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Novel Fluorescent Biosensor for α-Glucosidase Inhibitor Screening Based on Cationic Conjugated Polymers

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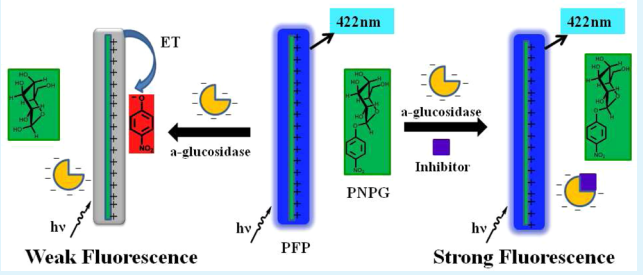
ABSTRACT: A new fluorescent biosensor has been designed to screen α-glucosidase inhibitors (AGIs) sensitively by utilizing signal amplification effect of conjugated polymers. The fluorescence of cationic poly(fluorenylene phenylene) (PFP) was quenched in the presence of para-nitrophenyl-α-D-glucopyranoside and α-glucosidase, and turned on upon addition of AGIs. Thus, a new method was developed for AGIs screening based on the fluorescence turn-off/turn-on. The IC_{50} values obtained for inhibitors were compared with that reported using absorption spectroscopy. All results present the new method is more sensitive and promising in screening AGIs and inhibitors of other enzymes whose hydrolysis product is 4-nitrophenol.



KEYWORDS: *biosensors, fluorescence spectroscopy, conjugated polymers, α-glucosidase inhibitor, diabetes, fluorescence turn-on*

INTRODUCTION

Diabetes is one of the most serious chronic diseases with high incidence around the world. In particular, the prevalence of diabetes increases rapidly among senior citizens and in a more affluent society. Recently, the diabetes has shown a new change trend that it increases exponentially and sickens younger people.[1−3] Globally, type 2 diabetes is the most common type among three broad categories. It is also called non-insulin-dependent diabetes or adult-onset diabetes, and accounts for at least 90% of all cases of diabetes.[1,4−7] Moreover, type 2 diabetes mellitus (DM2) is associated with an increased risk of Alzheimer's disease (AD) and vascular dementia.[8] Although there is no efficient treatment for diabetes, it can be controlled to relieve the harm to patients. One of the therapeutic approaches for type 2 diabetes is to inhibit the glycosidase activities. α-Glucosidase inhibitors (AGIs) are the main targets in the early treatment and prevention of diabetes. AGIs can retard α-glucosidase activities and thus delay the absorption of carbohydrates from small intestine. It plays a very important role in controlling patients' postprandial blood glucose levels and keeping glucose levels within a relatively normal range.[9−11] Therefore, it is of great importance to develop sensitive and easy-to-use assay methods to screen new and more effective α-glucosidase inhibitors.

Currently, the animal model of hyperglycemia[5,9] (in vivo screening) and enzyme−inhibitor model[12] (in vitro screening) are routinely employed for AGIs screening . The hyperglycemic animal model can provide relatively reliable results; however, it involves expensive and long-term animal experiments. The enzyme−inhibitor model, based on *para*-nitrophenyl-α-D-glucopyranoside (PNPG) substrate, has been widely used to screen active inhibitors from plants. Although the enzyme−inhibitor model offers a model to screen AGIs in vitro, its sensitivity is limited to some extent because the activity of AGIs was qualified by measuring absorbance of 4-nitrophenol at 400 nm released from PNPG.[13] Another drawback is that the detection was disturbed when the absorption of inhibitor overlaps with that of 4-nitrophenol.[14] The fluorescence spectroscopy is recognized as one of the more sensitive and selective among instrumental techniques. To the best of our knowledge, this is the first time to detect AGIs through fluorescence spectroscopy. In this study, we have designed and developed a cationic conjugated polymer (CP)-based fluorescent biosensor to screen AGIs.

CPs are highly renowned for their fluorescence signal amplification effects due to the presence of conjugated repeat units.[15,16] The long conjugation within the molecular backbone allows the excitation energy along the main chain transfer to an energy or electron acceptor after excited by UV light, which accounts for the fluorescence signal amplification.[17−20] In recent years, CPs have been applied widely in highly sensitive biosensors and chemosensors as an optical platform.[21−32] Taking advantage of the light-harvesting properties of the CPs, a biosensor for AGIs screening based on the conjugated polymers fluorescence turn-off/turn on mechanism has been developed with high sensitivity.

2. EXPERIMENTAL SECTION

2.1. Materials. All Chemicals were of analytical grade and were used as received. All aqueous solutions were prepared with

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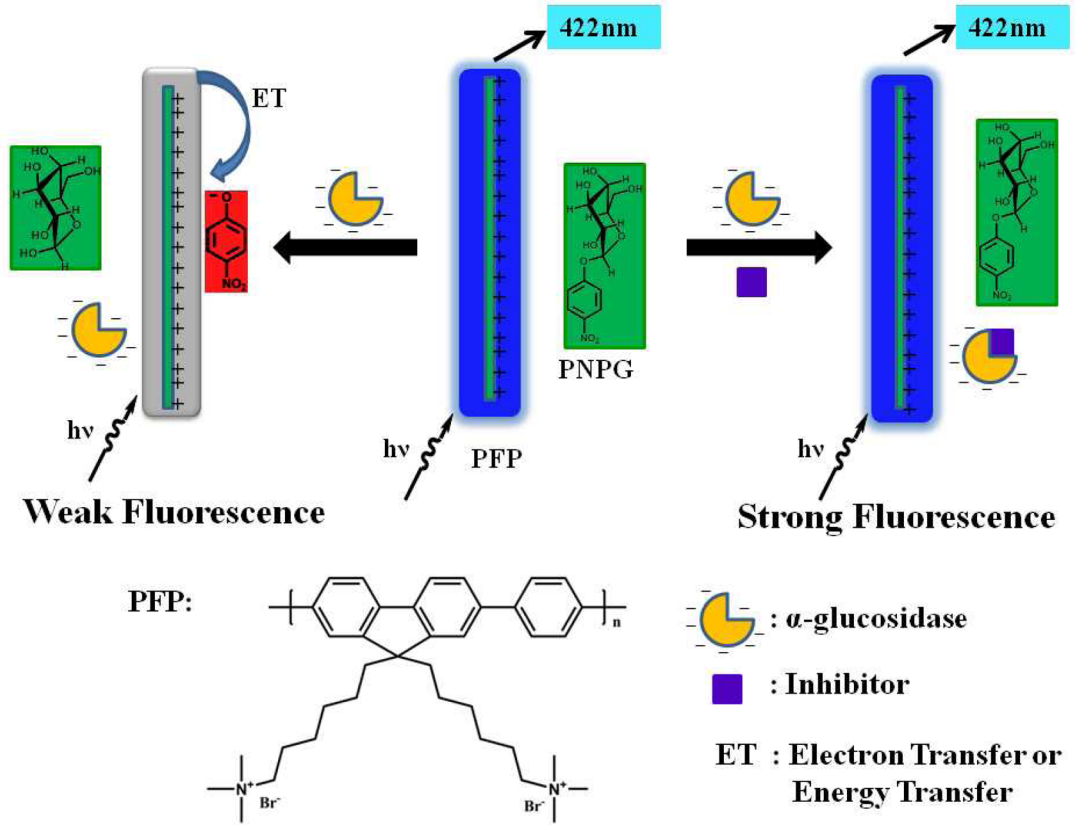
Scheme 1. Schematic Presentation of the Proposed Principal of α-Glucosidase Inhibitors Detection and the Chemical Structure of PFP



ultrapure water purified using a Millipore filtration. Cationic poly(9,9-bis(6′-N,N,N-trimethylammonium)hexyl)fluorene phenylene) (PFP) (M_w = 13500, PDI = 1.08) was synthesized according to the procedure in the literature.[33] KH_2PO_4 and 4-nitrophenol were purchased from Sangon Biotech (Shanghai, China) Co., Ltd. PNPG, 3-butylidenephthalide, β-glucosidase from almond, cellulase, and α-glucosidase from *Saccharomyces cerevisiae* were obtained from Sigma Aldrich. Acarbose was bought from SERVA (Heidelberg). 3-Propylidenephthalide was bought from Tokyo Chemical Industry Co., Ltd. 2, 4, 6-Tribromophenol was obtained from Aladdin Reagent Inc. (Shanghai, China). Fluorescence measurements were obtained in a 3 mL quartz cuvette at 37 °C using a Cary Eclipse fluorescence spectrophotometer equipped with a Xenon lamp excitation source (Varian INC.). The slit width and PMT voltage of the measurements were 5 nm and 600 V, respectively.

2.2. Fluorescence Quenching by 4-Nitrophenol. A solution with a total volume of 2.0 mL of KH_2PO_4 buffer (57 mM, pH 7.4) containing PFP ([PFP] = 2.0 μM in RUs (repeated units)) was prepared at 37 °C. The fluorescence spectra were measured before and after the addition of 4-nitrophenol successively (1.1×10^{-6}~2.5×10^{-5} M) at 37 °C with the excitation wavelength of 380 nm.

2.3. Assay of PNPG Cleavage by α-Glucosidase. A solution with a total volume of 2.0 mL of KH_2PO_4 buffer (57 mM, pH 7.4) containing PFP ([PFP] = 2.0 μM in RUs (repeated units), PNPG ([PNPG] = 20 μM) was prepared at 37 °C. An aliquot of α-glucosidase ([α-glucosidase] = 0.05−0.5 UN/mL) was then added. The fluorescence intensities of PFP at 422 nm were measured every 1 min within 30 min at 37 °C with the excitation wavelength of 380 nm.

2.4. Assay of PNPG Cleavage by Other Enzymes. A solution with a total volume of 2.0 mL of KH_2PO_4 buffer (57 mM, pH 7.4) containing PFP ([PFP] = 2.0 μM in RUs (repeated units), PNPG ([PNPG] = 20 μM) was prepared at 37 °C. β-Glucosidase or cellulase ([β-glucosidase] = [cellulase] = 0.5 UN/mL) was then added. The fluorescence intensities of PFP at 422 nm were measured after being incubated for 30 min at 37 °C with the excitation wavelength of 380 nm.

2.5. Assay of α-Glucosidase Inhibitors. A solution with a total volume of 2.0 mL KH_2PO_4 buffer (57 mM, pH 7.4) containing α-glucosidase (0.5 UN/mL) and a certain amount of 2, 4, 6-tribromophenol (concentrations from 5.0×10^{-6} M to 5.0×10^{-4} M) was prepared. After incubating at 37 °C for 20 min, PFP ([PFP] = 2.0 μM in RUs (repeated units) was added and the fluorescence spectrum was measured. Then PNPG ([PNPG] = 20 μM) was added to the solution and the fluorescence intensity of PFP at 422 nm was recorded after incubation for another 20 min at 37 °C with the excitation wavelength of 380 nm. Other α-glucosidase inhibitors (3-butylidenephthalide, acarbose and 3-propylidenephthalide) were assayed by the same protocol.

3. RESULT AND DISCUSSION

3.1. Design of Fluorescent Biosensor and Principle of Detecting AGIs. The proposed principle of AGIs screening based on conjugated polymers is illustrated in Scheme 1. Water-soluble cationic poly(9,9-bis(6′-N,N,N-trimethylammonium)hexyl)fluorene phenylene) (PFP) was used as the CPs in the biosensor.[33] PNPG was used as the

α-glucosidase substrate. As shown in Scheme 1, PFP can strongly emit blue fluorescence with the maximal emission at 422 nm after being excited by UV light of 380 nm. It is noted that the fluorescence of PFP was still strong in the presence of neutral PNPG. There was little interference in PFP fluorescence measurement by PNPG. In the prescence of α-glucosidase, PNPG was hydrolyzed by α-glucosidase and 4-nitrophenol was released. As reported by Simonian[34] and Ma,[35] 4-nitrophenol was used as a good quencher and electron or energy acceptor. Although the quenching mechanism is still not very clear in this case, it is reasonable to infer that the fluorescence of PFP was quenched efficiently by anionic 4-nitrophenol (pK_a = 7.16) presumably via the process of photo induced electron transfer (PET) or energy transfer under experimental conditions (pH 7.4), because the strong electrostatic interactions between cationic PFP and anionic 4-nitrophenol keep them in close proximity. Upon addition of AGIs into this system, α-glucosidase is inactivated by AGIs. So PNPG is not cleaved by α-glucosidase. Upon UV light excitation, the PFP emit the strong blue fluorescence with no significant interference. Thus a new method was developed to screen AGIs with high sensitivity.

3.2. Fluorescence Quenching. To study the quenching ability of 4-nitrophenol, the fluorescence quenching of PFP ([PFP] = 2.0 × 10^{-6} M in repeat units (RUs)) was thus examined by 4-nitrophenol in KH_2PO_4 buffer solution (57 mM, pH 7.4). Under the experimental conditions, 4-nitrophenol exhibits basic form because its pK_a value is below 7.4 (pK_a = 7.16), which provides the opportunity of 4-nitrophenol being high affinity to the cationic PFP via the dominant electrostatic interactions between them. As shown in Figure 1a, the fluorescence intensities of PFP are quenched gradually with the addition of 4-nitrophenol. A linear curvature in the Stern–Volmer plot was then obtained by taking I_0/I-1 value as *y*-axis and 4-nitrophenol concentration as *x*-axis, respectively (Figure 1b). At low concentration of 4-nitrophenol (Figure 1c), the linear quenching plot was obtained and the Stern–Volmer value (K_{sv}) of 3.7 × 10^5 M^{-1} was calculated from the Stern–Volmer eq 1.[36]

$$I_0/I = 1 + K_{sv}[Q] \quad (1)$$

Here I_0 and I are the fluorescence intensities in the absence and presence of quencher respectively, and [Q] is the concentration of quencher.

The K_{sv} value (3.7 × 10^5 M^{-1}) obtained indicates that conjugated polymer PFP can be quenched by 4-nitrophenol efficiently. Taking into account the fluorescence lifetime of PFP being around nanosecond magnitude (400 ps reported by Xu, et al),[37] the dynamic quenching rate constant k_q value can be around 9.2 × 10^{14} M^{-1} s^{-1}, which is several orders magnitude above the value (1.0 × 10^{10} M^{-1} s^{-1}) that is possible for collision-controlled quenching.[36] Hence, the static quenching dominates at low concentration of 4-nitrophenol in this case. This quenching experiment indicates the great possibility for us to design a sensitive biosensor for enzymes detection and relative inhibitors screening whose hydrolysis product is 4-nitrophenol.

3.3. α-Glucosidase Hydrolysis. Para-nitrophenyl-α-D-glucopyranoside (PNPG) has been widely used as a substrate to detect α-glucosidase via UV–vis spectroscopy in the traditional method. When PNPG is hydrolyzed by α-glucosidase, 4-nitrophenol is released and its absorption can be recorded through UV–vis absorption spectrophotometer.

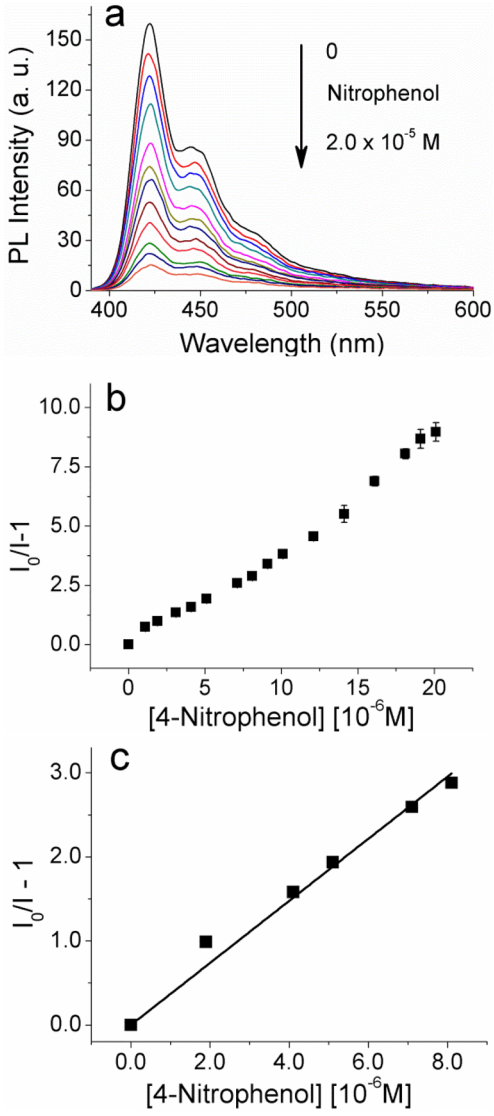


Figure 1. (a) Fluorescence emission spectra of PFP in KH_2PO_4 buffer solution (57 mM, pH 7.4) with successive addition of 4-nitrophenol. [PFP] = 2.0 × 10^{-6} M in RUs, [4-nitrophenol] = 0 to 2.0 × 10^{-5} M. (b) K_{sv} plot of PFP in the presence of 4-nitrophenol. The error bars represent the standard derivation of three measurements conducted at each time. (c) K_{sv} plot at low concentrations of 4-nitrophenol ([4-nitrophenol] = 0 to 8.1 × 10^{-6} M). The excitation wavelength is 380 nm.

To enhance the detection sensitivity, a new biosensor has been developed to detect α-glucosidase in this work, which contains PFP to increase sensing sensitivity. As shown in Figure 2a, the fluorescence emission intensity of PFP is quenched effectively upon the additions of PNPG and α-glucosidase. PNPG is a neutral molecule and no significant fluorescence quenching occurs between PFP and PNPG. Because the released 4-nitrophenol from PNPG has an exceptional quenching ability, the PFP fluorescence is quenched apparently after the cleavage of PNPG by α-glucosidase. This provides a new principle for us to detect α-glucosidase.

To investigate the concentration effect of α-glucosidase on PNPG hydrolysis, we carried out fluorescence quenching experiments in KH_2PO_4 buffer at pH 7.4 ([PFP] = 2.0 × 10^{-6} M, [PNPG] = 2.0 × 10^{-5} M) under various concentrations of α-glucosidase. As shown in Figure 2b, the

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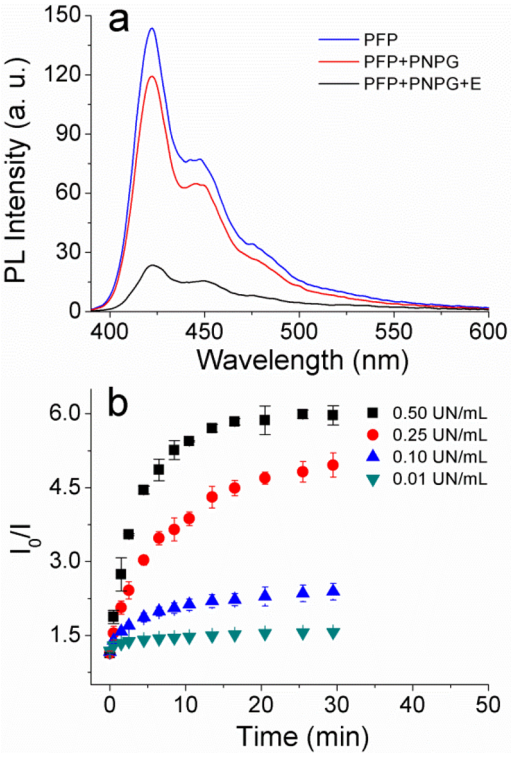

Figure 2. (a) Fluorescence emission spectra in the absence and presence of PNPG and α-glucosidase ([α-glucosidase] = 0.5 UN/mL). (b) Fluorescence intensity at 422 nm for PFP/PNPG as function of time at different concentrations of α-glucosidase. The error bars represent the standard derivation of three measurements conducted at each time. The excitation wavelength is 380 nm, and the measurements were conducted in KH_2PO_4 buffer solution (57 mM, pH 7.4). [PFP] = 2.0×10^{-6} M in RUs, [PNPG] = 2.0×10^{-5} M, [α-glucosidase] = 0−0.5 UN/mL.

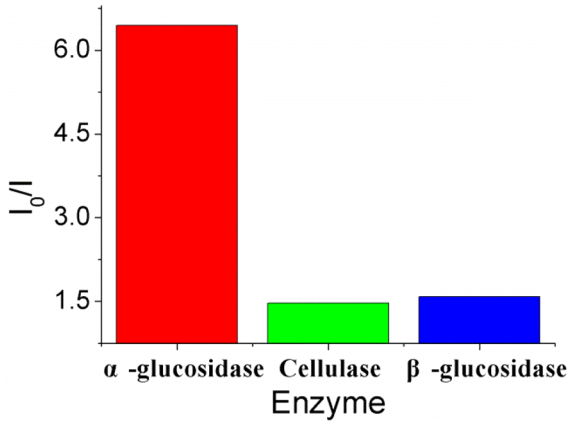

Figure 3. Fluorescence intensity ratio at 422 nm for PFP/PNPG in the presence of α-glucosidase, β-glucosidase, or cellulase. The measurements were conducted in KH_2PO_4 buffer solution after incubation for 30 min at 37 °C. (57 mM, pH 7.4). [PFP] = 2.0×10^{-6} M in RUs, [PNPG] = 2.0×10^{-5} M, [α-glucosidase] = [β-glucosidase] = [cellulase] = 0.5 UN/mL. The excitation wavelength is 380 nm.

fluorescence quenching shows the dependence on the concentration of α-glucosidase. When 0.01 UN/mL α-glucosidase is added into the solution, the ratio of I_0/I changes a little, which implies a small portion of PNPG is hydrolyzed by α-glucosidase at such low concentration after incubating for 30 min. Upon increasing the concentration of α-glucosidase to 0.1 UN/mL, the quenching efficiency enhances along the hydrolysis time; however, the ratio of I_0/I is still low. When the concentration of α-glucosidase increases to 0.5 UN/mL, the fluorescence intensity decreases quickly in the time range of 0 to 9 min and reaches a plateau after 13 min. As a result, around 85% fluorescence intensity of PFP was quenched in this case. The hydrolysis effect did not improve remarkably even increasing the α-glucosidase to 0.65 UN/mL (data not shown here).

3.4. Specificity. In addition to checking the specificity of PNPG for α-glucosidase, another two enzymes (β-glucosidase, cellulase) were studied in the KH_2PO_4 buffer solution at pH 7.4 ([PFP] = 2.0×10^{-6} M, [PNPG] = 2.0×10^{-5} M, [β-glucosidase] = [cellulase] = 0.5 UN/mL). As shown in Figure 3, the ratio of I_0/I in the presence of β-glucosidase or cellulase keeps much lower than that in the presence of α-glucosidase. It can be concluded that only α-glucosidase exhibits the significant hydrolysis ability to PNPG and the fluorescence of PFP is quenched distinctly, whereas β-glucosidase and cellulase do not show any efficient cleavage toward PNPG. These results demonstrate that the PNPG possesses the high selectivity for its specific α-glucosidase.

3.5. AGIs Screening. Figure 4a shows that the fluorescence of PFP turn-on is measured in the presence of α-glucosidase inhibitors. AGIs inactivate the α-glucosidase activity, and lead to the PNPG being cleaved barely, which was revealed by the nearly constant PFP fluorescence. It turns out that a novel and sensitive platform can be established to screen AGIs based on the fluorescence turn-off/turn-on. Several AGIs such as 2, 4, 6-tribromophenol, 3-butylidenephthalide and acarbose were also used to examine their inhibitory effects via the new method. As shown in Figure 4b, the inhibitory effect of 2, 4, 6-tribromophenol is dependent closely on its concentration in KH_2PO_4 buffer solution at pH 7.4 ([PFP] = 2.0×10^{-6} M in RUs, [PNPG] = 2.0×10^{-5} M, [α-glucosidase] = 0.5 UN/mL). When the concentration of 2, 4, 6-tribromophenol increases, the α-glucosidase activity decreases gradually, and the ratio of I_0/I decreases correspondingly after 20 min incubation. The maximal inhibitory effect is obtained when the concentration of 2, 4, 6-tribromophenol is 1.0×10^{-4} M.

Meanwhile, the inhibitory abilities of 3-butylidenephthalide and acarbose were examined in the same way. As shown in Figure 4b, when the ratio of I_0/I is around 3.5, the concentrations of 3-butylidenephthalide and acarbose are 3.0×10^{-4} M and 1.0×10^{-3} M respectively. They are much higher than that of 2, 4, 6-tribromophenol (5.0×10^{-5} M). It is obvious that the fluorescence turn-on induced by 2, 4, 6-tribromophenol is more efficient than those caused by other two inhibitors, which indicates the inhibitory ability of 2, 4, 6-tribromophenol is the strongest among the three inhibitors. The results are consistent with that reported in literatures.[9,10] The concentration required to inhibit the activity of α-glucosidase by 50% (IC_{50}) was obtained from the fluorescence turn-on experiments. As shown in the Figure 4b, the IC_{50} values obtained for 2, 4, 6-tribromophenol and 3-butylidenephthalide are about 0.037 mM and 0.21 mM respectively, which are lower than that reported using the absorption spectroscopy (IC_{50} for 2, 4, 6-tribromophenol and 3-butylidenephthalide were 60.3 μM and 2.35 mM, respectively in literatures).[9,10] In addition, The IC_{50} of acarbose against the *S. cerevisiae* is 0.60 mM. In this case, the inhibitory activity of acarbose is very weak, which is

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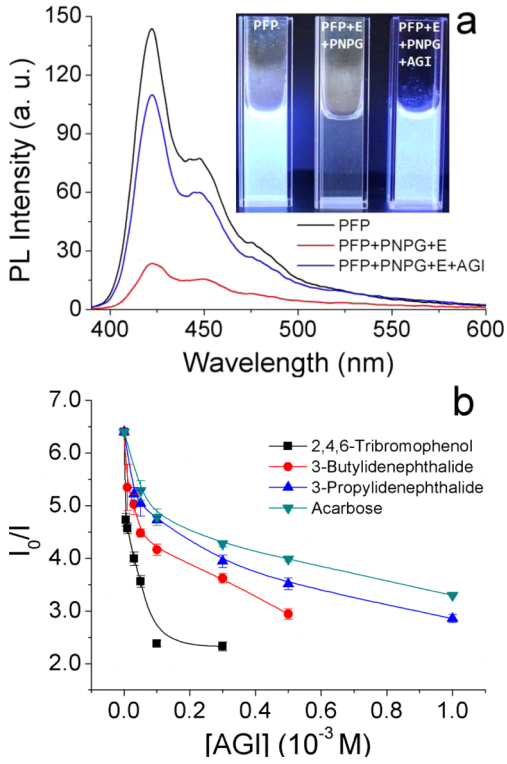

Figure 4. (a) Fluorescence emission spectra of PFP, PFP/PNPG /α-glucosidase, and PFP/PNPG /α-glucosidase/inhibitor (inset: photographs of PFP, PFP/PNPG /α-glucosidase, and PFP/PNPG /α-glucosidase/inhibitor.). (b) Fluorescence intensity ratio at 422 nm for PFP/PNPG/α-glucosidase as function of concentration in the presence of different inhibitors. The error bars represent the standard derivation of three measurements conducted at each time. The excitation wavelength is 380 nm, and the measurements were conducted in KH_2PO_4 buffer solution (57 mM, pH 7.4). [PFP] = 2.0×10^{-6} M in RUs, [PNPG] = 2.0×10^{-5} M, [α-glucosidase] = 0.5 UN/mL, and [inhibitor] = $0-1.0 \times 10^{-3}$ M.

consistent with the phenomenon reported by others.[10,12] The commercial inhibitor, acarbose exhibited high inhibitory effect on enzymes from mammalian species but weak activity against *S. cerevisiae*. Furthermore, a new inhibitor, 3-propylidenephthalide that its chemical structure is very similar to 3-butylidenephthalide was screened by the new method under the same experimental conditions ([PFP] = 2.0×10^{-6} M in RUs, [PNPG] = 2.0×10^{-5} M, [α-glucosidase] = 0.5 UN/mL). As shown in Figure 4b, 3-propylidenephthalide presents a similar inhibitory ability to 3-butylidenephthalide, and the IC_{50} value obtained for 3-propylidenephthalide is 0.35 mM. Therefore, the novel sensor based on the optical sign amplification effect of PFP provides reliable and sensitive sensing methods. It is potential to screen new α-glucosidase inhibitors sensitively with great convenience.

4. CONCLUSIONS

In summary, we have developed a novel biosensor to screen the α-glucosidase inhibitors sensitively. The sensor took advantage of the signal amplification effect of the cationic conjugated polymer PFP to enhance the detection sensitivity. The fluorescence intensity of PFP can be quenched in the presence of α-glucosidase and PNPG. Upon addition of AGIs, the fluorescence of PFP turns on. Thus a new sensor was developed to screen α-glucosidase inhibitors in vitro sensitively through measuring the fluorescence of PFP. The IC_{50} values obtained via the new method were lower than that the reported values calculated by using absorption spectroscopy. It is promising that the novel sensor can sensitively and real time screen new effective AGIs in vitro. Furthermore, it provides a new assay model for enzyme activities detection and inhibitors screening of other enzymes (such as α-galactosidase and β-glucuronidase).

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Notes

The authors declare no competing financial interest.

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