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Abstract. In renal transplant patients, immunosuppressive therapy may result in the reactivation of polyomavirus BK (BKV), leading to polyomavirus-associated nephropathy (PVAN), which inevitably causes allograft failure. Since the treatment outcomes of PVAN remain unsatisfactory, early identification and continuous monitoring of BKV reactivation and reduction of immunosuppressants are essential to prevent PVAN development. The present study demonstrated that the developed dual-channel heterodyne-based surface plasmon resonance (SPR) biosensor is applicable for the rapid detection of urinary BKV. The use of a symmetrical reference channel integrated with the poly(ethylene glycol)-based low-fouling self-assembled monolayer to reduce the environmental variations and the nonspecific noise was proven to enhance the sensitivity in urinary BKV detection. Experimentally, the detection limit of the biosensor for BKV detection was estimated to be around 8500 copies/mL. In addition, urine samples from five renal transplant patients were tested to rapidly distinguish PVAN-positive and PVAN-negative renal transplant patients. By virtue of its simplicity, rapidity, and applicability, the SPR biosensor is a remarkable potential to be used for continuous clinical monitoring of BKV reactivation. [DOI: 10.1117/1.JBO.19.1.011013]

Keywords: polyomavirus BK; surface plasmon resonance; biosensor; renal allograft nephropathy; renal transplantation.

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1 Introduction

Polyomavirus BK (BKV) is a nonenveloped DNA virus from the polyomaviridae family, which causes ubiquitous infection in early childhood and with seroprevalence in adults ranging from 60 to 100%.[1–5] Although BKV infection is of no consequence to the immune-competent host, it establishes persistent latent infections and is capable of reactivating in immunosuppressed hosts.[1,6] In contrast, renal transplant patients are treated with immunosuppressive therapy, which results in the reactivation of BKV.[6–8] The reactivation of the latent virus that can impair cellular immunity enables sustained viral replication in urothelial cells, which potentially leads to the development of polyomavirus-associated nephropathy (PVAN).[9] In addition, PVAN is now recognized as one of the major consequences associated with the infection of polyomaviruses.[7,9–13] Currently, 1 to 10% of renal transplant recipients are diagnosed to have PVAN, leading to graft loss in 20 to 80% of patients.[6,7,14–19] Since no established antiviral treatment is currently available and the immunosuppressed state is critical for renal transplant patients, a careful manipulation of immunosuppression to avoid rejection but early identification of BKV reactivation is probably the best option available for management at this time.[6,7] Thus, over the last several years, many studies have shown that screening by monitoring of the viral load in urine is able to predict patients at risk for the development of PVAN.[6,17,20] Therefore, a simple and rapid detection method for BKV monitoring is significantly important for clinical application.

Recently, several methods for the detection of BKV infections have been reported and some of them were not ready for clinical application. A polymerase chain reaction (PCR)-based technique, which has been widely used in clinical treatments, is the most effective and prevalent method for screening and monitoring active BKV infection.[1,15,17,20–24] Still, this method has some limitations because it is time-consuming, laborious, and expensive.[7,9,25] Urine cytology is frequently employed as a screening test for active viral infection, although the sensitivity and specificity of decoy cell measurement is disputable.[9,26–31] Loop-mediated isothermal amplification (LAMP)-based technique is another option. Even though LAMP is an effective and rapid method for amplification of nucleic acid, which typically occurs within 60 min under isothermal conditions, the complex primer design and standardization may limit its implementation in clinical settings.[32,33] Besides, there are other ways to detect BKV infection such as mass spectrometry-based and electron microscopy-based methods. But still, the requirement of highly skilled operators and costly equipment emerge as the major drawbacks.[9,25,34]

Surface plasmon resonance (SPR) biosensor is an effective alternative for virus detection. The highly localized electromagnetic fields render biosensor sensitive to changes in the effective

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refractive index (n_{eff}) of the dielectric medium near a metal film surface under the attenuated total reflection arrangement. Moreover, the capabilities for rapid, label-free, and real-time detection make it widely used to investigate biomolecular interactions.[35–42] However, limits on the detection sensitivity of conventional SPR biosensors make them incapable of detecting small changes in the n_{eff}, particularly in the measurement of biomolecular interactions at ultralow concentrations.[36] This evidence opens a fruitful area of research since SPR emanates as an outstanding platform for virus detection because virus could cause larger changes in n_{eff}.[36,43–46]

In this study, the developed dual-channel heterodyne-based SPR biosensor is utilized to rapidly detect BKV. Researches denoted that the limit of detection (LOD) of the biosensor for BKV detection is ~8500 copies/mL in urine, which is much lower than the threshold loads of a renal transplant patient who is probable to develop PVAN.[7,17,20,34,47] Also, the assay time of the biosensor is known to be ~20 min. It is noteworthy that this work may offer a great opportunity to develop an alternative PCR-free method enabling the detection of viral pathogens by incorporating an appropriate pathogen-specific antibody.

2 Material and Methods

2.1 *Clinical Samples*

This study was approved by the institutional review board of Chang Gung Memorial Hospital. Samples were taken from renal transplant recipients in the Department of Nephrology, Linkou Chang Gung Memorial Hospital, Taiwan, after their informed consent was obtained. A total of five patients were recruited in this study from July 2009 to June 2011.

2.2 *Materials*

The bare silver/gold chips (SPR chips) used in this study were produced by the semiconductor laboratory of Chang Gung University (Taoyuan, Taiwan). The immobilization buffer and amine coupling kit containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), and 1.0 M ethanolamine-HCl, pH 8.5 (ETH) was purchased from Biacore Inc. (Uppsala, Sweden). $C_{25}H_{44}O_6S_2$ and $C_{33}H_{58}O_{11}S_2$ were obtained from SensoPath Technologies (Bozeman, MT). All chemicals were used without further purification. The monoclonal BKV antibody (a-BKV) used as capture antibody and monoclonal nonspecific antibody used against influenza B virus were obtained from Abnova (Taipei, Taiwan) and Abcam (Cambridge, MA), respectively. The BKV (archetype strain) was purchased from American Type Culture Collection (CCL-137; Manassas, VA). Human proximal tubular cells, HK-2, were used for viral infection. The HK-2 cells were cultured in Dulbecco's modified Eagle medium/Ham's F12 (Life Technologies, Paisley, UK) supplemented with 5% fetal calf serum (Biological Industries Ltd, Cumbernauld, UK), 2 mM glutamine (Life Technologies Ltd), 20 mM Hepes buffer (Gibco BRL, Paisley, UK), 0.4 μg/mL hydrocortisone, 5 μg/mL insulin, 5 μg/mL transferring, and 5 ng/mL sodium selenite (Sigma Chemical Company Ltd, Poole, UK). The BKV copy number was determined at 2×10^7 copies/mL as described previously.[48]

2.3 *Optical Setup of the Dual-Channel Heterodyne-Based SPR Biosensor*

The optical setup of the dual-channel heterodyne-based SPR biosensor is illustrated in Fig. 1. The laser beam from a frequency-stabilized and linearly polarized He–Ne laser with wavelength of 632.8 nm was integrated with an electro-optic modulator driven at frequency ω. Afterward, the beam passes through a half-wave plate and a polarizer sequentially to produce two highly correlated P-polarized waves (TM wave, $P_{\omega 1}$ and $P_{\omega 2}$) at different frequencies. The P-polarized light was split into two parallel beams by passing it through a lateral displacement beam splitter and a homemade dual-channel SPR device consisting of two independent polydimethylsiloxane (PDMS) flow channels. Two lock-in amplifiers were used for simultaneously measuring the amplitudes of P-heterodyne signals from the reference chamber and signal chamber, respectively, for BKV detection.

2.4 *Preparation of SPR Chip*

The SPR chip used in this study was BK7 glass slide coated with a laminated Ag/Au (40/10 nm) metal layer. The SPR chips were cleaned with UV / Ozone Cleaning System (Novascan, Ames, IA) to purify the gold surface prior to self-assembled monolayers (SAMs) surface functionalization. Mixed SAMs of dithiols consisting of 90% $C_{25}H_{44}O_6S_2$ and 10% $C_{33}H_{58}O_{11}S_2$, used as a diluent at a mixing ratio of 1:9, were assembled at the Ag/Au substrates (SPR chip), generating a poly(ethylene glycol) (PEG)-based binding matrix optimized for the formation of an antibody monolayer via an amine-coupling protocol. The capture antibody and the nonspecific antibody were covalently immobilized to the signal chamber

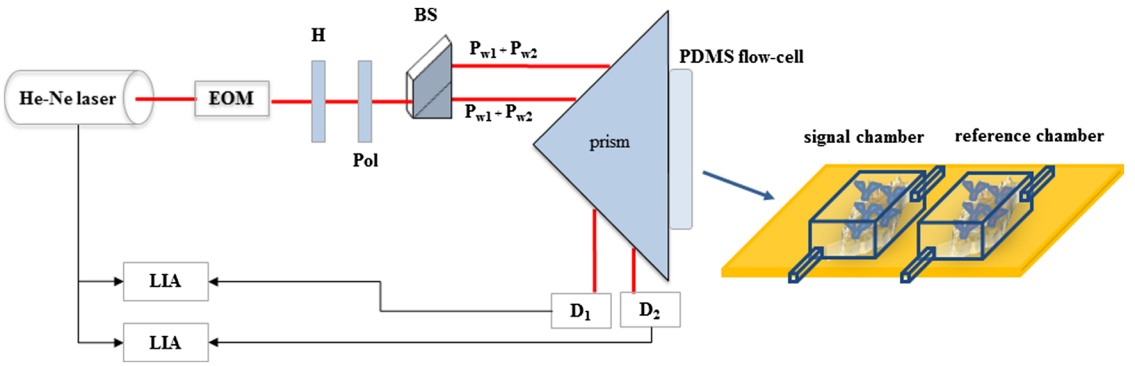

Fig. 1 The optical setup of the dual-channel heterodyne-based SPR biosensor. EOM, electro-optic modulator; H, half-wave plate; Pol, polarizer; BS, displacement beam splitter; D, photodetector; LIA, lock-in amplifier.

and reference chamber of SPR chip, respectively, by utilizing the homemade dual-channel PDMS flow-cell. The volume of each chamber was ~12 μL. It must be noted that the capture antibodies were only immobilized in the signal chamber, and the reference chamber only immobilized nonspecific antibodies. The amine-coupling protocol was performed as follows:

1. SAMs of 90% $C_{25}H_{44}O_6S_2$ and 10% $C_{33}H_{58}O_{11}S_2$ were activated by immersing the SPR chip in the mixed solutions, which included 0.4 M EDC and 0.1 M NHS, for about 10 min.
2. The capture antibody (or nonspecific antibody), at a concentration of 10 μg/mL prepared in immobilization buffer, was immobilized via the reaction of primary amine groups or other nucleophilic groups and then incubated with the sensor chip surface for ~30 min.
3. ETH was injected to block the active sites of non-reacted surface for ~7 min.

The covalent immobilization procedures of the capture antibody (a-BKV) were recorded by SPR intensity obtained by measuring the optical heterodyne signal of the reflected *P*-polarized waves as shown in Fig. 2. The sensorgram describes (1) the NHS/EDC activation, (2) a-BKV (10 μg/mL) immobilizations, and (3) deactivation by ETH.

2.5 *Measurement of BKV Using the Dual-Channel Heterodyne-Based SPR Biosensor*

The original concentration of the BKV culture supernatant was 2×10^7 copies/mL and the supernatant was irradiated by UV for 30 min before the experiment. In the mock experiments, the BKV isolate was 10-fold serial diluted by urine to mimic the *in vivo* isolation of BKV from a patient. In addition, five urine samples including two PVAN-negative renal transplant patients and three PVAN-positive renal transplant patients were tested. All the analytes were simultaneously injected into both signal and reference chambers to interact with the capture antibody (a-BKV) and nonspecific antibody, respectively.

3 Results and Discussion

3.1 *Performance of the Dual-Channel Heterodyne-Based SPR Biosensor on Detecting BKV in Urine*

The SPR chip was prepared using the method described above, and the original concentration of the UV-irradiated BKV culture supernatant before urine dilution was 2×10^7 copies/mL. In order to mimic the clinical situation, 10-fold serial dilutions of UV-irradiated BKV culture supernatant in 1 mL volume each, i.e., 2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 copies/mL, were spiked in urine (from a healthy adult) separately. After a-BKV and the nonspecific antibody were immobilized in the signal chamber and the reference chamber, respectively, each of the mimic solutions was injected into the signal and the reference chambers simultaneously to interact with the immobilized a-BKV and nonspecific antibody, respectively. Figure 3 shows the real-time SPR curve of interaction, where I_S is described as the heterodyne signal measured from the signal chamber. It can be seen that the curve exhibits a strange behavior after the washing step. This results from a higher flow rate during the washing process in order to reduce assay time. Finally, the flow rate returned to normal. It is remarkable that the sensing surface has low nonspecific protein adsorption after phosphate-buffered saline (PBS) washing. Therefore, it is observable that PEG-based SAMs provide a low-fouling ability that prevents nonspecific adsorption on the SPR chip. The inset in Fig. 3 presents the last 50 data points of the experiment. However, there is no correlation between the measured I_s and the concentration of the spiked BKV isolate over the range of 2×10^2 to

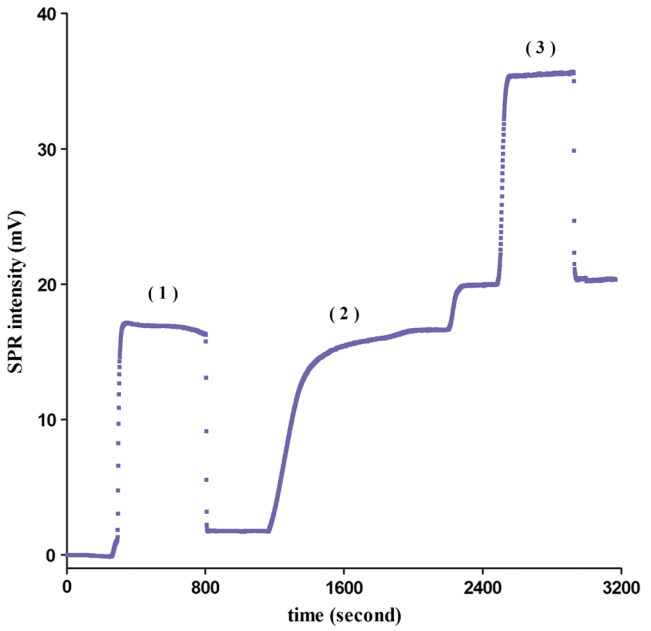

Fig. 2 Sensorgram of capture antibody immobilized on the PEG-based SPR chip. (1), (2), and (3) represent the NHS/EDC activation, the a-BKV (10 μg/mL) immobilization, and the deactivation by ETH, respectively.

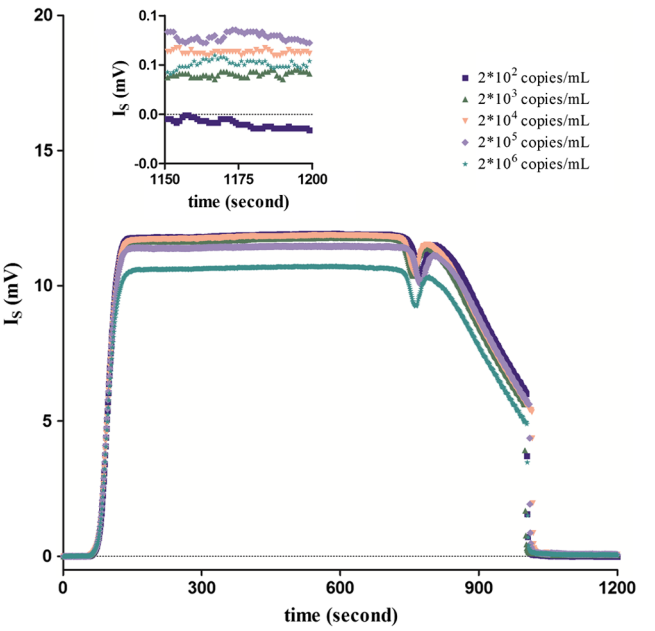

Fig. 3 Binding processes of a-BKV interaction with different concentrations of BKV over the range 2×10^2 to 2×10^6 copies/mL measured with single channel. Inset: zoom-in of the last 50 data points.

2×10^6 copies/mL. It is allegedly because the real signal coming from the specific binding of BKV is covered by the signal of nonspecific binding.

Accordingly, a differential method using a symmetrical reference channel is introduced to reduce the environmental variations and the nonspecific noise in order to promote the sensor sensitivity. The results are shown in Fig. 4, and in this experiment, the interaction was analyzed by subtracting I_R from I_S in which I_R is described as the heterodyne signal measured from the reference chamber. After the mimic solution and PBS injections, an abnormal phenomenon took place, associated with the different lengths of the flow channels during the transport processes. Nonetheless, the kinetic information is not the focus here. Hence, the end-point measurements are presented in the inset of Fig. 4. The values of $(I_S - I_R)$ seem to be able to differentiate the BKV concentrations. The correlation between the SPR signal and the concentration of the spiked BKV in urine over the range of 2×10^2 to 2×10^6 copies/mL is depicted in Fig. 5. The SPR signal was acquired by subtracting the background level from the average of $(I_S - I_R)$ over the last 50 data points of the experiment. The results were analyzed using a sigmoidal dose-response curve with variable slope, the so-called four-parameter logistic equation, found in GraphPad Prism software; the correlation coefficient (R^2) is 0.9999 and the error bar indicates one standard deviation in each measurement. Generally, the LOD is the concentration at which the signal corresponds to three times of the standard deviation positioned in the dose-response curve. Consequently, theoretical LOD of the dual-channel heterodyne-based SPR biosensor for BKV detection in urine was calculated to be 8500 copies/mL from both experimental data and the fitting curve for this experimental design. This value is well below the threshold level of the urine BKV of the renal transplant patient who is about to develop PVAN; the reasonable threshold loads considered to be clinically significant are always determined at 10^6 to 10^7 copies/mL in urine.[7,17,20,34]

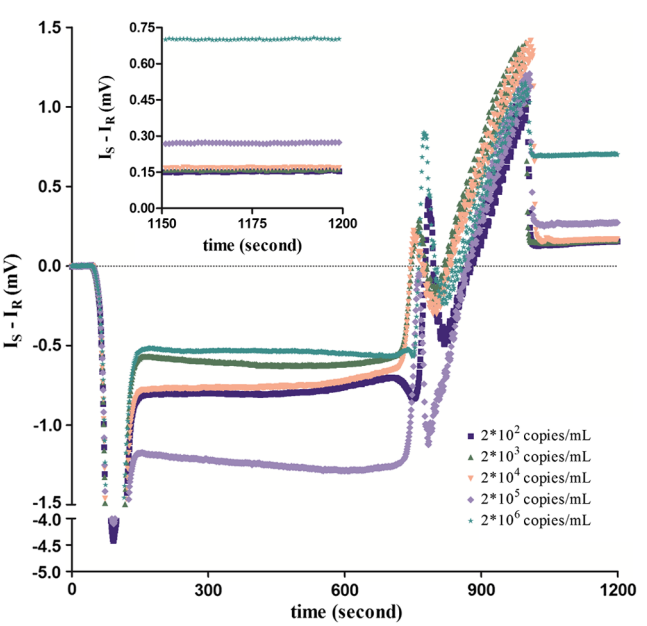

Fig. 4 Binding processes of a-BKV interaction with different concentrations of BKV over the range 2×10^2 to 2×10^6 copies/mL measured with dual channel. Inset: zoom-in of the last 50 data points.

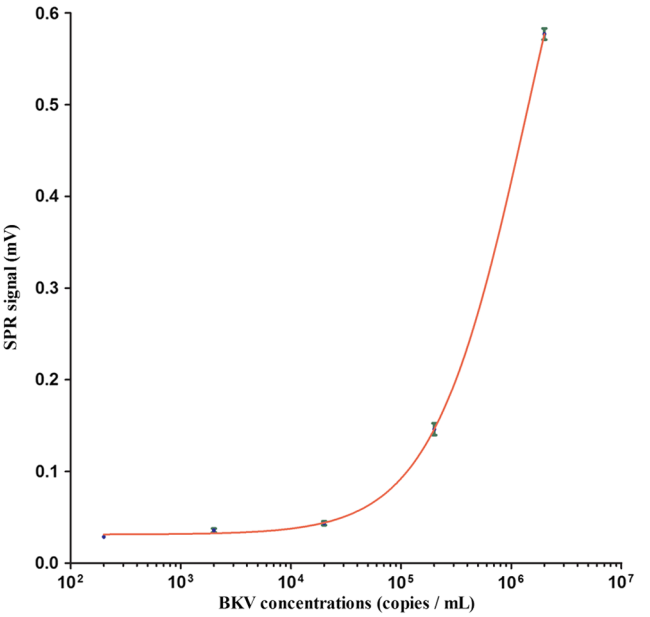

Fig. 5 Correlation ($R^2 = 0.9999$) between the SPR signal and the concentration of BKV over the range 2×10^2 to 2×10^6 copies/mL. The error bars indicate 1 SD in each measurement and are smaller than symbols.

3.2 *BKV Detection in Clinical Sample*

To prove that the dual-channel heterodyne-based SPR biosensor can be applied to detect clinical samples, urine from five renal transplant patients were checked by the developed biosensor. The results displayed in Fig. 6 reveal that the BKVs in the urine were successfully detected and PVAN-positive and PVAN-negative renal transplant patients were also clearly and promptly distinguished by this platform.

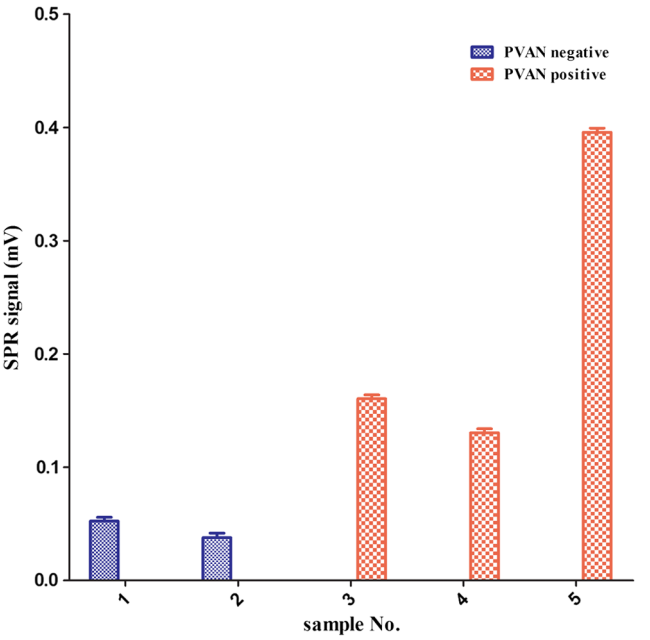

Fig. 6 Five urine samples detected by the dual-channel heterodyne-based SPR biosensor. SPR signals of the two PVAN negative were compared to those of the three PVAN positive renal transplant patients.

Table 1 Comparison of the LOD of BKV measurement in urine obtained from various techniques.

Methods	LOD (copies/mL)	Sample volume	Reference
PCR-based assay	40 to 80	25 μL	21
LAMP-based assay	~1600	60 μL	7
Electron microscopy-based assay	200	5 mL	34
Dual-channel heterodyne-based SPR biosensor	~8500	1 mL	Our system

In renal transplant patients, the use of immunosuppressive therapy may trigger the BKV reactivation, leading to PVAN in which its malignant stage is likely to conduct an inevitable allograft failure.[4,8,10] Based on this consideration, early identification and continuous monitoring of BKV reactivation and reduction of immunosuppressants are substantial to prevent the development of PVAN.[7,9,11–13,17,20] Current guidelines recommend regular observation of BKV reactivation by the detection of infected urothelial cells in urine (decoy cells) or viral nucleic acid in urine or blood.[9] On the contrary, the sensitivity and specificity of decoy cell measurement remain debated while PCR-based assays seem to be impractical owing to the needs of highly skilled operators and time-consuming processes.[7,9] Several groups hitherto reported advanced detection techniques wherein the sensitivity of BKV detection in urine reached >2000 copies/mL level. A comparison of the LOD of BKV measurement in urine of the various techniques is shown in Table 1. Although the dual-channel heterodyne-based SPR biosensor is not as sensitive as those advanced detection techniques, its simplicity, rapidity, and applicability do have valuable contributions in clinical continuous BKV monitoring.

In this study, the dual-channel heterodyne-based SPR biosensor could be employed to measure clinical urine samples where PEG-based low-fouling SAMs play an essential role. One of the main challenges for bioapplications of SPR biosensors is to maintain its high sensitivity in real-world complex media such as serum or urine due to the nonspecific adsorption on the sensing surface.[39] This nonspecific adsorption is attributed to high background noise that aggravates the LOD of the SPR biosensors.[39,42] Current studies point out that various low-fouling or nonfouling materials perform a great resistance to the nonspecific adsorption in SPR biosensor surface.[39–42,49] PEG, a water-soluble, nontoxic, and nonimmunogenic polymer, is regarded as an effective protein-resistant material and has been frequently used in biosensors, although it does not exhibit the best antifouling capability compared with other advanced nonfouling materials such as zwitterionic polymer.[49–54] Fortunately, the concentration of urine proteins is much lower than that of serum proteins.[55] In consequence of the availability and cost-effectiveness, the PEG-based SAMs would be a great option to withstand the nonspecific adsorption in urine as illustrated in Fig. 3.

4 Conclusions

In accordance with the differential method using a symmetrical reference channel integrated with the PEG-based low-fouling SAMs, we successfully showed that the developed dual-channel heterodyne-based SPR biosensor is applicable to rapid urinary BKV detection. The LOD of the biosensor for urinary BKV detection is estimated to be ~8500 copies/mL, which is much lower than the threshold loads of the renal transplant patient who is at the risk of developing PVAN. Even though the dual-channel heterodyne-based SPR biosensor sensitivity is not superior as compared to other detection techniques, yet, its simplicity, rapidity, and applicability have noteworthy contributions in continuous clinical BKV monitoring. Furthermore, when it was carried out in the detection of the urine samples from five renal transplant patients, our proposed SPR biosensor achieved a rapid determination of PVAN-positive and negative. This fact indicates that the developed dual-channel heterodyne-based SPR biosensor may be taken into account as a prospective biosensor in clinical applications in the future.

Acknowledgments

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