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Wanted Dead or Alive: Novel Technologies for Measuring Infectious Norovirus Particles

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Foreword

Noroviruses are common causes of gastroenteritis in humans. As with all pathogens with an oral – faecal transmission path, food can easily become contaminated. Oysters in particular can become contaminated with norovirus due to effluent flows into estuaries. Hence, the importance of fast and accurate tests for the presence of norovirus in oysters is constantly increasing.

Currently, testing for foodborne viruses are laboratory based molecular methods, which although sensitive, do not discriminate between infective and non-infective viral particles. Biosensors (biological sensors) may be able to overcome this barrier and might be suitable for real-time sensing and on-site monitoring.

This project aimed to develop a functional biosensor for the detection of norovirus in shellfish.

Because human noroviruses cannot be grown *in-vitro*, this project used cultivatable murine norovirus as a substitute.

Two sensing platforms, electrochemical and gold nanoparticle, were developed for the detection of murine norovirus using a homologous aptamer as the bioreceptor. Both biosensor platforms gave similar sensitivity of detection in buffer and had significantly greater sensitivity than previously reported biosensors for norovirus.

Furthermore, the electrochemical biosensor was found to be functional in diluted oyster extract and was able to quantify murine norovirus in seeded oyster tissue at concentrations of relevance to industry. Importantly, detection and quantification of murine norovirus using the electrochemical aptasensor correlated with the detection of infectious virus by *in-vitro* cell culture.

This project has proven the concept of biosensor testing for foodborne noroviruses. Further work is now needed to develop and commercialise a biosensor test for human norovirus.

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Abbreviations

AFM	atomic force microscopy
cfu	colony forming units
DLS	dynamic light scattering
DMEM	Dulbecco's modified eagle's medium
DPV	differential pulse voltammetry
DT	digestive tissue
EC	European commission
EFSA	European Food Safety Authority
EIS	electrochemical impedance spectroscopy
ELISA	enzyme linked immunosorbent assay
EU	European Union
FCV	feline calicivirus
FBS	fetal bovine serum
GAR-HPR	goat anti rabbit horse radish peroxidase
GNP	gold nanoparticles
GSB	general sensing buffer
HACCP	hazard analysis critical control points
HAV	Hepatitis A virus
HBGA	histoblood group antigen
ID_{50}	median infectious dose
LB	Luria-Bertani media
LBA	Luria-Bertani agar
LOD	limit of detection
LOQ	limit of quantification
MNV	murine norovirus
MOI	multiplicity of infection
MS2	MS2 bacteriophage
MW	molecular weight

NAA	nanoporous anodic alumina
NoV	human norovirus
ORF	open reading frame
PBS	phosphate buffered saline
PBST	phosphate buffer saline tween
PCR	polymerase chain reaction
pfu	plaque forming units
pSi	porous silicon
qRT-PCR	quantitative reverse transcription polymerase chain reaction
R_{ct}	charge transfer resistance
RNA	ribonucleic acid
RU	response units
SM-PBST	skim milk phosphate buffered saline tween
SPR	surface plasmon resonance
TCID	tissue culture infective dose
TEM	transmission electron microscopy
TIRF	total internal reflection fluorescence
TMB	3,3',5,5'-Tetramethylbenzidine
TYGB	tryptone yeast glucose broth
VLP	virus like particles
VP1	viral protein 1

Executive Summary

Scientific researchers in the fields of foodborne virology, electrochemical transducer development and nanotechnology from SARDI Food Safety and Innovation, The University of South Australia and RMIT University have been able to develop rapid and sensitive proof-of-concept biosensors for the detection of cultivable murine norovirus (MNV). Currently, the identification of human norovirus in foods (oysters, leafy greens and berries) is reliant on detection of the viral genome by PCR technology. Although this method offers the best sensitivity available for detection of foodborne contamination (<100 virus genomes per gram food matrix), it has the disadvantage that it does not distinguish between infective and non-infective particles, and may not be directly associated with foodborne risk. The methodology is also specialised, requiring samples to be sent to a laboratory with the appropriate capability and infrastructure, as well as being costly. Two successful biosensing platforms, (1) electrochemical gold electrode porous alumina and (2) gold nanoparticle, were developed for the detection of MNV using an aptamer bioreceptor. MNV was used as a surrogate for human NoV in this study as, until recently, it has been the only norovirus which has been able to be cultured *in-vitro*, hence enabling investigations of infective virus to be undertaken. Both developed biosensor platforms had similar sensitivities for detection of virus in buffer and were more sensitive than reported for other norovirus or norovirus surrogate biosensors. The developed electrochemical biosensor also demonstrated application for detection of MNV in diluted oyster extract, although further work to optimise oyster extraction methodology is required to minimise sample matrix effects on sensor performance. Detection and quantification of MNV using the electrochemical aptasensor correlated with the detection of infectious virus by cell culture.

Background

Foodborne gastroenteritis is a significant cost to society. Norovirus (NoV) is the leading cause of non-bacterial gastroenteritis worldwide and is highly contagious, spread primarily via the faecal to oral route. Faecal contamination of shellfish production areas, especially near highly urbanised locations, results in increased risk of NoV accumulating in filter feeding shellfish. NoV has been implicated as the leading cause of viral gastroenteritis associated with consumption of raw or undercooked oysters. Ensuring product safety is crucial in maintaining consumer confidence, as well as maintaining and growing export markets. The European Commission (EC) regulations on the microbiological criteria for foodstuff state that "criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently". Currently, the gold standard for testing for foodborne viruses is quantitative reverse transcription PCR (qRT-PCR). Despite the increased sensitivity of qRT-PCR methods for detecting NoV, the risk associated with illness from consuming contaminated food is not clear as viral copies detected by PCR are not directly related to infective viral particles. Furthermore, the current methodology requires laborious sample preparation and testing in a diagnostic laboratory by specialised personnel. Internationally, there is considerable research being undertaken developing rapid diagnostics based on biosensors (biological sensors). Potentially, biosensors provide a powerful means of detecting pathogens with the advantages of high sensitivity, high specificity, real-time sensing and on-site monitoring.

Aims/objectives

Generation of a functional biosensor for the detection of norovirus.

Methodology

Human NoV has not been able to be reliably cultivated *in-vitro*, *w*hich has significantly hampered investigation on its infectivity. Murine norovirus (MNV), a species of norovirus affecting only mice and easily cultured *in-vitro*, was used as a NoV surrogate in this study. In addition, non-infectious human NoV virus like particles (VLP) (empty virus particles morphologically and antigenically indistinguishable from

native virus yet unable to infect and cause disease due to the lack of a viral genome) were engineered in the laboratory to a clinical human strain of NoV (GII.4 Sydney) circulating within the Australian community in 2012. Both the non-infective NoV VLP and cultivable MNV were used as targets for the biosensor development. Bioreceptors to NoV VLP and MNV were developed or identified and evaluated for target capture. These included polyclonal antibodies developed in-house, synthetic histoblood group antigens and aptamers. Various transducer sensor platforms were investigated and included commercial and experimental optical fibre surface plasmon resonance (SPR), electrochemical and gold nanoparticles (GNP).

Results/key findings

This project has been successful in the development and demonstration of functional biosensors for the detection and quantification of MNV. More importantly, functionality has been demonstrated using oyster tissue extracts. Two different sensing platforms, electrochemical and GNP, were developed for the detection of MNV using a homologous aptamer as the bioreceptor. Both developed biosensor platforms reported similar LODs (10 pfu/mL; electrochemical and 240 pfu/mL; GNP) in buffer. The sensitivity of detection of the developed biosensors are significantly greater than for previously reported biosensors for norovirus or virus surrogate detection. Furthermore, the electrochemical biosensor developed demonstrated application for detection in diluted oyster extract with an LOD of 52 pfu/mL and was able to quantify MNV in seeded oyster tissue at concentrations greater than 1,000 pfu/mL. Further work is required to optimise oyster extraction methodology to eliminate the need for oyster extract dilution prior to sensor application. Detection and quantification of MNV using the electrochemical aptasensor 6,300 virions/mL or 3,073 virions/g oyster DT) correlated with the detection of infectious virus by *in-vitro* cell culture (5,103 pfu/mL or 2,298 pfu/g oyster DT). Quantification of MNV by the qRT-PCR of a sub-sample of this material resulted in an overestimation by several fold, indicating a misrepresentation when trying to investigate infectivity by PCR based methods.

Implications for relevant stakeholders

Current PCR technology for measuring NoV in oysters is relatively expensive and time consuming to be used for routine monitoring in Australia; it is more commonly used in the management of sewage spills in oyster growing areas and trace back in the event of an oyster-related foodborne outbreak. Many contamination events arise from treated or partially treated sewage, where a significant portion of the viral particles present are likely to be non-infectious, and PCR is likely to overestimate the risk.

This project has developed a rapid, sensitive new technology for the detection and quantification of potentially infectious norovirus (murine) in oysters. With further development and extension to human NoV, the technology could be used to ensure food safety standards are met, in turn increasing export potential and market access of product. The developed technology could also provide opportunity to market point-of-care diagnostic devices, enabling industry to be more proactive in managing food safety issues. The developed biosensor(s) would be a rapid diagnostic with potential for improved sensitivity of detection, eliminating the need and cost associated with sending samples to a specialised laboratory for analysis. Rapid on-site analysis would allow instantaneous decision making throughout the supply-chain, potentially avoiding the large-scale outbreaks often associated with NoV in bivalve shellfish. The biosensor technology could also be marketed internationally, generating revenue to the Australian industry.

Specific advantages of a biosensor able to detect only infectious virus would be the reduction of “false” positives from non-infectious virus, which do not pose a health risk, and the potential for on-site real-time monitoring. If progressed and commercialised this new tool would improve risk management options available to industry and regulators and decrease the risk of illness to consumers. Furthermore, such a diagnostic tool could be adapted for other food matrices commonly associated with NoV, such as leafy greens and berries.

Recommendations

Further work to refine and optimise the developed electrochemical aptasensor is recommended, as it has shown the greatest potential for use as a rapid on-site diagnostic for the quantification of MNV in oyster samples. However, this biosensor is still a proof-of-concept as detection of infective human NoV has not been realised. The major hurdle in realising this has been the inability to culture human NoV strains until very recently (2016). With the significant advance in this knowledge further modification of the sensor for human NoV detection should be possible.

Further work required to achieve this is to:

- Establish capability for human NoV culture at SARDI Food Safety and Innovation via a sabbatical in a collaborating laboratory with significant knowledge in the technique.
- Develop human NoV aptamer bioreceptors to cultured human NoV.
- Refine virus extraction in the oyster matrix to reduce interference on biosensor performance.
- Identify new modification strategies, based on differential functionalisation of external and internal surface of the electrochemical aptasensor, to minimise matrix effects when analysing oyster samples.
- Investigate the impact of decreased pore size and thickness of the nanoporous anodic alumina membrane of the electrochemical aptasenor in achieving improved analytical performance in sensitivity, limit of detection and analysis time.
- Undertake virus inactivation experiments to confirm specificity of methods for detection of infectious NoV.
- Investigate options for commercialisation of electrochemical aptasenor platform for NoV detection and quantification.

Keywords

Norovirus, Murine norovirus, Aptamer, Biosensor, Oyster

1. Introduction

A systematic review of global shellfish related viral foodborne outbreaks between 1980 and 2012 reported NoV (83.7%) and HAV (12.8%) as the most common viral pathogens and oysters (58.4%) as the most frequently consumed shellfish associated with outbreaks (Bellou et al., 2013). NoV outbreaks following shellfish consumption are attributed to contamination of growing waters by human faeces and consumption of raw or lightly cooked product (Bellou et al., 2013). In 2012 the Codex Alimentarius Commission released guidelines on general principles of food hygiene to control viruses in food, with Annex I specifically focussing on control of HAV and NoV in bivalve molluscs (FAO/WHO, 2012). It recommended that countries monitor for NoV and HAV in bivalves following shellfish-related foodborne outbreaks and high-risk pollution events (heavy rainfall and overflow from sewage treatment plants).

The European Commission (EC) regulations on the microbiological criteria for foodstuff states that "criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently" (EC, 2005). With the development of the ISO/TS 15216 method *Microbiology of food and animal feed- horizontal method for the determination of hepatitis A virus and norovirus in food using real-time RT-PCR*, virus methods have become available that may be considered suitable for use in regulation (ISO/CEN, 2013). Hence, risk managers are considering establishing virus limits for high-risk live bivalve molluscs. The EFSA Scientific Opinion on NoV in oysters recommended: the establishment of an acceptable limit for NoV in oysters to be harvested and placed on the market; NoV testing of oysters to verify compliance with the acceptable NoV limits established; and for food businesses to verify their hazard analysis and critical control points (HACCP) plans and demonstrate compliance with the acceptable levels (EFSA, 2012). In 2012 the EU Community Reference Laboratory recommended that if virus standards are introduced then standards for NoV should be quantitative (i.e. a maximum acceptable level be determined) and standards for HAV be qualitative (i.e. presence/absence) (CEFAS, 2013). It also considered and made recommendations on possible levels for a NoV standard in the context of both end-product and production area monitoring applications (CEFAS, 2013). The EC recently sponsored a European baseline survey of NoV in raw oysters at production and dispatch centres, to be undertaken over a two year period (EFSA, 2016). Pending the results of that survey, the EC will determine if and what NoV microbiological criteria will be introduced for oysters. A national survey of foodborne viruses in Australian oysters has already been undertaken at production during 2014-2015 (Torok et al., 2015). A total of 33 oyster production regions were involved in the survey and samples were only collected from leases that were fit for human consumption from an enteric virus perspective. During the survey period, no NoV or HAV were detected in Australian oysters resulting in an estimated prevalence of NoV and HAV of <2% with a 95% confidence interval. These results show that the Australian oyster industry is producing a safe product with limited risk of viral contamination.

The genus *Norovirus* (family *Caliciviridae*) contains a single species, Norwalk virus (de Graaf et al., 2016). This species is divided into at least six genogroups and further sub-divided into at least 30 genotypes. Viruses in the genus *Norovirus* have been found in a wide range of hosts including humans, pigs, cattle, sheep, felines, canines, sea lions and bats. Human strains capable of causing viral gastroenteritis are classified into genogroups GI, GII and GIV, with the GI and GII strains more commonly known to infect humans than the GIV strains. Ovine and bovine strains are classified into genogroup GIII, while porcine and murine strains are classified into genogroups GII and GV, respectively (Karst, 2010). The nomenclature of genogroups GIV, GVI and GVII have not been consistent; genogroups GIV and GVI have been previously classified as genogroup GIV, while strains of NoV in genogroup GVII have also been classified as belonging to genogroup GVI (de Graaf et al., 2016).

Noroviruses are single stranded ribonucleic acid (RNA), non-enveloped viruses. Genogroups comprise a range of genetically and antigenically diverse strains which can be further divided into genotypes or genetic clusters (Karst, 2010). This high degree of variability is likely one factor that complicates

protective NoV immunity. The human NoV consists of a 7.7 kb RNA genome and includes three open reading frames (ORFs). When visualised by electron microscopy the virus is 26 to 34 nm in diameter, round, with an amorphous surface and ragged outer edge (Donaldson et al., 2010; Le Pendu et al., 2006). ORF1 is located in the first two-thirds of the genome and encodes a ca. 200 kDa polyprotein that is co-translationally cleaved by the viral protease to yield the non-structural and replicase proteins essential for viral replication (Donaldson et al., 2010; Parra et al., 2012). ORF2 encodes the 57 kDa major structural capsid protein (VP1) while ORF3 (ca. 0.6 kb) encodes a 22 kDa minor basic structural protein (VP2). The NoV capsid is composed of 90 dimers of the VP1 monomer (Hardy, 2005; Le Pendu et al., 2006). The variable region of the VP1 interacts with synthetic carbohydrates corresponding to various ABH histoblood group antigens (HBGA) (Donaldson et al., 2010; Le Pendu et al., 2006; Parra et al., 2012). The expression of ORF2 (VP1 gene) in insect cell lines results in the formation of non-infectious (empty) virus-like particles (VLPs) which have been shown to be morphologically and antigenically similar to native NoV virions.

The majority of all non-bacterial gastroenteritis outbreaks are caused by human NoVs (de Graaf et al., 2016). These viruses are highly infectious. Transmission occurs by the faecal-oral route, either through contact with infected individuals or through exposure to contaminated food and water or to infectious aerosols (vomitus). The median infectious dose (ID_{50}) of NoV is low and has been estimated to be between 18 to 1,015 genome copies (Teunis et al., 2008). More recently, the ID_{50} has been reported to be higher (1,320 to 2,800 genome equivalent copies) than previously estimated and similar to that of other RNA viruses (Atmar et al., 2014). The virus is shed at high levels (10^4-10^{11} viral genomic copies/g) in the faeces and (10^4 viral genome copies/mL) in the vomitus of infected individuals (Atmar et al., 2008; Atmar et al., 2014). In Human NoV challenge experiments, susceptible subjects demonstrated a dose-dependent probability of becoming ill, ranging from 0.1 (at a dose of 10^3 genomes) to 0.7 (at 10^8 virus genomes) (Teunis et al., 2008).

Currently, the gold standard for testing for foodborne viruses is quantitative reverse transcription PCR (qRT-PCR). Despite the increased sensitivity of qRT-PCR methods for detecting NoV, the risk associated with illness from consuming contaminated food is not clear as viral copies detected by PCR are not directly related to infective viral particles as non-encapsidated RNA and degraded viral RNA are also detected (EFSA, 2011). Furthermore, the relationship between the number of infectious virus particles and the number of virus genome copies detected by qRT-PCR is not a constant and may vary depending on environmental conditions including time from the initial release from the host (EFSA, 2012). As the number of genome copies detected by qRT-PCR are not directly related to infectious NoV particles, these methods can only be used to provide an indirect measure of risk. When considering what is an acceptable level of NoV in oysters it is also important to realise that the infection risk associated with low level contaminated oysters as determined by qRT-PCR may be overestimated (EFSA, 2011). Detection of high levels of norovirus RNA in oysters is indicative of a significantly elevated health risk. However, illness may not necessarily be reported after consumption when detection of NoV RNA indicates low levels in the consumed food (Lowther et al., 2010). This may be due to the fact that the low levels of NoV RNA detected may not have been obtained from infectious virus particles or that the epidemiological surveillance systems used were not sensitive enough to capture associated illness. Despite these limitations, qRT-PCR is the most advanced and sensitive methodology currently available.

Any new diagnostic methodology for detection of NoV in oysters (and other food matrices) will need to have a level of sensitivity which is greater than, or at least equal to qRT-PCR; ideally, the detection limit should be lower than the median infective dose. Any new methodology should also give a clearer association between detection of NoV and the presence of infectious virus particles. It would also be desirable that the methodology be faster to perform than qRT-PCR as currently the best-case scenario for NoV testing is two to three working days due to the multistep process required (dissection of the digestive tissues, purification of the virus from tissue matrix, extraction of nucleic acid and qRT-PCR setup). New methodologies would need to be less costly than the current qRT-PCR detection technique to encourage uptake and pro-active monitoring by industry. Achieving all of these criteria would be ideal, however, achieving any one or a combination of them would be a significant advance on existing methodology.

Internationally, there is considerable research being undertaken into developing rapid diagnostics based on biosensors. Potentially, biosensors provide a powerful means of detecting pathogens with the advantages of high sensitivity, high specificity, real time sensing and portability, and allowing on-site monitoring. A biosensor or biological sensor is a device which converts certain properties of the target of interest into a detectable response with real-time application. The main components of a biosensor are the "bioreceptor", which directly interacts with the target, and the "transducer", which transforms this interaction into a physical or chemical response. The transducer determines the effectiveness of the signal processing and the output of the biosensor, while the bioreceptor determines the selectivity of the biosensor. The ultimate advantages of using biosensors for detection of foodborne pathogens would be their real-time application in the field along with their ability to discriminate between infectious and non-infectious NoV, enabling immediate decision making on product safety. The challenges with developing a biosensor will be sensitivity of detection and application to real-life sample matrices.

This project investigated several bioreceptors for the capture of NoV including synthetic histoblood group antigens, polyclonal antibodies and apatmers (single-stranded oligonucleotides that fold into distinct three-dimensional conformations capable of binding strongly and selectively to a target molecule). Norovirus targets investigated were non-infective human NoV VLP and the cultivable MNV surrogate. A significant challenge with studying human NoV biology has been the recalcitrant nature of its culture *in-vitro,* unlike MNV, which has been used as a surrogate in numerous studies on virus infectivity. Although, infective human NoV culture has not been widely possible, the production of NoV VLP have been widely reported since their first production in 1992 (Jiang et al., 1992).

2. Objectives

The original objective of the project was to:

- Develop sensor surface functionality of the surface plasmon technology to improve discrimination between infectious and non-infectious noroviruses.

A variation to the original objective was sought and granted with the new objective being:

- Generation of a functional biosensor for the detection of norovirus.

3. Methods

3.1 Literature review

Two independent literature reviews were undertaken at the onset of the project. The topics were:

- Foodborne viruses in shellfish and current detection methodologies
- Biosensing technologies for virus detection.

The purposes of these reviews were to identify:

- previous research into diagnostic methods available for foodborne viruses in shellfish;
- challenges associated with their application for NoV detection in shellfish; and
- promising biosensing technologies which could be utilised for infective virus detection in shellfish samples.

3.2 Antigenic targets for sensor development

3.2.1 Cultivable murine norovirus (MNV) surrogate

The murine norovirus 1 (MNV-1, ATCC® number PTA-5935™) was propagated in RAW 264.7 (ATCC® number TIB-71) mouse macrophage cells (Gonzalez-Hernandez et al., 2012). The RAW 264.7 cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM)-10 containing 10% fetal bovine serum (FCS), 1% Glutamax and 1% penicillin/streptomycin (Gibco reagents). MNV-1 was propagated by infecting flasks of confluent RAW 264.7 cells with virus suspension with a multiplicity of infection (MOI) of 1 and incubation for 60 min at 37°C and 5% CO_2 with gentle rocking. DMEM-10 (15 mL) was then added to each flask and incubated for a further 24 to 48 h until cell lysis. Media and debris were collected and centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was collected and the virus titre determined by plaque forming assay as follows. RAW 264.7 cells were seeded into 6-well plates at a density of 1×10^6 viable cells/mL in DMEM-10 and incubated overnight at 37 C and 5% CO_2. The above MNV-1 virus preparation was serially diluted ten-fold to 10^{-8} in a 24-well plate in DMEM-5 (DMEM, 5% FCS, 10 mM HEPES, 1% Glutamax, 1% penicillin/streptomycin). Medium was removed from RAW 264.7 cells and 0.5 mL of the virus dilution series were added to the wells in duplicate and incubated for 1 h at room temperature. Following incubation, the virus inoculum was removed and 2 mL of molten agarose overlay preparation was added to each well and incubated for 48-72 h at 37°C and 5% CO_2. Neutral red staining solution was added to each well and incubated for 1-3 h at room temperature and the virus titre calculated in plaque forming units per mL (pfu/mL).

3.2.2 Non-infectious human norovirus virus like particles (VLP)

3.2.2.1 NoV GII.4 Sydney VLP

VLP are empty, non-infectious NoV capsids which are morphologically and antigenically similar to the native infectious virus. VLPs can be produced in the laboratory by expression of the viral coat protein gene in eukaryotic cell lines. Under these conditions the expressed viral coat protein self-assembles into VLP.

Generation of NoV ORF2

A characterised NoV strain (GII.4 Sydney) isolated from an Australian clinical faecal sample was provided by Dr Rodney Ratcliff (SA Pathology). Nucleotide sequence (2,045 bp) data encompassing the entire ORF2 (encoding the major VP1 coat protein) and flanking regions were also provided by Dr Ratcliff. FramePlot 2.3.2 (http://www0.nih.go.jp/~jun/cgi-bin/frameplot.pl) was used to identify the beginning and end of ORF2 and translate the nucleotide sequence from ORF2 into VPI coat protein sequence. RT-PCR primers were designed to amplify NoV GII.4 Sydney ORF2 and assist cloning into the expression vector pFastBac (Invitrogen Life Technologies). Reverse transcription (RT) was done by initially incubating 4 µL of total nucleic acid, 0.5 µM RAH-NoV-RT primer (Table 1) and 1 mM dNTP in a total volume of 6.5 µL at 65°C for 5 min and then rapidly cooling to 4°C. To this 1 X strand buffer, 10 mM DTT, 20 U RNase Out and 100 U Superscript III (Invitrogen Life Technologies) were added to a total volume of 10 µL and incubated at 42°C for 60 min, followed by cooling to 4°C. PCR was done using 2 µL cDNA, 1 X HF buffer, 1 µM dNTP, 0.5 µM each of RAH-NoV-F-EcoRI and RAH-NoV-R-XbaI (Table 1), 3% v/v DSMO, 0.02 U Phusion DNA Polymerase (New England Bio Labs) in a final volume of 20 µL. PCR cycling conditions were initial denaturation at 98°C for 1 min followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, with a final extension of 72°C for 10 min. The resulting amplification product was electrophoresed on a 1% agarose gel in 1 X TAE and a DNA band corresponding to approximately 1,700 bp was excised, purified using an Isolate PCR and Gel Kit (Bioline) according to the manufacturer's recommendation and the recovery of the desired fragment confirmed by gel electrophoresis.

Production of recombinant pFastBac NoV ORF2 plasmid and bacmid

Competent *Escherichia coli* JM109 cells (Promega) were transformed by heat shock with pFastBac vector and pFastBac Gus control vector (Bac-to-Bac Baculovirus Expression System; Invitrogen Life Technologies) according to the manufacturer's recommendations, plated onto Luria-Bertani agar (LBA) plates containing 100 µg/mL ampicillin and incubated overnight at 37°C. Single colonies were picked and grown in 10 mL Luria-Bertani (LB) media containing 100 µg/mL ampicillin and incubated shaking overnight at 37°C. Plasmids were purified from 3 mL overnight culture using the Isolate Plasmid Mini Kit (Bioline) according to the manufacturer's recommendations. Recovery of plasmid was confirmed visually using gel electrophoresis.

Both the pFastBac vector (8 µL) and the purified NoV GII.4 Sydney ORF2 PCR product (20 µL) were digested with restriction endonucleases *Xba*I (10 U) and *Eco*RI (10 U), 1 X NEB4 buffer (New England Biolabs) in a total volume of 30 µL and incubated at 37°C overnight. Residual endonuclease activity was deactivated by incubation at 65°C for 10 min. Restricted PCR product was purified directly, while restricted plasmid was gel purified using the Isolate PCR & Gel Kit (Bioline) according to the manufacturer's recommendation. Recovered products were confirmed by gel electrophoresis. The NoV GII.4 ORF2 was ligated into the pFastBac by adding 1 X ligase buffer, 1 µL restricted pFastBac, 5 µL restricted NoV GII.4 ORF2, 3 U T4 DNA ligase (Promega) in a total volume of 10 µL and incubated at 4°C overnight. Following ligation, plasmids were transformed into competent JM109 *E. coli* cells and plated on ampicillin containing LBA as outlined above. Several single bacterial colonies were grown in 10 mL LB supplemented with ampicillin and plasmids purified as outlined above.

Successful ligation of NoV GII.4 ORF2 into pFastBac was checked by PCR using primers (pFastBac Fwd and pFastBac Rev; Table 1) targeting the plasmid and flanking the insertion site. PCR was done by diluting plasmids 1/100 and adding 5 µL to 1 X reaction buffer II, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.4 µM each primer and 1 U AmpliTaq (Applied Biosystems) in a total volume of 25 µL. PCR cycling conditions were as outlined above and amplification products were checked by gel electrophoresis.

Plasmids confirmed as containing the desired insert size were sent for confirmation by Sanger sequencing. Three individual plasmids (clones) were sent to the Australian Genome Research Facility

(Adelaide) for sequencing. Each plasmid was sequenced with pFastBac Fwd, pFastBac Rev, NoV 1765R, NoV 624F and NoV 1104F (Table 1).

Recombinant pFastBac NoV ORF2 plasmid was transformed into DH10Bac (Invitrogen) competent *E. coli* using heat shock according to the manufacturer's recommendations and plated onto LBA plates containing 50 µg/mL kanamycin, 7 µg/mL gentamycin sulphate, 10 µg/mL tetracycline, 0.5 µM IPTG and 80 µg/mL X-Gal. The DH10Bac competent *E. coli* cells contain a bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid can transpose to the mini-attTn7 site on the bacmid in the presence of the transposition protein provided by the helper plasmid. Colonies containing recombinant bacmid were identified by disruption of the lacZ gene resulting in white colonies. Confirmed white clones were grown in 10 mL LB containing 50 µg/mL kanamycin, 7 µg/mL gentamycin and 10 µg/mL tetracycline at 37°C overnight. Recombinant bacmid was purified from 3 mL of overnight culture using PureLink HiPure plasmid DNA purification kit (Invitrogen) according to the manufacturer's recommendations. Transformed bacmids were checked by PCR for the presence of the expected size insert. Bacmid diluted 1/10 (5 µL) was added to 1 X buffer II, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 0.25 µM each pUC/M13 forward and pUC/M13 reverse primers (Table 1) and 1U AmpliTaq (Invitrogen) in a total volume of 20 µL. PCR cycling conditions were initial denaturation of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 45 min and extension at 72°C for 3 min. PCR products were checked by gel electrophoresis for expected transposed insertion size.

Table 1: Primers used in construction and validations of NoV ORF2 recombinant plasmid and bacmid.

Primer	Sequence
RAH-NoV-RT	GACATCAGATGCCAATCCAGC
RAH-NoV-R-XbaI	TCTTCTAGATTATAGTGCACGTCTACGC
RAH-Nov-F-EcoRI	AGCGAATTCAAATGGCGTCGAGTGAC
NoV 2012 1752 R	GTGTAAAACTGGTTGACCCAGG
NoV 2012 624 F	CCCCAGCCAGGTCACTATGTTC
NoV 2012 1104 F	CATATCACAGGTAGTCGTAACTACACAATG
pFastBac Fwd	TATTCCGGATTATTCATACCGTC
pFastBac Rev	GTATGGCTGATTATGATCCTC
pUC/M13 forward	CCCAGTCACGACGTTGTAAAACG
pUC/M13 reverse	AGCGGATAACAATTTCACACAGG

Production of baculovirus recombinants and insect cell infection

Insect (*Spodoptera fugiperda*) cells (Sf9) passage 2 (P2) at a concentration of 20 x 10^6 cell/mL in 7.5% DSMO were provided by Dr Kelly Hill (SARDI). Cryopreserved Sf9 cells were thawed and diluted to 1 X 10^6 cells /mL in sf-900™II SFM (Life Technologies) media containing 50 U/mL penicillin and 50 µg/mL streptomycin and incubated at 27°C in an orbital shaker. Cells were passaged every 2-3 days to maintain a cell count of 0.5-1 X 10^6 viable cells/mL. Transfection was done by incubating 1-2 µg bacmid in 2 mL sf-900™II SFM and 15 µL Cellfectin® (Invitrogen) at ambient temperature for 15-45 min. Following incubation the solution was added to a pellet of Sf9 cells, re-suspended and incubated shaking at 27°C for 5 h. The solution was centrifuged at 3000 rpm for 5 min, supernatant removed and pellet re-suspended in sf-900™II SFM medium containing penicillin and streptomycin and incubated 48-72 h shaking at 27°C. Cells were centrifuged at 5000 rpm for 5 min and supernatant retained as P1 baculovirus stock. Stock was amplified by passage to increase virus titre. Cells were infected using a MOI of 2 into 200 mL of sf9 cells (2 x 10^6 cells/mL) containing penicillin, streptomycin and 4% FBS and incubated at 27°C with shaking for 5 days until viability of cells dropped below 30%. Cells were centrifuged at 1,500 rpm for 5 min and supernatant collected.

VLP purification

5 ml of 25% sucrose was added to 25 mL supernatant as an underlay and centrifuged at 18,000 rpm (JA-18 rotor, Beckman Avanti J-25I) for 5 h at 4°C. The supernatant was discarded and pellets re-suspended in 1.5 mL PBS overnight at 4°C. A further 1.5 mL CsCl (1.6 g/mL) was added and the solution was ultra-centrifuged at 48,000 rpm (TLA 100.3 rotor, Beckman TL-100) for 18 h at 15°C. Following centrifugation, the opaque VLP band was collected with a syringe under gradient light. Samples were dialyzed in 10,000 MWCO dialysis tubing, floating in 2 L PBS at 4°C for 4 h stirring and then overnight in fresh PBS.

3.2.2.2 Electron microscopy

VLP were visualised using transmission electron microscopy (TEM) by Lyn Waterhouse, Adelaide Microscopy, University of Adelaide. Commercial copper (GSCu 200CH-100) grids were used and the virus preparations were stained with 2% uranyl acetate.

3.2.2.3 Quantification of VLP

As VLP are non-infective, the concentration cannot be estimated using a plaque forming assay. The concentration of VLP is measured by determining the protein concentration and the following formula (Le Guyader et al., 2006; White et al., 1996):

$$\text{VLP}/\mu\text{L} = \frac{\text{Protein concentration (g/}\mu\text{L)} \times 6.023 \times 10^{23}\ \text{(molecules/mol)}}{\text{Molecular Weight of VLP (g/mol or Daltons)*}}$$

*MW of one VP1 monomer is 58,000 Da and a VLP is made up of 180 monomers.

3.2.3 MS2 bacteriophage

Bacteriophage MS2 (NCO12487) was propagated in *Salmonella enterica* subsp enterica, WG49, NCTC 12484 (CEFAS, 2007). The MS2 phage titre was determined by plaque assay. Briefly, the host bacteria, *Salmonella* WG49 was cultured to early log phase in 50 mL tryptone yeast glucose broth (TYGB) containing 1% calcium/glucose solution, to a cell count of 7-40 × 10^7 colony forming units per mL (cfu/mL) within 2-6 h at 37°C. The MS2 phage stock was serially diluted to 10^{-10} in 0.1% peptone water. TYGA agar overlay aliquots, were aseptically dispensed into tubes and maintained at 48°C in a water bath. Following incubation of the *Salmonella* WG49, 1 mL of host *Salmonella* WG49 culture and 1 mL of the MS2 phage dilution was added to warm calcium/glucose supplemented TYGA agar overlay, mixed and poured onto the surface of calcium/glucose supplemented TYGA agar plates and incubated for 18 h at 37°C. The plaques were counted and the titre expressed as pfu/mL.

3.3 Generation of bioreceptors to norovirus

3.3.1 Direct Capture

3.3.1.1 Antibodies

Polyclonal NoV GII.4 Sydney antisera was raised in rabbits using NoV GII.4 VLP as an antigen. Pre-immune sera was collected from the animals prior to injection with the antigen (NoV VLP). Rabbits received an initial subcutaneous injection of purified VLP (ca. 150 µg) in Freud's complete adjuvant.

At 3, 6 and 9 weeks post initial dose of antigen, the animals received booster injections of the same VLPs in Freund's incomplete adjuvant. Polyclonal antibody production and collection was done by the South Australian Health and Medical Research Institute, Antibody Production Services in Gilles Plains, Australia. On receipt, the pre-immune and polyclonal antisera were stored at −80°C.

3.3.1.2 Histoblood group antigens

Biotinylated synthetic histoblood group antigens (HBGA) A, B and H (GlycoTech Corporation) were investigated as bioreceptors for NoV VLP.

3.3.1.3 Aptamers

The AG3 aptamer (Figure 1) specific for MNV (Giamberardino et al., 2013) was investigated as a bioreceptor for NoV GII.4 VLP capture, and used as the bioreceptor for MNV in the biosensor development. The affinity of AG3 to MNV was investigated by fluorescence anisotropy and the apparent dissociation constant (EC50) been shown to be in the low pM range (Giamberardino et al., 2013). AG3 was also shown to be selective for noroviruses over other targets i.e. ED50 for FCV was in the low µM range. AG3 was also shown to have high affinity to NoV GII.3 VLP by anisotropy, although the EC50 was not determined (Giamberardino et al., 2013).

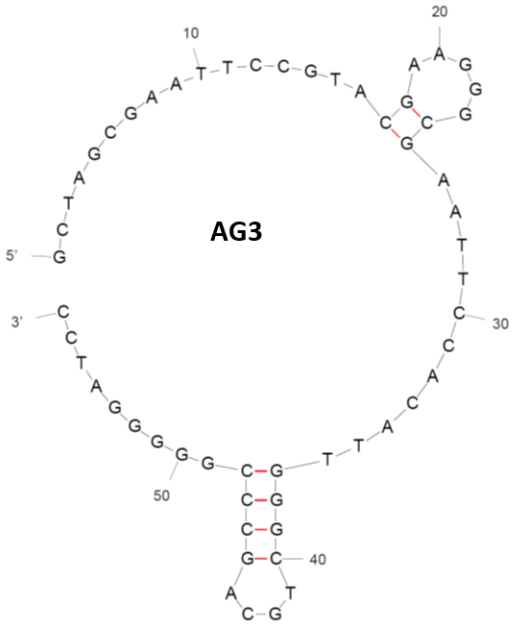

Figure 1: Mfold secondary structure prediction of AG3 aptamer (taken from Giamberardino et al., 2013).

3.3.2 In-direct Capture

3.3.2.1 Protein A

Biotinylated Protein A (Thermo Scientific) was used to capture polyclonal antibodies to NoV.

3.4 Evaluation of bioreceptors for NoV detection using ELISA

Enzyme linked immunosorbent assay (ELISA) was used to evaluate bioreceptor – target interactions. The approach taken to evaluate biotinylated bioreceptors (HBGAs, AG3 and protein A) was to coat streptavidin coated ELISA plates (Greiner bio-one) with each of these ligands (2 ug/mL in PBS) overnight at 4°C. Plates were then washed with phosphate buffered saline containing 0.05% Tween 20 (PBST), blocked with PBST containing 10% skim milk powder (10% SM-PBST). Following blocking all plates, except those coated with biotinylated protein A, were incubated with NoV GII.4 VLP target (1 µg/mL or equivalent to 5.77 X 10^7 VLP/µL). All plates were then incubated with primary NoV GII.4 antibody diluted 1:100,000 in 1% SM-PBST followed by secondary goat anti-rabbit IgG conjugated horse radish peroxidase (GAR-HPR; Thermo Scientific) diluted 1:10,000 in 1% SM-PBST and TMB substrate solution (ELISA Systems). Following each incubation step, plates were washed with 10% SM-PBST, and following substrate incubation the reaction was stopped by addition of 1M H_2SO_4. Plates were read on an ELISA reader at 450 nm.

The approach taken to evaluate pre-immune rabbit sera and polyclonal rabbit NoV GII.4 antisera was to coat VLP (1 µg/mL) in 0.06 M Na_2CO_3/$NaHCO_3$ directly onto ELISA plates (F96 maxisorp Nunc-immuno plate) overnight at 4°C. This was followed by incubation with primary sera (pre-immune or polyclonal) of various dilutions, secondary GAR-HPR antibody and substrate incubation as above.

3.5 Transducer technologies investigated

3.5.1 Experimental optical fibre surface plasmon resonance

An experimental optical fibre SPR platform developed by the University of Adelaide was used for NoV VLP detection. Sensor fabrication and detection experiments were done by Ms Elizaveta Klansataya (PhD candidate). The approach was based on that previously published for ApoE detection (Sciacca et al., 2013). The architecture of the optical fibre SPR sensor is shown in Figure 2. Functionalised silver-coated fibres were mounted in a flow cell with a hole to enable collection of light scattering at the intersection between fibre and test analyte. The flow cell was also connected to a syringe containing analyte at 90° to the fibre. A white LED light source was focused and coupled into the fibre samples and the light which scattered out from the silver-coated region was collected and analysed using a spectrometer.

Both F2 glass and silica fibres were used for sensor production. In brief, fibres were coated with silver using the Tollens reaction, followed by a polyelectrolyte layer, biotin and neutravidin layer. Subsequent functionalisation steps varied depending on the bioreceptor being used for NoV VLP capture. When polyclonal sera was used as the bioreceptor, the fibres were coated with biotinylated protein A, rabbit polyclonal NoV antisera and blocking solution. When HBGA was used as the bioreceptor, the fibres were coated in biotinylated HGBA-B. VLP analyte was then passed over the sensor surface. SPR shifts were measured at each step of the sensor fabrication using a spectrometer and LED light source.

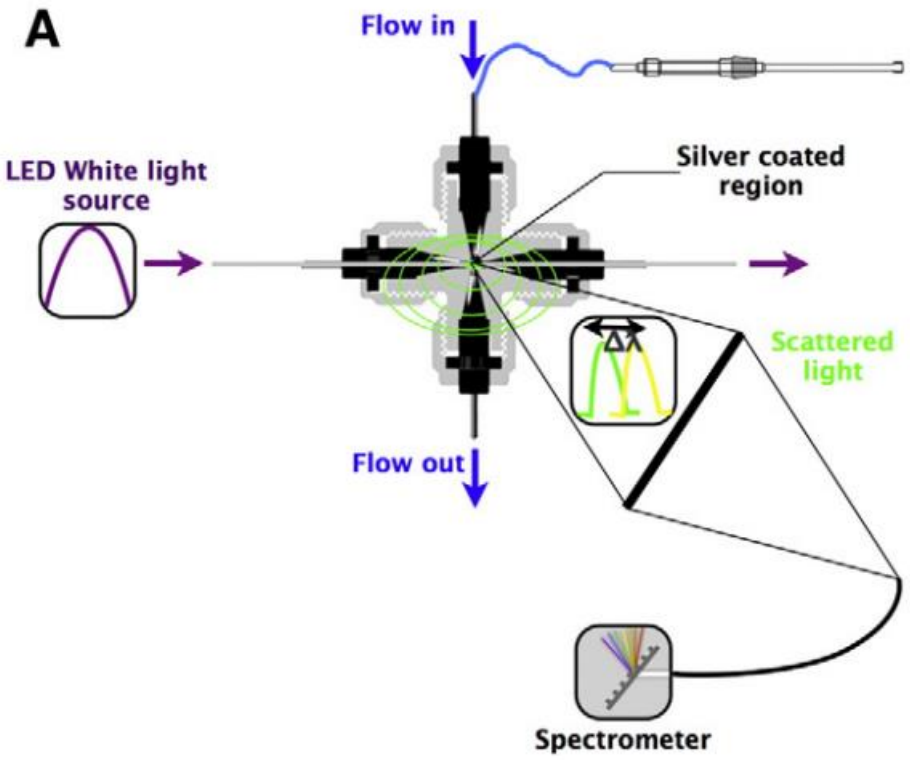

Figure 2: Representation of the optical fibre SPR transducer architecture.
The re-scattered plasmonic wave from the silver coated optical fibre is collected and analysed using a compact spectrometer (taken from Sciacca et al., 2013).

3.5.2 Commercial surface plasmon resonance

A commercial SPR sensing platform, ProteOn XPR36 array instrument (Bio-Rad Laboratories) was utilised to investigate binding affinity of various NoV bioreceptors to VLP. This work was done by Dr Olan Dolezal at the CSIRO Manufacturing Flagship, Melbourne. The bioreceptors and target used in these experiments were the same as those used in the experimental optical fibre SPR development, with the exception that MNV apatamer (AG3) was also investigated for NoV VLP capture.

Assays were performed by injecting NoV VLPs over immobilised norovirus bioreceptors. Biotinylated versions of HBGA-B and MNV aptamer AG3 were captured onto the SPR chip (GLC, Bio-Rad Laboratories) surface containing immobilised amine-coupled streptavidin. NoV rabbit polyclonal antisera were immobilization onto the sensor chip surface using a double capture approach, whereby a biotinylated Protein A was first anchored onto the streptavidin chip surface followed by the capture of antibodies.

Detailed methodology is provided in the confidential report provided by Dr Dolezal, CSIRO Manufacturing Flagship held at SARDI Food Safety and Innovation and available on request.

3.5.3 Electrochemical

Electrochemical biosensor development was done by Dr Beatriz Prieto Simon, University of South Australia. Electrochemical biosensors for the detection of norovirus were developed using graphite, gold, porous silicon-modified gold or porous alumina-modified gold electrodes and impedimetric and voltammetric measurements. A schematic of the porous silica or porous alumina modified gold

transducer is shown in Figure 3. Surfaces of electrodes were functionalised with oriented and non-oriented immobilised polyclonal NoV GII.4 antibody for the detection of NoV VLP, or immobilised AG3 aptamer for the detection of MNV. Detailed methodology is provided in the confidential reports provided by Dr Prieto Simon held at SARDI Food Safety and Innovation and available on request.

Oyster samples spiked with MNV were also analysed using the developed electrochemical sensor to determine influence of sample matrix on sensor performance and sensitivity of detection. Detection of infective virus was also confirmed by cell culture (see Evaluation of developed biosensors for detection of infective norovirus).

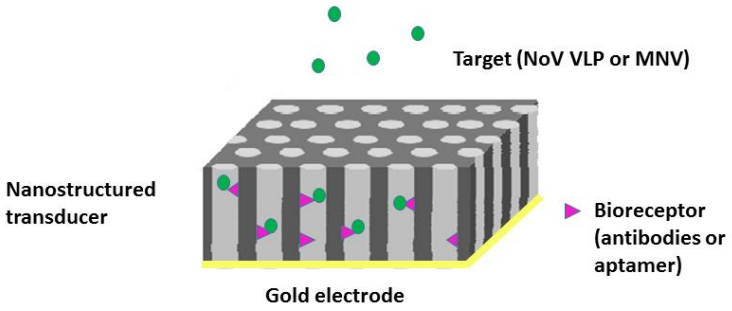

Figure 3: Schematic of nanostructured transducer

3.5.4 Gold nanoparticles

Nanoparticle sensor development was done by Professor Vipul Bansal's group at RMIT University. Recently, his group developed a 'turn-off/turn-on' biosensing approach for detection of small molecules such as antibiotics (Sharma et al., 2014) and pesticides (Weerathunge et al., 2014). The assay combines the target recognition ability of aptamers with the peroxidase enzyme-like activity (nanozyme activity) of gold nanoparticles (GNPs). Using this approach, GNPs are coated with aptamer molecules and show no nanozyme activity. However, in the presence of appropriate target, aptamers dissociate from the GNP surface to interact with the target (Figure 4). This turns-on the nanozyme activity of gold, which provides a visual colorimetric read-out in a quantitative manner. This approach was investigated for the detection of MNV, as a surrogate for human NoV, using the AG3 aptamer developed by Maria DaRosa's group (Carlton University, Ottowa) (Giamberardino et al., 2013).

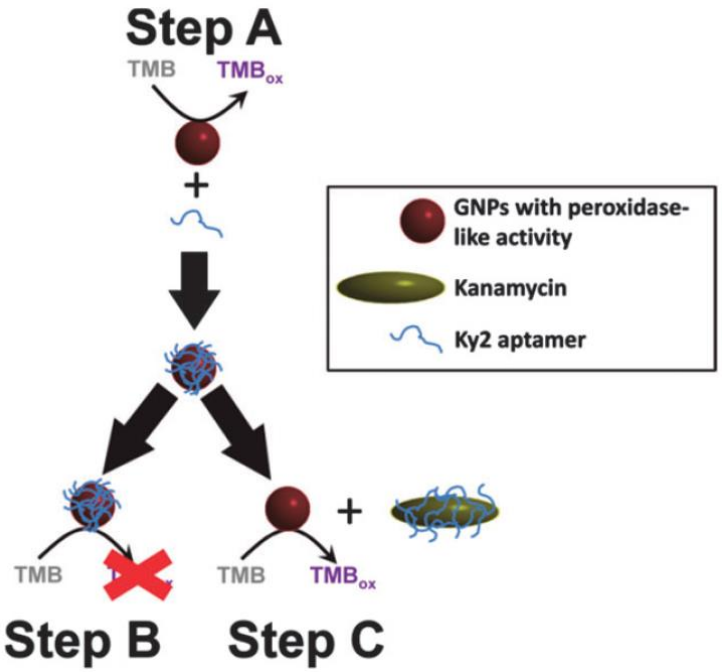

Figure 4: Schematic representation of the 'turn-off/turn-on' nanozyme activity of aptamer-functionalised GNP for the detection of kanamycin.
Step A shows the intrinsic peroxidase-like activity of GNP which are 'turned-off' after functionalisation with the Ky2 aptamer and remain 'turned-off' in the absence of kanamycin (step B). However, in the presence of kanamycin (step C) the intrinsic peroxidase-like activity of GNP is 'turned-on' again as aptamers attach to their target. Taken from Sharma *et al*., 2014.

3.6 Evaluation of electrochemical aptasensor for detection of norovirus

3.6.1 Seeding oysters with MNV

Oyster digestive tissue (DT) was removed, pooled and finely chopped. Two lots of 4 g of DT were each spiked with 20 µL of MNV at two concentrations; sample 1 (3 x 10^6 pfu/mL) and sample 2 (3 x 10^5 pfu/mL). This material was used to investigate various virus extraction methodologies to preserve virus infectivity. Replicate samples were then used to compare MNV detection and quantification using the developed electrochemical biosensor, cell culture (quantification of infective virus) and qRT-PCR (quantification based on viral genome copies).

3.6.2 Purification of MNV from oyster matrix to preserve infectivity

All purification methods investigated were variants of the glycine-threonine extraction and ultracentrifugation approach (Deqing et al., 2013; Myrmel et al., 2004; Uhrbrand et al., 2010). The method of choice was the addition of 6.7 mL chilled sterile glycine buffer (0.05 M glycine and 0.14 M NaCl buffer, pH 7.5) to the 4 g chopped DT spiked with MNV, followed by thorough mixing and centrifugation at 5,000 × g at 4°C for 20 min. The supernatant was collected, retained and the pellet was re-suspended in 5 mL of threonine buffer (0.5 M threonine, 0.14 M NaCl, pH 7.5), vortexed for 60 sec and centrifuged at 5000 × g at 4°C for 20 min. These steps were done in duplicate for each sample. The resulting supernatant was collected and combined with the first lot of supernatant and the duplicates were pooled. Three mL aliquots of pooled supernatant were centrifuged at 190,000 × g and 4°C for 2 h.

Each pellet was re-suspended in 300 µL GSB. This resulted in a total of 3.9 mL virus extract for sample 1 and 3.6 mL virus extract for sample 2, from 8 gm of oyster digestive tissue each.

3.6.3 Quantification of MNV

3.6.3.1 Cell culture

The viable MNV titre in the DT extracts was determined by plaque assay. In brief, RAW 264.7 cells were seeded into 6-well plates at a density of 1×10^6 viable cells/mL in DMEM-10 and incubated overnight at 37°C and 5% CO_2. The oyster extracts were serially diluted ten-fold to 10^{-4} in a 24-well plate in DMEM-5 (DMEM, 5% FCS, 10 mM HEPES, 1% Glutamax, 1% penicillin/streptomycin). Medium was removed from the RAW 264.7 cells and 0.5 mL of the oyster extract dilution series added to the wells in duplicate and incubated for 1 h at room temperature. Following incubation, the virus inoculum was removed and 2 mL of molten agarose overlay preparation added to each well and incubated for 48-72 h at 37°C and 5% CO_2. Neutral red staining solution was added to each well and incubated for 1-3 h at room temperature and the virus titre calculated in pfu/mL.

3.6.3.2 MNV plasmid standard for quantification

End-point MNV RT-PCR was done using the MNV-1 strain and the primers of Hewitt et al., 2009 (Hewitt et al., 2009). Five µL of nucleic acid was added to a final reaction volume of 25 µL containing 0.5 µM MNV-Fw primer (TGCAAGCTCTACAACGAAGG), 0.9 µM MNV-Rev primer (CACAGAGGCCAATTGGTAAA) in 1 X reaction mixture (RNA Ultrasense™, Invitrogen). RT-PCR cycling parameters were reverse transcription at 55°C for 1 h, 95°C for 5 min, followed by 45 cycles of 95°C for 15 sec, 60°C for 60 sec and 65°C for 60 sec. The resulting PCR product was electrophoresed on a 1.5% agarose gel and the 127 bp MNV fragment excised and purified using the Purelink Quick Gel Extraction Kit (Invitrogen) according to the manufacturer's instructions. The MNV dsDNA plasmid was constructed by ligating the purified MNV PCR product into pGEM-T vector (pGEM-T, Promega) and incubating overnight at 4°C. Competent *E. coli* (JM109) was transformed by heat shock and plated onto LBA plates containing 100 µg/mL ampicillin, 80 µg/mL X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 0.5 mM IPTG (isopropyl β-D-thiogalactopyronoside) plates. Single recombinant white colonies were inoculated into LB containing 75 µg/mL ampicillin and incubated overnight at 37°C. Plasmid DNA was extracted using the QIA prep Spin Miniprep kit (Qiagen) as per manufacturer's instructions. Recombinant MNV plasmids were confirmed by Sanger sequencing (AGRF, Adelaide).

Quantification of the dsDNA plasmid was determined using spectrophotometry (Eppendorf biospectrophotometer) from the absorbance at 260 nm. The concentration of dsDNA in copies/µL was calculated using the following formula:

$$\frac{\text{concentration of dsDNA (ng/µL)} \times 10^{-9} \times \text{Avogadro's constant}}{\text{plasmid length} \times \text{relative molecular mass of average base pair}}$$

where the recombinant plasmid length = 3,324 bp, relative molecular mass of an average base pair = 607.4 and Avogadro's constant = 6.02×10^{23}

3.6.3.3 Quantitative real-time PCR

qRT-PCR was done using the primers and probe of Hewitt et al., 2009. Five µL of nucleic acid was added to a final reaction volume of 25 µL containing 0.5 µM MNV-Fw primer, 0.9 µM MNV-Rev primer and 0.25 µM MNV-p probe in 1 X reaction mixture (RNA Ultrasense™, Invitrogen). qRT-PCR cycling parameters were reverse transcription at 55°C for 1 h, 95°C for 5 min, followed by 45 cycles of

95°C for 15 sec, 60°C for 60 sec and 65°C for 60 sec. The MNV plasmid generated above was used for quantification. qRT-PCR was done on the QuantStudio 6 (Applied Biosystems).

4. Results

4.1 Literature review

The literature review "Foodborne viruses in shellfish and current detection methods" was prepared by Valeria Torok (SARDI Food Safety and Innovation) and can be found on the SafeFish website (http://safefish.com.au/wp-content/uploads/2013/03/6.Review-of-Food-Borne-Viruses.pdf) (Appendix A). The literature review "Biosensing technologies for virus detection" was prepared by Elizaveta Klansataya (University of Adelaide, PhD candidate) and is attached as Appendix B. At the time of the reviews, detection methodologies for NoV in food and environmental samples were based on amplification and detection of the viral genome. Published reports of biosensors for NoV detection were limited to commercial SPR platforms using antibody, HBGA or HBGA-like bioreceptors and did not demonstrate the sensitivity required for detection of foodborne contamination or application to food matrices (de Rougemont et al., 2011; Shang et al., 2013; Yakes et al., 2013).

4.2 Norovirus targets

4.2.1 Non-infectious NoV GII.4 Sydney VLP

The cloned ORF2 of NoV GII.4 Sydney was sequenced resulting in 1,617 bp (Figure 5).

ATGGCGTCGAGTGACGCCAACCCATCTGATGGGTCCGCAGCCAACCTCGT
CCCAGAGGTCAACAATGAGGTTATGGCTCTGGAGCCCGTTGTTGGTGCCG
CCATTGCGGCACCTGTAGCGGGCCAACAAAATGTAATTGACCCCTGGATT
AGAAATAATTTTGTACAAGCCCCTGGTGGAGAGTTTACAGTATCCCCTAG
AAACGCTCCAGGTGAAATACTATGGAGCGCGCCCTTGGGCCCTGATCTAA
ATCCCTACCTATCCCATTTGGCCAGAATGTACAATGGTTATGCAGGTGGT
TTTGAAGTGCAGGTAATTCTCGCGGGGAACGCGTTCACCGCCGGGAAGGT
CATATTTGCAGCAGTCCCACCAAATTTTCCAACTGAAGGCTTGAGCCCCA
GCCAGGTCACTATGTTCCCCCATATAGTAGTAGATGTTAGGCAACTAGAA
CCTGTGTTGATTCCCTTACCCGATGTTAGGAATAATTTCTATCATTACAA
TCAATCAAATGACCCCACCATTAAGTTGATAGCAATGTTGTATACACCAC
TTAGGGCTAATAATGCTGGGGATGATGTCTTCACAGTTTCTTGCCGAGTT
CTCACGAGACCATCCCCCGATTTTGATTTCATATTTCTAGTGCCACCCAC
AGTTGAGTCAAGAACTAAACCATTCTCTGTCCCAGTCTTAACTGTTGAGG
AGATGACCAATTCAAGATTCCCCATTCCTTTGGAAAAGTTGTTCACGGGT
CCCAGCAGTGCCTTTGTTGTCCAACCACAAAACGGCAGGTGCACGACTGA
TGGCGTGCTCCTAGGCACCACCCAACTGTCTCCTGTCAACATCTGCACCT
TCAGAGGAGATGTCACCCATATCACAGGTAGTCGTAACTACACAATGAAT
TTGGCTTCTCAAAATTGGAACAATTATGACCCAACAAAAGAAATCCCAGC
CCCTCTAGGGACTCCAGATTTTGTGGGGAAGATTCAAGGTGTGCTCACCC
AAACCACAAGGACAGATGGCTCAACACGCGGCCACAAAGCTACAGTGTAC
ACTGGGAGCGCCGATTTTGCTCCAAAACTGGGTAGAGTTCAATTTGAAAC
TGACACAGACCATGATTTTGAAGCTAACCAAAACACAAAGTTCACCCCAG
TTGGTGTCATCCAAGATGGTGGCACCACCCACCGAAATGAACCCCAACAG
TGGGTGCTCCCAAGTTACTCAGGCAGAAATACTCCTAATGTGCACCTGGC
CCCTGCTGTAGCCCCCACTTTTCCGGGTGAGCAACTTCTCTTCTTCAGAT
CCACCATGCCCGGATGCAGCGGGTACCCCAACATGGATTTGGACTGTCTG
CTCCCCCAGGAATGGGTGCAGTACTTCTACCAAGAGGCAGCCCCAGCACA
ATCTGATGTGGCTCTGCTAAGATTTGTGAATCCAGACACAGGTAGGGTTT
TGTTTGAGTGTAAGCTTCATAAATCAGGCTATGTTACAGTGGCTCACACT
GGCCAACATGATTTGGTTATCCCCCCCAATGGTTATTTTAGGTTTGATTC
CTGGGTCAACCAGTTTTACACGCTTGCCCCCATGGGAAATGGAACGGGGC
GTAGACGTGCACTATAA

Figure 5: Nucleotide sequence of ORF2 of NoV GII.4 Sydney.
The start (AGT) and stop codons (TTA) of ORF2 are underlined.

The nucleotide sequence of NoV GII.4 Sydney strain ORF2 was translated *in-silico* into a 538 amino acid sequence with a calculate MW of 58,719 Da (Figure 6).

```
MASSDANPSDGSAANLVPEVNNEVMALEPVVGAAIAAPVAGQQNVIDPWI
RNNFVQAPGGEFTVSPRNAPGEILWSAPLGPDLNPYLSHLARMYNGYAGG
FEVQVILAGNAFTAGKVIFAAVPPNFPTEGLSPSQVTMFPHIVVDVRQLE
PVLIPLPDVRNNFYHYNQSNDPTIKLIAMLYTPLRANNAGDDVFTVSCRV
LTRPSPDFDFIFLVPPTVESRTKPFSVPVLTVEEMTNSRFPIPLEKLFTG
PSSAFVVQPQNGRCTTDGVLLGTTQLSPVNICTFRGDVTHITGSRNYTMN
LASQNWNNYDPTKEIPAPLGTPDFVGKIQGVLTQTTRTDGSTRGHKATVY
TGSADFAPKLGRVQFETDTDHDFEANQNTKFTPVGVIQDGGTTHRNEPQQ
WVLPSYSGRNTPNVHLAPAVAPTFPGEQLLFFRSTMPGCSGYPNMDLDCL
LPQEWVQYFYQEAAPAQSDVALLRFVNPDTGRVLFECKLHKSGYVTVAHT
GQHDLVIPPNGYFRFDSWVNQFYTLAPMGNGTGRRRAL
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Figure 6: Amino acid sequence of VPI of NoV GII.4 Sydney

Figure 7 shows the morphology of the generated NoV GII.4 Sydney VLP as determined by TEM

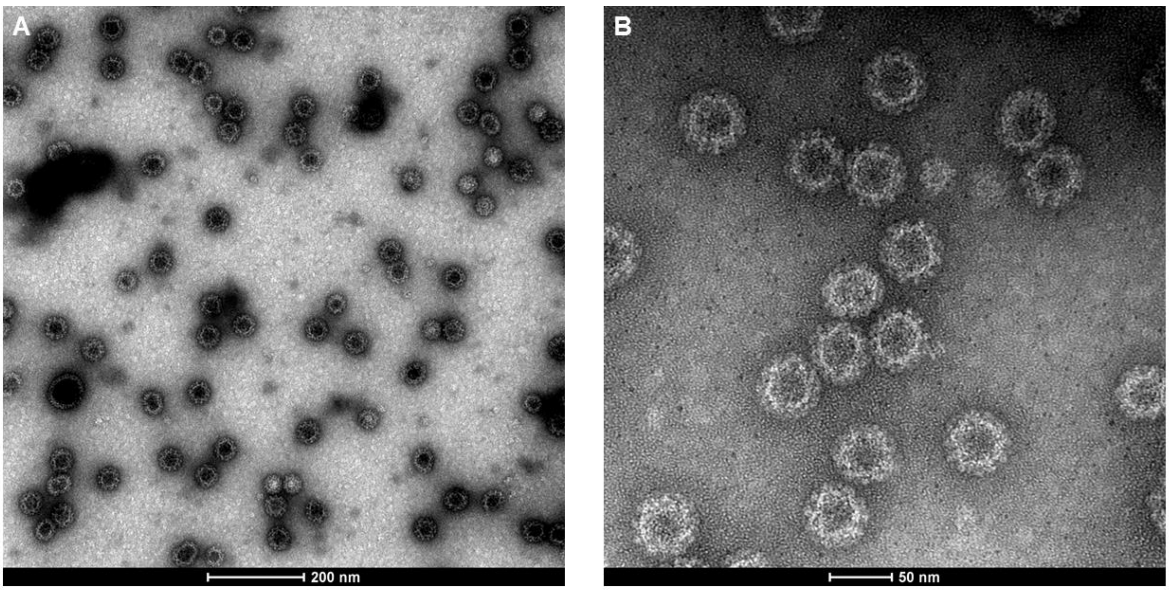

Figure 7: Electronmicrographs of NoV GII.4 Sydney VLP at two magnifications.

4.3 Evaluation of various bioreceptors for NoV detection

ELISA was used to screen the potential of various bioreceptors for the detection of NoV using NoV GII.4 VLP. Polyclonal antibodies to NoV GII.4 VLP were successfully raised in rabbits. Pre-immune rabbit sera showed no cross-reactivity with VLP. Polyclonal sera were able to detect VLP even when diluted at greater than 1:2 million. Of the HBGA antigens investigated (A, B and H type 1) for the detection of NoV GII.4 VLP, it was found that only HBGA-B showed some specificity. The AG3 MNV specific aptamer also showed some specificity for NoV GII.4 VLP. The specificity observed was greater for AG3 than HBGA-B based on ELISA absorbance readings, although this was not quantified. The biotinylated protein A was also shown to capture polyclonal NoV antisera by ELISA.

4.4 Biosensor development

4.4.1 Experimental optical fibre SPR

An example of the kinetic analysis for the experimental optical fibre SPR sensor is presented in Figure 8. It shows the positive SPR shift in nm (y axis) as compounds are attached to the sensing fibre. In this example, a functional sensor for NoV VLP capture was not achieved as no positive SPR shift was demonstrated when VLP were added at 250 min.

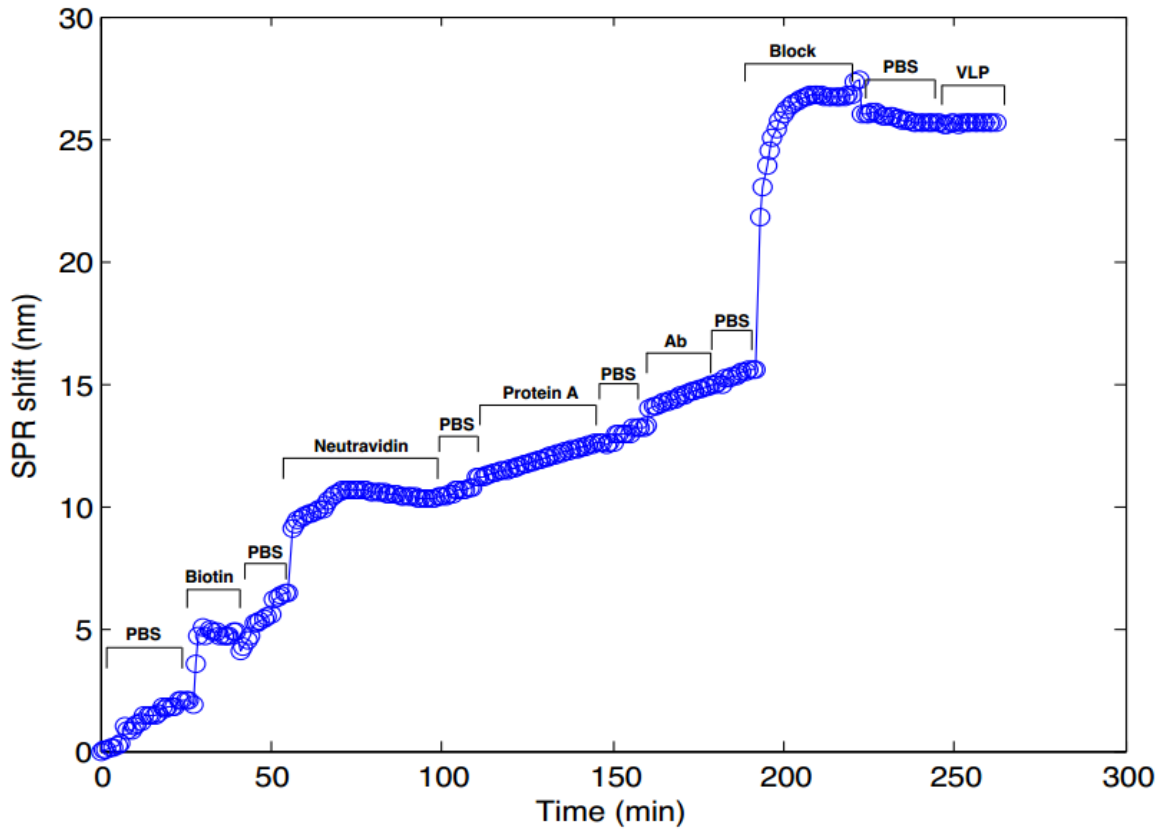

Figure 8: Example of kinetic analysis for NoV VLP detection using a silver coated F2 glass fibre SPR sensor.
PBS is a wash step following each functionalisation step. Functionalisation steps included in this example were biotin, neutravidin, biotinylated protein A, anti-NoV rabbit polyclonal sera (Ab) and blocking of unbound sites, followed by application of the NoV VLP test analyte. As each component is successfully added to the sensor surface there should be a positive SPR shift (nm).

In total, 21 attempts were made to generate a functional optical fibre sensor using biotinylated protein A to capture rabbit polyclonal NoV antisera and NoV VLP target (Table 2). Three further attempts were made to generate a functional sensor using the biotinylated HBGA-B approach for NoV VLP capture. Neither approach resulted in the generation of a functional SPR sensor.

Table 2: Summary of kinetic analysis for SPR NoV detection using biotinylated protein A capture approach.

	Biotin	Neutravidin	Biotinylated Protein A	Polyclonal sera	Block	VLP (10^3-10^5/mL)	VLP (10^6-10^{10}/mL)
Fibre	Average wavelength shift ± standard deviation (nm)						
F2	3.01 ± 2.98	6.08 ± 2.19	4.88 ± 6.23	2.92 ± 0.35	8.73 ± 4.10	0.38 ± 0.54	0.77 ± 0.63
n =	14	11	7	5	7	2	4
Silica	9.15 ± 7.62	9.71 ± 2.58	11.53 ± 7.41	6.49 ± 5.04	8.31 ± 6.82	-0.30 ± 0.88	0.52 ± 0.63
n =	7	6	6	5	5	6	5

4.4.2 Commercial SPR

4.4.2.1 NoV VLP interaction with HBGA-B and AG3 aptamer

Binding experiments (Chip101GLC) were designed to reduce the extent of nonspecific binding. NoV VLP interactions were analysed with biotinylated HBGA-B, biotinylated MNV AG3 aptamer, biotinylated protein A and control (human Vanin) rabbit polyclonal sera which were captured onto streptavidin surfaces (Figure 9). NoV VLP showed a response with streptavidin at concentrations >3 μg/mL (Fig. 9A). No significant binding (above streptavidin background) of NoV VLP to HBGA-B was seen (Fig. 9C). NoV VLP bound to AG3 aptamer (Fig. 9B, 9E) in a dose dependant manner with the lowest binding signal being detectable at 0.333 μg/mL (2 x 10^7 VLP/μL). The control surface consisting of streptavidin, minimally biotinylated Protein A and control polyclonal sera, did not show significant interaction with NoV VLP injected at 9 μg/mL (Fig. 9D). This observation showed that unwanted interactions with the streptavidin surface could be significantly reduced when appropriate control surfaces were prepared.

4.4.2.2 NoV VLP interaction with rabbit polyclonal NoV antibodies

Detection of NoV VLP with rabbit polyclonal NoV antisera involved the capture of antibodies with biotinylated Protein A onto a streptavidin surface. This particular functionalisation approach was taken to allow comparison with the approach investigated by the University of Adelaide in their experimental optical fibre SPR development. However, the assay using polyclonal NoV antisera was also demonstrated to be functional without the need to use the streptavidin and biotinylated Protein A steps. Antibodies could be reversibly captured onto the non-biotinylated Protein A that was amine-coupled directly onto the chip surface and resulted in a functionalised surface for detection of NoV VLP. Results indicated that some level of non-specific binding occurred when NoV VLP were injected at concentrations >3 μg/mL over the streptavidin and biotinylated Protein A surfaces. To overcome this, an unrelated control (human vanin) rabbit polyclonal sera was included. CSIRO also prepared an in-house biotinylated Protein A, as it was thought the commercial preparation was over biotinylated and, therefore, relatively inefficient in antibody capture.

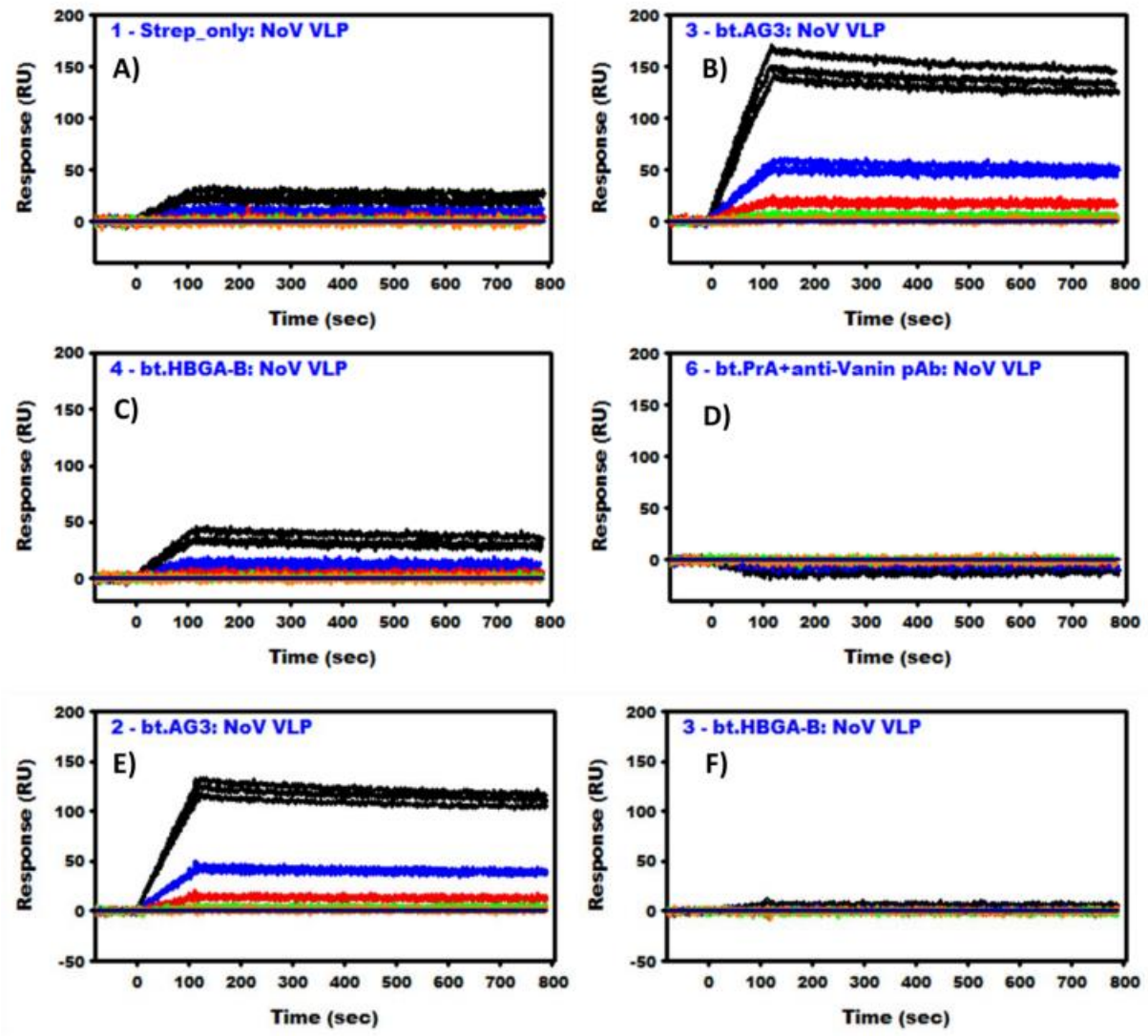

Figure 9: Binding of NoV VLP to immobilised AG3 and HBGA-B (triplicate experiment).
Panels A-D) show NoV VLP binding interactions that were referenced against an interspot surface (unmodified chip) whilst panels E-F) were referenced against streptavidin only surfaces. NoV VLP interacting with (A) streptavidin only, (B) strep + biotinylated AG3 aptamer, (C) strep + bt HBGA-B, (D) strep. + bt. Protein A + control pAb, E) strep. + bt. AG3 aptamer and F) strep. + bt. HBGA-B. Injected VLP concentrations were 9 μg/mL (black), 3 μg/mL (blue), 1 μg/mL (red), 0.333 μg/mL (green) and 0.111 μg/mL (orange). strep. = streptavidin, bt = biotinylated, anti-vanin polyclonal antibody (pAb)

Highly specific interactions between NoV VLP and polyclonal NoV antisera were demonstrated via commercially (Figure 10) and minimally biotinylated Protein A (Figure 11). Some non-specific interactions were observed with control Vanin antisera when VLP were injected at 9 μg/mL but these interactions were not significant and were unlikely to affect accurate estimation of kinetic binding parameters. Although, minimally biotinylated Protein A captured higher amounts of serum antibodies, no significant advantage was observed when it came to obtaining adequate binding responses and overall data quality. Nevertheless, higher amounts of captured polyclonal antibody would be beneficial if a biosensor assay was to be used for NoV detection and for establishing 'low-end' cut off limits. No extensive attempts were made to accurately determine VLP detection limits, as this study was aimed at establishing and optimising the NoV VLP detection assay in a commercial biosensor using specific detection reagents supplied by SARDI. The ProteOn biosensor assay reliably detected NoV VLP at ≥0.111 μg/mL (6.4 x10^6 VLP/μL) when polyclonal NoV anitsera was used as a detection reagent. If the current assay was to be further refined, then the VLP detection limit could be 1 x10^6 VLP/μL.

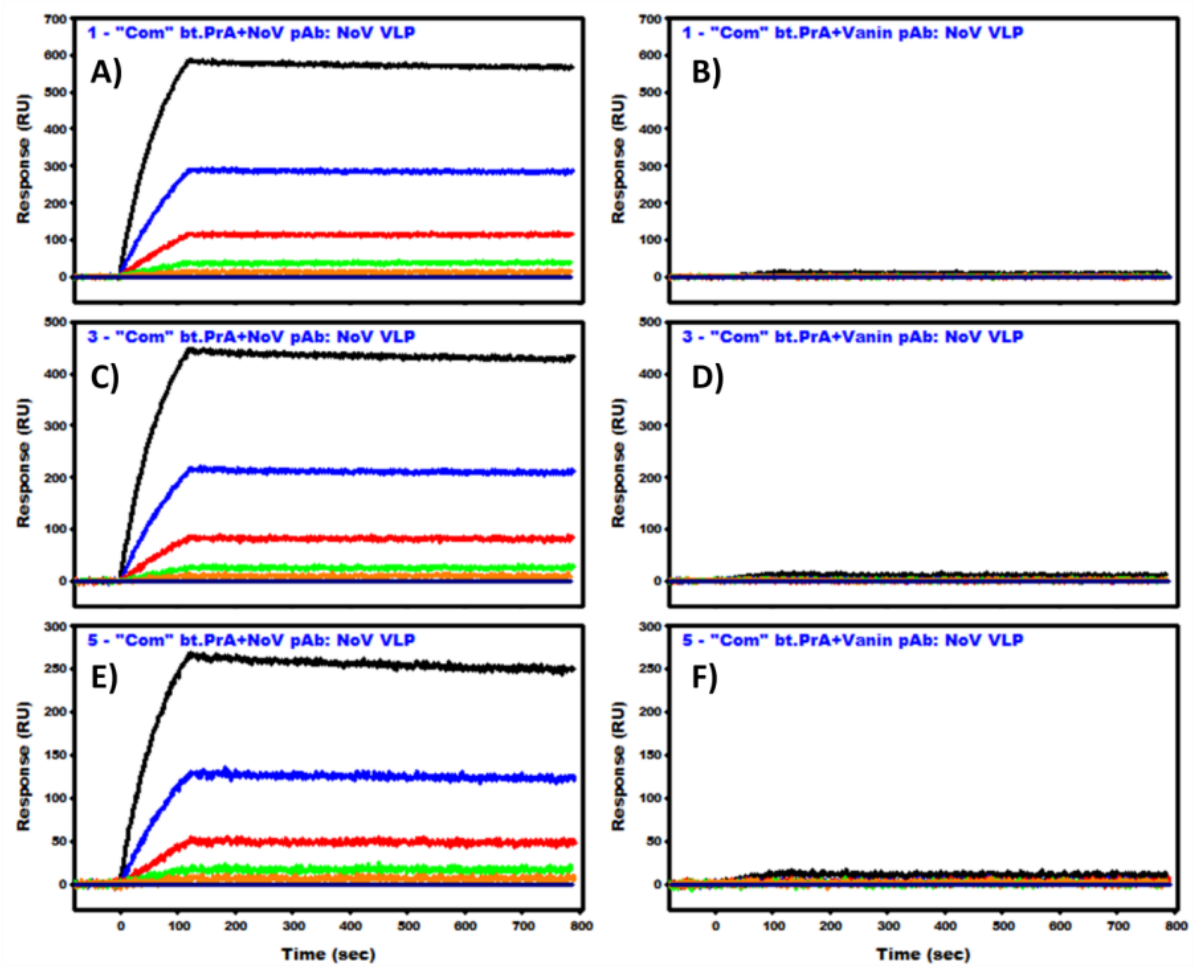

Figure 10: Binding interactions of NoV VLP with NoV and control antibodies captured via commercially biotinylated Protein A.
Triplicate repeat experiments are shown in panels A, C and E and B, D and F with the exception that different serum antibody levels were captured onto the Protein A surface prior to NoV VLP injection. Hence, overall NoV VLP binding responses are not identical. Rabbit serum antibody levels captured onto Protein A were: A) NoV - 360 RU, B) Vanin pAb - 350 RU, C) NoV pAb - 210 RU, D) Vanin pAb - 190 RU, E) NoV pAb - 80 RU and F) Vanin pAb - 80 RU. Injected VLP concentrations were 9 µg/mL (black), 3 µg/mL (blue), 1 µg/mL (red), 0.333 µg/mL (green) and 0.111 µg/mL (orange).

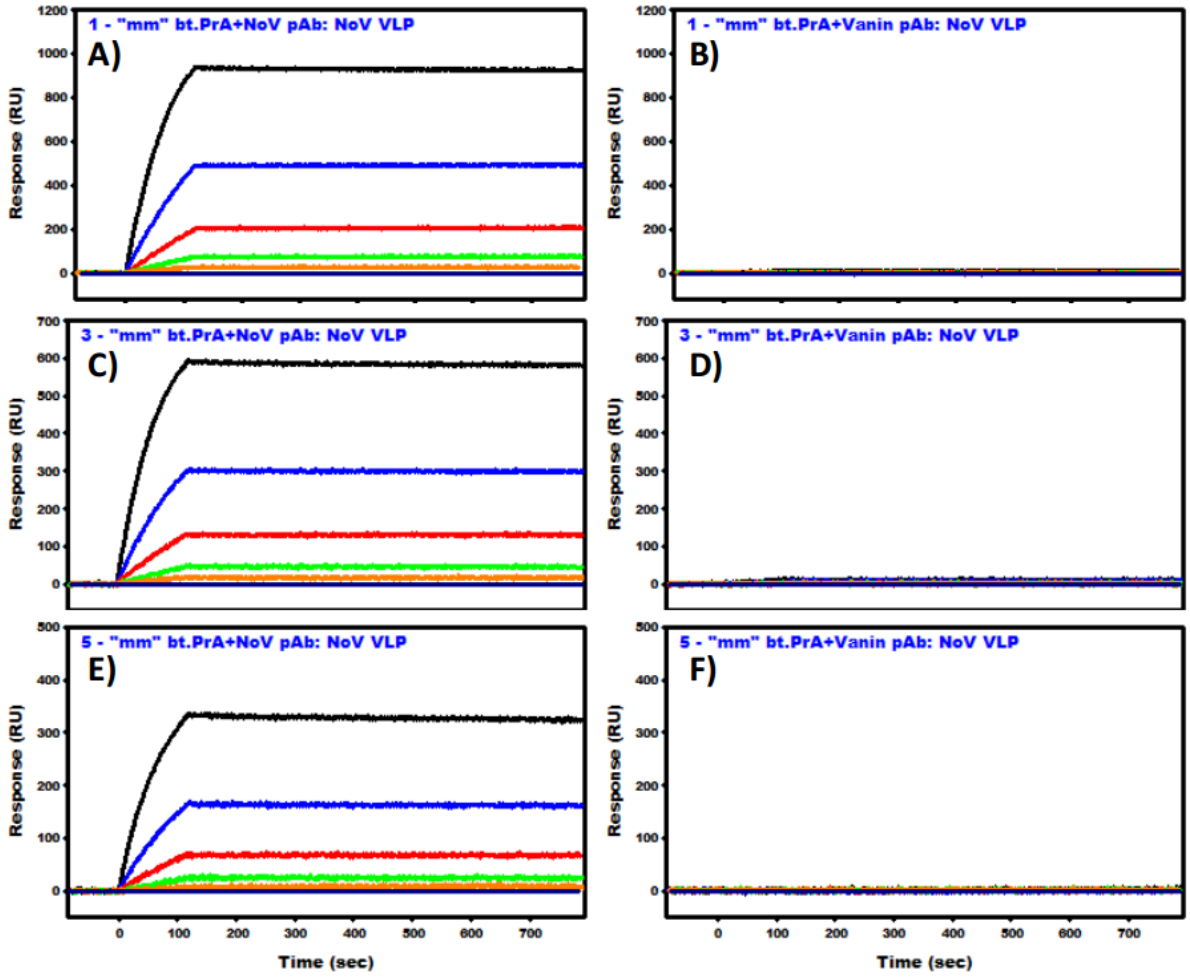

Figure 11: Binding interactions of NoV VLP with NoV and control antibodies captured via minimally biotinylated Protein A.
Triplicate repeat experiments are shown in panels A, C and E and B, D and F with the exception that different serum antibody levels were captured onto the Protein A surface prior to NoV VLP injection. Hence, overall NoV VLP binding responses are not identical. Rabbit serum antibody levels captured onto Protein A were: A) NoV pAb - 1450 RU, B) Vanin pAb - 1400 RU, C) NoV pAb - 600 RU, D) Vanin pAb - 560 RU, E) NoV pAb - 210 RU and F) Vanin pAb - 210 RU. Injected VLP concentrations were 9 µg/mL (black), 3 µg/mL (blue), 1 µg/mL (red), 0.333 µg/mL (green) and 0.111 µg/mL (orange).

4.4.2.3 Estimations of kinetic binding parameters and binding affinity

Binding kinetics of NoV VLP with rabbit polyclonal NoV antibodies were investigated. To enable this the concentration of NoV VLP needed to be converted from µg/mL to molar concentration. The molecular weight (MW) of VLP is approximately 10,260 kDa (assuming 180 repeats of 57 kDa monomer). Consequently, the top injected concentration of 9 µg/mL was assumed to be equivalent to 877 pM. Binding data were fitted globally with a 1:1 binding (Langmuir) model. Estimated kinetic binding parameters are listed in Table 3. It should be noted that these binding kinetic estimates are a simplification as they provide 1:1 binding equivalent to what, due to the multiple VP1 monomers making up the viral capsid, is a multivalent binding interaction.

Table 3: Binding paramenters of NoV GII.4 VLP and rabbit polyclonal NoV antisera. Mean ± standard deviation.

k_a ($M^{-1}s^{-1}$) association rate	k_d (s^{-1}) dissociation rate	K_D (pM) equilibrium dissociation
$(1.33 \pm 0.06) \times 10^7$	$(7.1 \pm 2.1) \times 10^5$	5.3 ± 1.4

Results have demonstrated that specific binding of NoV VLP to rabbit polyclonal NoV antisera and to AG3 aptamer can be detected on the surface of the functionalised commercial SPR biosensor. NoV VLP bound in a dose response manner to immobilised rabbit polyclonal NoV antisera and AG3 aptamer, whilst no binding was observed with immobilised HBGA-B. NoV VLP bound to AG3 aptamer with the lowest binding signal being detectable at ≥0.3 μg/mL (2×10^7 VLP/μL). VLP bound to rabbit polyclonal NoV antisera at ≥0.1 μg/mL (6.4×10^6 VLP/μL).

4.4.3 Electrochemical

4.4.3.1 NoV VLP and polyclonal NoV antisera

Gold, graphite and porous silicon-modified gold electrode electrochemical sensors were developed for detection of NoV using the rabbit polyclonal NoV antisera bioreceptor and NoV VLP target. Preliminary results showed the feasibility to electrochemically detect NoV VLP from 10^3 to 10^7 VLP/μL using direct and competitive immune-based assays. However, the observed trend in charge transfer resistance (R_{ct}) and current intensity as measured by electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV), respectively were not as expected. NoV VLP aggregation was suspected as a potential cause. Atomic force microscopy (AFM) and dynamic light scattering (DLS) studies confirmed aggregation of NoV VLP. AFM showed single particles of 20-30 nm and clusters of particles, approximately 110 nm (Figure 12A). As particles may behave differently on dried surfaces as opposed to in solution, DLS measurements were also done to confirm findings (Figure 12B). Whilst a decrease in R_{ct} is expected when the concentration of NoV VLP increases (less free antibody available and thus less binding to the immobilised NoV VLP), an increase in R_{ct} was observed, likely due to the aggregation of NoV VLP in solution with the immobilised NoV VLP. The potential aggregation of NoV VLP, along with presence of potentially interfering species within the polyclonal sera, made it difficult to conclude whether the detection of NoV VLP was solely based on NoV VLP specifically bound to antibodies, or to NoV VLP non-specifically adsorbed to other NoV VLP (aggregation). Further work would have been required to ensure the dispersion of NoV VLP as single particles by testing different working solutions (i.e. buffers, ionic strength, pH, addition of a detergent etc.). Aggregation of NoV particles has previously been observed by TEM and shown to be resistant to sonication (Teunis et al., 2008). Viruses may be "sticky" in suspension, depending on the ionic strength, pH, and properties of the viral protein coat. Solutions high in protein tend to promote viral aggregation (Teunis et al., 2008).

4.4.3.2 MNV and AG3 aptamer

Electrochemical electrodes were prepared by placing porous silicon (pSi) or nanoporous anodic alumina (NAA) membranes onto a gold surface. pSi membranes were then modified in order to immobilise the MNV AG3 aptamer. 5´-biotinylated and 5´-aminated aptamers were tested as immobilised bioreceptors onto pSi membranes. Binding of MNV to the immobilised aptamer would cause the partial blockage of nanochannels and, therefore, hinder the diffusion towards the gold surface of the electroactive species added in solution. Blockage is measured by the decrease in the current intensity produced (Figure 13). MNV solutions were incubated on the biosensor surface for 1 h. To verify that the current changes were caused only by the specific binding of MNV to the AG3 aptamer, a control aptasensor was prepared with a random ssDNA sequence and evaluated under identical conditions. All current intensity values

were normalised as follows: $\Delta I = (I_0-I_i)/I_0$, where ΔI is the normalised current change and I_0 and I_i are the current intensity values measured prior and after MNV binding. Figure 14A shows the response obtained after incubating the aptasensors prepared with the aminated AG3 aptamer in solutions of 10^3 and 10^5 pfu/mL MNV. DPV was used to measure pore blockage as a decrease in current intensity. The oxidation current decreased as the MNV concentration increased. Results clearly showed a dramatic decrease in current intensity for the sensor modified with the MNV aptamer, while no decrease was observed for a sensor modified with a control ssDNA (Figure 14b). It was also observed that changes in current were larger for aminated aptamer than for sensors prepared using biotinylated aptamer (data not shown). The developed aptamer-modified sensors successfully detected MNV (from 1 to 10^3 MNV/µL) in buffer (Figure 15). Further optimisation of the aptasensor will need to focus on effect of the pore diameter, aptamer concentration, sample incubation time and external surface functionalisation.

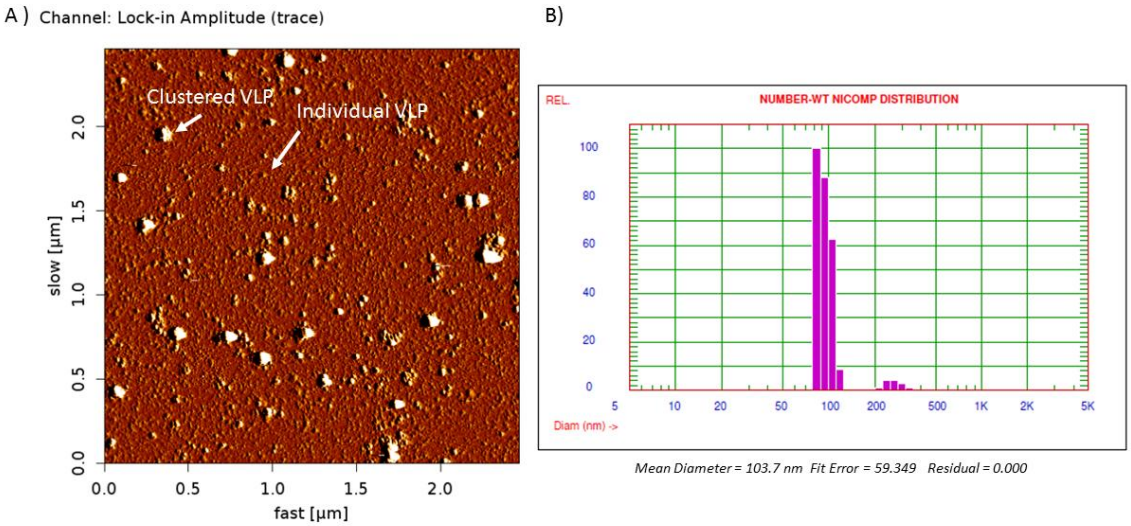

Figure 12: Investigation of VLP aggregation.
A) Atomic force microscopy of 10^9 VLP/µL deposited onto a mica surface showing individual VLP (approximately 20-30 nm) and clustered VLP (approximately 110 nm). B) Dynamic light scattering plot showing aggregation of VLP (10^8 VLP/uL), forming clusters of approximately 100 nm.

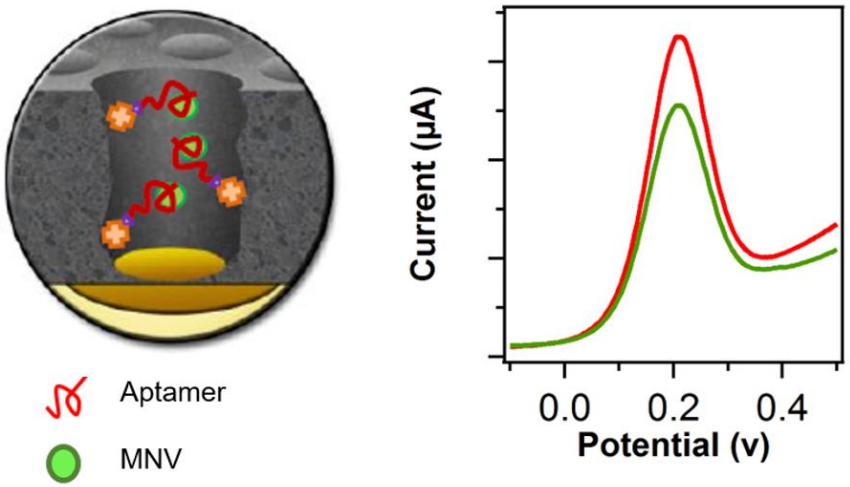

Figure 13: Sensing principle of the pSi membrane-based aptasensor for the label-free detection of MNV and the corresponding DPV traces prior (red) and following (green) MNV binding.

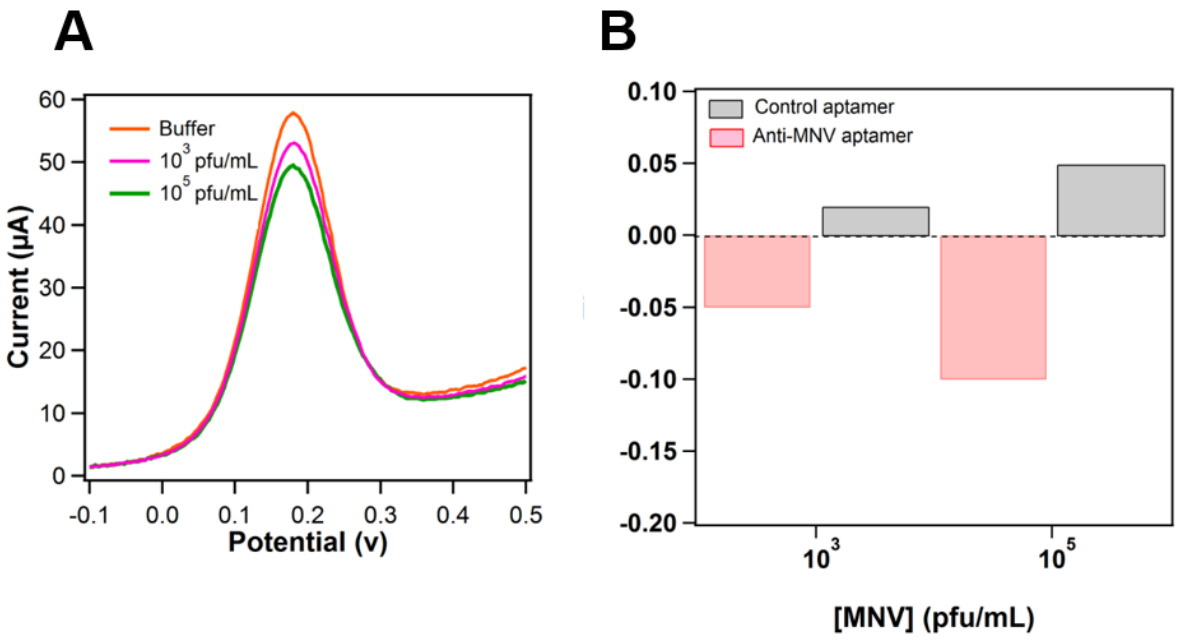

Figure 14: DPV traces using aptasensor for MNV functionalised with animated AG3 (A) and normalised oxidation current versus MNV concentration for the aptasensor functionalised with animated AG3 and control random ssDNA sequence.

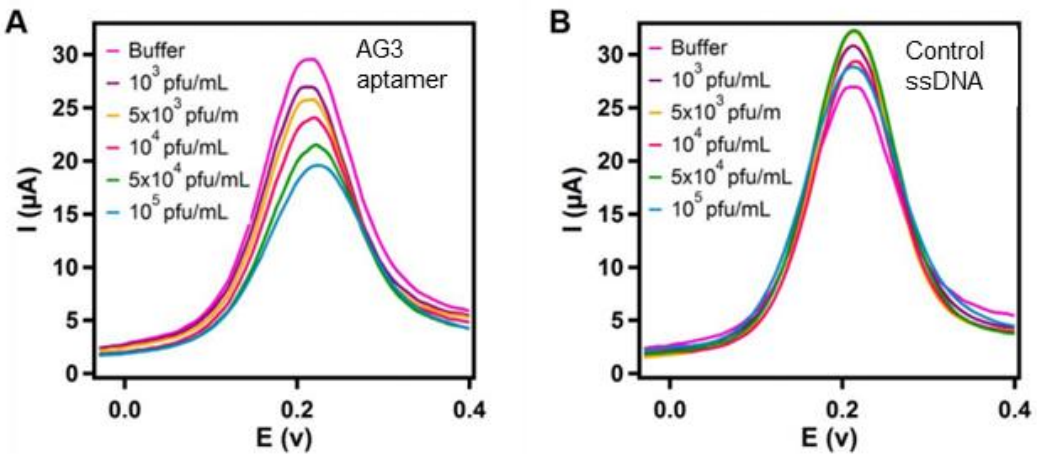

Figure 15: Square wave voltammetry traces for the increasing concentration of MNV in buffer. Aptasensor prepared using porous NAA membranes modified with (A) AG3 aptamer and (B) a random ssDNA sequence (control).

4.4.3.3 Detection and quantification of norovirus in oyster matrix

Having successfully demonstrated the ability to detect MNV spiked into buffer, detection of MNV spiked into oyster DT extracts was attempted. Dilutions of MNV-free oyster extract (1:10, 1:100, 5:1,000, 1:1,000) were prepared in general sensing buffer (GSB; 50 mM NaCl, 20 mM Tris-HCl pH 7.4, 3 mM MgCl2, 5 mM KCl) which was followed by filtration using a 0.2 µm filter. These dilutions were then incubated on the AG3 aptamer-modified NAA surface for 1 h to investigate oyster matrix effects.

Figure 16 shows the electrochemical response for different oyster dilutions in GSB buffer. It was observed that changes in current were significant when aptasensors were incubated with 1:10, 1:100 and 5:1000 oyster dilutions while, changes were negligible for the highest dilution (1:1000) and similar to the changes produced in buffer alone. Thus, a 1:1000 dilution was chosen to prepare MNV spiked oyster extracts and attempt MNV detection using the developed electrochemical aptasensor. The need to dilute the oyster extract 1:1000 indicates that some further improvements in sample preparation are required to optimise sensor performance.

Figure 17 shows the dose response curves after incubating samples of MNV spiked into buffer and into 1,000 times diluted oyster extract. A linear correlation was found between normalised current change (ΔI) and the MNV concentration for the AG3 aptasensor prepared using NAA membranes with a specific pore size. The linear fitting for both curves: $\Delta I = 0.061 \log[MNV] - 0.14$ with a $R^2 = 0.95$ (MMV spiked in oyster extract) and $\Delta I = 0.067 \log[MNV] - 0.13$ with a $R^2 = 0.99$ (MNV spiked in buffer), respectively are shown. The percentage of slope deviation between dose response curves for MNV spiked into buffer and into diluted oyster extract were quite similar, showing the ability to detect MNV spiked in diluted oyster extract without significant matrix effects.

The limit of detection (LOD) and limit of quantification (LOQ) values were calculated using the equation $3S_a/b$ and $10S_a/b$, where S_a is the standard deviation of the y-axis and b is the slope. For MNV in buffer the LOD and LOQ was 10 pfu/mL and 1,872 pfu/mL, respectively. For MNV spiked into diluted oyster matrix (1/1000) the LOD and LOQ was 52 pfu/mL and 53,000 pfu/mL, respectively.

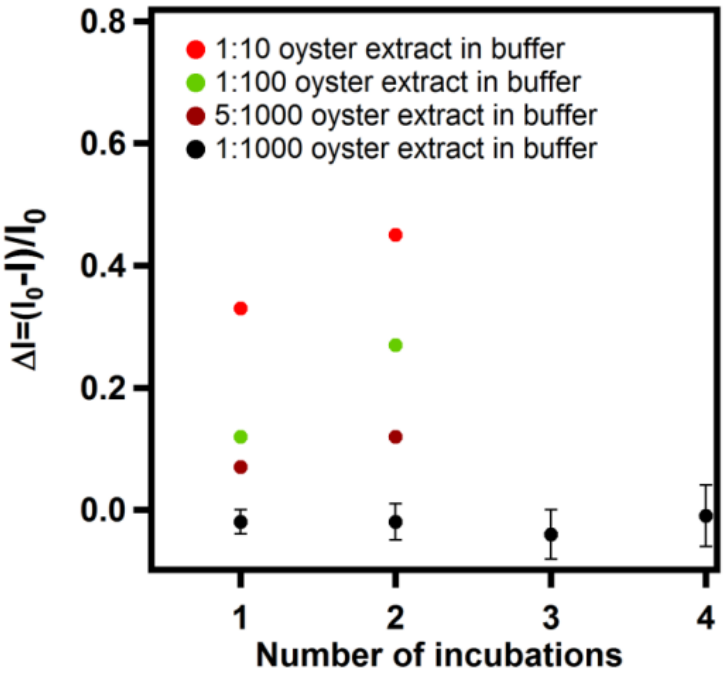

Figure 16: Electrochemical response for MNV free oyster extract dilutions

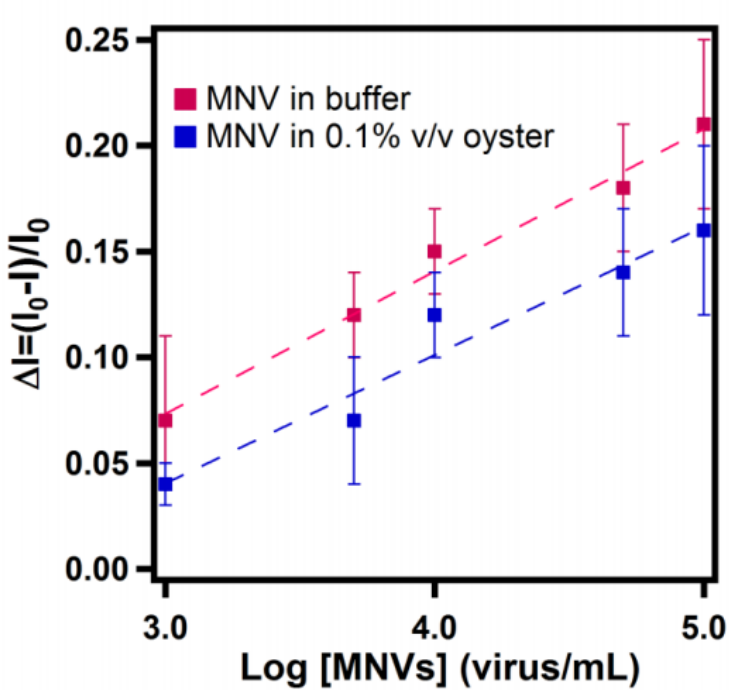

Figure 17: Dose response curve of MNV spiked into oyster matrix and buffer

The concentration of MNV in two blind oyster extracts provided by SARDI FSI were determined using the dose response curve shown in Figure 17. Both samples were first diluted 1,000 times in GSB buffer and then filtrated using a 0.2 µm filter. These samples were incubated with NAA membranes modified with AG3 aptamer for 1 h and the current intensity values for the oxidation peak were measured prior to (I_0) and following (I_i) sample incubation. Changes in current (ΔI) were then interpolated into the linear dose response curve obtained for 1:1,000 diluted oyster extract (ΔI= 0.061log[MNV] - 0.14) and the concentration of MNV estimated (Table 4). The infective concentration of MNV in these blind samples was independently determined by cell culture, while the concentration of viral genomes was calculated by qRT-PCR (Table 4). Not surprisingly, quantification by qRT-PCR gave an overestimation of virus concentration as it is based on the detection of viral RNA which may or may not represent infective (encapsulated) virus. Due to the need to dilute the oyster samples 1:1,000 to eliminate the negative effects of sample matrix on sensor performance, the MNV quantification of sample 2 was out of linear range (<1,000 virions/mL) and corresponded to 500 pfu/mL (roughly equivalent to 225 pfu/g oyster DT) as determined by cell culture. The MNV quantification in sample 1 was determined to be 6,300 virions/mL by the sensor (approximately to 3,073 virions/g oyster DT) and 5,103 pfu/mL by cell culture (approximately to 2,298 pfu/g oyster DT).

Table 4: Determination of MNV concentration from spiked oyster DT samples as determined by the pourous alumina electrochemical aptasensor, cell culture and qRT-PCR.

	MNV quantification			
Spiked oyster DT	Sensor virions/mL	Cell culture pfu/mL	qRT-PCR genome copies/mL[a]	qRT-PCR genome copies/mL[b]
Sample 1	6,300	5,103	1.49×10^6	1.02×10^7
Sample 2	Out of linear range ($<10^3$)	500	1.40×10^5	9.11×10^5

[a] Based on standard prepared using infective MNV
[b] Based on dsDNA MNV plasmid standard

4.4.4 Extraction efficiency of oyster purification

The virus extraction efficiency of the sample purification method adopted was evaluated by determining the percentage recovery of MNV spiked into the oyster matrix prior to sample purification. The glycine-threonine extraction and ultracentrifugation method used resulted in approximately a 15% recovery of MNV, as determined by cell culture (Table 5).

Table 5: Viable MNV extraction efficiency from spiked oyster DT based on plaque assay.

	Spiked viable MNV	Recovered viable MNV	Viable MNV recovery (%)
Sample 1	1.2×10^5	1.99×10^4	16.6
Sample 2	1.2×10^4	1.8×10^3	15.0
Control	0	0	-

4.4.5 Gold nanoparticle

The developed MNV GNP aptasensor was evaluated for specificity using homologous and non-homologous targets; MNV (target of interest), MS2 bacteriophage (a virus morphologically similar to MNV), a gram positive bacteria (*Streptococcus aureus*) and a gram negative bacteria (*Eschericia coli*). A fixed number of virus particles or cells (10^3) were used to obtain the sensor response. Serum was also included to assess the stability and potential cross-reactivity of the sensor probe. This control is not of relevence for detection in food or clinical samples and it would have been better to include oyster matrix as the control. Figure 18 shows the characterstic response corresponding to each particle/cell type obtained from the sensor probes. A positive response was specifically obtained from MNV, while other particle/cell types showed minimal responses, with no cross reactivity with sera observed.

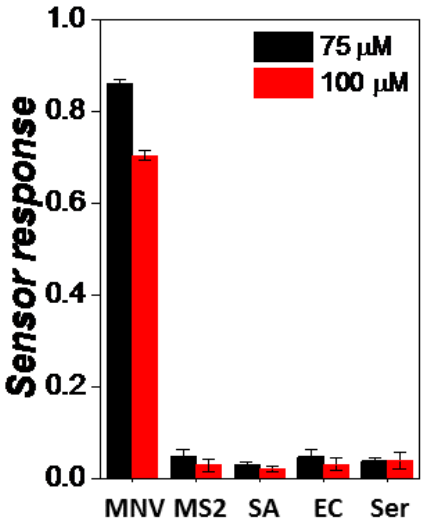

Figure 18: GNP (75 µM and 100 µM) aptasensor response to 1,000 particles/cells of MNV, MS2 phage (MS2), *S. aureus* (SA), *E. coli* (EC) and sera (Ser).

The sensitivity of the sensor was evaluated by incubating the sensor probe with increasing amounts of MNV. This enabled calcluation of sensor parameters, such as LOD, LOQ, linearity, dynamic range, precision and accuracy. A visible response was observed with as few as 20 MNV virus particles in 100 µL. However, the sensitivity was dependent on the concentration of the GNP used for the fabrication of the sensor probe (Figure 19). When the sensor probe was fabricated with 75 µM GNP, the LOD was

240 pfu/mL with a linear dynamic range of 200-9,900 pfu/mL. The precision and accuracy of the sensor was assessed by performing 20 repeats using 100 virus particles (Table 6). A similar calculation yielded similar sensor performance for the 100 µM GNP concentration.

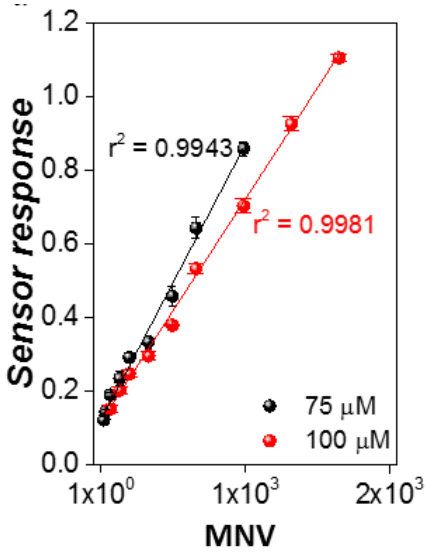

Figure 19: Linear fit of the nanozyme activity obtained with different concentrations of MNV and sensor probes.

Table 6: Calculated sensor paramenters for the nanozyme aptasensor in buffer

GNP (µM)	LOD (pfu/mL)	LOQ (pfu/mL)	Linear dynamic range (pfu/mL)	Accuracy (%)	Precision (%)
75	240	7,30	200-9,900	100	98.7
100	1,300	3,950	1,320-16,500	100	98.6

5. Discussion

This project has been successful in the development and demonstration of functional biosensors for the detection and quantification of murine norovirus. More importantly, functionality has been demonstrated in oyster tissue extracts. Two different sensing platforms, electrochemical and GNP were developed for the detection of MNV using a homologous aptamer as the bioreceptor. Both developed biosensor platforms reported similar LOD (10 pfu/mL; electrochemical and 240 pfu/mL; GNP) in buffer. The sensitivity of detection of the developed biosensors are significantly greater than for previously reported biosensors for norovirus or virus surrogate detection (Table 7). The LOD of the qRT-PCR ISO/TS 15216 method for NoV in oysters is 100 copies/g DT for NoV GI and 52 copies/g DT for NoV GII[1]. The developed electrochemical biosensor also demonstrated application for detection of MNV in diluted oyster extract with an LOD of 52 pfu/mL. Further work to optimise oyster extraction methodology will be required to eliminate the need for oyster extract dilution prior to sensor application. As a result of the current need to dilute oyster extracts, the developed electrochemical sensor could only quantify MNV in seeded oyster tissue at concentrations greater than 1,000 pfu/mL. Quantification of MNV in seeded oysters was comparable using the electrochemical aptasensor (6,300 virions/mL or 3,073 virions/g oyster DT) and *in-vitro* cell culture of infective virus (5,103 pfu/mL or 2,298 pfu/g oyster DT). Quantification of MNV by qRT-PCR of a sub-sample of this material resulted in an overestimation by several fold, indicating a misrepresentation when trying to investigate infectivity by PCR based methods.

In total, four technologies were investigated in this study: experimental optical fibre SPR, commercial SPR, electrochemical and GNP. Development of an experimental optical fibre SPR sensor for NoV VLP detection using a polyclonal NoV antisera was unsuccessful. Commercial SPR sensor functionalisation using the same SARDI developed polyclonal NoV antisera and NoV VLP target was successful, detecting NoV VLP at $\geq$ 0.111 µg/mL (6.4 x10^6 VLP/µL). Furthermore, bind kinetic studies showed that the polyclonal NoV antisera had a K_D of 5.3 pM for the homologous NoV VLP. Although, the sensitivity of the commercial SPR was lacking for application in foodborne virus diagnosis and quantification, it demonstrated the ability to successfully functionalise a sensor surface using our bioreceptors to NoV VLP. Development of an electrochemical biosensor was investigated using the NoV GII.4 VLP and polyclonal NoV antisera. Issues of VLP aggregation and potential interfering species within the polyclonal sera were observed, necessitating further work to investigate different working solutions and antibody purification. However, further optimisation of conditions in an ideal environment wouldn't have been representative of a food sample nor would it have enabled quantification of an infective viral dose, as there would have been no way of validating results based on non-infectious NoV VLP. Therefore, a cultivable virus surrogate, MNV, and specific aptamer bioreceptor, AG3, were used to further develop the electrochemical biosensor for virus detection.

Several biosensors for the detection of NoV VLP or cultivable virus surrogates, such as feline calicivirus (FCV) and MNV, have been described using both commercial and experimental sensing platforms since the first reports appeared six years ago (Table 7). However, the majority of these studies have not focussed on optimisation of sensitivity or application as a diagnostic in food samples. The majority of reports have used SPR and electrochemical platforms and antibody or HBGA bioreceptors. de Rougemont et al. (2011) investigated the binding of NoV VLP to synthetic HGBAs using a commercial SPR platform. As their aim was not to develop a sensitive diagnostic, but rather investigate variability in NoV binding to different HBGAs, VLP binding was only investigated at 2 ng/µL, representing 10^8 VLP/µL (de Rougemont et al., 2011). Likewise, Shang et al. (2013) investigated the binding of NoV VLP to human milk glycans using a commercial SPR. They were not investigating sensitivity of detection either and used 10 µg/mL VLP, representing 5.8 x 10^8 VLP/µL (Shang et al., 2013). Yakes et al. (2013) also used a commercial SPR platform to study infectious FCV detection using an antibody detection approach. They found that a 10^4 50% tissue culture infective dose ($TCID_{50}$) FCV/mL could be detected from purified cell culture lysate. Performance of this biosensor for detecting FCV in oyster

[1] www.cfs.gov.hk/english/whatsnew/whatsnew.../B_David_LEES.pdf

extract was also investigated and inhibition was observed in the absence of dilution or use of a secondary antibody (Yakes et al., 2013). Ashiba et al. (2016) used SPR on a V-trench chip with a double antibody sandwich approach to capture and quantify NoV VLP. The reported LOD was 0.01 ng/mL, corresponding to approximately 10^5 VLP/mL. However, performance was not evaluated in a matrix representative of a food sample (Ashiba et al., 2016).

Bally et al. (2013) used total internal reflection fluorescence (TIRF) to detect NoV VLP with HBGA as the bioreceptor and they reported a LOD in the low fM levels, corresponding to approximately 10^6 VLP/mL (Bally et al., 2013). Hargstrom et al. (2015) developed a lateral flow assay using labelled phage particles for the detection of NoV VLP with a LOD of 10^7 VLP/mL (Hagström et al., 2015). None of these investigations evaluated detection performance in food sample matrices.

Hong et al. (2015) developed an electrochemical biosensor for the detection of NoV (isolated from human faeces and quantified by qRT-PCR) using concanavalin A (a carbohydrate binding protein) attached onto a gold electrode to capture NoV with subsequent detection using a specific antibody (Hong et al., 2015). They reported a LOD of 35 particles/mL, although methodology used for quantification was ambiguous. We have observed that quantification of MNV by qRT-PCR resulted in a significant over estimation as compared to *in-vitro* cell culture. The study of Hong et al. (2015) also investigated detection of NoV in lettuce extract and reported an LOD of 60 particles/mL. However, the methodology used was not representative of a procedure demonstrating extraction of virus from food samples, as virus was spiked into a prepared extract rather than into a sample prior to extraction. The method outlined used 10 g of lettuce leaves which were homogenised in 50 mL buffer, centrifuged to remove cellular debris with NoV spiked into the retained supernatant. If an LOD of 60 copies/mL is assumed and a minimum of 50 mL supernatant recovered, then the sample would have contained 3,000 viruses/10 g of lettuce sample. The theoretical limit of detection for NoV using the ISO/TS 15216 method is 10 copies per 25 g of leafy greens sample (ISO/CEN, 2013).

Giamberardino et al. (2013) developed an aptamer to MNV (AG3) and used this to develop a gold nanoparticle-modified screen-printed carbon electrode electrochemical sensor with a LOD of 180 MNV particles (10 aM). This is the most sensitive biosensor developed to date for a NoV surrogate, and the first using an aptamer as the bioreceptor. This sensor, however, was not evaluated in food matrices or clinical samples. This same aptamer was used as the bioreceptor on our electrochemical and GNP sensor platforms, and both achieved an improved level of sensitivity. The AG3 aptamers have been reported to capture NoV GII.3 VLP with affinity at concentrations as low as 240 fM (Giamberardino et al., 2013). We have also shown, by ELISA and commercial SPR, that the AG3 aptamer non-specifically captured NoV GII.4 VLP, at levels $\geq$0.3 μg/mL (2 x 10^7 VLP/μL). Specific capture of NoV VLP GII.4 with our homologous polyclonal NoV antisera was only slightly better at $\geq$6.4 x10^6 VLP/μL. Development of a human specific NoV aptamers should significantly improve sensitivity, as compared to anybody approaches. Aptamers have several advantages over antibodies in that they have high specificity and affinity to target; are able to target small molecules, large proteins and cells; once selected can be synthesised with high reproducibility and purity from commercial sources (cost effective to produce); are highly chemically stable; and can be chemically modified (McKeague et al., 2011).

Only one publication has previously investigated nanoparticle sensors for NoV detection (Ahmed et al., 2017). Graphite GNP conjugated with NoV antibody were developed for detection of NoV VLP with a LOD of 92.7 pg/mL (10^3 VLP/mL). Detection was done in sera but this is not biologically relevant for NoV detection in either clinical or food samples. The GNP sensor developed for our project used aptamers instead of antibodies for antigen capture and achieved a LOD of 240 MNV pfu/mL making it superior in sensitivity. Further development of our GNP sensor would have to evaluate performance in oyster or other food matrices, which was not achieved in this project.

Table 7: Summary of biosensors for norovirus and cultivable surrogate virus detection.

Transducer	Bioreceptor(s)	Target	LOD	Matrix	Reference
Commercial SRP	HBGA	NoV VLP (10^{11} VLP/mL)	ND*	Buffer	de Rougemont et al., 2011
Commercial SPR	Milk glycans	NoV VLP (5.8×10^{11} VLP/mL)	ND	Buffer	Shang et al., 2013
Commercial SPR	Antibodies	FCV	10^4 $TCID_{50}$ /mL	Purified cell lysate	Yakes et al., 2013
		10^5 $TCID_{50}$ /mL	ND	Oyster extract	
Electrochemical	Aptamer	MNV	180 pfu	Buffer	Giamberardino et al., 2013
TIRF	HBGA	NoV VLP	10^6 VLP/mL	Buffer	Bally et al., 2013
Lateral flow	Phage	NoV VLP	10^7 VLP/mL	Buffer	Hagstrom et al., 2015
Electrochemical	Concanalvin A & antibody	NoV from faeces & quantified by PCR	35 copies/mL	Buffer	Hong et al., 2015
			60 copies/mL (3,000 copies/10 g lettuce)	Lettuce extract	
V-trench chip SPR	Antibodies	NoV VLP	10^5 VLP/mL	Buffer	Ashiba et al., 2016
GNP	Antibodies	NoV VLP	10^3 VLP/mL	Serum	Ahmed et al., 2017
Electrochemical	Aptamer	MNV	10 pfu/mL	Buffer	This study
			52 pfu/mL	Oyster extract	
GNP	Aptamer	MNV	240 pfu/mL	Buffer	This study

* ND, not determined

Published biosensor studies for NoV or virus surrogate detection tend to state LOD, but do not state LOQ or linear dynamic range. For a signal at the LOD, the probability of a false positive is small but the probability of a false negative is 50% for a sample that has a concentration at the LOD. This means for a sample containing an impurity at the LOD, there is a 50% chance that a measurement would give a result less than the LOD. At the LOQ, there is minimal chance of a false negative. The linear dynamic range of the assay is also important in using it for quantification. Using the qRT-PCR ISO/TS 15216 methodology, the LOD and LOQ for NoV GI in pacific oyster (*C. gigas*) is reported to be 100 copies/g DT, while for NoV GII it is reported to be 52 copies/g DT. The linear range for NoV GI and GII is reported to be 100-20,407 copies/g DT and 52-9,603 copies/g DT, respectively[1].

Only one previously published biosensing paper has investigated cultivable virus detection using FCV in oyster matrix and they observed inhibition in the absence of dilution (Yakes et al., 2013). The importance of sample extraction methodologies which limit negative impact on biosensor performance, while still effective in virus concentration and preservation of virus infectivity, have not been investigated and are overlooked in previous publications (Hong et al., 2015; Yakes et al., 2013). The ISO/TS 15216 method includes the use of a process control virus which is spiked into the sample prior to extraction to enable the determination of virus recovery (virus extraction efficiency). In our experience, significant process control virus is lost during the extraction step for lettuce matrix, with virus recovery on average 23% (median=11%, n=137; SARDI unpublished) using the ISO/TS 15216 method. The average virus recovery from oyster matrix is higher at 70% (median =100%, n=293; SARDI unpublished). The virus extraction method used in the ISO/TS 15216, would not be appropriate for sample preparation to be used on a biosensor for infective virus detection, as it contains a proteinase K step which could remove the protein capsid of the virus and render it non-infective. The method we adopted for spiked oyster sample preparation was based on glycine-threonine extraction and ultracentrifugation, resulting in virus recovery of approximately 15% as confirmed by cell culture. However, this method also resulted in the need to dilute the sample 1:1,000 so as not to impact negatively on the developed electrochemical aptasensor. This resulted in contaminated samples containing <1,000 pfu/mL going undetected. The dynamic range of this sensor was much broader (1,000-100,000) than for the GNP aptasenor. Further work to optimise virus purification from oyster samples, hence reducing interference to the electrochemical aptasensor, is required to achieve improved detection. This is feasible using the MNV model to compare impact of extraction on infective virus recovery. Not only was the electrochemical aptasensor direct, simple and highly sensitive for MNV quantification, but potential was also demonstrated to shorten analysis time and easily and quickly regenerate the sensor surface by either incubating in a solution of certain ionic strength or applying a short pulse potential.

Since the identification of human norovirus in the early 1970s a barrier to understanding its biology has been the inability to culture human strains in the laboratory. The discovery of MNV in the early 2000s and the ability to culture it *in-vitro* has provided a valuable tool in NoV surrogate studies. Consistent infection and replication of human NoV viruses *in-vitro*, has not been demonstrated to any great extent (Straub et al., 2011). Several human and animal cell lines, methods for differentiation, and treatments to identify cell lines and conditions supporting replication have been investigated with little success. Straub et al. (2007) reported replication of human NoV in three-dimensional culture of small intestinal epithelial cells, however, this has not been able to be reproduced in other laboratories. Jones et al. (2014) demonstrate that human NoV GII.4 Sydney and MNV could infect B cells *in-vitro*, but required enteric HBGA bacteria as a stimulatory factor for infection (Jones et al., 2014). Recently, Ettayebi et al. (2016) reported successful cultivation of multiple human NoV strains in stem cell-derived, non-transformed human intestinal enterocyte monolayer cultures (Ettayebi et al., 2016). Bile was identified as a critical factor of the intestinal milieu, and required for strain dependent NoV replication. Filtered (removal of contaminating bacteria) stool from infected individuals was used as the NoV inoculum in this study, and bacterial lipopolysaccharides were not required for infectivity. These results are in contrast with those of Jones et al. (2014) in the cultivation of a single NoV strain in B cells, where unfiltered stool inocula or commensal gut bacteria were required as cofactors for infection. The report from Robert Atmar's group at Baylor College of Medicine, Houston, Texas, is the most promising to date in being able to replicate multiple human NoV strains (Ettayebi et al., 2016). This human NoV culture methodology has

now been reported to have been reproduced in other laboratories (personal communication, Marion Koopmans) and will be an invaluable tool in studying NoV biology and infectivity.

Having a human NoV culture system established would be the next step in further developing the "proof-of-concept" murine norovirus electrochemical aptasensor. Human NoV specific aptamers would need to be developed as bioreceptors for a range of relevant human strains. Sample preparation methodologies would also need to be optimised. Finally, virus inactivation studies could be undertaken to demonstrate detection of infective versus non-infective human NoV. This would be a significant advance in the detection of NoV in shellfish and determination of foodborne risk.

6. Conclusion

The key objective of the project was to develop a functional biosensor for the detection of norovirus. Internationally, a major hurdle in human NoV diagnostic development has been the ability to quantify infective virus, as *in-vitro* culture has been recalcitrant. Diagnostics of NoV in foods has been based on qRT-PCR detection, which although sensitive may also detect non-infective virus. The objective of the project was realised with the following findings and outcomes:

- Electrochemical, GNP and SPR (experimental and commercial) transducers were investigated in this project using both non-infectious NoV VPL and a cultivable MNV surrogate target.

- NoV VLP were produced to the NoV GII.4 Sydney strain and polyclonal rabbit antisera raised to this target. Both NoV VLP and sera were evaluated in biosensor development.

- Commercial SPR detected NoV VLP using both homologous polyclonal NoV antisera and non-homologous MNV (AG3) aptamer at $\geq 6.4 \times 10^6$ VLP/µL and 2×10^7 VLP/µL, respectively. The K_D of the polyclonal sera to NoV VLP was determined to be 5.3 pM. Although a functional biosensor was achieved, the sensitivity was not sufficient for detection in food matrices. Development of an experimental optical fibre SPR was unsuccessful and failure to detect NoV VLP was likely to be due to inefficient surface functionalisation of the optical fibre, rather than a failure for the bioreceptor to detect NoV VLP, as demonstrated by the results of the commercial SPR sensor.

- During this project, capability was developed and utilised for the *in-vitro* culture of MNV, enabling the use of a cultivable virus for biosensor development and evaluation.

- Two proof-of-concept biosensors (electrochemical and GNP) using the same aptamer (AG3) bioreceptor were developed for MNV detection, with both platforms showing equivalent sensitivity in test buffer.

- The GNP nanozyme atpasensor reported a LOD of 240 pfu/mL in test buffer, although performance in oyster sample extract was not evaluated.

- The developed electrochemical gold nanoporous alumina aptasensor reported a LOD of 10 pfu/mL in test buffer. Furthermore, the electrochemical biosensor demonstrated application for detection in diluted oyster extract with an LOD of 52 pfu/ml, and was able to quantify MNV in seeded oyster tissue at concentrations greater than 1,000 pfu/mL. Further work is required on oyster sample extraction methodology to reduce the need for dilution. The detection and quantification of MNV in seeded oysters was comparable to that as determined by *in-vitro* cell culture, implying that detection it is likely to be of infectious virus particles, although further work is required to confirm this. In comparison to published literature, this is the most promising data to date for detection of infective virus in food matrices (oyster) using a biosensor. The sensing strategy taken was direct, simple and highly sensitive for MNV quantification, with great potential demonstrated to shorten analysis time and easily and quickly regenerate the sensor surface by either incubating in a solution of certain ionic strength or applying a short pulse potential. Taking this strategy to the next level for detection of infectious human NoV in foods will be the next hurdle, but one which should be less challenging than at the onset of this project for two reasons: multiple human NoVs have been recently demonstrated to be cultivable *in vitro;* and substitution of AG3 for human specific NoV aptamers should be easily achievable as aptamer production and selection is becoming more automated and streamlined.

7. Implications

This project has developed rapid, sensitive new technology for detection and quantification of murine norovirus virus in oysters. With further development the technology could be used to ensure food safety standards are met, in turn increasing export potential and market access of product. The developed technology could also provide opportunity to market point-of-care diagnostic devices, enabling industry to be more proactive in managing food safety issues. The developed biosensor(s) would be a rapid diagnostic with potential for improved sensitivity of detection, eliminating the need and cost associated with sending samples to a specialised laboratory for analysis. Rapid on-site analysis would allow instantaneous decision making throughout the supply-chain, avoiding the large-scale outbreaks often associated with NoV in bivalve shellfish. The biosensor technology could also be marketed internationally, generating revenue to the Australian industry.

8. Recommendations

- Further develop the electrochemical aptasensor for infective human NoV detection and quantification as this appears to be the most promising technology developed.
- Investigate options for commercialisation of electrochemical aptasenor platform for NoV detection and quantification.

8.1 Further development

- Establish capability for human NoV culture at SARDI Food Safety and Innovation via a sabbatical in a collaborating laboratory with significant knowledge in the technique.
- Develop human NoV aptamer bioreceptors to cultured human NoV.
- Refine virus extraction in the oyster matrix to reduce interference on biosensor performance.
- Identify new modification strategies, based on differential functionalisation of external and internal surface of the electrochemical aptasensor, to minimise matrix effects when analysing oyster samples.
- Investigate the impact of decreased pore size and thickness of the nanoporous anodic alumina membrane of the electrochemical aptasenor in achieving improved analytical performance in sensitivity, limit of detection and analysis time.
- Undertake virus inactivation experiments to confirm specificity of methods for detection of infectious NoV.

9. Extension and Adoption

The results of the GNP aptasensor will be submitted for publication in a scientific journal. A draft manuscript is in preparation.

The results of the electrochemical aptasensor will be submitted for publication in a scientific journal.

Results of the project will be presented at the 9th Australasian Virology Society Meeting to be held in Adelaide, 4th-7th December, 2017.

Future discussions with commercialisation companies, other researchers and investors will be sought to identify how the developed proof-of-concept electrochemical aptasensor can be taken to the next step of demonstrating infective human norovirus detection. Collaboration with Robert Atmar, Baylor Medical School, USA, or another international group where human NoV culture has been demonstrated, will be sought to establish human NoV *in-vitro* culturing capability at SARDI FSI.

9.1 Project coverage

None

Glossary

Aptamers are termed “chemical antibodies”. They are single-stranded oligonucleotides that fold into distinct three-dimensional conformations and are capable of binding strongly and selectively to a target molecule.

A ***biosensor*** or “biological sensor” is a device made up of a bioreceptor or biological element that may be an enzyme, an antibody or a nucleic acid and a transducer. The bioreceptor interacts with the analyte being tested and the biological response is converted into an electrical signal by the transducer.

Electrochemical impedance spectroscopy (EIS) is a perturbative characterisation of the dynamics of an electrochemical process. A tool for unravelling complex non-linear processes.

Project Materials Developed

- Torok V.A. (2013) "Foodborne viruses in shellfish and current detection methods". http://safefish.com.au/wp-content/uploads/2013/03/6.Review-of-Food-Borne-Viruses.pdf) (Appendix A).
- Klantsataya E. (2013) "Biosensing technologies for virus detection" (Appendix B).
- NoV GII.4 Sydney strain VLP. ORF2 nucleotide and amino acid sequences.
- Rabbit polyclonal antisera to NoV GII.4 Sydney strain VLP

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Appendices

Appendix A: Foodborne viruses in shellfish and current detection methodologies

Appendix B: Biosensing technologies for virus detection

Appendix C: List of researchers and project staff

Valeria Torok, Kate Hodgson, Ali Turnbull and Catherine McLeod (South Australian Research and Development Institute, Food Safety and Innovation).

Klaus Oldach (South Australian Research and Development Institute, Crop Improvement).

Rodney Ratcliff (SA Pathology, Adelaide).

Elizaveta Klantsataya, Alexandre Francois and Tanya Monro (University of Adelaide).

Olan Dolezal (CSIRO Manufacturing Flagship).

Beatriz Prieto Simon, Nico Voelcker, Nekane Reta Murua and Zikri Budi (University of South Australia, Adelaide).

Vipul Bansal and Rajesh Ramanathan (RMIT University, Melbourne).