



Novel peptides functionalized gold nanoparticles decorated tungsten disulfide nanoflowers as the electrochemical sensing platforms for the norovirus in an oyster

Seung Hoon Baek^{a,1}, Chan Yeong Park^{a,1}, Thang Phan Nguyen^b, Min Woo Kim^a, Jong Pil Park^c, Changsun Choi^d, Soo Young Kim^e, Suresh Kumar Kailasa^f, Tae Jung Park^{a,*}

^a Department of Chemistry, Institute of Interdisciplinary Convergence Research, Research Institute of Chem-Bio Diagnostic Technology, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul, 06974, Republic of Korea

^b Department of Chemical and Biological Engineering, Gachon University, Seongnam-si, Gyeonggi-do, 13120, Republic of Korea

^c Department of Food Science and Technology, Chung-Ang University, Ansung, 17546, Republic of Korea

^d Department of Food and Nutrition, School of Food Science and Technology, Chung-Ang University, Ansung, 17546, Republic of Korea

^e Department of Materials Science and Engineering, Korea University, 145 Anam-ro, Seongbuk-gu, Seoul, 02841, Republic of Korea

^f Department of Applied Chemistry, Sardar Vallabhbhai National Institute of Technology, Surat, 395007, Gujarat, India

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ABSTRACT

Human norovirus is one of the potential foodborne pathogens causing acute gastroenteritis, diarrhea, and food poisoning. For norovirus detection, we developed the norovirus specifically capturable peptides functionalized gold nanoparticles (AuNP) decorated tungsten disulfide nanoflower (WS₂NF/AuNP). Based on the 3D-structured WS₂NF acting as a supporting material, AuNP were immobilized on its surface to assist the conductivity of WS₂NF and provide effective immobilization sites for bioreceptors. Since norovirus is composed of protein that functions as an insulant, as the virus bind with WS₂NF/AuNP, the impedance is elevated by hindering the charge transfer between the working electrode and redox species ([Fe(CN)₆]^{3-/4-}). The prepared nano-bio hybrid sensor was assessed by electrochemical impedance spectroscopic technique with spiked samples and deliberately infected oyster samples, respectively. In the case of the former, the electrochemical sensor represents a low detection limit (2.37 copies/mL) with a detection range from negative control to 10⁴ copies/mL, and it was shown a detection limit of 6.21 copies/mL even in the oyster samples. Besides, when it applies to the different types of viruses such as rotavirus and dengue virus to verify the selectivity of this system, there were no effective signals except the norovirus. Therefore, it revealed that combining WS₂NF and AuNP enhances electrochemical activity and stability via mutual synergetic effects.

1. Introduction

As one of the potent pathogens to induce acute foodborne illness for humans, norovirus is a major threat to the health problem in the world (Payne et al., 2013; Vinjé, 2015). The virus is belonged to *Caliciviridae* viruses and classified into five groups (from GI to GV types) as following the sequence of capsid protein and polymerase (Rooney et al., 2014). Among them, type GI and GII viruses are especially fatal due to more infectibility toward humans (Vinjé, Hamidjaja, & Sobsey, 2004). Moreover, even 10² copies/mL of the viruses outbreak the disease easily (Atmar et al., 2008; Teunis et al., 2008), it is highly demanded that the development of sensitive and selective analytical sensing system for

food samples. In this respect, several molecular diagnostic methods such as loop-mediated isothermal amplification (Jeon, Seo, Oh, Kingsley, & Choi, 2017), xTAG GPP (McHugh et al., 2018), and reverse transcription-polymerase chain reaction with real-time PCR (Kulis-Horn & Tiemann, 2019) have been utilized for this purpose. Although these methods identify and quantify the norovirus accurately, requiring both multiple experimental steps and high cost restricts their wider uses in clinical microbiology (Shieh, Calci, & Baric, 1999).

Recently, applicating nanomaterials to the electrochemical sensing platform have attracted as an alternative to the traditional molecular biological tools due to its simplified sample preparations and high specificity (Hong, Kwon, Kim, & Yang, 2015; Li et al., 2014;

* Corresponding author.

E-mail address: tjpark@cau.ac.kr (T.J. Park).

¹ These authors contributed equally to this work.

Maduraiveeran, Sasidharan, & Ganesan, 2018). Another advantage of the electrochemical sensor is it can be able to realize the miniaturized devices, and these properties make this technology to promisingly adapt to the point-of-care testing system like as a portable glucose sensor (Quesada-González & Merkoçi, 2018). So far, various nanomaterials such as gold nanoparticles (AuNP) (Khater, de la Escosura-Muñiz, Quesada-González, & Merkoçi, 2019), CdZnSeS quantum dots (Adegoke et al., 2016), and other nanomaterials (Mayorga-Martinez et al., 2015) were studied for the electrochemical biosensors. Furthermore, metal nanoparticle (metal NP) conjugated two-dimensional (2D) WS_2 have been received much attention in various fields owing to their synergistic effect of photonic/electronic properties through the coupling of exciton-plasmons (Polyakov et al., 2014; Radisavljevic, Radenovic, Brivio, Giacometti, & Kis, 2011; Wang et al., 2015). For these reasons, we choose the WS_2 as a supporting material, and AuNP as the decorating metal NPs to enforce the conductivity, charge carrier mobility, thermal conductivity, and mechanical properties (Su, Chen, & Fan, 2018; Wang, Xiong, Xiao, & Duan, 2017). Moreover, as the mediators, the anchored AuNP can be facilitated for the immobilization of bioreceptor attached cysteine at C-terminal onto the WS_2 surface through the strong interaction between Au-S (Chi et al., 2017).

In this regard, we envisioned that AuNP-decorated tungsten disulfide nanoflowers ($WS_2NF/AuNP$) can be utilized for the electrochemical biosensors to detect the norovirus in an oyster sample. As a platform material, the morphological property of WS_2 was developed under various experimental conditions such as the reaction time and temperature to extend its flat 2D structure to 3D flower-like shape (Lee et al., 2013). After that, the surface of WS_2NF was decorated by AuNP to bolster the electrochemical conductivity and offer the site for the peptide fixation (Scheme 1a). The prepared norovirus detectable nanobio composite modified onto the carbon-screen-printed electrode (C-SPE) with Nafion was evaluated by electrochemical impedance spectroscopy (EIS) (Scheme 1b). Because the constituent of norovirus is mainly protein that acts as an insulant, the impedance can be elevated by hindering the charge transfer between the working electrode and redox species ($[Fe(CN)_6]^{3-/4-}$) as the virus binding on the $WS_2NF/AuNP$ (Grossi & Riccò, 2017). The developed electrochemical biosensor exhibited high selectivity and sensitivity toward the norovirus even in an oyster sample.

2. Experimental

2.1. Chemicals

Tungsten (VI) chloride (WCl_6 , 99.9%), thioacetamide (TA, 98%),

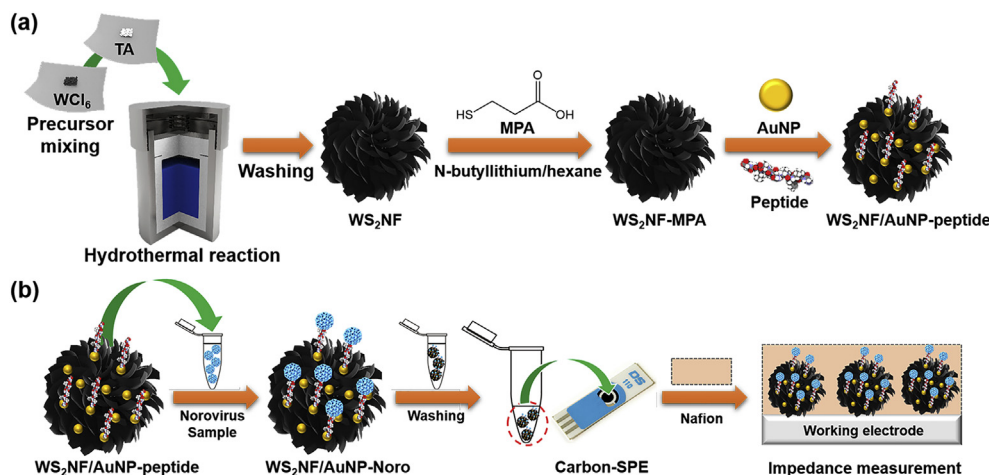
fetal bovine serum (FBS), potassium ferrocyanide (II), potassium ferricyanide (III), Nafion, 3-mercaptopropionic acid (MPA), gold (III) chloride trihydrate ($HAuCl_4$), n-butyllithium and phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without any further purification. Sodium borohydride and hexane were purchased from Daejung Chemical (Siheung, Korea). The carbon screen-printed electrode (C-SPE) was received from EG Technology (C220AT, Seoul, Korea). Dulbecco's modified Eagles medium (DMEM) was purchased from GenDEPOT (Katy, TX, USA). The sequence of norovirus affinity peptide (QHMKHKKPHKNTKEKEKEKEGGSGGGGGSC) and gold-binding polypeptide (GBP peptide) (QHMKHKKPHKNTKEKEKEKEGGSGGGGSMHGKTQATSGTIQS) were obtained from Peptron (Daejeon, Korea). Norovirus type GI, GII, Rotavirus and Dengue virus type I were obtained from the Korea Center for Disease Control (Cheongju, Korea), and Dengue virus type II, III and IV were obtained Korea Bank for Pathogenic Viruses (Seoul, Korea). Human norovirus was extracted from norovirus-infected humans (Chung-Ang University, Seoul, Korea), and the detailed procedure for extraction and culture was described in the literature (Baek et al., 2019). All reagents were used as received, and deionized (DI) water prepared by the Direct-Q® Water Purification System (Millipore, Billerica, MA, USA) was used for the whole experiment.

2.2. Synthesis of WS_2 nanoflower (WS_2NF)

The WS_2NF was prepared by the hydrothermal method (Hasani et al., 2018). Typically, 0.4 g of WCl_6 and 0.375 g of TA were dissolved in 10 mL of DI water via agitation for 1 h. After transferring the blended solution into a Teflon-lined stainless-steel autoclave, it was sealed and heated up to various temperatures (260, 270, 280, and 290 °C) with 10 °C/min in box furnace (C-A14P, Hanteck, Gunpo, Korea). The vessel was cooled down to room temperature, then black powder lies at the bottom was collected. The product was dispersed in DI water and washed by centrifugation (4500 ×g, 10 min) for three times. Finally, WS_2NF powder was obtained after drying in the oven at 60 °C for 10 h. To investigate the influence of reaction time on the growth aspect for WS_2NF , this experiment was carried out with different heating times (2–20 h).

2.3. Functionalization of MPA onto the surfaces of WS_2NF ($WS_2NF-MPA$)

The surface modification of WS_2NF was carried out by the described procedure in the literature with slight modifications (Zhou, He, Yang, & He, 2014). Briefly, 20 mg of prepared WS_2NF was added into the n-butyllithium/hexane solution (1.25 M, 2 mL) and then stirred for 1 h.



Scheme 1. Schematic illustration for the detection of norovirus by using $WS_2NF/AuNP$ -peptide electrochemical biosensor. (a) The preparation of $WS_2NF/AuNP$ -peptide. (b) The fabrication of C-SPE with $WS_2NF/AuNP$ -noro and detection of norovirus through the EIS analysis.

The excess butyllithium was washed with hexane twice and then centrifuged at $4500 \times g$. After drying, Li^+ -loaded materials were treated with a specified concentration of MPA/DI ranging from 25 mM to 150 mM. Finally, unreacted MPA was removed by dialyzing against DI water using Spectra/Por dialysis membrane (MWCO = 1000 Da) for 3 d.

2.4. Decoration of AuNP on the WS_2NF -MPA ($\text{WS}_2\text{NF}/\text{AuNP}$)

To optimize the proper fabrication of AuNP onto the WS_2NF , the different amount of HAuCl_4 was added into the solution of WS_2NF -MPA and then vortexed for 2 h. After that, 1 mL of NaBH_4 (0.3 M) was added into the above solution, and the AuNP decorated WS_2NF ($\text{WS}_2\text{NF}/\text{AuNP}$) was washed twice with DI water by centrifuging at $4500 \times g$ for 10 min. The morphologies of WS_2NF and $\text{WS}_2\text{NF}/\text{AuNP}$ were characterized by FE-SEM (SIGMA instrument from Carl Zeiss, Cambridge, UK) and TEM (Tecnai G2 F30 S-TWIN from FEI, Hillsboro, OR, USA). The powder XRD patterns of the samples were analyzed by D8-Advance instrument from Bruker AXS (Karlsruhe, Germany) with $\text{Cu-K}\alpha$ as an X-ray source and the 2θ range from 5 to 70° . The surface area was recorded by Brunauer-Emmett-Teller (BET) analysis with Nova 1200e (Quantachrome, Boynton Beach, FL, USA). After dehydration at 60°C for 6 h, BET multipoint analyses were performed with N_2 gas adsorption.

2.5. Immobilization of norovirus affinity peptide on the surfaces of $\text{WS}_2\text{NF}/\text{AuNP}$ ($\text{WS}_2\text{NF}/\text{AuNP-peptide}$)

In order to optimize an effective immobilization of bioreceptors onto the surfaces of $\text{WS}_2\text{NF}/\text{AuNP}$, the different concentrations of norovirus specific peptide (100 μL ; 0.1, 0.3, 0.5, 0.7, 1.0 and 2.0 mg/mL) was mixed with $\text{WS}_2\text{NF}/\text{AuNP}$ for 120 min. After the reaction, the reactants were washed by centrifugation ($4500 \times g$, 7 min). To figure out the adequate time for immobilization, 100 μL of the peptide was reacted with $\text{WS}_2\text{NF}/\text{AuNP}$ from 60 to 360 min. After synthesis of $\text{WS}_2\text{NF}/\text{AuNP-peptide}$, it was stored at 4°C for the further use. The optimization process was confirmed by EIS as described below.

To prove that the $\text{WS}_2\text{NF}/\text{AuNP}$ represents more better performance than bare AuNP, AuNP was synthesized by conventional citation methods than functionalized with GBP-peptide. Briefly, 7.2 mL of sodium citrate and 5 mL of HAuCl_4 (25 mM) solutions were added to 150 mL of DI water, and the mixture was heated up to 90°C for 10 min. After the yellow-colored solution is turned into red, 250 μL of 10% of PEG 8000 was added to 5 mL of AuNP (9 nM) and stirred for 1 h to prevent the aggregation of AuNP. After that, peptide (30 μL , 300 $\mu\text{g}/\text{mL}$) was added into the above AuNP solution to obtain AuNP-peptide. The unbounded species were removed by centrifuging the solution at $4500 \times g$ for 7 min.

2.6. Preparation of norovirus conjugated $\text{WS}_2\text{NF}/\text{AuNP-peptide}$ ($\text{WS}_2\text{NF}/\text{AuNP-noro}$)

To find optimum time for the conjugation between $\text{WS}_2\text{NF}/\text{AuNP-peptide}$ and norovirus, $\text{WS}_2\text{NF}/\text{AuNP-peptide}$ (1 mg/mL) was treated with norovirus (100 μL ; 10^5 copies/mL) at different times (20, 30, 40, 50, 60, 90, and 120 min). To remove the unbounded virus and impurities, norovirus conjugated $\text{WS}_2\text{NF}/\text{AuNP-noro}$ was washed with a centrifuge at $4500 \times g$ for 7 min. Based on the confirmed optimum reaction time, the $\text{WS}_2\text{NF}/\text{AuNP-peptide}$ was reacted with different concentrations of norovirus (100 μL ; 10^1 , 10^2 , 10^3 , 10^4 , and 10^5 copies/mL). After then, 10 μL of each manufactured $\text{WS}_2\text{NF}/\text{AuNP-noro}$ solution is dropped onto the working electrode region of C-SPE and dried. To modify the $\text{WS}_2\text{NF}/\text{Au-noro}$ on the C-SPE surface, 0.1% Nafion diluted by 2-propanol was casted on the working electrode.

To analyze the norovirus in the oyster sample, we follow the ISO 15216-1 procedure. In brief, after gently washing mud on the surface of

the shell, carefully open it with a sterile knife and dissected its digestive glands. The flesh of oyster (2.0 g) was transferred to a clean Petri dish and evenly chopped until it changed to the paste-like consistency. To the homogenized sample, 10 μL of virus samples with different concentrations and 2.0 mL of proteinase K were sequentially added. After this sample incubated for 1 h at 37°C , it was placed in a water bath for the secondary incubation for 15 min at 60°C . To remove the protein debris, it was centrifugated ($3000 \times g$, 5 min) and decant the supernatant into a clean tube. The obtained samples were retained in the refrigerator (4°C) for the analysis.

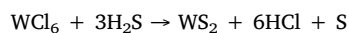
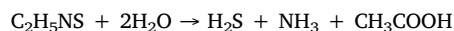
2.7. Impedance measurement of $\text{WS}_2\text{NF}/\text{AuNP-noro}$

As shown in Scheme 1, after C-SPE was modified with the $\text{WS}_2\text{NF}/\text{AuNP-noro}$, EIS was monitored by a CHI 750 E instrument (CH Instruments, Austin, TX, USA) connected to a computer data analysis system. The electrochemical responses were measured in a PBS solution (25 mM, pH 7.4) supplemented with $[\text{Fe}(\text{CN})_6]^{3-/4-}$ as a redox couple. The EIS was recorded by applying AC potential in the range of 0.09–1.3 V through frequency from 10 kHz to 1 Hz. The components of the system are composed of a three-electrode system, and we adopted the Randles equivalent circuit for the model things (Fig. S1). The obtained EIS was fitted by using Z-MAN software (Wonatech, Seoul, Korea). For probing reproducibility, calculating error bars, and detection limit, three samples were equally prepared and then measured. The limits of detection (LOD) of this developed sensor for the norovirus in the spiked and oyster samples were calculated by the equation of three-sigma rule ($\text{LOD} = 3.3\sigma/S$). Where σ is a standard deviation of the blank, S is the slope of the calibration curve (Shrivastava & Gupta, 2011).

3. Results and discussion

3.1. Synthesis and characterization of WS_2NF

Considering that the morphology of WS_2 is closely related to both effective electron transfer of WS_2 and the carrying amount of AuNP which is anchoring sites for the bioreceptor, controlling the shape of WS_2 is of paramount importance for the sensing performance. Therefore, the influences of the synthetic conditions for WS_2 such as different reaction temperatures and reaction times were investigated. The precursor solution for WS_2 is composed of both WCl_6 for the tungsten source and TA acting as the reducing and sulfurizing reagent, respectively, and it was simply synthesized through the one-pot hydrothermal process. The formation of WS_2 was proceeded by two-steps. Firstly, TA is decomposed by H_2O at high temperature then generate H_2S gas. Next, the gas is subsequently reacted with WCl_6 and develops the WS_2 . The chemical reaction formula for WS_2 (Ansari, Ansari, Parveen, Cho, & Song, 2018) is shown below:



First of all, the influence of reaction temperature was conducted under 260°C – 290°C , and each product was investigated by XRD (Fig. 1a) and FE-SEM (Figs. 1b–e). Since the upper-temperature limit for Teflon is up to 290°C , and the reported reaction temperature for WS_2NF was 280°C (Hasani et al., 2018). While XRD patterns of the sample prepared at 260°C are matched with hexagonal WO_3 (WO_3 , ICSD No.80634, hexagonal, $P6/mcm$, $a = b = 7.32 \text{ \AA}$, $c = 7.66 \text{ \AA}$), it was observed that the mixtures both beginning of the formation hexagonal WS_2 structure (WS_2 , ICSD No. 08-0237, hexagonal, $P6_3/mmc$, $a = b = 3.15 \text{ \AA}$, $c = 12.36 \text{ \AA}$) and hexagonal WO_3 at 270°C . When the reaction temperature is over 280°C , hexagonal WS_2 was completely formed without WO_3 (Fig. 1a). The major crystal planes of WS_2 consisted of (002), (004), (101) and (103). Among them, (002) peak

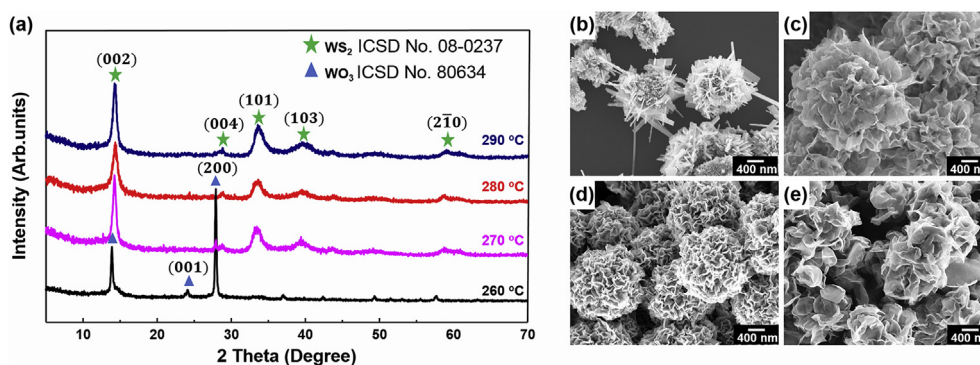


Fig. 1. Characterization of synthesized WS_2 under different temperatures with fixed time at 16 h. (a) Powder XRD patterns. FE-SEM images of WS_2 at different reaction temperatures of (b) 260 °C, (c) 270 °C, (d) 280 °C, and (e) 290 °C.

representing the highest intensity indicates that the material is constructed in a direction as 2D nanosheets (Yu, Zhou, & Wang, 2016). It was also reported elsewhere that low temperature (below 240 °C) cannot induce the conversion WO_3 to WS_2 when it synthesized via a hydrothermal process (Shang, Xia, Xu, & Chen, 2005; Yang et al., 2013). According to the reaction mechanism, the amount of H_2S gas within the reactor is essential to the formation of WS_2 . Based on these reasons, it was speculated that why WS_2 is not fully formed below 280 °C is the temperature is not enough to decompose TA and insufficient to possess H_2S gas within the solution. The solubility of the gas in an autoclave like the closed system is proportional to pressure, thus, it was revealed that temperature affects complexly to the formation of WS_2 .

The FE-SEM images of the above corresponding products are illustrated in Figs. 1b–e. Excepting for the 260 °C, their morphologies generally appeared a curly flower-like shape, and it means that hexagonal WS_2 was developed by changing its crystalline structure (Wang, Lee, & Ma, 2009). The average size of nanomaterials prepared at 260 °C is 2.0–4.0 μm with rectangular shaped petals, which have 500–800 nm length and 50–180 nm width. In the case of prepared at 270 °C, the formed WS_2 nanomaterials are indistinct flower shape with an average size of 6.0–7.5 μm . Finally, when it prepared at 280 °C, it exhibits an obvious flower shape with an average size of 4.0–5.0 μm . Although the XRD pattern of 290 °C sample shows a hexagonal WS_2 structure, its appearance looks like the slightly collapsed shape of WS_2NF due to thermal degradation (Mai et al., 2006). To ascertain the detailed morphological and structural features of WS_2NF , each sample was ultrasonicated for 1 h and then studied by TEM and HR-TEM (Fig. S2). The growth direction of WS_2 sheet is along with the (002) direction, the lattice distance calculated from XRD through the Bragg's equation is a coincidence with the value of interplanar distance in HR-TEM image (0.63 nm) (Ratha & Rout, 2013).

The crystal growth aspect of WS_2NF as following the reaction time (2–20 h) was studied by tracking their crystal structure (Fig. S3) and morphology (Figs. S4a–f) at 280 °C. At 2 h, monoclinic WO_3 (WO_3 , ICSD No. 38832, monoclinic, $P121/n1$, $a = 7.3 \text{ \AA}$, $b = 7.53 \text{ \AA}$, $c = 7.68 \text{ \AA}$) was formed with irregularly shaped particle (Fig. S4a). In the case of 4 and 8 h, monoclinic WO_3 transforms to hexagonal WO_3 , simultaneously, their shape is changed to the aggregated bundle of nanorods (Figs. S4b and S4c). In the early stage of progression, monoclinic WO_3 is priority formed due to insufficient decomposition of TA, and then its phase is changed to a thermodynamically stable hexagonal phase. As shown in Figs. S3 and S4d, the sample is starting to metamorphosis to hexagonal WS_2 after 12 h, and it was thoroughly converted to flower shapes at 16 h. However, when it reacts over 20 h, the XRD patterns are broadened and disappeared except (002) peak, revealing the collapse of their shape by thermal decomposition (Fig. S4f). To verify the surface area of WS_2 is influenced by their morphology, the multi-point BET analysis was conducted on the formed WS_2NF excepting both

monoclinic and hexagonal phase of WO_3 . The surface area was estimated over a relative pressure (P/P_0) range from 0.05 to 0.30. Figs. S5 and S6 show the BET analysis data of WS_2NF at different temperatures with the fixed reaction time (16 h), and the different reaction times with fixed temperature (280 °C), respectively. It was noticed that the synthesized WS_2NF at 280 °C for 16 h exhibited the largest surface area (14.8 m^2/g). Because many exposed well-separated petals of WS_2NF can hold nitrogen molecules more (Park et al., 2017). As a result, the temperature of 280 °C with 16 h was chosen for the optimal synthetic condition for the WS_2NF .

3.2. Preparation and optimization of norovirus detection system ($\text{WS}_2\text{NF}/\text{AuNP-peptide}$)

The pristine WS_2NF cannot take the norovirus directly, its surface was decorated by AuNP to immobilize the bioreceptor for norovirus. The norovirus capturable peptide was improved by attaching cysteine on the C-terminal to facilitate the anchoring on the $\text{WS}_2\text{NF}/\text{AuNP}$ surface. Before AuNP decoration, the surface of WS_2NF was modified with MPA through Li-ion intercalation method. As modifying MPA on WS_2NF , the surface chemistry of WS_2NF is converted to the $-\text{COOH}$ group. The $-\text{COOH}$ group on WS_2NF can effectively coordinate with gold (III) ion, it is the nucleation site for AuNP growth.

Before identifying the optimal amount of MPA, the effects of Nafion used for entrapping WS_2NF on the working electrode without losing electrochemical activity was investigated with different concentrations in a range of 0.01–1.0%. As shown in Fig. S7, the redox current by $\text{Fe}(\text{CN})_6^{3-/4-}$ was declined when it was higher or lower than 0.1% of Nafion. This is because of the receded distance between redox species and electrode by the augmented thickness of Nafion layer on the electrode. On the other hand, the thickness of polymer films is too thin, the covered materials which are what we want to investigate were able to diffuse away from the electrode due to either incomplete coating or cracks (Hoyer, Florence, & Batley, 1987). Therefore, 0.1% Nafion was selected for further experiments. In succession, different concentration of MPA was reacted with WS_2NF then prepared $\text{WS}_2\text{NF}/\text{AuNP}$ with the 2.4 mg of HAuCl_4 . The evaluation of the differently prepared $\text{WS}_2\text{NF}/\text{AuNP}$ is implemented by EIS (Fig. S8). Since AuNP is a highly conductive material, the impedance value will be decreased as increasing the amount of AuNP on WF_2NF . Whereas the impedance is decreased as raising the concentration of MPA until 60 mM, the value was increased over the 60 mM of MPA. Thus, 60 mM of MPA was selected as the best concentration. Furthermore, the effect of HAuCl_4 concentration (0.6–10.0 mg) for the formation of AuNP onto WS_2NF was investigated by EIS (Fig. 2a). Unlike the MPA, the impedance value of $\text{WS}_2\text{NF}/\text{AuNP}$ is decreased until 2.4 mg of HAuCl_4 , but there are no longer changes even over that concentration. To apprehend that phenomena, the surface of each prepared $\text{WS}_2\text{NF}/\text{AuNP}$ were analyzed by FE-SEM (Figs. 2b–g). These images demonstrated that the AuNP is well

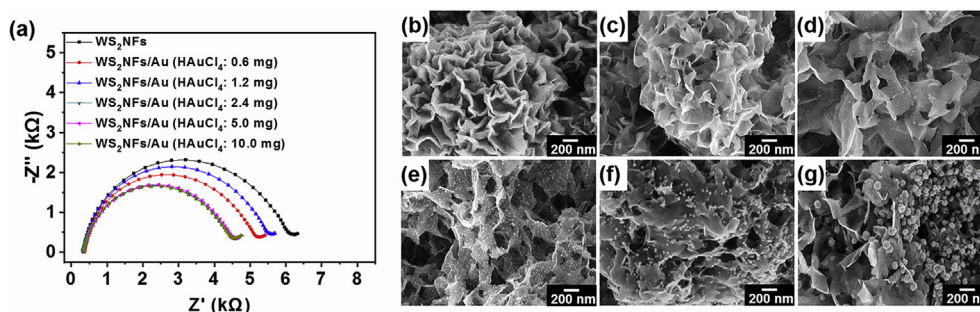


Fig. 2. (a) Impedance spectra of WS₂NF/AuNP with different concentration of HAuCl₄. (n = 3) FE-SEM images of WS₂NF/AuNP with the different concentration of HAuCl₄, i.e., (b) control, (c) 0.6 mg, (d) 1.2 mg, (e) 2.4 mg, (f) 5.0 mg, and (g) 10.0 mg.

decorated onto the surfaces of WS₂NF in a size excepting the 0.6 mg of HAuCl₄. The sizes of AuNP were found to be 11 ± 3 , 30 ± 5 , 50 ± 10 and 98 ± 20 nm for 1.2, 2.4, 5.0, and 10.0 mg of HAuCl₄. These results indicate that the higher concentration of HAuCl₄ is used the larger size of AuNP is formed. However, it leads to a diminished number of AuNP onto the surfaces of WS₂NF. Before WS₂NF/MPA react with different concentration of HAuCl₄, MPA served as the nucleation site for AuNP evenly exist on the WS₂NF surface, the number of nucleation site for the whole samples must be similar. However, as the size of AuNP is larger, contacting area ratio between AuNP and WS₂NF is declined and reduce the mutual interaction, thus, easily detachable from the WS₂NF during experimental processes (Li et al., 2013). The release of AuNP especially occurred during washing steps, this unexpected error affects the accuracy and sensitivity of the sensors. Consequently, 2.4 mg of HAuCl₄ were chosen for the optimum concentration to the WS₂NF/AuNP due to low impedance and uniform size distribution of AuNP. In addition, the formation of AuNP on the WS₂NF was verified by XRD, the characteristic peak for AuNP was appeared in all the samples of WS₂NF/AuNP (Fig. S9).

For utilizing this nanocomposite to norovirus sensors, WS₂NF/AuNP was conjugated with norovirus bindable peptide. To figure out the optimal conditions, the concentration of peptide, immobilization time for peptide on WS₂NF/AuNP and, reaction time between WS₂NF/AuNP-peptide and norovirus were sequentially investigated. As shown in Fig. 3a, the impedance is increased until 0.7 mg/mL then saturated due to the insulation effect of the peptide. It means that most of the peptides are completely conjugated on the WS₂NF/AuNP surface, 0.7 mg/mL was selected. The adequate reaction time for conjugating peptide on the WS₂NF/AuNP was also determined by EIS after reacted with them from 60 to 360 min (Fig. 3b). The impedance value is raised until 240 min, there are no changes from that time due to the completion of the reaction. In succession, the optimal incubation time between WS₂NF/AuNP-peptide and norovirus was monitored with 10^5 copies/mL of norovirus (Fig. 3c). The value was gradually increased as the time from 10 to 60 min, and it was saturated after that time. From this result, we decided 60 min as the optimum incubation time for the interaction of WS₂NF/AuNP-peptide with norovirus.

3.3. Analytical performance of WS₂NF/AuNP-peptide as an electrochemical biosensor

With the optimized analytical conditions, the impedance spectra of WS₂NF/AuNP-noro prepared by different concentrations of norovirus (0 , 10^1 , 10^2 , 10^3 , and 10^4 copies/mL) were measured. The results for the spiked samples are illustrated in Figs. 4a and b, and they show that the impedance increased gradually with the amount of norovirus. The logarithm (Log) transformed impedance values of WS₂NF/AuNP-noro (Z' (kΩ)) were dependent on the Log of the concentration of norovirus (C), and the linear regression equation was $\log(Z'(\text{k}\Omega)) = 0.0697 \times \log C + 0.0220$ ($R^2 = 0.9875$; $n = 3$). The LOD for spiked norovirus samples was 2.37 copies/mL, which is calculated by

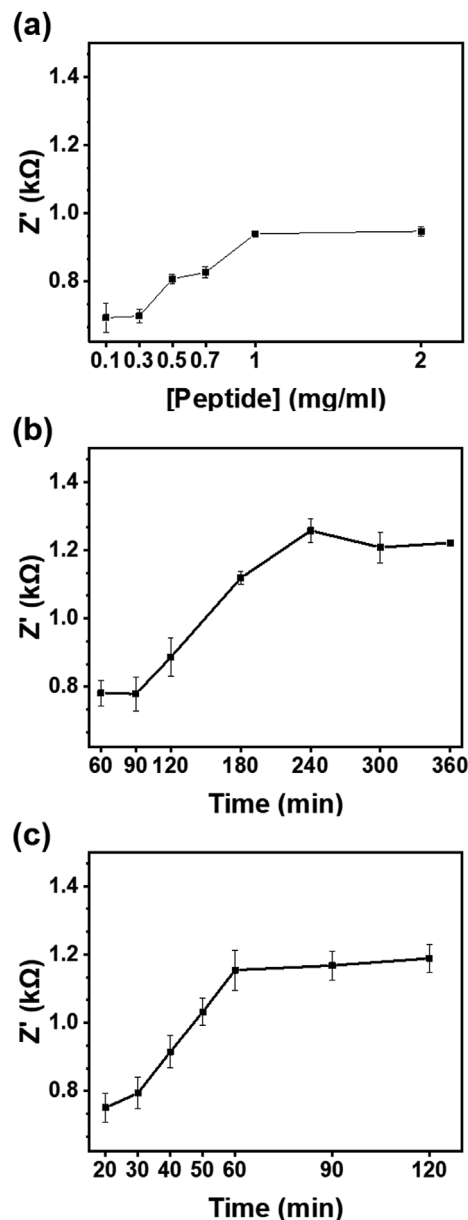


Fig. 3. Optimization process for the nano-bio sensor through impedance value. (a) The concentration of peptide for WS₂NF/AuNP-peptide. Conjugation time between (b) WS₂NF/AuNP and peptide and (c) WS₂NF/AuNP-peptide and norovirus. These experiments were carried at WS₂NF/AuNP (0.1 mg/mL) and norovirus (10^5 copies/mL) (n = 3).

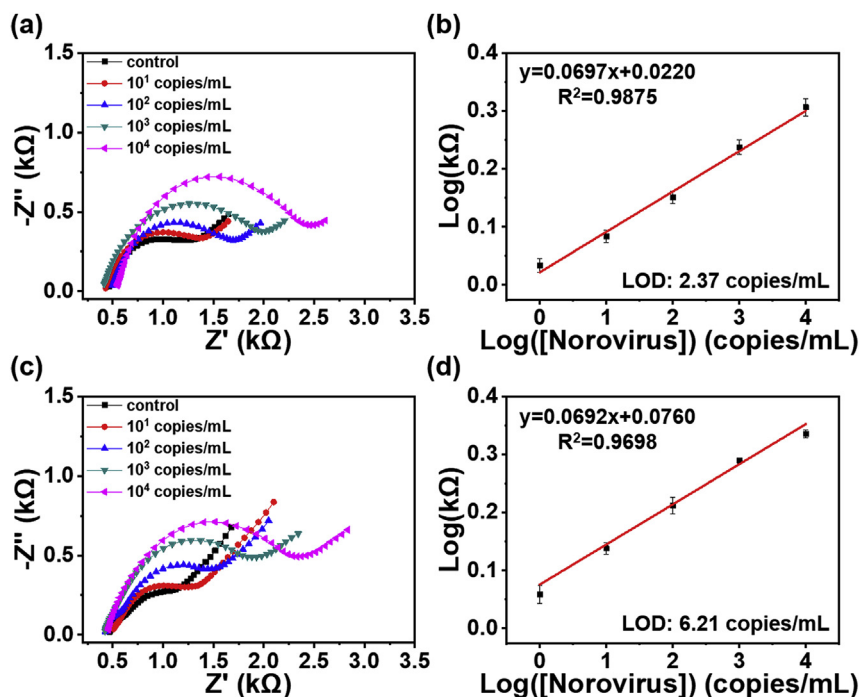


Fig. 4. The detection of norovirus under the optimized WS₂NF/AuNP-noro system by EIS. Spiked sample test for (a) impedance spectra and (b) calibration graph against the concentration of norovirus. Real sample test for (c) impedance spectra and (d) calibration graph against the concentration of norovirus originated from an oyster (n = 3).

3 σ -rule. To find out whether adopting WS₂NF/AuNP is improving the detection limit or not, AuNP-GBP peptide-based detection system was implemented as following the above procedure. The LOD of AuNP-GBP peptide system was 5.59 copies/mL, which is lower than WS₂NF/AuNP (Fig. S10). As shown in Fig. S11, to evaluate the time-dependent stability of the prepared WS₂NF/AuNP-peptide electrode, 10^2 copies/mL of norovirus was analyzed. After 15 days, the impedance value still retains approximately 92% for detection of the same concentration of norovirus due to the supported nanomaterials helping the long-term stability. Moreover, to verify the performance of the developed sensor for the point-of-care test, the real norovirus samples which were prepared by extracting norovirus from the deliberately infected oyster with different concentration of virus as following the standard protocol (ISO TS 15216-1) was adopted to this system. The oyster extracts were reacted with WS₂NF/AuNP-peptide as same as the above procedure (Figs. 4c and d). The linear regression equation for the oyster sample was $\log(Z' (k\Omega)) = 0.0692 \times \log C + 0.0760$ ($R^2 = 0.9698$; n = 3). The LOD for norovirus in oyster samples was 6.21 copies/mL. When it compares with the equation of the spiked sample, both slopes are similar, but the y-intercept of the oyster sample is higher than the spiked sample result. This result is due to the ingredients containing oysters that may influence the background signal. Even though the detection performance of the system is slightly lower in the oyster sample, the proposed WS₂NF/AuNP-peptide-based electrochemical biosensor demonstrates that the wider linear dynamic range and higher sensitivity than those previous reports (Table S1). Furthermore, the selective response of WS₂NF/AuNP-peptide was investigated with different viruses such as dengue virus (I, II, III, IV type) and rotavirus (10^4 copies/mL). In the case of rotavirus, its symptom seems like norovirus, but it is important to rapidly and accurately diagnose the disease separately because it occurs mainly in infants (Chen et al., 2012). The dengue virus was chosen because it has less connection with norovirus. As shown in Fig. 5, there is no significant impedance response except norovirus, and these results convinced that the developed sensor system has promising analytical advantages with high sensitivity and selectivity for the detection of norovirus even at low concentrations.

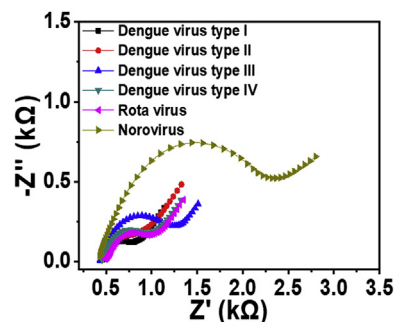


Fig. 5. Selectivity test for WS₂NF/AuNP-noro system by EIS. The EIS spectra were measured in the presence of various viruses, rotavirus, and dengue virus type I, II, III and IV, (10^5 copies/mL) as negative controls.

4. Conclusion

In summary, a novel and unique WS₂NF/AuNP-peptide was developed for the specific and sensitive electrochemical biosensors for norovirus in the oyster. To enhance the electrochemical property, AuNP were decorated on the surfaces of WS₂NF and then modified with norovirus specific peptide. To fabricate an effective electrochemical biosensor, various parameters such as shape control of WS₂, the effects of MPA and HAuCl₄ concentration, reaction time, reaction temperature, and concentration of peptide were investigated. The prepared WS₂NF/AuNP-peptide was successfully applied to detect norovirus in both spiked and real samples within 60 min. The developed electrochemical biosensor exhibited tremendous performance in terms of selectivity and sensitivity (LOD = 2.37 copies/mL). Moreover, even different types of viruses were introduced into the system, the only norovirus exhibited the specific signal change, which confirms that the developed biosensor is high selectivity towards norovirus. To the best of our knowledge, this is the first study applying the AuNP-decorated WS₂NF with novel affinity peptides to the electrochemical sensors. Although the pretreatment of an oyster and reaction with sample consume much time, if suitable sample kit would be developed, the WS₂NF/AuNP-peptide biosensor has a strong potential in food control and point-of-care testing of norovirus in shellfish.

CRedit authorship contribution statement

Seung Hoon Baek: Conceptualization, Methodology, Investigation. **Chan Yeong Park:** Methodology, Validation, Visualization. **Thang Phan Nguyen:** Resources, Investigation. **Min Woo Kim:** Resources, Data curation. **Jong Pil Park:** Writing - review & editing. **Changsun Choi:** Resources. **Soo Young Kim:** Resources, Writing - review & editing. **Suresh Kumar Kailasa:** Visualization, Writing - review & editing. **Tae Jung Park:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2020.107225>.

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