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Paper-Based Enzyme Biosensor for One-Step Detection of Hypoxanthine in Fresh and Degraded Fish

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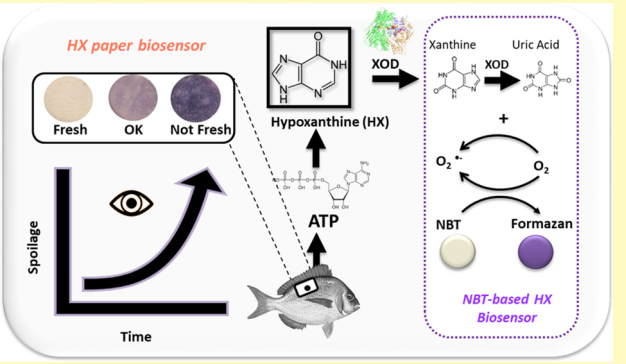
ABSTRACT: Food freshness monitoring, which can reflect the quality of the product at the time of use, remains a great challenge for consumers and the food industry. Herein, we report the development of a cost-effective enzyme-based paper biosensor, which can monitor fish freshness and predict spoilage. The biosensor measures the release of hypoxanthine (HX), an indicator of meat and fish degradation, using the enzymatic conversion of HX by xanthine oxidase (XOD). We demonstrate that the entrapment of XOD and an organic dye, nitro blue tetrazolium chloride (NBT), within a sol−gel biohybrid enables their stabilization on paper and promotes the enzymatic reaction with further retention of the reaction products within the cellulosic network . Linearity in the micromolar concentration range with a detection limit of 3.7 μM for HX is obtained. The biosensor has high selectivity toward HX and is manufactured in few steps from inexpensive widely available materials. The applicability of the biosensor is demonstrated by following fish degradation over time and measuring HX concentrations ranging from 117 (±9) to 198 (±5) μM within 24 h of degradation, at levels that are comparable with those measured by a commercial enzymatic kit for HX detection. As compared to the commercial kit, our biosensors are more cost-effective, do not require addition of exogenous reagents and are portable, having all of the reagents needed for analysis embedded within the sensing platform. This proof-of-concept work demonstrates that the paper-based HX biosensor has potential as a robust reagentless device for real-time monitoring of food freshness and for other applications in which HX plays an important role.



KEYWORDS: *hypoxanthine, paper biosensor, fish freshness, xanthine oxidase, nitro blue tetrazolium chloride*

Hypoxanthine (HX) is an important product of purine metabolism present in biological fluids, cells, and tissues. HX is often used as a biochemical marker to provide information about the aging of biological cells, to diagnose diseases in patients with gout, cardiac, and neoplastic conditions,[1] and to quantify the quality and degradation status of meat and fish products.[2] Karube et al. proposed a model to estimate the freshness of fish, based on the content of I, X, and HX,[3] establishing the role of these metabolites as degradation (or freshness) biomarkers. Products of adenosine triphosphate (ATP) degradation include monophosphate (AMP), inosine-5′-monophosphate (IMP), inosine (I), hypoxanthine (HX), xanthine (X), and uric acid (UA). ATP and AMP decrease rapidly within 24 h, IMP increases around 5−24 h after death, and I and HX increase significantly at the later stages of degradation.[4]

These metabolites can be determined using liquid chromatography,[5] mass spectrometry,[6] and biochemical methods. Conventional chromatography and spectroscopy techniques cannot be directly used in the field because of sampling requirements, the complexity, and the cost of analysis. The majority of biochemical methods involve the use of xanthine oxidase (XOD) that converts HX or X[7] to hydrogen peroxide (H_2O_2), X, and uric acid, in the presence of molecular oxygen (O_2). The amount of HX and X can then be estimated by measuring the consumed O_2,[88] and the produced H_2O_2[7,97,9] or by estimation of UA,[9a,10] using electrochemical or colorimetric means. Electrochemical biosensors prepared by attaching XOD to electrode surfaces[7b,11] can be used to determine the HX and assess meat degradation by measuring the O_2 consumed or the H_2O_2 released, but these sensors require a potentiostat and an energy source to measure the voltammetric current related to the electrochemical reduction or oxidation of these species. Colorimetric sensors can be designed as alternatives.[12] Yan et al.[13] developed a colorimetric sensor based on copper nanoclusters with peroxidase-like activity. The nanocluster was able to oxidize 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of H_2O_2

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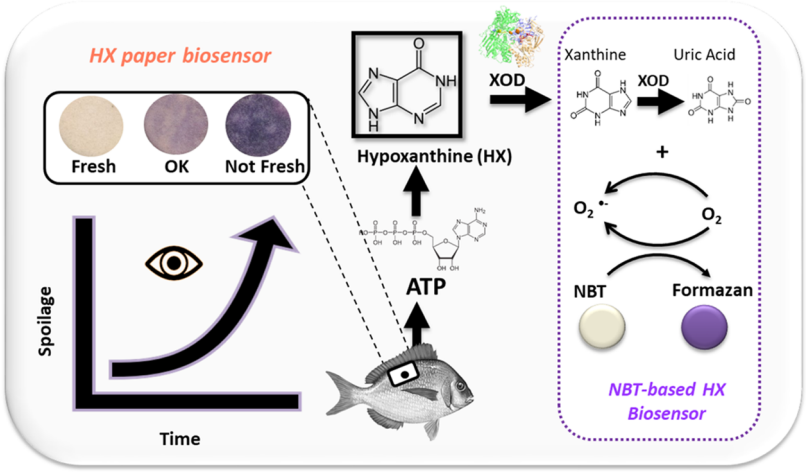

Figure 1. Concept of HX detection by a paper-based NBT/XOD biosensor and its potential application for monitoring HX in fish samples.

generated from the enzymatic reaction of XOD and X producing a blue color. Pu et al.[14] found that citrate-stabilized AuNPs aggregate in the presence of X through a ligand-exchange process. As a result, a blue-shift color occurred and the ratio of absorbance at 630/520 nm was measured spectrophotometrically and related to the X concentration. Chen et al. used Au nanorods[12b] to measure the H_2O_2 produced from the HX oxidation by XOD through the Fenton reaction producing hydroxyl radicals in the presence of Fe^{2+} and etching the Au nanorods, further changing the color of the dispersion. Despite progress, available methods are still complex, require sample collection, trained personnel, and specialized equipment for signal quantification. This limits the number of samples that can be analyzed and are out-of-reach for the nonexpert user, in terms of operation, cost, and remote usability. Therefore, easy-to-use tests are highly desirable to measure the products of the metabolic degradation of ATP in biochemical, clinical, and food applications.

To overcome these limitations, paper-based devices have been proposed as alternative platforms. Paper is highly abundant, inexpensive, and highly porous and can be easily functionalized.[15] Paper sensors regained popularity in 2007,[16] and various advancements have been reported with notable examples from Suslick, Swager, and Whiteside groups and others demonstrating the effective integration of sensing components on paper. Whiteside's group designed diagnostic microfluidic paper-based analytical devices (μPADs) for point-of-care (POC) analysis,[16] providing healthcare users and people in resource-limited settings with inexpensive user-friendly diagnostic devices.[17] Suslick's group reported a colorimetric sensor array detecting various VOC, toxic gases, aromas, and freshness markers,[18] for odor visualization functioning similarly to the mammalian olfactory system.[19] Our group has previously developed enzyme-based paper biosensors with integrated biomolecular recognition and colorimetric transduction for detection of glucose with immobilized glucose oxidase,[20] bisphenol A with immobilized tyrosinase,[21] and phenolic compounds with enzyme-mimetic nanocatalysts.[22] Herein, we report a self-integrated paper-based enzyme biosensor that can measure the amount of HX in an easy, one-step, and cost-effective manner. The presence of HX is seen as a color change that can be quantified by the naked eye within minutes of contact with the sensor. An application for HX detection to measure changes in HX concentration in fresh and degraded tilapia fish over time is described.

To develop the biosensor, we use nitro blue tetrazolium chloride (NBT), which reacts with the HX/XOD-generated superoxide, producing a strongly adsorbing formazan product via the reduction of NBT, generating a clearly detectable deep violet colorimetric response.[23] NBT has been used previously to measure XOD from microorganisms[24] and determine XOD inhibitors and superoxide radical scavengers,[25] but to our knowledge, it has never been used as a colorimetric reagent to construct an integrated enzyme-based biosensor for HX detection. The conceptual design and operation of the XOD biosensor are shown in Figure 1. To produce an integrated device, the biosensor is constructed by coimmobilizing the NBT dye with the XOD enzyme on filter paper, proving both biorecognition and colorimetric detection. Our proof-of-principle tests demonstrate that the biosensor can effectively measure the HX content and differentiate between fresh and degraded fish through color-based measurements. The biosensor is sensitive, demonstrating good correlation with commercial solution-based HX tests, and it is portable and easy-to-use. A potential application of this easy-to-use biosensor is as a freshness indicator to measure the HX evolution over time to quantify the quality and potential degradation of fish and meat products. An application for detection of HX in fish measured by placing the paper biosensor in contact with a small sample of the fish is demonstrated in this work. The principle can be applied in the manufacturing of portable biosensors for the estimation of the fish freshness status and in other applications in which HX plays an essential role.

EXPERIMENTAL SECTION

Materials and Reagents. Xanthine oxidase (XOD) from bovine milk, poly(sodium-4-styrenesulfonate) (PSS) (1 mg/mL in phosphate-buffered saline, pH 7.4, PBS), poly(ethyleneimine) (PEI) solution 50% w/v in water (10 mg/mL), uric acid, and the HX colorimetric kit were obtained from Sigma-Aldrich (MAK 186). Hypoxanthine (HX) was obtained from Acros Organics. *p*-Nitroblue tetrazolium chloride (NBT) was obtained from MP Biomedicals. Chitosan (Chit) from shrimp's shells (1% solution was prepared in 0.05 M acetic acid (J.T. Baker)), 3-(trimethoxysilyl)propyl methacrylate (TMESPMA), and inosine (I) were obtained from Aldrich. Sodium phosphate dibasic (Na_2HPO_4) and sodium phosphate monobasic (NaH_2PO_4) were obtained from Sigma-Aldrich. 3-

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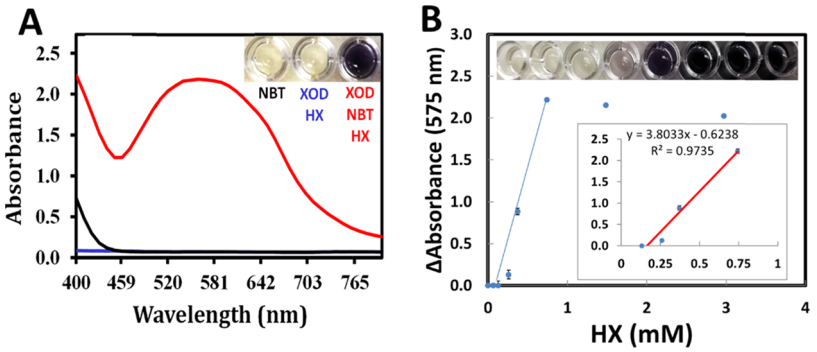

Figure 2. (A) UV−vis spectra showing comparative absorbance readings of NBT, XOD with HX, and XOD, NBT, and HX in the same reaction cell showing the color formation only when all reagents are present. (B) Colorimetric response and calibration curve showing Δ absorbance vs HX concentration measured at 575 nm for different HX concentrations in the presence of XOD (0.13 U/mL) and NBT (0.33 mg/mL).

Aminopropyltriethoxysilane (APTS), quantitative cellulose filter paper, and sodium hydroxide were purchased from Spectrum Chemical. A 0.45 μm polytetrafluoroethylene (PTFE) filter membrane was purchased from Whatman, and 10 kD centrifuge tubes were obtained from Pall. Hydrochloric acid was obtained from J.T. Baker. D-glucose was obtained from Fisher Scientific, and L-actic acid and dopamine were purchased from Sigma. All reagents were used without further purification, and all solutions were prepared with ultrapure water (Millipore, Direct-Q system, 18.2 MΩ·cm).

Instrumentation. UV−vis absorption spectra were recorded on a SpectraMax i3x 96-well plate spectrophotometer (Molecular Device). Images were scanned using an EPSON scanner (Perfection V600 Photo). The violet color resulting from the reaction of NBT with the enzymatically produced H_2O_2 from HX was analyzed by ImageJ software. The RGB color was evaluated by measuring the red color intensity of the dried paper strips upon exposure to HX. An area was selected for each sensor to cover about 18 × 18 (width × height). The surface morphology of the biosensors was evaluated by field emission scanning electron microscopy (FESEM) with a JEOL JSM-7400F instrument. For analysis, the biosensor was attached to an aluminum stub using carbon tape (Ted Pella).

NBT-Based HX Detection in Solution. HX solution was prepared by dissolving 6.1 mg in 100 μL of 1 M NaOH, neutralized by 1 M HCl, and then diluted up to 5.0 mL (4.5 mM). HX detection in solution was performed by mixing 50 μL of 0.4 U/mL XOD with 50 μL of HX in the concentration range of 0.39−8.9 mM and incubated for 15 min. Thereafter, 50 μL of 1 mg/mL NBT was added, and the mixture was incubated for 10 min. UV−vis spectra were recorded in the range of 400−800 nm, and HX was quantified at the maximum absorbance of 575 nm.

Fabrication of the Self-Integrated Biosensor for HX Detection. The biosensor was fabricated by coimmobilizing XOD and NBT within several sequential layers deposited on silanized paper, which was utilized as a platform to construct the biosensor. Cellulosic filter paper was punched into 0.6 cm diameter disks. The silanization of paper consisted in applying a sol−gel mixture prepared based on the procedure by Wang et al.[26] with modification. In a typical experiment, 360 μL of methanol was mixed with 100 μL of TMESPMA and a chitosan solution consisting of 150 μL of 1% chitosan mixed with 60 μL of H_2O and 20 μL of 5 mM NaOH to obtain a final pH of 6.5. The mixture was vortexed for 5 min. On a glass dish, 10 μL of the sol−gel mixture was deposited on each paper disk and dried for about 1 h. Then, 5 μL of aqueous NBT solution (1 mg/mL) was added to the silica paper and dried for 15 min, followed by another 5 μL and drying for about 45 min (a total of 10 μg per sensor). XOD (prepared in 50 mM potassium phosphate buffer pH 7.5) was immobilized through successive addition of 5 μL of 4 U/mL XOD and drying for about 1.5 h. The sequentially deposited layers were sol−gel-chitosan/NBT/XOD, and the generated sensor was designated as an NBT paper biosensor throughout the manuscript. To optimize the biosensor and determine the figures of merit, 20 μL of different concentrations of HX covering the range of 0.019−4.5 mM was added. Paper sensors were dried for about 45 min prior to color reading.

Real Sample Analysis. Tilapia fish fillets were obtained from a local store. 1.5 g were sampled from each fillet in triplicate and left to decay at room temperature (RT) for 0, 4, and 24 h. To demonstrate the ability of the biosensor to detect HX evolved from fish, biosensor disks were placed directly on the fish sample for 5 min and then removed, dried, and scanned. Comparative analysis was performed on digested homogenized samples using the (1) NBT solution assay and (2) the commercial XOD kit and (3) by applying the extract on the paper sensor. For analysis of digested samples, 5 g of sample was left to degrade in duplicate. Samples were taken at the corresponding degradation time (0, 4, 12, 24 h). Each sample was homogenized with a mortar and pestle, sonicated for 15 min in 15 mL of water, centrifuged at 13.4 krpm for 10 min, and filtered through the 0.45 μm PTFE filter membrane. To eliminate the existing enzymes, the samples were further filtered using a 10 kDa MWCO ultrafiltration tube at 13.4 krpm for 15 min. Samples were stored at −20 °C till the day of analysis. To perform analysis with the NBT assay, 50 μL of the fish extract was mixed with 50 μL of 0.4 U/mL XOD (prepared in 50 mM potassium phosphate buffer pH 7.5 buffer) and allowed to stand for 15 min. Then, 50 μL of 1 mg/mL NBT was further added, and the mixture was incubated for 10 min. Finally, the absorbance was measured spectrophotometrically at 575 nm. Evaluation of the HX content in the homogenized samples was also performed with the paper biosensor. For this analysis, fish extract samples must be diluted five times prior to the application to the sensor surface due to the higher sensitivity of the method. The fish extract was also analyzed using the commercial kit and the protocol provided by the vendor (Sigma-Aldrich).

RESULTS AND DISCUSSION

Proof-of-Concept of the NBT-Based HX Detection in Solution. The sensing mechanism relies on the oxidation of HX by XOD producing xanthine that is further oxidized to uric acid. In the presence of NBT, the superoxide anion radicals produced by HX/XOD reduce the NBT into a strongly absorbing insoluble NBT formazan complex with a violet color (Figure 1).[23] The feasibility of this concept was first established in solution. When tested alone, XOD, HX, or NBT does not show any colorimetric response. Upon addition of HX (1.5 mM) to a mixture of NBT (0.33 mg/mL) and XOD (0.13 U/mL), a very strong violet color was developed, with an absorbance peak with a maximum intensity at λ_{max} = 575 nm (Figure 2A). The color formed due to complex formation progresses over time, reaching a plateau after about 10 min [Figure S1 in the Supporting Information (SI) section]. After ∼15 min, the color intensity starts to decrease, and a precipitate is formed. Therefore, experiments to optimize the

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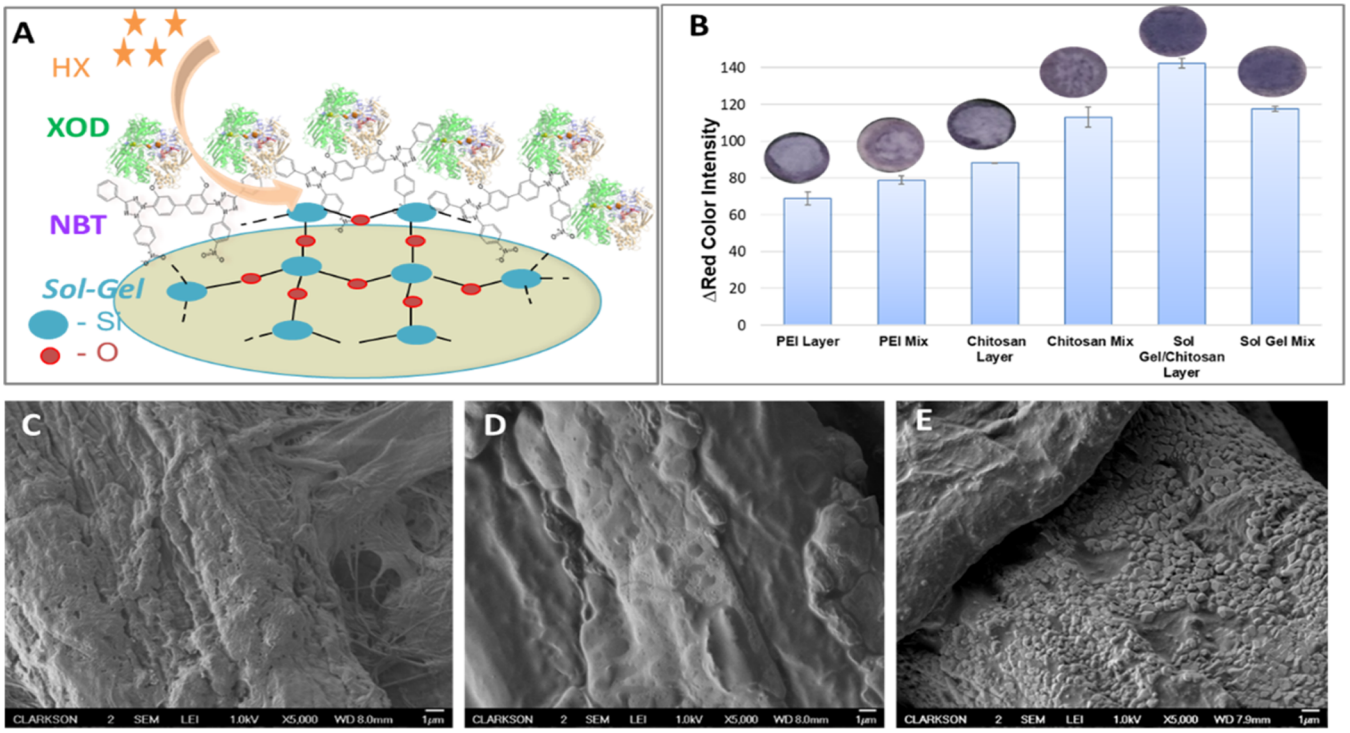

Figure 3. (A) Surface of the paper-based NBT biosensor consisting of a sol−gel/chitosan layer, NBT, and XOD. (B) Signal intensity and color homogeneity of biosensors prepared with immobilization materials including PEI, chitosan, and sol−gel/chitosan deposited as sequential layers or in mixture with NBT followed by XOD. The graph shows triplicate measurements and standard deviation of color intensity after addition of 4.5 mM HX (the color was quantified using ImageJ software). The SEM images of (C) bare unmodified and sol−gel/chitosan-modified cellulosic filter paper before (D) and after (E) reaction with HX.

assay were performed using a 10 min reaction time. The results showing the calibration curve for different concentrations of HX are illustrated in Figure 2B. This NBT solution assay is able to detect HX concentrations within the range 10−700 μM with a limit of detection (LOD) of 3.1 μM, calculated using 3 times the standard deviation (SD) of the blank divided by the slope (3 × SD (blank)/slope) of the calibration curve. The lower limit of quantification (LLQ) quantified as 10 × SD (blank)/slope was 10 μM.

Fabrication and Optimization of the Integrated NBT-Based Biosensor Label. The next set of experiments focused on the translation of the solution assay to a solid platform, e.g., paper, to produce an integrated biosensor capable of measuring HX released from decayed food with our sample treatment or addition of exogenous reagents. The biosensor is configured to store both the NBT dye and the enzyme and to produce a colorimetric output signal that is visible to the naked eye in the presence of HX. The biosensor design is illustrated in Figure 3A. To manufacture the sensor, chitosan is cross-linked with TMESPMA producing a sol−gel-like material, which impregnates the pores of the cellulose paper enhancing its permeability and rigidity and increasing the stability of the enzyme[26] and preventing its leaching. The deposited sol−gel/chitosan has hydrophilic properties. Thus, when applying a hypoxanthine sample, the sample is absorbed and the color is developed homogeneously. This matrix provides high functionality for XOD immobilization increasing the enzyme stability and preventing its leaching.[26] We used chitosan in the composition of the sol−gel to increase biocompatibility and impart hydrophilicity and enzyme-binding characteristics through its abundant amino groups that are compatible with biomolecules. Chitosan is also known to facilitate strong attachment of enzymes to cellulose paper and reduce swelling.[27]

Essential to the fabrication and reproducibility of the biosensor are the composition and the sequence of the layer deposition, both of which dictate the homogeneity and sensitivity of the colorimetric response. To optimize the biosensor, several configurations were tested including the use of sol−gel/chitosan and chitosan alone deposited either as a layer or mixed with NBT followed by enzyme immobilization to create the HX-responsive surface. In addition to these materials, we have tested PEI, a branched polyamine known for its cross-linking abilities and abundant amino groups that have been used for the entrapment of various enzymes.[28] The PEI was applied either as a separate layer followed by NBT or as a mixture with NBT. Figure 3B shows the sensor response for the different configurations tested. The biosensors constructed with a hybrid sol−gel/chitosan layer showed the best homogeneity and highest color intensity. Color formation confirms the occurrence of the sequential enzymatic and chemical reactions within these layers. A summary of the optimization experiments and composition of the biosensing layers is provided in the SI. To optimize the biosensor, we also tested the sensor response to different color readings in the RGB scheme including red, gray, green, and blue. The highest color intensity was measured on the red channel (Figure S2), and therefore this configuration was used to determine the analytical figures of merit and for real sample measurements.

The biosensor works as a colorimetric readout that recognizes HX released when this reaches the active sensing surface. Once the HX is in contact with the XOD biosensor, this is converted to H_2O_2. The superoxide anion radicals produced by HX/XOD reduce the NBT dye into a strongly

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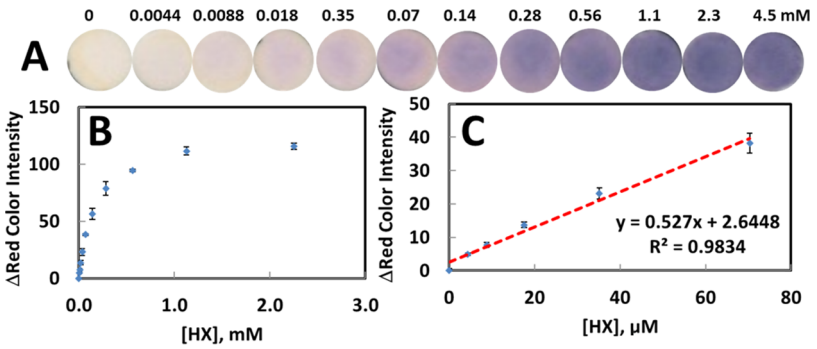

Figure 4. Colorimetric responses of NBT biosensors to increasing concentrations of HX (A). Increase of Δ red color intensity vs HX concentration with the corresponding (B) calibration curve and (C) linearity range of the biosensor. Error bars represent standard deviation for three replicates. Biosensors were coated with XOD (0.04U) and NBT (10 μg).

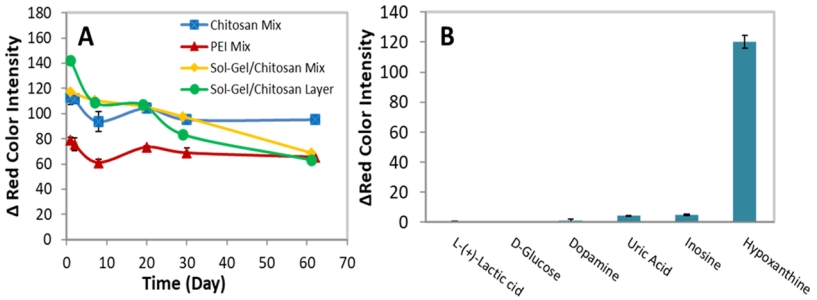

Figure 5. (A) Storage stability at −20 °C of NBT-based biosensors fabricated using chitosan, PEI, and sol−gel/chitosan layered or mixed with NBT and XOD. Color intensity was measured upon addition of 4.5 mM HX. (B) Comparative responses of the biosensor for HX versus uric acid and inosine interfering compounds tested at the following concentrations: 2 mM glucose, 2 mM uric acid, 2 mM lactic acid, 2 mM dopamine, 2 mM inosine, and 2 mM hypoxanthine.

absorbing insoluble NBT formazan complex with a violet color. The method is able to measure the accumulative amount of HX and X with this enzymatic reaction catalyzed by XOD. It was shown previously that X competes with HX at pH close to the experimental condition,[29] and both X and HX are related to freshness as described by Karube et al. who proposed a model to estimate the freshness of fish, based on the content of I, X, and HX.[3]

As compared to the solution assay, the formazan complex is retained and stabilized within the micropores of the silanized paper. The morphology of the paper surface changes with each of these steps as can be seen from the SEM micrographs shown in Figure 3C−E. The silanized paper (Figure 3D) shows a smoother surface as compared to the unmodified paper (Figure 3C), confirming successful modification. Following reaction with HX, the insoluble formazan product is seen as microscopic deposits onto the cellulosic paper (Figure 3E). Addition of HX to a sensor containing NBT/XOD generates a change in color from white to intense violet, with a similar rate as the solution assay. Since both the enzyme and the color-generating agent are fixed within the biosensor and the only step necessary for analysis is contact with the HX-containing sample, this biosensor functions as a "reagentless" self-contained device.

Analytical Characteristics of the Optimized Biosensor Label. To determine the analytical figures of merit of the HX biosensor, optimized biosensors were exposed to increasing concentrations of HX, in the concentration range that is typically found in degraded fish. For analysis, the sensors were left to dry and color analysis as a function of HX concentration was performed at the same time point. As seen in Figure 4, increasing concentrations of HX resulted in stronger color intensity proportional to the HX concentration. No change in color was observed in the absence of the enzyme or NBT, indicating that the color formed is due to the XOD-generated formazan product. The color formation starts gradually within a few minutes, and the color is very deep and easily detected by the naked eye. For more precise quantification, the color intensity can be measured by scanning the dried paper (∼45 min). The linear range of the optimized biosensor was between 4 and 35 μM. The LOD was 4.1 μM, which is comparable to the solution-based approaches for HX detection, summarized in Table S1. As compared to other approaches, our biosensor is reagentless, easy-to-use, and portable (e.g., one-step reagentless analysis on a paper strip). The preparation steps and drying could be speeded up by incubating the sensor under vacuum at RT. Moreover, the color analysis could be accelerated by taking a picture of the sensor using a mobile phone app and determining the HX amount based on a color-based calibration curve.

Stability and Reproducibility. Shelf life, reproducibility, and stability are essential for the development of a practical biosensor. All experiments were performed with triplicate biosensors, and the average color intensity was consistently below 5%. Figure S3A in the SI shows color responses obtained with independent sensors exposed to varying HX concentrations, indicating uniform and consistent responses. To further demonstrate reproducibility, Figure S3B illustrates replicate analysis for n = 10 independently produced biosensors showing an average color intensity of 80.7(±7.2) upon addition of 4.5 mM Hx. The storage stability was determined by measuring the colorimetric response of

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biosensors stored at −20 °C and refrigerated at (2−8 °C). A concentration of 4.5 mM HX was used in these tests. Figure 5 shows the stability of biosensors stored at −20 °C over a period of 60 days. The results show that the biosensors retain ∼84% activity after 60 days of storage with a coefficient of variation below 3%. The biosensors stored in the refrigerator at (2−8 °C) retained ∼35% residual activity after 10 days and ∼30% in the next 20 days of storage. Since enzyme stability is dependent on the immobilization material, we also compared the stability of biosensors prepared with different materials including chitosan, PEI, and the sol−gel/chitosan deposited as a layer or mixed with NBT. As seen in Figure 5A, stability of the biosensors stored at −20 °C varies. While the biosensors incorporating chitosan and PEI showed stable responses over the time of study, the responses of the sensors with sol−gel-chitosan (layer or mix) decline more rapidly (Figure S4A). We selected the sol−gel/chitosan configuration for this work due to the enhanced sensitivity and homogeneity of the colorimetric response (Figure 3B).

Interferences. Further, the selectivity of the HX biosensors was investigated in the presence of uric acid and inosine, as these compounds are products of ATP degradation in addition to D-glucose, L-lactic acid, and dopamine that could coexist in meat and biological samples.[3] A concentration of 2 mM of HX and interfering compounds were used in these tests. Figure 5 shows the comparative responses of the biosensor exposed to equal concentrations (2 mM) of HX, inosine, and uric acid. The response to I, UA, D-glucose, L-lactic acid, and dopamine is below 4% of the HX signal, demonstrating high selectivity toward HX, which is attributed to the specificity of XOD for HX.

Monitoring HX Levels in Fish Samples. We further tested the ability of the biosensors to measure the HX released naturally from fresh and degraded fish. HX is one of the major products of ATP degradation mediated by bacterial activity and an indicator of meat and fish freshness, and therefore spoilage. After ATP degradation, HX increases gradually[12b,30] at levels that vary with the storage conditions, time and temperature, and the fish species.[11] To assess fish freshness with our biosensors, tilapia fish samples were left to decay at room temperature, and the HX content was monitored over a period of 24 h. To test the sample, fish was left to deteriorate for 0, 4, and 24 h at room temperature. For comparison (Figure S4), the sample was digested, and the homogenate was tested with the solution-based NBT assay (A), a commercial HX assay kit consisting of XOD, peroxidase enzyme solutions (B), and the biosensor applied on the surface (without applying any additional pressure) (C). A summary of the comparative protocols used to test fish samples is illustrated in the SI (Figure S4).

The results shown in Figure 6 demonstrate the capability of the NBT biosensor to track HX content over a period of 24 h. The HX levels measured by the biosensor were found to increase from 117 (±9) to 198 (±5) μM within 24 h. The results shown in Figure S5 are comparable to HX values found in tilapia fish reported in the literature.[30,31] An increased HX level for degraded samples was consistently measured by all tests. As compared to the commercial HX kit with spectrophotometric/fluorescence detection, our biosensor is easier to use and cost-effective as it uses only one enzyme and both the enzyme and dye are stabilized within a paper-based label. Moreover, the analysis does not involve the use of a spectrophotometer, and the biosensor is robust and can be fabricated in large quantities.

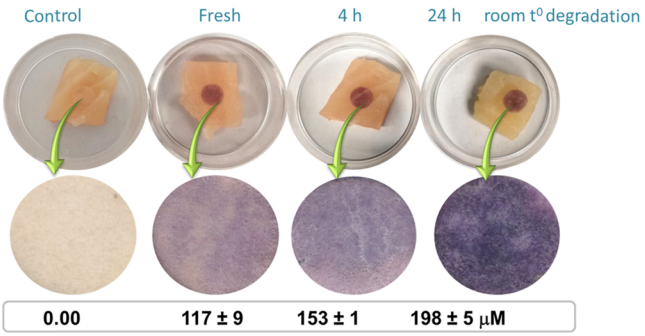

Figure 6. Fish freshness assessment in real samples. Images show color change of biosensors placed on the top of 1.5 g of fish samples tested as purchased and left to undergo deterioration for 4 and 24 h at RT. Measurements were taken with the biosensors placed for 5 min in contact with a small fish sample. The control sample shows the color of the biosensor at 0 contact time on top of the nondeteriorated fish sample.

Due to the requirements of the enzyme for an aqueous environment, the biosensor responds to HX when the biosensor is applied on a wet fish sample. In the proof-of-concept work, we have found that a contact time of ∼5 min of the sensor with the fish sample is sufficient to obtain a colorimetric response that lies within the linear range of the curve. Analysis does not require addition of any reagent, water, or buffer solution, other than placing the sensor on a small portion of the fish, freshly removed from the package. To measure HX, the user would simply apply the biosensor on a small portion of the sample and visually see the color formed (about 5 min).

After being harvested, the rate of accumulation of ATP degradation metabolites including HX in fish muscles varies among species.[32] Karube et al. proposed a model to estimate the freshness of fish, based on the content of I, X, and HX,[3] establishing the role of these metabolites as degradation (or freshness) biomarkers. The HX level in fish is not regulated as it is for biogenic amine (histamine), for example, for which a 5 mg/100 g fish was set.[2] However, several studies relate the concentration of HX to freshness such as the study conducted by Chen et al.[12b] who have suggested that *Miichthys miiuy* fish is fresh if containing HX of 0.25 mM, under deterioration at 0.375−0.53 mM, and spoiled at >0.63 mM. Qiong et al. described that the total concentration of HX/X/U of ≤7.2 mg/100 g indicates fresh fish, while subfresh fish has 7.2−11.8 mg/100 g and fish starts to spoil at ≥11.8 mg/100 g for *Mylopharyngodon piceus*, *Clenopharyngodon idellus*, and *Hypophthalmichthys molitrix*.[33] Table S2 shows the amounts of HX found in tilapia fish measured using our NBT-based solution and paper sensor formats as compared to the commercial Sigma-Aldrich kit and data reported in the literature. The concentrations measured by the different methods are comparable and within the range. These levels are dependent on the fish species and therefore further practical implementation of the biosensor for freshness assessment would require a database of HX content developed for different types of fish and storage conditions.

Future developments could be envisioned toward the translation of this technology as a food freshness indicator. This could be achieved by incorporating the paper biosensor

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within a porous permeable membrane placed inside the food package. The membrane could be opened at the time of use, allowing migration of the fish degradation products (HX) toward the enzyme layer. The user can check the fish status by pulling the sensor cover permitting the active compartment to get in contact with the HX present at the surface of the fish and trigger the enzymatic reaction. Another scenario would be to apply the biosensor on a small sample of the fish to check for quality at the time of use. These biosensors could be further developed as freshness indicators as a convenient user-friendly alternative to the "use-by-date" to assess the loss of food quality and provide information about the release of HX, a freshness biomarker, in packaged food.[34] It is known that HX formation can be used to quantify freshness during the first few days of storage.[35] Such biosensors can provide a solution for industry and consumers to assess the quality of food products rapidly and inexpensively at the time of use. Conventional packaging technologies are not equipped with systems for monitoring markers of biochemical degradation, and biosensors for monitoring of packaged food do not currently exist.[3634c] These can be used alone or in conjunction with other freshness labels for monitoring volatile amines[37] using the total volatile basic nitrogen (TVBN) evolved from spoiled fish to establish comparability of the different degradation markers at different time points. This could complement existing labels that are designed primarily to measure the environment of packed food such as changes in temperature, humidity, pH, or oxygen through time−temperature indicators,[38] RFID,[39] or gas indicators.[40] This integration will allow not only assessment of freshness of fish stored at different temperatures but during different storage times such that HX could be detected earlier than TVBN, which reflects later stages of fish spoilage.[37,41] Further developments can also include the large-scale manufacturing of these biosensors and the design of permeable membranes that would allow degradation products to migrate from the fish to the biosensor surface into the headspace and the deployment and use of this technology inside packaging. Other developments could include the use of the biosensor to establish a database of HX degradation pathways to predict freshness in different types of fish.

CONCLUSIONS

In summary, the enzymatic biosensor described in this paper provides a novel fish freshness monitoring test that allows real-time tracking of a degradation biomarker, e.g., HX, released by stored and degraded fish over time without requiring the addition of exogenous reagents or the use of sophisticated laboratory equipment. The biosensor has several features that make it ideally suited for the monitoring of fish and meat products, such as (i) the ability to detect a degradation biomarker without/with sampling, (ii) simple analysis with easy colorimetric readout, e.g., contact of the biosensor with a portion of the sample and visual detection of color change, (iii) portability, resembling a pH paper, and a structure that can, in principle, be fabricated in large numbers using roll-to-roll manufacturing methods such as printing, (iv) good stability covering the length of time and storage conditions of stored fish products, and (v) rapid response time and stability of the color once the product is formed. In the future, portable designs compatible with smartphones equipped with color reading software can be added to facilitate analysis. Integration of such biosensors that can monitor degradation markers will provide opportunities for real-time assessment of the food quality and freshness status and identifying spoilage at a large scale, providing convenience and accessibility to the general public to verify the quality of meat and fish products for everyday use. In addition to detecting HX, the biosensor could also be applied for a wide range of applications in the biomedical field, for example, for measuring HX levels in tumors and samples from patients with cardiac disorders or leukemia.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.0c02350.

Kinetics of color formation of the NBT solution assay; fabrication and optimization of the NBT-based biosensor; selection of the color reading scheme; biosensor reproducibility; comparative real sample analysis using solution assay and comparison of analytical parameters with other hypoxanthine biosensors reported in the literature; and movie of color formation with the biosensor label placed on fish (PDF)

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Notes

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