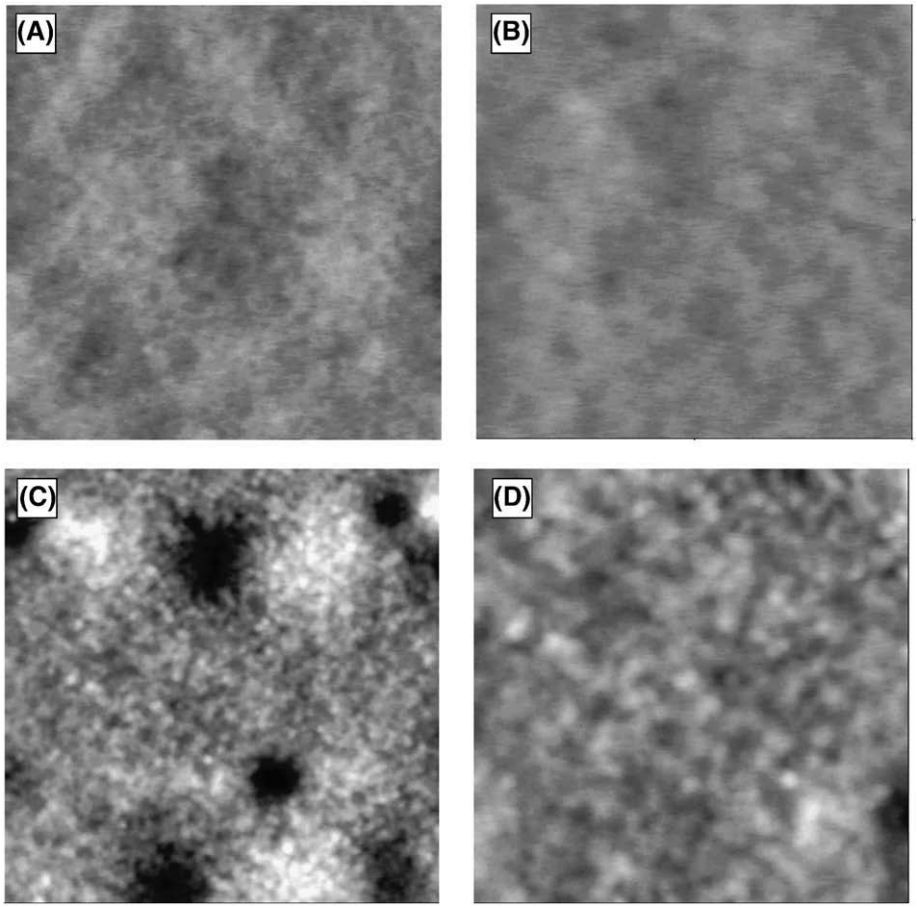


Fig. 2. (A, C) 1 × 1 and (B, D) 0.5 μm AFM morphology images of (A, B) PEI modified PGMA film and (C, D) after adsorption of BCIN. Substrate is a model surface, nylon thin film deposited on a silicon wafer. Vertical scale of all images is 5 nm.

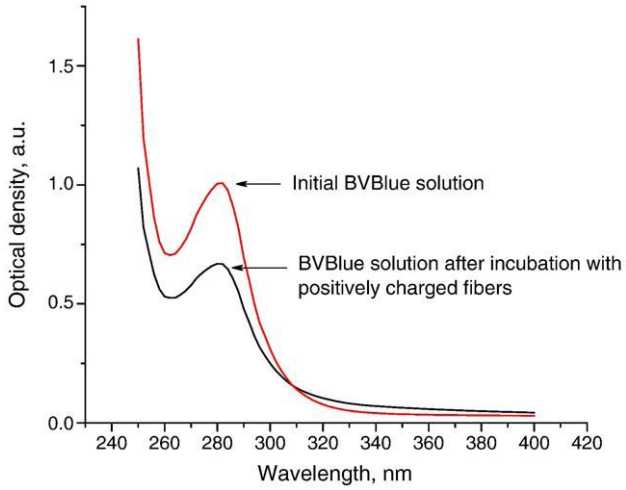


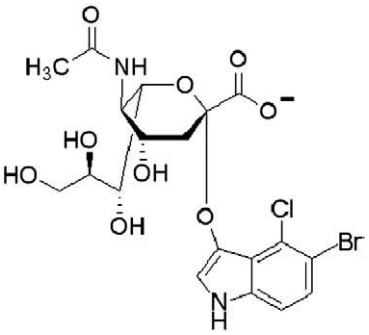
Fig. 3. UV spectra of 0.5 mg/mL BVBlue® reagent before (red line) and after (black line) its incubation with a positively charged nylon fiber. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

case of BCIN-modified fiber, no alkaline solution was added, and incubation time consisted of 2 h.

3. Results and discussion

In our experiments we attempted electrostatic immobilization of colorimetric sialidase substrates on a conventional 0.3 mm thick nylon yarn. Before conducting experiments with the yarn, the procedure for deposition of nanocoating was carried out on the model surface, nylon thin film deposited on a silicon wafer [23]. Ellipsometric measurements for the model sample showed that thickness of the PGMA and PEI layers deposited were about 5–15 and 9–13 nm for PGMA and PEI layers, respectively. The AFM images (Fig. 2A,B) clearly show that PEI macromolecule chains uniformly coat the model surface. After treatment with BCIN solution and rinsing with HPLC water we observed change in the surface morphology indicating deposition of the active substrate on the surface (Fig. 2C,D). Ellipsometric measurements for the model substrate after the BCIN adsorption did show scattered changes within the error for the measurements on the model nylon film used (±0.5–1 nm) suggesting that amount of BCIN anchored to the model substrate was on the order of 0.1 μg/cm^2.

Fig. 4. Nylon fibers with attached BVBlue reagent change color from white to blue in the presence of 0.06 U of sialidase from *Arthrobacter ureafaciens* after treatment with 0.1 M sodium hydroxide. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



Scheme 1. Structure of BCIN.

Fig. 3 shows UV spectra of BVBlue® reagent solution before and after its incubation with the fiber. An approximately 30% decrease of the absorbance maximum at ~280 nm after incubation indicates that significant amount of BVBlue reagent was immobilized on the fiber (0.25 μg/cm^2 or 0.34 mg/g). Dried fiber was tested in reaction with sialidase. After addition of alkaline solution, the fiber turned bright blue immediately after adding the alkaline solution (Fig. 4).

The experiment with BVBlue reagent shows that colorimetric sialidase substrate can be readily and with high yield immobilized on polymeric fibers surface-modified by covalent attachment of a cationic polymer. The fibers change color from white to bright blue in the presence of sialidase. However, BVBlue reagent does not appear to be the ideal colorimetric substrate for this test because of the necessity to stop the reaction and reveal the color by alkaline solution. Having in mind our ultimate goal to embed the fibers into female hygienic products, such necessity would make them much less attractive for the end users, and would reduce the effectiveness of the test due to compliance issues.

To achieve color change at slightly acidic pH (close to that of vaginal fluid), a fiber chemically modified by covalent attachment of positively charged polymer was treated by aqueous solution containing another colorimetric sialidase substrate, cyclohexylammonium salt of 5-bromo-4-chloro-3-indolyl-a-D-*N*-acetylneuraminic acid (BCIN, Scheme 1). The choice of this substrate is dictated by its negative charge, prior successful use for detection of BV, long-term chemical stability, and its robustness at slightly acidic pH [25].

Electrostatic attachment of BCIN to positively charged nylon fiber also occurs readily upon treatment of PEI-modified fiber with BCIN

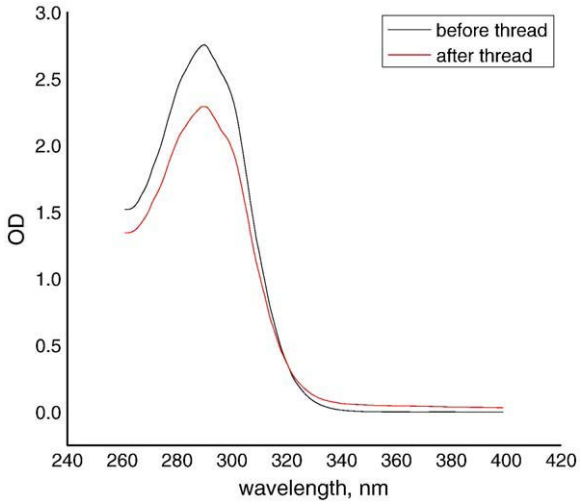


Fig. 5. UV spectra of 0.58 mg/mL BCIN solution before (black line) and after (red line) its incubation with a positively charged nylon fiber. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

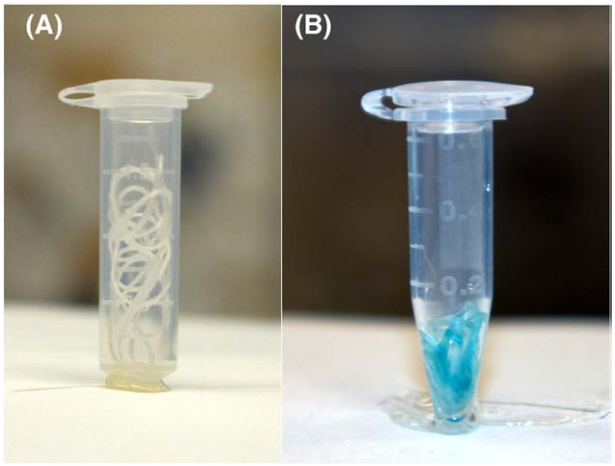


Fig. 6. BCIN-treated nylon fiber changes color from white (A) to blue (B) in the presence of 0.06 U of sialidase from *Arthrobacter ureafaciens* in phosphate buffer saline (pH 5.5). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solution. Spectroscopic studies of the solution before and after incubation with the fiber show ~18% drop of BCIN concentration (Fig. 5). This drop of concentration corresponds to adsorption of 0.17 μg/cm^2 (0.23 mg/g) of BCIN. Assuming molecular weight of BCIN anion of 536.9, surface density of BCIN coating can be estimated at 1.9 anions/nm^2, or 53 Å^2 per BCIN anion, indicative of a monolayer coating. Fig. 6 shows the photograph of the fiber after 2 h incubation with sialidase at 37 °C. Notably, use of the fibers treated by BCIN did not require reaction with an alkaline solution to reveal the color, which makes them much more suitable for the proposed applications then those treated by BVBlue reagent.

Our ultimate goal is to prepare nanofibers with high sensitivity, specificity, and positive predictive value for detection of BV. It means that the amount of BCIN immobilized on nanofibers should be adjusted in such a way that nanofibers change color in contact with vaginal fluid from BV patients, but remain unchanged when in contact with fluid from healthy patients. Such optimization of the concentration of sialidase substrate has previously been achieved in solution-based assays such as BVBlue [16], supporting the feasibility of our goal of similar optimization for BCIN-loaded nanofibers. Experiments on in vitro testing of the fibers with different sialidase concentrations modeling those in BV patients and healthy women, as well as experiments on clinical testing with samples of vaginal fluid from real patients are currently in progress.

In conclusion, we electrostatically immobilized colorimetric sialidase substrate on positively charged polymeric fibers. The fibers change color from white to bright blue in the presence of sialidase. Further research is needed to optimize the amount of the substrate attached to the fibers to achieve higher specificity of the test. More broadly, the results obtained during this study will serve as the springboard for the development of a novel family of active biosensors embeddable in everyday household items. Biosensors to various conditions can be incorporated into items that routinely contact our body on a day-to-day basis including, but not limited to napkins and handkerchiefs (analysis of nasal conditions), chewing gum or floss (dental conditions), toilet paper (sensor for occult blood), cloth (analysis of sweat), and many others.

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