

Sensitive SERS Glucose Sensing in Biological Media Using Alkyne Functionalized Boronic acid on Planar Substrates

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Abstract

In this work, we propose a novel glucose binding mechanism on a highly sensitive SERS substrate, in order to overcome challenges in specific glucose detection in bio-fluids. We make use of phenylboronic acid as a receptor for saccharide capture onto the substrate and the ability of the captured glucose molecule to undergo secondary binding with an alkyne-functionalized boronic acid to form a glucose-alkyne-boronic acid complex. The formation of this complex shows high selectivity to glucose, over other saccharides. In addition, the alkyne group of the alkyne-functionalized boronic acid exhibits a distinct Raman peak at 1996 cm^{-1} in a biological silent region ($1800\text{-}2800\text{ cm}^{-1}$) where most endogenous molecules, including glucose, show no Raman scattering, thus offering a high sensitivity over other SERS glucose sensing. The substrate offers long-term stability, as well as high SERS enhancement to the glucose-alkyne boronic acid complex on substrate. In addition, the reversibility of SERS signals at various incubation stages also show reusability capabilities, whereas positive results in clinical urine samples demonstrate clinical feasibility. All these strongly suggest that this newly developed SERS-based assay offers great potential in glucose sensing.

Key Words: Surface-enhanced Raman spectroscopy; alkyne; boronic acid; glucose; biosensing.

1. Introduction

Surface-enhanced Raman scattering (SERS) is a highly sensitive spectroscopic technique discovered several decades ago, which has been well studied (Nie et al. 1997). It has significantly improved the inherent low sensitivity of Raman spectroscopy by virtue of the fact that the Raman signals of molecules on a nanostructured surface can be enhanced by several orders of magnitude (typically 10^6 to 10^{14}), due to the strong surface plasmon resonance of the nanostructured surface (Albrecht et al. 1977). SERS offers higher specificity in detection, since it typically produces spectra with sharp peaks, whereas fluorescence spectra are generally broad and overlapping. Thus, SERS has been proposed as a highly sensitive and specific technique for chemical sensing applications with higher detection limits (Nie and Emory 1997) and is exemplified by its use in DNA detection (Barhoumi et al. 2010), cancer diagnosis (Sha et al. 2007) and cellular molecules detection (Kneipp et al. 2002). In particular, nanostructured substrates provide unique advantages in biological environments over nanoparticles, which may undergo typical aggregation in a medium having high proteins and ionic strength (Du et al. 2012; Zakaria et al. 2013; Zhang et al. 2009). Moreover, the SERS signal from nanoparticles can be susceptible to noise in a high salt medium. Thus, the high stability of SERS substrates has proven valuable for nano-biosensor development, which in turn allows analysis of high salt samples including blood and urine. Furthermore, a SERS substrate also allows for the immobilization of various bio-receptors without aggregation issues, as well as the ability of performing measurements on low-volume samples ($\sim \mu\text{L}$) with sub-nanometer resolution.

One important application for SERS biosensing is glucose detection. This is due to the direct association of blood glucose level with the common metabolic disorder, diabetes, which affects 371 million people worldwide according to the World Health Organization (WHO) report in 2012. Therefore, extensive research efforts have been made to develop SERS-based glucose sensors (Camden et al. 2008; Dieringer et al. 2006; Haynes et al. 2005; Lyandres et al. 2010; Ma et al. 2011; Shafer-Peltier et al. 2003; Shah et al. 2007; Stuart et al. 2005; Stuart et al. 2006; Vangala et al. 2010; Yonzon et al. 2004; Yonzon et al. 2006; Yuen et al. 2010; Zhang et al. 2006). Van Duyne's group has done extensive work in the field of direct glucose detection using SERS. SERS-based glucose detection had been developed, where an alkanethiol monolayer is

self-assembled on a silver film over nanosphere (AgFON) surface and acts as a partition layer, preconcentrating glucose near the SERS-active surface (Haynes et al. 2005; Lyandres et al. 2005; Yonzon et al. 2004). This technique was further improved in later years by using a mixed decanethiol/mercaptohexanol partition layer instead, which offers higher temporal stability, reversibility and ease of control and thus, allows *in vivo* monitoring of glucose concentrations in the interstitial fluid of rats (Dinish et al. 2011; Stuart et al. 2005; Vangala et al. 2010; Yonzon et al. 2005). Even more recently, other groups have also attempted to use other types of SERS substrates for direct glucose detection, such as bimetallic silver-gold substrates (Kanayama et al. 2000), photonic crystal fibers (Barriet et al. 2007), silver/gold nanoparticle-based substrates (Piergies et al. 2013). However, the development of a highly sensitive SERS-based glucose sensor has been plagued by many challenges, mainly due to the low Raman cross section of the glucose molecule (Dinish et al. 2011). In order to circumvent this problem, one method is to use carbohydrate recognition molecules, such as boronic acid, to capture glucose molecules onto the nanostructured surface. This increases the concentration of captured glucose, thus improving the detection sensitivity (Kanayama and Kitano 2000). However, the SERS spectra of the glucose molecule and functional groups of the carbohydrate recognition molecules, such as boronate (1370 cm^{-1}) and aromatic (1580 cm^{-1}) group in phenylboronic acid are in the $400 - 1800\text{ cm}^{-1}$ region, which can be prone to interference by the Raman signals of inherent biomolecules (Reinemann et al. 2011). Thus, it is still challenging to develop a simple, reliable, and highly sensitive SERS-based glucose sensor.

One potential solution to the low sensitivity and signal interference is the use of an alkyne-based probe. A very useful characteristic of alkyne compounds is the strong alkyne stretching vibrations in the mid-IR ($1800-2200\text{ cm}^{-1}$), a region that is relatively free of interference from the Raman signals of biomolecules. This has been utilized in cell imaging, with Raman and SERS spectroscopy detection techniques (Palonpon et al. 2013; Yamakoshi et al. 2011; Yamakoshi et al. 2012). In this work, we design a novel mechanism for specific glucose detection with alkyne-functionalized boronic acids immobilized on a planar SERS substrate. This substrate is made up of an optimized bimetallic (Au on top of Ag) coating on top of polystyrene nanospheres immobilized on the base glass material. The SERS enhancement due to the bimetallic coating on the planar SERS substrate was also stimulated by finite element method (FEM). On top of

providing an interference-free SERS signal, another advantage of employing alkyne-functionalized boronic acid is its high tendency to form a stable boronic acid-diol complex with syn-periplanar hydroxyl groups (Bielecki et al. 1999; Eggert et al. 1999; James 2007; Nicholls et al. 2004). Furthermore, this assay also offers reusability capabilities in clinical urine samples, thus offering great potential towards glucose sensing in biofluids.

2. Materials and Methods

2.1 Fabrication of Substrates

Monodisperse polystyrene (PS) colloidal suspension ($\varnothing = 384$ nm, 2.5 wt%) was purchased from Kisker and stored at 4 °C. During preparation, 15 wt% surfactant sodium dodecyl sulphate (SDS) was added to the PS sphere solution to form a composite solution containing 2.36 wt% PS spheres and 0.85 wt% SDS. Clean microscope glass slides to be used as supporting substrates were cut into square pieces (10 mm x 10 mm x 1 mm). The glass slides were first sonicated in a bath of ethanol for 20 min before being dried with argon gas. Using spin-coating, PS sphere monolayers were assembled onto the clean glass slides. This was done by dispersing 10 μ L of the prepared colloidal solution onto the center of a glass slide. Each glass slide was spin coated in the spin coater preset at 2000 rpm for 20 s. Due to the Bragg diffraction given by the closely-packed PS spheres, a green reflective surface was then formed. The coated glass slides were then dried in vacuum desiccators overnight at a pressure of 0.6 PA. The substrates were first coated with Ag (99.999% purity, JEOL) using 120 s of sputtering (JEOL JFC-1600 Auto fine coater) before being sputtered with a layer of Au (99.999% purity, JEOL) for 80 s. Each metal layer was deposited at a rate of 1.33 nm/s.

2.2 Immobilization of 4-Mercaptophenylboronic Acid on BMFON Substrates

4-mercaptophenylboronic acid was dissolved in ethanol to form an aqueous solution of 10 mM. Fabricated BMFON substrates were then incubated in this solution for 2 hours. Following that, these substrates were then washed thoroughly with pure ethanol and dried in air.

2.3 Saccharide Detection (Glucose, Fructose and Galactose)

4-mercaptophenylboronic acid-functionalized BMFON substrates were incubated with saccharides (~20 μ l) for 1 hour. The incubated substrates were then washed with water thrice (3 x 2 ml) prior to incubation of alkyne-functionalized phenylboronic acid solution (2 mM) for 1 hour. The excess alkyne-functionalized phenylboronic acid solution was then removed and the substrates were washed with water. Subsequently, the substrates were placed on a glass slide and under the microscope objective lens for measurements.

2.4 SERS Measurements

Raman measurements were carried out using a Renishaw InVia Raman (UK) microscope with a Peltier cooled CCD detector and a laser excitation wavelength at 785 nm. The laser beam was directed onto the sample for excitation through a 50x objective lens, which in turn was used to collect the scattered Raman signal. All Raman spectra were processed with WiRE3.0 software. The maximum laser power at the sample was measured to be 300 mW and the exposure time was set at 10 s throughout the measurements. Prior to each measurement, the instrument was calibrated with a silicon standard whose Raman peak is centered at 520 cm^{-1} .

2.5 Detection of Glucose in Clinical Urine Samples

In this experiment, samples used for the study were from clinical specimens and stored at -20°C . Specimens were used in accordance with procedures approved under the local ethics committee (CIRB Re 2011/558/C) and informed consent was given by all patients. 2 mM of glucose was added to 20 ml of the urine sample, which was then agitated. 4-mercaptophenylboronic acid-functionalized BMFON substrates were incubated with the glucose-urine sample, washed, incubated with alkyne-functionalized phenylboronic acid solution, washed again, before SERS measurements were acquired.

3. Results and Discussion

3.1 Characterization of SERS Substrate

As shown in the SEM image (Fig. 1A), large domains of hexagonally-packed two dimensional colloidal crystals are formed, mainly due to the self-assembly of polystyrene (PS) beads. The undulating curve on the metal layer created by the underlying periodic nanostructures contributes to the plasmonic coupling. Higher metal thickness helped to reduce the inter-bead gaps and forms a uniform structure (Fu et al. 2012). This will lead to high SERS enhancement and as well as reduced point to point intensity variation. At the optimized metal coating, the inter-bead gap is only $\sim 20\text{nm}$. If the thickness of the upper Au layer is further increased, it may lead to smoothing of crevices between the beads and will eventually limits the SERS sensitivity. The finite element method (FEM) calculations suggest that the SERS enhancement is originates from the hot-spots distributed primarily at the junctions of interconnected PS beads as well as over the surface of beads with Au and Ag layers (Fig. 1B). It shows the simulated electric field distribution ($|E|/|E_0|$) around the gap with sphere diameter at 200 nm and gap distance at ~ 100 nm. The Au/Ag coating layer was excited with an incident plane wave at 785 nm, propagating in the z-direction. Results showed the presence of SERS hotspots with high enhancement factors of up to $\sim 10^4$ distributed around the junctions of interconnected PS beads with Au and Ag layers (Fig. S1, SI).

Fig. 1 here

3.2 Phenylboronic Acid (Carbohydrate Capture Receptor) Functionalized SERS Substrate

Phenylboronic acid molecules were immobilized on the substrate via Au-S covalent linkages to act as carbohydrate receptors. The spatial and temporal distribution of the receptors on the substrate was examined before and after incorporating phenylboronic acid on substrates, via SERS mapping ($200\ \mu\text{m} \times 200\ \mu\text{m}$) based on the Raman peak corresponding to the arene group ($1580\ \text{cm}^{-1}$) (Fig. S2, SI). As shown in Fig. 2, the SERS spectrum displays identical peaks over 3 days and the SERS mapping demonstrates spatial and temporal uniformity in SERS signal. The

high binding affinity of the phenylboronic acid layer on the substrate is mainly due to the strong Au-S bond, which demonstrates the substrate stability for repeated measurements.

Fig. 2 here

3.3 Glucose Measurement

The mechanism of this assay is that phenylboronic acid receptor functionalized substrate was incubated with the analyte (glucose, fructose or galactose) to allow binding of the analyte molecules onto the boronic acid receptor via an ester linkage (Fig. 3). Following that, the substrate was then incubated with alkyne-functionalized boronic acid to allow binding to glucose occur. The binding ability of alkyne-functionalized boronic acid to glucose was determined by HR ESI-MS (Fig. S3, SI). The high affinity of alkyne-functionalized boronic acid to the glucose molecules is mainly due to the formation of a bidentate complex, which is not the case for fructose and galactose. In this way, it is possible to selectively quantify the concentration of glucose via the alkyne signal which is enhanced by the SERS substrate. This forms the basic mechanism for indirect glucose detection in this newly developed assay.

Fig. 3 here

In order to examine the detection specificity of this glucose sensor, SERS measurements were acquired and compared with one another, when the phenylboronic acid-functionalized substrate is incubated with different types of sugars, namely glucose, fructose and galactose. As shown in Fig. 4A, a significant alkyne peak at 1996 cm^{-1} is observed in the substrate incubated with glucose, but not with fructose and galactose. Thus, this strongly suggests that the alkyne-functionalized boronic acid selectively binds to glucose to form a bidentate complex, allowing the alkyne SERS signal to be detected. This selectivity over glucose based on bidentate complex formation can be attributed to the higher tendency of glucose to form a bidentate complex with boronic acid; this is reminiscent of a recent report which utilised the greater affinity of fructose for binding to monoboronic acids and of glucose to form 1:2 adducts with the two sets of syn-periplanar diol groups present in the α -D-glucopyranose ring (Huang et al. 2013; Nicholls and

Paul 2004). Presumably, therefore, all three carbohydrates interacted with the phenylboronic acid-functionalized substrate, but there is preferential binding of alkyne-functionalized boronic acid with the surface-bound glucose.

Fig. 4 here

For demonstration of the sensor's quantification capabilities in glucose detection, concentration dependence studies were conducted, in which SERS measurements were acquired when the phenylboronic acid-functionalized substrate was incubated with different glucose concentrations. As shown in Fig. 4B, there is an almost linear increasing trend in the SERS intensity of alkyne peak at 1996 cm^{-1} with increasing glucose concentration. Repeated experiments with varying glucose concentration showed that the lower limit of detection is approximately $100\text{ }\mu\text{M}$. As a control, SERS measurements were also collected with different fructose or galactose concentrations, which showed little or no increasing trend in terms of SERS intensity. Thus, these results present an almost linear relationship between glucose concentration and SERS intensity, demonstrating quantification in detection.

For cost-effectiveness and ease of operation, an ideal glucose sensor should also be reusable, offering the capability for repeated measurements. Boronic acids are known to be able to form diols reversibly. In order to investigate the reversibility in the SERS signal, SERS measurements were acquired and compared with one another at different experimental stages: phenylboronic acid-functionalized substrate before glucose incubation, after initial glucose incubation, after washing of incubated substrate with slightly acidic solution ($\text{pH} = 5$), as well as after glucose re-incubation of substrate. The alkyne SERS signal at 1996 cm^{-1} is diminished after the washing step, but restored after re-incubation with glucose, demonstrating the reusability of this glucose sensor (Fig. S4, SI).

3.4 Glucose Sensing in Glucose Doped Clinical Urine Sample

Finally, in order to demonstrate the feasibility of glucose detection in clinical samples, the functionalized substrates were incubated with human urine samples spiked with a known amount

of glucose and corresponding SERS measurements were acquired. As shown in Fig. 5, the alkyne SERS peak at 1996 cm^{-1} can be detected in a biofluid such as urine, without spectral interference from inherent biomolecules. Based on a SERS intensity calibration curve, we calculated the glucose concentration in the urine samples to be 1.8 mM, which is in good agreement with the actual amount of glucose (2.0 mM) added into urine. Thus, the effective and accurate determination of glucose concentration in urine samples strongly suggests that this newly developed assay offers great potential in clinical diagnosis.

Fig. 5 here

4. Conclusions

In this work, we present a novel perspective towards glucose detection assays that uses the phenylboronic acid as the recognition element, and SERS as the signal transduction element for direct glucose detection. It has always been challenging to detect glucose directly using SERS, as glucose has a small Raman cross-section and adsorbs weakly or not at all to bare silver surfaces. Nevertheless, research groups have developed partition techniques to preconcentrate glucose near the SERS-active surface for better SERS enhancement, or SERS techniques that study the SERS signature of phenylboronic acid instead of that of glucose, for improved SERS signal. However, these techniques inherently still suffer from limitations in terms of non-specificity in detection, especially for diabetes-glucose monitoring in biological samples, due to SERS interference from other biomolecules. In order to overcome this drawback, we have approached this problem from a different angle, by attaching the alkyne moiety to the phenylboronic acid for secondary binding, in order to enable indirect glucose detection. In particular, the alkyne bond exhibits a distinct Raman peak at 1996 cm^{-1} in a biological silent region ($1800\text{-}2800\text{ cm}^{-1}$) where most endogenous molecules, including glucose, show no Raman scattering, thus offering a competitive edge over direct glucose detection. Validation studies have been conducted to prove the selectivity of this assay for glucose over fructose and galactose, while other experiments have also been performed to demonstrate quantification, reusability, as well as clinical feasibility. In summary, these studies have formed the foundational framework for this newly proposed assay

to be further developed into a more robust, integrated, highly sensitive biosensor for glucose monitoring and detection in future.

Author Contributions: ‡These authors contributed equally.

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Figure Captions

Figure 1

(A) SEM image of substrate.

(B) Simulated electric field distribution ($|E|/|E_0|$) ($\times 10^4$) in the Au/Ag coated polystyrene substrate.

Figure 2

Monitoring of SERS spectra corresponding to the phenylboronic acid-functionalized substrate with no analyte, over 3 days.

Figure 3

Schematic for glucose detection mechanism, using the phenylboronic acid-functionalized substrate.

Figure 4

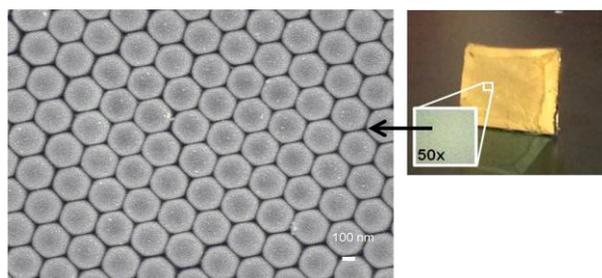
(A) Comparison of SERS spectra of phenylboronic acid-functionalized substrate with (I) no analyte, and 10 mM of (II) fructose, (III) galactose and (IV) glucose.

(B) SERS intensity of alkyne peak at 1996 cm^{-1} with various concentrations of glucose, galactose and fructose. Insert is the Raman spectra, showing the alkyne peak with various concentrations of glucose.

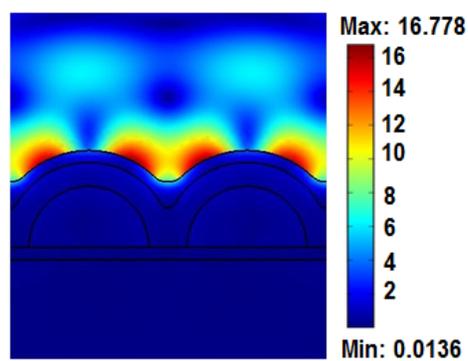
Figure 5

SERS spectrum of substrate incubated in urine sample spiked with urine show alkyne peak, demonstrating clinical feasibility; (I) urine, (II) urine spiked with 1.5 mM of glucose, and (III) urine spiked with 2 mM of glucose.

Figure 1



(A)



(B)

Figure 2

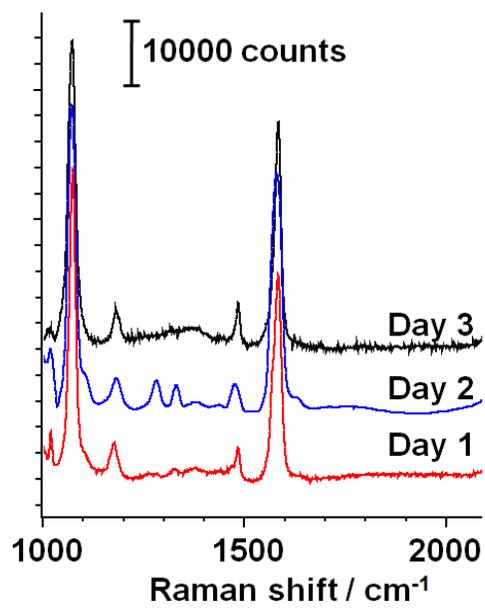


Figure 3

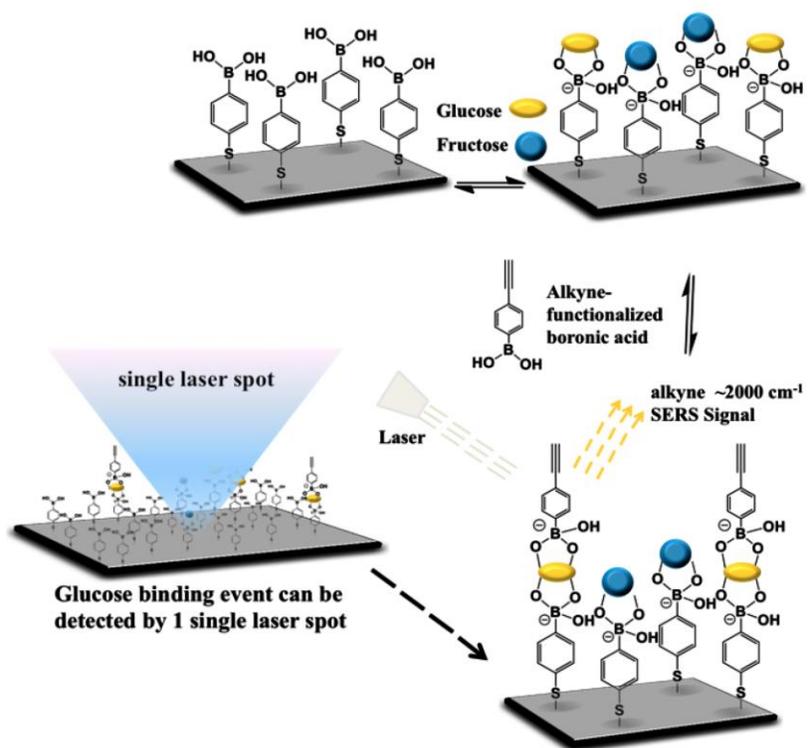
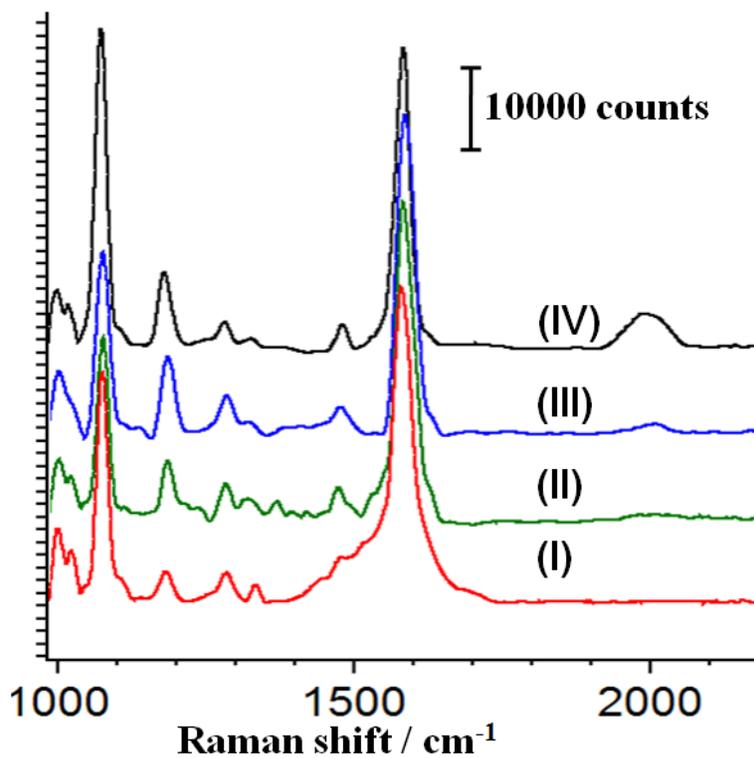
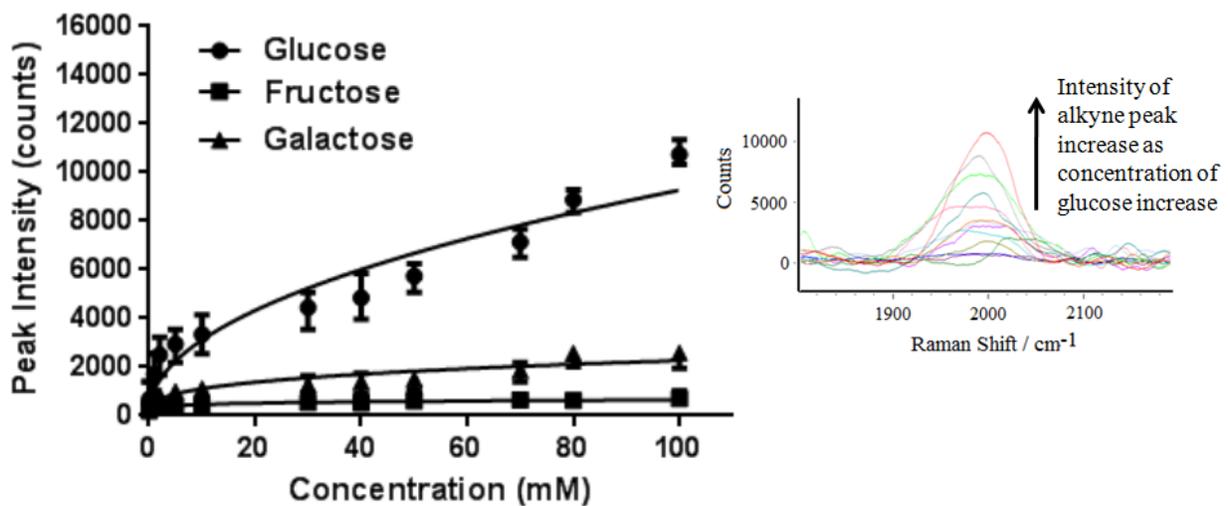


Figure 4



(A)



(B)

Figure 5

