The study presented in this thesis deals with the development and characterization of nanobioprobes based biosensing platforms for the specific and ultra-sensitive detection of *S.* Typhimurium in the environmental samples. The first and foremost requirement for developing an immunosensor for *S.* Typhimurium detection is the generation of specific bioreceptor molecules which can specifically bind with *S.* Typhimurium cells and shows very less cross reactivity with *S.* Typhi and other closely or distantly related food and water borne pathogens. Antibodies are one of the major classes of proteins used as specific bioreceptor molecules for the detection of analyte based on their high affinity interaction with the analyte. In this study, the generated bioreceptors molecules labeled with different nanostructures and nanocomposite were used as nanobioprobes for the development of ultra-sensitive and specific biosensing platforms for *S.* Typhimurium detection. OPS, OmpC, Vi and the H antigens are the commonly used biomarkers for the detection of *Salmonella.* Patients infected with *Salmonella* show a detectable antibody response against the OPS and H antigens during the infection. However, the antibody titer against these molecules exist for prolong period, even after the cure of the infection (House et al. 2005; House et al. 2008; House et al. 2001), resulting in a high false positive results. Again, the structural and sequence similarity of OPS and OmpC antigen among the closely related species results in cross reactivity and thus, limits its use for accurate diagnosis at the species level (Barclay and Scott 1987). However, the diagnosis at species level is important for the treatment as well as for prevention of the development of multi drug resistance in erroneously diagnosed pathogen. The commonly used Widal test for detecting the *S.* Typhi infection is based on the detection of antibody titer against the OPS and H antigens (Parry et al. 1999) and hence, associated with all the above mentioned limitations. Commercially available kits such as

Tubex and Typhidot (Islam et al. 2016; Tam and Lim 2003) also use IgM and IgG against the above mentioned antigens and thus, having the same limitations (Dutta et al. 2006; Ochiai et al. 2008). Vi polysaccharide is specifically present in *S.* Typhi and thus has been used for its specific detection. However, no such specific biomarkers for *S.* Typhimurium are reported so far. Bacterial porins are one of the interesting candidates with reported immunogenic properties. The use of OmpC porin for *Salmonella* detection has been reported. However, studies for specific detection of *S.* Typhimurium using porins have not been explored yet. Here, we have made an attempt to characterize and compare the porins of *S.* Typhi, *S.* Typhimurium and *S.* Paratyphi along with other related pathogens such as *K. pneumoniea* and *E. coli*, to screen new specific biomarker for *S.* Typhimurium. Before screening specific biomarker against *S.* Typhimurium, growth profiling of *S.* Typhimurium and *S.* Typhi was performed. *S.* Typhimurium showed maximum growth in NB medium at 37°C, pH 7.0 in the absence of NaCl. While, *S.* Typhi showed maximum growth in NB medium at 37°C, pH 7.0 and 200 mM NaCl concentration.

Some already known biomarkers such as OPS, CMP, OmpC were extracted and purified for the generation of bioreceptor molecules. For OPS isolation, LPS was extracted, hydrolyzed and the purity of the OPS was checked using silver stained SDS-PAGE and agarose gel electrophoresis. Sugar analysis of the OPS was done by KDO and phenol sulfuric acid assay. The extracted OPS, characterized by phenol sulfuric acid assay, KDO, fluorescence and mass spectrometry techniques was conjugated with carrier protein (BSA) in an optimum molar ratio of protein: hapten (1:20) for immunization in New Zealand white rabbit for antibodies generation. Before immunization, the conjugates were characterized by SDS-PAGE, native PAGE, fluorescence spectroscopy and MALDI mass spectral analysis. A

different carrier protein (OVA) was used to prepare OPS-OVA conjugate, as a coating antigen for the characterization of OPS antibodies.

The generated antibodies were characterized by UV-Vis spectroscopy, SDS-PAGE, and they showed a very high cross reactivity of around 80% with LPS, however negligible cross reactivity was seen with BSA, Vi, OmpC, and OmpD antigens. Using the generated anti-OPS antibody, detection upto 10^1 CFU mL^{-1} was observed with a good titre of 1:128K but high cross reactivity of around 60% was observed with the closely related species. CMP of *S.* Typhimurium was extracted and further used for the isolation of OmpC. Both CMP and OmpC were used for immunizing New Zealand white rabbit. Antibodies generated against CMP showed a detection limit of 10^2 CFU mL^{-1} with an antibody titre of 1:64K and high cross reactivity with other serovars of *Salmonella.* Similar results were obtained with anti-OmpC antibody. Since, antibodies generated against OPS, OmpC and CMP showed cross reactivity within the genus *Salmonella*, these were used as capture probe during immunoassay development.

For screening new biomarker, CMP was fractioned into membrane and cytosolic proteins, followed by solubilization of membrane proteins in different detergents to separate the solubilized proteins (supernatant) from unsolubilized proteins fraction (pellet), both of which were screened for the search of specific and novel biomarker. Comparison of protein fractions between *S.* Typhimurium and *S.* Typhi showed the presence of one protein band specifically in *S.* Typhimurium. By N-terminal sequencing and LC-MS/MS analysis, the protein was found to be OmpD. The protein band was extracted from SDS-PAGE and was used for immunization. To obtain high yield of the screened OmpD protein, the OmpD gene sequence was synthesized from genescript and expressed in *E. coli* BL21 and BL21 c43

using pET 28a expression vector. The expressed protein was found to form inclusion bodies, which were solubilised, purified and partially refolded for immunization purposes. The expression of protein was checked at different temperature and pH, and was estimated by densitometric analysis of the SDS-PAGE gel containing whole cell proteins. The expression of protein was found maximum at 25°C and pH 5.0. Further, specific epitopes of OmpD were screened using different bioinformatics tools to further resolve the cross reactivity issues. Eight different epitopes were screened that can be used in future for bioreceptor development with even more specificity than OmpD. Also, while screening specific biomarker for *S.* Typhimurium detection, marker for *S.* Paratyphi, *E. coli, K. pneumoniae* were found. The generated anti-OmpD antibodies showed very good specificity towards *S.* Typhimurium, showing very less percentage cross-reactivity towards *S.* Typhi cells. The specific binding of anti-OmpD antibodies with *S.* Typhimurium cells was also confirmed by confocal microscopy, FACS and TEM analysis with proper controls.

Nanotechnology is playing an important role for the development of new diagnostics approaches in the field of environmental monitoring and clinical diagnostics. A number of nanostructures such as GNPs, SNPs, G-GO nanocomposites, Gold nanorods have been synthesized and utilized as bionanoprobes by conjugating them with bioreceptors for the detection of pathogens in environmental samples. For the stabilization of negatively charged GNP, antibody concentration was optimized by calculating the critical flocculation concentration. The critical flocculation concentration was determined by shift in the peak from 525 nm to 610 nm as a result of aggregation of GNPs. The GNP-Ab conjugate was confirmed by TEM, DLS and agarose gel electrophoresis. The aggregation profile of the antibody functionalized GNPs was confirmed by observing the shift in peak maxima from

520 nm to 610 nm. The specific nanobioprobes have successfully been used for the development of different immunoassays for the detection of *S.* Typhimurium in environmental samples. Similarly, SNP and gold nanorods were also characterized.

G-GO nanocomposite was synthesized from graphite and the structural aspects of rG-GO nanocomposite were investigated by using UV-Vis spectroscopy, FESEM, TEM, AFM, FTIR and Raman spectroscopy for confirming the deposition of rG-GO on SPE's. The sheet-like structure of the graphene was preserved after chemical modification as confirmed by FESEM which showed well defined and interlinked three-dimensional graphene sheets. The G-GO nanocomposite was also used for the detection of *S.* Typhimurium in environmental samples.

Different immunoassay platforms for the detection of *S.* Typhimurium in environmental samples were developed using the specific bionanohybrid receptors. Sandwich immunoassay using GNP was developed for the detection of *S.* Typhimurium. In sandwich format, anti-OmpC antibody was used as the capture molecule in dot blot format while GNP conjugated anti-OmpD antibodies were used as the detector probe. Depending upon the concentration of pathogen present, difference in binding of GNP labeled anti OmpD Ab was observed. Using this developed immunoassay technique, *S.* Typhimurium cells were detected with a sensitivity of 10^2 CFU mL^{-1}. Though the sensitivity achieved using the developed immunoassay was good, but the aim of the present study was the detection of pathogens even at low concentration. Therefore, in order to further improve the sensitivity, enhanced chemiluminescence assay was developed. 10 fold enhancement in the sensitivity was observed using HRP labeled OmpD antibody. Metallic nanoparticles such as GNPs, gold nanorods, and SNPs of different sizes and aminophenyl boronic acid (APBA) were tested for

further enhancement of chemiluminescence immunoassay. Maximum enhancement of 10 fold was achieved using APBA, i.e overall 100 fold enhancement in sensitivity was achieved using the developed immunoassay. The generation of phenoxyl radical from phenyl ring can be considered as one of the main factor for enhancement, which might help to increase the radiative decay during the reaction and thus affect the HRP-catalyzed chemiluminescent oxidation of luminol in the presence of H_2O_2. Also, amine groups present at the 4-substituent group of aminophenyl boronic acid, acts as electron donor groups which enhance the electron density in the oxygen of the phenolic OH group and through the inductive effect of para-orientation it influences the stabilization of the phenoxy radical (Navas DÃ-az et al. 1996; Wu et al. 2013). Presence of nitrogen groups of amine further adds to enhancement by providing a resonance stabilization of the phenoxyl radicals through π-delocalization. The developed chemiluminescence immunosensor was also tested for its specificity by conducting some negative control experiments with *S.* Typhi, *E. coli, K. pneumoniea* and *S.* Paratyphi, results showed very low cross reactivity. Validation of the developed platform was done in spiked juice samples. Photographic detection was also performed in the similar manner in real and spiked juice samples.

Since, in all the above developed immunoassays formats, two antibodies are required for detection of *S.* Typhimurium. But to reduce the cost of assay, and also to decrease the total assay time, use of single antibody is always preferred. Hence, different assay platforms were developed, which require only a single Ab (anti-ompD antibody) as the detector probe using electrochemical impedance spectroscopy and Square wave voltametry. The Ab/rGO/SPE electrochemical impedance developed immunosensor was capable of detecting 10^1 *S.* Typhimurium CFU mL^{-1}. We observed a significant increase in impedance with the

increase in the number of cells. The Ab/rGO/SPE immunosensor proposed in this study was also tested for its specificity by conducting some negative control experiments with *S.* Typhi, *E. coli, K. pneumoniea, S.* Paratyphi and *Bacillus* species, demonstrating very less cross reactivity with all above closely related pathogens. The developed immunosensor was also validated in spiked juice and milk samples. Amperometric based electrochemical immunoassay format for the specific detection of *S.* Typhimurium using specific anti-OmpD antibodies was developed with a sensitivity of 10^1 CFU mL^{-1}. Anti-OmpD antibodies were used as a capture molecule for *S.* Typhimurium and ferrocene boronic acid as a tracer molecule due to well known binding of boronic acid with oligosaccharides present in the cells by the formation of cis diol linkage. Large window clearly indicated that lower detection was also possible.

Our results represent a choice between different suitable method for detection and monitoring of *S.* Typhimurium in environmental samples depending upon the requirement of desired sensitivity and time kinetics. The developed immunosensors presented in this thesis, demonstrated very high sensitivity, besides good specificity and reproducibility in comparison to the existing methods. We have shown the use of OmpD as a biomarker in diagnostics for the detection of *S.* Typhimurium in environmental samples for the first time. This simplistic approach uses specific screened biomarkers with stable low cost nano materials to develop immunosensing techniques that can be used for real samples analysis.

Future perspectives:

Modern biosensing techniques are gaining importance these days, due to the increased cases of food and water borne diseases which demands for the development of inexpensive, reliable assay techniques for qualitative and quantitative monitoring of

pathogens in environmental samples. Immunosensors are replacing the current culturing and other established techniques for pathogen monitoring since antibodies can be produced against specific biomarkers for immunoassay development for qualitative and quantitative analysis with higher sensitivity, specificity, accuracy and precision than other conventional analytical methods. However, as the world becomes more concerned about the impact that environmental contamination may cause on public health and the ecosystem, the demand for rapid detecting biosensors will further increase.

Specificity is of major concern while detecting pathogens in environmental samples due to similarity in genes of various food borne pathogens. Using the developed immunosensors, we can detect *S.* Typhimurium in environmental samples. Also, the other screened biomarkers can be used for generating antibodies or aptamers for the specific detection of *E. coli, K. pneumoniae, S.* Typhi, *S.* Paratyphi and immunosensors can be developed for the detection of these pathogens. Therefore, a combo card can be developed in future using the same approach for the simultaneous detection of different food and water borne pathogens.