Polysulfone/Pyrene Membranes: A New Microwell Assay Platform for Bioapplications

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The use of PSU-Py prepared by click chemistry as a platform in membrane-bottom microwell plates for oxidase and hydrolase/oxidase-based enzyme assays is studied. For the GOx assay, the postulated fluorescence mechanism is based on the consumption of glucose by dissolved oxygen and GOx in the microwell plates covered with the PSU-Py membrane. For the AG-GOx assay, maltose is used as AG substrate and hydrolyzed to glucose which is then oxidized by the GOx activity. It is shown that the PSU-Py membrane acts as a fluorescence indicator of the enzymatic reactions, and both GOx and AG/GOx enzyme assays are successfully applied for glucose, maltose and acorbose analysis in the range 0.125–2.0 $\times$ $10^{-3}$ M glucose, 0.05–0.5 $\times$ $10^{-3}$ M maltose, and 0.0125–0.1 mg mL$^{-1}$ acorbose, respectively.

Introduction

High-throughput screening assays enable the analysis and testing of large numbers of chemical compounds for activity in diverse areas of biological sciences. Membranes with functional groups could be good candidates as solid support materials for integration into existing high-throughput plastic microwell formats due to their versatility and physicochemical properties. This integration yielded membrane-bottom plates and extended the possible applications of these materials by using them in conjunction with preexisting accessories designed for microwell formats and detection in commercially available radioisotope, colorimetric, and chemiluminescence plate readers. Assay development in such microwell formats thus offers a versatile tool for high-throughput sample processing on membranes. Polysulfone (PSU) polymers are widely used in tissue engineering as biomaterials as biocompatible and non-degradable materials, these polymers have been used in a variety of bio-applications such as hemodialysis, ultrafiltration, filtration and bioreactor technology as well as drug delivery and cell culture applications. In recent years, the main scientific and applied interest is focused not only on the synthesis of new types of polymeric materials but also on the modification of...
existing polymers to alter their properties to meet requirements for new applications.[10] As a fluorescence probe, pyrene (Py) has been attractive because of its high fluorescence yield, stability and characteristic excimer formation. Particular attention has been directed on the integration of Py units into polymers. For instance, living anionic, conventional radical polymerization, atom transfer radical polymerization, and ring-opening metathesis polymerization processes were successfully used to prepare polymers with Py groups.[11–19] Py functionalization showed that the strong fluorescence properties of such polymers makes them valuable candidates for a variety of applications in biomolecular interactions including those between antigen and antibody, enzymes and substrates, and nucleic acids and their complementary sequences. A versatile method to synthesize Py-functional poly(vinyl alcohol) (PVA) directly from the PVA using the click chemistry approach has been previously reported[20] and successfully applied as fluorescence probe for both oxidase-based enzyme assay and in vitro cancer diagnosis.[21,22] PSUs have also been successfully functionalized with Py by successive chloromethylation, azidation, and click chemistry processes (Scheme 1).[23]

Click chemistry has been reported as a promising approach for the construction of various bioconjugates, biomaterials as well as functional bioactive surfaces.[24] In a previous work,[24] we demonstrated that PSUs can be successfully post-functionalized by using click chemistry on the example of fluorescent Py molecule. It was considered that incorporation of such molecules might bring remarkable fluorescence regulated behavior when subjected to irradiation which makes them useful candidates for various applications in biomolecular interactions. Herein, we describe the use of Py-functional polysulfone (PSU-Py) obtained this way as an indicator matrix for monitoring of bio-catalytic reactions as well as cell adhesion that would allow their interaction to be monitored as a change in fluorescence signals. It is well known that fluorometric assays have the advantage of high sensitivity and are recommended for detection of low enzyme activities.[25] As it will be shown below, PSU-Py has been applied as a new microwell assay platform for the oxidase and hydrolase/oxidase-based assays and cell detection.

**Experimental Section**

**Materials**

PSU (Udel P-1700) was used as received. Propargylpyrene was synthesized according to literature procedure.[23] Dichloromethane (CH₂Cl₂, 99% Lab-Scan) was distilled over P₂O₅. N,N-dimethylformamide (DMF, 99%, Aldrich), sodium azide (99%, Merck), copper(I) bromide (98%, Acros), sodium hydride (98%, Fluka), propargyl bromide (80 vol% in toluene, Fluka), 1-pyrenemethanol (98%, Aldrich), 2,2-bipyridyl (Acros Organics 99%), paraformaldehyde (95%, Aldrich), chlorotrimethylsilane (99%, Aldrich), methanol (99%, Riedel-de Haen), diethyl ether (98%, Carlo-Erba), and tetrahydrofuran (THF, 99% Lab-Scan) were used as received. Glucose oxidase (GOx; 21 200 U g⁻¹, type II-S, from Aspergillus niger), β-glucosidase (AG; 84 000 U g⁻¹ from Saccharomyces cerevisiae), β-D-glucose (99.5%) were purchased from Sigma. Acorbose was obtained from Bayer and used as AG inhibitor.

**Cell Line**

Hutu-80 cells were obtained from ATCC (The Global Biosource Center) and cultured in 75 cm² flask with minimum essential medium (Eagle) with 2.0 × 10⁻³ M L-glutamine and Earle’s balanced salt solution (BSS), 1.5 g L⁻¹ sodium bicarbonate, 10⁻⁴ M non-essential amino acids, and 10⁻³ M sodium pyruvate, 90%; fetal bovine serum, 10% at 37 °C in a humidified atmosphere of 5.0% CO₂ and 95% air. Cells were cultured to 60–80% confluent. Then, aspirate the medium, rinse the cells three times with 5.0 mL of phosphate-buffered saline (PBS) and cover the cells with 2.0 mL of trypsin/ethylenediaminetetraacetate (EDTA) (0.05/0.02%) solution for

![Scheme 1. Py functionalization of PSU by click chemistry.](image-url)
splitting the monolayer. 10 mL of medium was added for trypsin neutralization and centrifuged at 400g and 4°C.

**Preparation of PSU-Py Membranes**

Side-chain PSU-Py was synthesized and characterized as described previously.[23] For the membrane formation, PSU-Py (1.0 mg) was dissolved in chloroform (1.0 mL). Then, 50 μL of polymer solution was cast either into the 96-well (or 24-well) tissue culture plates or glass slides and allowed to dry at room temperature.

**Microscopic Characterization**

Differential interference contrast microscopy (DICM, Zeiss, Axio Vision Microscope with differential interference contrast-mode) and atomic force microscopy (AFM, NanoMagnetics Instruments, UK) were used for the microscopic images of the PSU-Py membrane which were prepared on the glass slides as described before. AFM imaging was carried out at ambient temperature in non-contact mode.

**Bioassays**

Adherence Assay for Cell Detection

Hutu-80 cells were prepared in cell culture media at concentrations of 10^5, 10^6, 10^7, and 10^8 cells per mL. Polymer-coated 24-wells were assayed fluorometrically at the beginning of the assay and basal fluorescence of polymers was registered. Different concentration of Hutu cells was seeded into the wells. Wells were incubated for 24 h and washed with PBS and fluorometrically assayed in a multiwell fluorescence plate reader (Thermo, Milford, MA). Media were added on to the wells, incubated for 48 h and again assayed fluorometrically. This procedure was repeated twice with incubation times of 72 and 96 h. Change in fluorescence unit exhibits amount of adhered cells related to concentration and time variables. Additionally, fluorescence microscopy was used for the imaging of the PSU-Py membrane before and after 24 h incubation with the Hutu cells. 9 cm² tissue-culture Petri dishes were covered with a PSU-Py coated slide and fluorescence mounting medium (Daco, Copenhagen, Denmark), and visualized with 100× magnification and photographed through an epifluorescence microscope (Olympus, Tokyo, Japan).

Detection of GOx and AG Activities

GOx catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. Since oxygen quenches photoexcited fluorescent molecules, the increase in fluorescence intensity of Py in the matrix is observed due to the oxygen consumption during the enzymatic reaction depending on the glucose concentration.[26]

The emission fluorescence spectra have been collected by means of a fluorescence spectrophotometer purchased from Varian Cary Eclipse (USA). Sample excitation was performed at 340 nm, while the emission spectrum was recorded in the range 340–420 nm by using a glass slide covered with PSU-Py polymer which was placed in 1 × 3 cm² cells at 25°C. The spectra have been acquired with entrance and exit slits fixed at 5 nm and with a scan speed of 10 nm s⁻¹.

In the fluorescence emission assay, the emission intensities were registered at 400 nm by using a proper filter after excitation at 340 nm in a multiwell fluorescence plate reader (Thermo, Milford, MA). All measurements were performed in 96-well plates at 25°C. Initially an appropriate amount of GOx and glucose from the stock solutions were transferred into the wells covered by PSU-Py and containing oxygen saturated working buffer solutions and allow to incubate at 25°C. Then, fluorometric signals of PSU-Py as a result of enzymatic activity in microwells were measured against the blank containing the same reaction mixture except the glucose substrate.

In the case of AG assay, AG and GOx enzymes in the ratio of 1:10 and maltose which is AG substrate were used. Initially, maltose was hydrolyzed by the activity of AG and glucose was released as product that was the substrate of GOx enzyme. Therefore, fluorometric response of PSU-Py was monitored due to the bi-enzymatic reaction against the blank containing the same reaction mixture except the maltose as described before. For the effect of AG inhibitor on AG assay, acarbose was used as a model compound and AG was incubated for 5 min with the inhibitor in the reaction mixture before adding maltose substrate and GOx. The response signals were defined as the difference in fluorescence signals in the presence and absence of substrate and registered as arbitrary units (Δa.u.). For the observation of effect of pH and enzyme amount on GOx assay maximum signal value was assumed as 100% and other values calculated relative to this value. All data were presented as the average of 4–5 measurements ± standard deviation (SD).

**Glucose Analysis in Samples**

PSU-Py was used to analyze glucose content in real samples such as fizzy and cherry juice that were purchased from the local markets. Additionally, high-performance liquid chromatography (HPLC) was used as a reference method for independent glucose analysis. HPLC column [GL Sciences Inc. Inertsil NH 2 5.0 μm (4.6 id × 250 mm), Japan] was used for the chromatographic separation of glucose at 30°C. Injection volume was 20 μL. The mobile phase was H₂SO₄ (5.0 × 10⁻³ m). The flow rate was 0.6 mL min⁻¹. Initially, calibration curve for glucose was constructed in a 10⁻⁵–10⁻¹ range. The spectra have been acquired with entrance and exit slits fixed at 5 nm and with a scan speed of 10 nm s⁻¹.

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**Results and Discussion**

**Characterization of PSU-Py**

Membrane-bottom microwell plates facilitate exploitation of novel matrices and they are now used as much more than merely solid supports for reactants. Membranes offer a higher surface area than traditional plastic surfaces. Their porosity increases their versatility. Surface-modification
capabilities for reactive groups and charges on membranes allow ionic, hydrophobic, or covalent binding with target molecules. PSU is one of the most important polymer materials, and the PSU-based films showed outstanding thermal and hydrolytic stability as well as good mechanical and film-forming properties. \[27\] In this paper, potential use of PSU-Py prepared by click chemistry was searched as a platform in membrane-bottom microwell plates in cell and enzyme based bio-assay applications. Initially, microscopic characterization was performed by using AFM and DICM. AFM which provides morphological information was used in non-contact mode. According to the surface area coefficient (sa) from the histogram roughness, average was obtained as 25.7 nm. Homogeneous surface morphology for the modified PSU was evidenced by phase (Figure 1A) and 3D topography images (Figure 1B). The roughness could be mainly due to the presence of Py units in the structure.

DICM is an optical microscopy illumination technique used to enhance the contrast in unstained, transparent samples. DICM images of the transparent PSU-Py membrane was shown in Figure 2.

![AFM phase images of PSU-Py in non-contact mode, 3D topography of PSU-Py](image-url)
Bioassays

Cell Detection

In the cell assay, Hutu-80 which is known as adherent were used as the model for the eukaryotic cells and prepared in cell culture media in PSU-Py-coated 24-wells at various amounts (10⁶, 10⁵, 10⁴, and 10³ cells per well). Polymer coated 24 wells were fluorometrically assayed and changes in fluorescence unit correlated amount of adhered cells related to concentration and time variables. In a previous work, eukaryotic, and prokaryotic cell adhesions as well as protein adsorption on the amphiphilic membranes possessing hydrophobic PSU backbone and hydrophilic side chain were studied and compared to unmodified PSU.¹⁰ It seemed, therefore, appropriate to use bare PSU as a versatile platform for cell adhesion and easily adapt to membrane-bottom microwell plates for high throughput screening assays. In the present case, PSU structure enables the cells to adhere on the surface which results in covering Py units and consequently, decrease in fluorescence intensity depending on the amount of cell drop in fluorescence signals due to the adhesion of Hutu cells (cell amount: 10⁶) by the time is shown in Figure 3. Maximum adhesion was observed after 24 h incubation, and then rather slight decreases in the signals were registered. On the other hand, a good correlation was found between the logarithm of cell amount adhered on the membranes and the decrease in the fluorescence signals which refers as (Δ(a.u.)) and linearity was defined by the equation of \( y = 57.3x - 135.6 \) \( (R^2 = 0.997) \) (Figure 4). Additionally, fluorescence microscopy was used for the imaging of the PSU-Py membrane before and after 24 h incubation with the Hutu cells (Figure 5). In accordance with the spectroscopic measurements, diminution of fluorescence intensity arising from the cell adhesion is detected. These findings showed that PSU-Py could be a promising culture material and utilized for both cultivation and detection in a one step without requiring any other labels. In this connection, it should be pointed out that additional functionalization with suitable side chains may enhance or prevent cell adhesion as was demonstrated for hydrophobic poly(tert-butyl acrylate) and hydrophilic poly(ethylene glycol) segments.¹⁰,²⁸

Detection of GOx Activity

In this fluorescence sensing approach, glucose is oxidized by the GOx in the reaction medium and oxygen is simultaneously consumed and the change in dissolved oxygen causes an increase in fluorescent intensity of Py bound to the PSU. Figure 6 shows the fluorescence emission spectra of PSU-Py cast onto the glass slide in the absence and in the presence of GOx (80.5 U) and glucose \( (10^{-2} \text{ m}) \). An increase in
fluorescence signal was observed when glucose was added into the reaction medium. Higher fluorescence signals were obtained in the presence of GOx without substrate in comparison to the signals in the presence buffer. This could be due to the contribution of fluorescence properties of amino acids in the GOx structure.

The pH dependency of the bio-assay system was investigated by monitoring the fluorescence emission signals via microplate reader by using sodium acetate (pH = 4.5–5.5; 5 × 10^{-2} M) and sodium phosphate buffers (pH = 6.0–8.0; 5 × 10^{-2} M) in the presence of GOx (10 mg·mL^{-1} which equals to 80.5 U) and glucose 10^{-2} M after incubation of the reaction mixture for 5 min at 25 °C. Maximum increase in the signal due to the bio-catalytic activity was observed at pH = 6.5 and 7.0 and as the pH increases further the decrease in fluorescence signals was observed (Figure 7). For further trials pH = 7.0, the point where enzyme exhibited the highest activity was used.

Prior to optimization of enzyme activity in the assay, optimum incubation time was verified by monitoring the fluorescence emission signals of the reaction mixture containing GOx (10 mg·mL^{-1}, 80.5 U) and glucose 10^{-2} M during 5 min at 25 °C. As shown in Figure 8, signals reached to the maximum in 60 s, then remain constant. Therefore, further experimental steps conducted with this incubation time.

To optimize enzyme concentration, substrate was taken in an excess amount, i.e., reaction is independent of the substrate concentration. Different concentrations of GOx (40.23, 80.5, 120.7, and 160.9 U) were allowed to react with 10^{-2} M glucose. As can be seen in Figure 6, saturation was observed after 80.5 U enzyme activity and for further increments the signals remained constant (Figure 9).

It has been observed that when the GOx activity was kept constant at 80.5 U while the substrate concentration was gradually increased, the signals were found to be increased until it reached a maximum at 2.0 × 10^{-3} M (Figure 10). After this point saturation was observed at 5.0 × 10^{-3} M glucose and then signals remained constant as expected due to Michael-Menten kinetics. The linear response of the system...
was observed between 0.125 and 2.0 \times 10^{-3} \text{ M} glucose with the equation of \( y = 1.215x \) \( (R^2 = 0.996) \), where \( x \) is the glucose concentration in \( 10^{-3} \text{ M} \) and \( y \) is an arbitrary unit (a.u.) that is defined as the difference in fluorescence emission signals (inset in Figure 10). Also, by applying the \( S/N = 3 \) criterion, the limits of detection (LOD) was calculated as \( 3 \times 10^{-5} \text{ M} \).

The investigated system was also applied for glucose analyses in real samples. For the glucose sensing in fizzy and cherry juice, samples with the appropriate dilutions were added into the reaction mixture containing GOx (80.5 U) in phosphate buffer (\( 5 \times 10^{-2} \text{ M}, \text{pH} = 7.0 \)) in polymer covered microwells instead of substrate. Then, the signals were recorded as already described for fluorometric assay, and data were calculated from the linear curve between glucose concentration and fluorescence intensities. According to the procedure glucose content in cherry juice and fizzy samples were found as 13.55 \pm 0.09 and 10.2 \pm 0.04 g per 100 mL. Additionally, HPLC was used as the reference method and the amounts were calculated as 13.64 \pm 0.19 and 9.99 \pm 0.02 g per 100 mL. Obtained results were very good agreement with the reference method. These results clearly confirm that the possibility of applying the proposed system to real sample analysis with high accuracy. Hence, PSU-Py could be used as a suitable assay component that may adapt to high throughput screening of oxidase based enzyme activities as well as bioanalysis of such important analytes as glucose in a 60 s of response time without using any other reagents in the reaction medium. It should also be pointed out that, the fluorescence signals of PSU-Py remained constant even after 200 uses. This stability is also advantageous as an assay platform.

Detection of AG Activity

AG (\( \alpha-\text{D-glucoside glucohydrolase, EC 3.2.1.20} \)), also called maltase, is a membrane-bound enzyme at the epithelium of the small intestine that catalyzes the cleavage of glucose from disaccharides. Maltose is the main substrate for AG. In this part, the proposed GOx-based PSU-Py assay was applied for the detection of AG activity and AG inhibitors. Initially, AG hydrolyses maltose to 2 mol \( \text{D-glucose} \). Then, glucose is oxidized by the GOx in the reaction medium and oxygen is simultaneously consumed through the enzymatic reaction that reduced the quenching effect by the oxygen. Calculations were made by using standard curve between the fluorescence signals (\( \Delta \text{a.u.} \)) and maltose concentrations. During the measurements of bienzyme assay, AG/GOx was used in ratio of 1/10 to avoid
the presence of excess glucose in the medium. Hence, the reaction mixture containing 8.5 U of AG and 80.5 U GOx in working buffer (300 mM L) in microwell plates were incubated for 5 min and changes in fluorescence signal was registered when maltose was added into the reaction medium. The signal intensity increases linearly up to $5 \times 10^{-4}$ mM maltose concentration and remains stable over the range from 0.5 to $5 \times 10^{-4}$ mM (Figure 11). Linearity was defined by the equation of $y = 3.696x$ ($R^2 = 0.997$), where $x$ is the maltose concentration in $10^{-3}$ mM and $y$ is an arbitrary unit (a.u.) and defined as the difference in fluorescence emission signals (inset in Figure 11).

This bi-enzyme approach was also used to observe the analysis of AG inhibitors and Acorbose was used as a model compound and prior to the measurement, AG enzyme (8.5 U) was pre-incubated with Acorbose for 5 min in the plates and then, maltose substrate at the saturation level ($5 \times 10^{-4}$ mM) and GOx (88.5 U) were subsequently added and after 5 min incubation, the response signals were registered toward the blank including the reaction mixture except maltose. For the observation of effect of inhibitor concentration on AG/GOx assay, decrease in the signal values that was registered in the absence of the inhibitor was monitored versus inhibitor concentration and shown in Figure 12. The linear inhibition graph between the signals and the negative logarithm of the inhibitor amount [–log ($C$/mg · mL$^{-1}$)] was obtained in the range between 0.0125 and 0.1 mg · mL$^{-1}$ acorbose with the equation of $y = -34.586x + 120.86$ ($R^2 = 0.990$) (inset in Figure 12).

In recent years, the importance of biologically active substances has been revitalized and many physiological effects that have been reported in the presence of AG inhibitors in many plants were screened for antidiabetic principles of natural medicine.[30] The inhibition effect of the acorbose can be easily monitored via PSU-Py based bi-enzyme reaction and this might be also applied for the high throughput screening of the possible drug candidates in a short time.

**Conclusion**

In conclusion, as an extension of the previous work[23] in which PSUs had been successfully functionalized with Py units by click chemistry approaches, here we described potential use of Py functional PSU membranes as a microwell assay platform for bio-assay development. In the enzymatic assays, when PSU-Py was interacted with the GOx or AG/GOx in the presence of their substrates, fluorescence of Py has been changed and this enables us to monitor the bio-catalytic activities as well as analysis of their substrates and inhibitors. With this approach, oxidase-based enzyme mutants can also be screened in a reasonably short time. Moreover, with the assay platform introduced, a tool for detecting cell by following diminishes in fluorescence as a result of cell adhesion on the membrane is implemented. Apart from, the possible use of PSU-Py membrane in high throughput screening assays, it would be a promising material for the cell culture applications.

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