2016

Development of a biosensor system to detect bacteria in food systems

Luis Alonso Alfaro Sanabria

Louisiana State University and Agricultural and Mechanical College, luisalonsoalfaro77@gmail.com

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Part of the Engineering Commons

Recommended Citation

https://digitalcommons.lsu.edu/gradschool_dissertations/4472

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradtd@lsu.edu.
DEVELOPMENT OF A BIOSENSOR SYSTEM TO DETECT BACTERIA IN FOOD SYSTEMS

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Biological and Agricultural Engineering

by

Luis Alonso Alfaro Sanabria
B.S., Zamorano University, 2007
M.S., Louisiana State University, 2012
December 2016
ACKNOWLEDGEMENTS

First, I want to thank God for being my provider and opening doors for unique opportunities in life, thank you God for giving me health and being my shelter in difficult times. I want to thank Dr. William Richardson and the LSU AgCenter for supporting me financially during my Masters and Ph.D. studies at LSU, without their support I would not have been able to obtain these achievements in my career. I want to thank Dr. Subramaniam Sathivel for being my advisor these years at grad school and always encouraging me to do my best academically and personally, thank you for having patience with me and for teaching me professional and life lessons. I also want to express gratitude to my committee members Dr. Jin-Woo Choi, Dr. Joan King, Dr. Joseph Bankston, and graduate dean’s representative Dr. Gerald Baumgartner for their support in my research and their help in reviewing my dissertation; special thanks to Dr. Choi for having patience with me and always open his door for answering all my questions during the project. Special thanks to Mr. Chris O'Loughlin and Mr. Evan Wong from the Department of Electrical and Computer Engineering for their kind help at the EMDL laboratory during my research.

Sincere thanks to all my lab mates during my Masters and Ph.D. studies for the enjoyable times at work and classes. My deepest appreciation to my friends from Zamorano Agricultural Society (ZAS) at LSU for all the good times. My great appreciation and thanks to my parents for always believing in me, thank you Mother and Father (R.I.P.) for teaching me humility, for your advice, prayers, support, and love. I also want to thank my sister-in-law Carolina Avellaneda for her friendship and fun moments at LSU. Finally and not least, I want to thank my wife Kimberly Avellaneda for believing in me, for always being there in the good and bad times, for helping me being a good man, for your love and patience, I love you.
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS.................................................................................................................. ii
LIST OF TABLES ............................................................................................................................. vii
LIST OF FIGURES ........................................................................................................................... viii
ABSTRACT........................................................................................................................................ xv
CHAPTER 1 ......................................................................................................................................... 1
LITERATURE REVIEW ...................................................................................................................... 1

1.1 Sample collection ....................................................................................................................... 2
  1.1.1 Use of cellulose membranes for sample collection ............................................................. 4
    1.1.1.1 Cellulose structure ......................................................................................................... 4
    1.1.1.2 Morphology of cellulose ............................................................................................... 5
    1.1.1.3 Cellulose membranes for collection of bacteria ............................................................ 5

1.2 Bacteria concentration and immobilization for detection ......................................................... 7
  1.2.1 Dielectrophoresis ................................................................................................................ 8
  1.2.2 Field-flow fractionation ....................................................................................................... 12
  1.2.3 Dielectrophoresis Field-flow fractionation (DEP-FFF) ........................................................ 15
  1.2.4 Other concentration methods ............................................................................................. 16
    1.2.4.1 Microfluidics and meniscus dragging effect ................................................................. 16
    1.2.4.2 Nanoimprinted holes ................................................................................................... 18

1.3 Sample detection ....................................................................................................................... 19
  1.3.1 Biosensors for food safety ................................................................................................. 19
  1.3.2 Definitions and classification of biosensors ...................................................................... 20
  1.3.3 Biorecognition elements in biosensors ........................................................................... 21
    1.3.3.1 Antibodies .................................................................................................................... 21
    1.3.3.2 Enzymes ...................................................................................................................... 22
    1.3.3.3 DNA based recognition elements ................................................................................. 22
  1.3.4 Electrochemical biosensors ............................................................................................... 23
  1.3.5 Optical biosensors ............................................................................................................ 30
  1.3.6 Surface plasmon resonance (SPR) based biosensors ....................................................... 35
  1.3.7 Enzyme based biosensors ............................................................................................... 41
  1.3.8 Mechanical biosensors ..................................................................................................... 47
1.3.9 Other novel sensors – nanobiosensors .............................................................. 53
1.4 Point-of-care (POC) detection and recent developments in POC ................................ 55
1.5 Listeria monocytogenes .......................................................................................... 60
1.6 Teichoic acids in cell wall of Gram-positive bacteria .............................................. 61
1.7 Carbon nanotubes .................................................................................................. 64
  1.7.1 Definitions and electrical properties of carbon nanotubes ......................... 64
  1.7.2 Use of carbon nanotubes in electrochemical biosensors ............................ 67
  1.7.3 Incorporation of carbon nanotubes as transduction elements in potentiometric biosensors .......................................................... 68
1.8 Objectives .............................................................................................................. 70
1.9 References ............................................................................................................ 72

CHAPTER 2 ................................................................................................................. 81
DEVELOPMENT OF A SAMPLE COLLECTION PROTOCOL AND RECOVERY SYSTEM FOR LISTERIA INNOCUA FROM FOOD SURFACES ......................................................... 81
Abstract .................................................................................................................. 82
2.1 Introduction ............................................................................................................ 82
2.2 Materials and methods ....................................................................................... 84
  2.2.1 Inoculum and inoculation............................................................................. 84
  2.2.2 Sampling protocol and optimization of sample collection with cellulose membrane..... 85
  2.2.3 Scanning electron microscopy of cellulose membranes .................................. 86
  2.2.4 Statistical analysis ....................................................................................... 86
2.3 Results and discussion ....................................................................................... 87
  2.3.1 Optimization of sampling time with cellulose membrane ....................... 87
  2.3.2 Sample recovery efficiency of cellulose membrane .................................... 89
  2.3.3 Characterization of cellulose membranes by SEM ................................... 93
2.4 Conclusion ............................................................................................................ 94
2.5 References ............................................................................................................ 96

CHAPTER 3 ................................................................................................................. 99
DEVELOPMENT OF A MICROFLUIDIC DEVICE FOR CONCENTRATION OF BACTERIA BASED ON VOLUME REDUCTION BY HYDROGELS ......................................................... 99
Abstract .................................................................................................................. 100
3.1 Introduction ............................................................................................................ 100
3.2 Materials and methods .......................................................... 103
  3.2.1 Microfluidic device fabrication .............................................. 103
  3.2.2 Fabrication and characterization of hydrogel films .................. 105
  3.2.3 Concentration visualization with fluorescent beads .................. 107
  3.2.4 Bacteria preparation .......................................................... 107
  3.2.5 Concentration of bacteria and recovery efficiency determination .... 108
  3.2.6 Statistical analysis .......................................................... 108
3.3 Results and discussion ........................................................... 109
  3.3.1 Hydrogel films characterization ......................................... 109
  3.3.2 Fluorescent beads concentration and quantification .................. 111
  3.3.3 Concentration of bacteria samples ....................................... 114
3.4 Conclusion .................................................................................. 119
3.5 References .................................................................................. 120
CHAPTER 4 ....................................................................................... 122
DEVELOPMENT OF A CARBON NANOTUBE POTENTIOMETRIC BIOSENSOR FOR A
QUANTITATIVE DETECTION OF LISTERIA INNOCUA FROM FOOD SAMPLES ........ 122
Abstract ......................................................................................... 123
4.1 Introduction ............................................................................... 123
4.2 Materials and methods .............................................................. 126
  4.2.1 SWCNTs oxidation ............................................................... 126
  4.2.2 Oxidation characterization by Fourier Transform Infrared Spectroscopy (FTIR) .... 127
  4.2.3 Preparation of biosensor ....................................................... 127
    4.2.3.1 Glassy carbon electrode activation .................................... 127
    4.2.3.2 SWCNTs deposition on GCE ........................................... 128
    4.2.3.3 Activation of CNT layer on GCE by EDC/Sulfo-NHS chemistry .......... 129
    4.2.3.4 SWCNTs-antibody functionalization ................................... 129
  4.2.4 Characterization of SWCNT-antibody functionalization .............. 130
  4.2.5 Electrochemical measurements ............................................. 131
  4.2.6 Bacteria preparation ............................................................ 132
  4.2.7 Characterization of the biosensor’s selectivity ............................ 133
  4.2.8 Testing of biosensor with food samples ................................... 134
4.2.9 Statistical analysis ............................................................................................................. 136
4.3 Results and discussion ....................................................................................................... 137
  4.3.1 Characterization of oxidation of SWCNTs by FTIR ................................................. 137
  4.3.2 Characterization of SWCNTs modified electrode by Scanning Electron Microscopy (SEM) ......................................................................................................................... 139
  4.3.3 SWCNT-antibody functionalization characterization by TEM ................................... 140
  4.3.4 Detection of bacteria using the carbon nanotube based potentiometric biosensor ...... 143
  4.3.5 Selectivity of the biosensor .......................................................................................... 148
  4.3.6 Testing of biosensor with food samples ......................................................................... 150
  4.3.7 Design and fabrication of biosensor system ................................................................. 154
4.4 Conclusion .......................................................................................................................... 157
4.5 References ........................................................................................................................ 159
CHAPTER 5 ............................................................................................................................... 161
GENERAL CONCLUSIONS ...................................................................................................... 161
VITA ........................................................................................................................................ 163
LIST OF TABLES

Table 1.1 Comparison of the characteristics between traditional techniques and biosensors (Van Dorst et al., 2010) ........................................................................................................................................ 20

Table 1.2 Table of diffusion coefficient (D) of the redox probe for L. monocytogenes (Susmel et al., 2003) ........................................................................................................................................ 27

Table 1.3 Concentrations of Rac (ng g\(^{-1}\)) detected by SPR biosensor and UPLC-MS/MS (Lu et al., 2012) ........................................................................................................................................ 41

Table 1.4 Effect of untreated food samples on the activity of AChE in comparison to isooctane extracts of corresponding foodstuff (unspecific inhibition) (Schulze et al., 2002) ................. 46

Table 4.1 EMF values for final concentrations of L. innocua ................................................................. 144

Table 4.2 EMF values for final concentrations* of L. innocua from meat samples ...................... 151

Table 4.3 EMF values for final concentrations* of L. innocua from milk samples ..................... 153
LIST OF FIGURES

Fig. 1.1 Functional components of food microbiological analysis (Brehm-Stecher et al., 2009) ................................................................. 2

Fig. 1.2 Repeating units of a cellulose chain (O'Sullivan, 1997) ................................................................................................................. 4

Fig. 1.3 Electron micrographs of cellulosic microfibrils of different origins (Kennedy, Phillips, & Williams, 1993) ........................................ 5

Fig. 1.4 Comparison of electron micrographs at 7000 magnification (scale bars = 2 µm), for the different membranes (A) PVDF, 0.45 µm; (B) mixed cellulose, 0.45 µm; (C) polycarbonate, 0.4 µm. Arrow indicates where the bacteria are buried inside tortuous and non-uniform structure of (A) PVDF or (B) cellulose, but not (C) polycarbonate (Pettipher, Mansell, McKinnon, & Cousins, 1980) ......................................................... 6

Fig. 1.5 Principle and operation of the dielectrophoresis-based deviation and capture of cells in the bioprocessor (Gomez-Sjoberg et al., 2005) ................................................................. 8

Fig. 1.6 3-D schematic of (a) the impedance based biosensor for pathogens detection. (b) Magnified view of the focusing region, and detection region (Dastider et al., 2013) ................. 10

Fig. 1.7 (A) The non-flowthrough chip used in this study for DEP enhanced immunocapture of Salmonella cells, which consists of a chamber formed by PDMS and a set of interdigitated microelectrodes (IMEs) on a glass substrate and (B) the experiment setup (Yang, 2009) .......... 11

Fig. 1.8 Schematic representation of the FFF separation principle. Separation depends on the combined action of an axial flow and a field applied in a perpendicular direction. The axial flow is governed by an ideally parabolic flow profile along the channel thickness. Separation depends on interaction of the sample components with an externally generated field, which is applied perpendicularly to the direction of the longitudinal flow. The role of the field is to drive different species into different regions of the parabolic flow profile, from where they are eluted at different velocities (Rambaldi et al., 2011) .................................................................................. 12

Fig. 1.9 Schematic of GrFFF-CL immunometric method for determination of Y. enterocolitica. B) Presentative calibration curve, obtained by averaging 10 calibration curves from different days. RLU= relative light units (Magliulo et al., 2006) ........................................................................... 14

Fig. 1.10 Separation principle of DEP-FFF. a) A conventional DEP-FFF device with interdigitated electrodes levitating particles by nDEP force. b) Combined pre-focusing and fractionation functions using both nDEP and pDEP (P. Gascoyne, Satayavivad, & Ruchirawat, 2004; Holmes, Green, & Morgan, 2003) ........................................................................... 16

Fig. 1.11 Working principle for the meniscus dragging effect (Zhang et al., 2010). ............... 17

Fig. 1.12 (a) Fabrication process of nanoimprinted holes. (a-i) Photoresist coating on ITO glass slide. (a-ii) development. (b-i) SU-8 coating. (b-ii) Micro holes patterning by nanoimprint. (b-iii)
Plasma etching. (iv) Fluidchannel patterning. (v) Thermo compression bonding. (vi) Completion (Kano et al., 2013).

Fig. 1.13 Basic representations of the antibody’s structure (Afaneh, Aull, & Kapur, 2012; Tasman & Jaeger, 2010).

Fig. 1.14 Aptamer-target binding mechanism (Bio-Resource, 2016).

Fig. 1.15 Diagram of the functioning of a biosensor for detection of inulin in foods (Javier Manso et al., 2008).

Fig. 1.16 DAO and PAO enzyme reactions with amines (Esti et al., 1998).

Fig. 1.17 calibration curves for amperometric detection of Escherichia coli (a), Listeria monocytogenes (b), and Campylobacter jejuni (c) (Chemburu et al., 2005).

Fig. 1.18 Fiber optic spectrometer based biosensor (Chang et al., 2001).

Fig. 1.19 Diagram of the FRET immunosensor. Left; recognition element. Right; transduction element (Ko & Grant, 2006).

Fig. 1.20 Optimization of detection limit (a) and specificity (b) of the fiber optic biosensor (Geng et al., 2006).

Fig. 1.21 Experimental setup for SPR biosensor (Subramanian et al., 2006).

Fig. 1.22 Sensitivity of detection of S. aureus on alkane monothiol surface (Subramanian et al., 2006).

Fig. 1.23 Calibration curves for 11benzimidazole carbamates in bovine milk matrix (Keegan et al., 2009).

Fig. 1.24 The immobilization of Rac derivatives and inhibition response SPR curve (1) activation; (2) immobilization or Rac derivatives; (3) inactivation; (4) Rac containing Rac antibody injected on the chip surface (Lu et al., 2012).

Fig. 1.25 Residual activity after repeated glucose analysis with biosensor using GOD immobilized with different PBSA (Gouda et al., 2002).

Fig. 1.26 Residual activity after repeated sucrose analysis with biosensor with different PBSA (Gouda et al., 2002).

Fig. 1.27 Calibration curve for AChE inhibition caused by different paraoxon concentrations (Schulze et al., 2002).
Fig. 1.28 AChE-inhibition caused by incubation in isooctane-extracts of different food samples and in buffer spiked with 10 µg/kg of paraoxon, compared with incubation in isooctane extracts of unspiked food samples and in pure isooctane (negative control) (Schulze et al., 2002)........ 47

Fig. 1.29 Schematic diagram for sensor fabrication and bacterial binding (Su & Li, 2004)........ 49

Fig. 1.30 Cyclic voltammograms of the same Au electrode in the presence of 10 mM Fe(SCN)63-/4- (scan rate 100 mV/s). a) Bare Au electrode; b) after formation of MHDA-monolayer; c) after activation with EDC/NHS; d) after immobilization of the antibodies; and e) after binding of E. coli O157:H7 cells (Su & Li, 2004).............................................................. 49

Fig. 1.31 Response of the biosensor to different concentrations of Pseudomonas aeruginosa. (a) 1.3x10⁷, (b) 2.0x10⁷, (c) 3.1x10⁷, (d) 6.3x10⁷, and (e) 1.3x10⁸ CFU/mL (Kim et al., 2004)......... 52

Fig. 1.32 Principal component analysis (PCA) plot for the six wines (Santos et al., 2005)........ 53

Fig. 1.33 a) IDAM chip with gold microelectrodes on a glass wafer, b) microchannel with detection microchamber, and inlet and outlet channels, c) assembled microfluidic flow cell with embedded IDAM and connection wires (Varshney et al., 2007). ................................................................. 54

Fig. 1.34 A) gel electrophoresis for the DNA double-tagged amplicon. B) Rapid electrochemical verification of PCR amplification of Salmonella enterica serovar Typhimurium (Brasil de Oliveira Marques et al., 2009) ...................................................................................... 55

Fig. 1.35 Schematic illustration of a fabricated multiplex immunochromatographic strip. The strip is composed of four different functional membranes: (A) sample-application pad, (B) conjugate-release pad containing the reporter probes, (C) signal generation pad, and (D) absorption pad. The signal-generation pad contains four dots to detect (a) Salmonella typhimurium, (b) Staphylococcus aureus, (c) L. pneumophila, and (d) E. coli O157:H7, and one dot for control signal (e). The arrow indicates vertical flow of the immunoreaction (Park et al., 2010).................................................................. 57

Fig. 1.36 Schematic diagram of the paper-based bacteria sensor. A) Sensing strategy for detection of single bacterial species, in which either XG or CPRG (color reaction or CR zone) and FeCl₃ (Fe zone) are entrapped within sol–gel-derived silica materials in the two dashed regions on a Whatman no. 1 paper strip (0.5×8 cm) via inkjet printing. A hydrophobic barrier (HB zone) composed of MSQ is layered at the top of the sensing zone to prevent leaching of color and thus increase signal intensity. The sensor is dipped into a cell lysate until the liquid front reaches the substrate/sensing region. Color appearance in the CR zone is indicative of the presence of bacteria. B) Bidirectional multiplexed sensing strategy in which XG, CPRG, and FeCl₃ are entrapped within sol–gel-derived silica materials in separate regions on a paper strip. A HB barrier is introduced between two sensing zones. The bottom of the paper strip is first placed into a cell lysate (E. coli BL21 in this case) and allowed to flow. The sensor is then inverted, immersed into the same lysate, and allowed to flow. The color intensity is monitored after 30 min at room temperature, unless otherwise stated. C) Optional preconcentration step in which cells are first isolated from samples via magnetic pulldown, resuspended in a minimal volume of a lysing reagent (10–100-fold less volume than initial sample) and then tested (Hossain et al., 2012) ........................................................................ 58
Fig. 1.37 Specificity testing using samples of (a) *S. aureus*, (b) *P. aeruginosa*, (c) *S. aureus + P. aeruginosa*, (d) *E. coli*, (e) *S. aureus + E. coli*, and (f) *P. aeruginosa + E. coli*. Left: the testing lines of the strips were modified by anti-*S. aureus* antibody. Right: the testing lines of strips were modified by anti-*P. aeruginosa* antibody. Sample (d) served as a negative control (Li et al., 2011).

Fig. 1.38 Wall teichoic acid structure (Neuhaus & Baddiley, 2003).

Fig. 1.39 Schematic of the gram-positive cell wall showing the wall teichoic acids (Brown, Santa Maria Jr, & Walker, 2013).

Fig. 1.40 Model for the role of WTA in proton binding and control of autolysin activity. (A) The negatively charged WTA phosphate groups retain protons in the cell wall, which creates an acidic environment keeping the activity of the major autolysin AtlA low. (B) In the absence of WTA protons are not retained, which avoids local acidification and leads to higher activity of AtlA (Biswas et al., 2012).

Fig. 1.41 Single wall carbon nanotube (SWCNT) structures (Weber, 1999).

Fig. 1.42 Multiwall carbon nanotubes (MWCNTs) structure (JCrystalSoft, 2016).

Fig. 1.43 System for specifying nanotube chiralities. (a) The tube is specified by the circumferential vector na1 + ma2 denoting a vector joining two equivalent points on the graphene lattice in terms of the unit vectors a1 and a2. The vector notation is simplified to (m, n). The tube is generated by rolling the graphene lattice so that the vector lies on a circumference, as shown by the arrows, and joining the start and end points. (b) An example of an (8,8) tube, also called an armchair tube because of the pattern of carbon atoms at the end. Any (n, n) tube will have an armchair configuration. (c) An example of a (12,0) tube, also called a zigzag tube because of the pattern of carbon atoms at the end. Any (n, 0) tube will have a zigzag configuration (Binns, 2010).

Fig. 1.44 Schematic description of the ion-to-electron transduction mechanism of the GC/SWCNT/Electrolyte system. C⁺ = cation, A⁻ = anion, e⁻ = electron (G. A. Crespo et al., 2008; G. n. A. Crespo et al., 2008).

Fig. 2.1 *Listeria innocua* counts for different sampling times with cellulose sampling membrane. Values expressed as mean ± standard deviation of three determinations. ABC Means within columns with different letters are significantly different (p<0.05).

Fig. 2.2 Absorption efficiency (%) of cellulose membrane at different sampling times from inoculated meat samples with *Listeria innocua*. Values expressed as mean ± standard deviation of three determinations. AB Means within columns with different letters are significantly different (p<0.05).
Fig. 2.3 Scheme of sample introduction after sampling with cellulose membrane. (A) Cellulose membrane containing sampled bacteria before detection. (B) Sampling membrane is placed into biosensor and bacteria is carried out from membrane into the detection zone by forced fluid flow.

Fig. 2.4 Scanning electron micrographs of cellulose membranes after sample collection. A) Cellulose membrane after sampling *L. innocua* $10^1$ CFU/mL concentration, bar = 10 µm, 2,000X. B) Cellulose membrane after sampling *L. innocua* $10^5$ CFU/mL concentration, bar = 10 µm, 2,000X. C) Close up micrograph for *L. innocua* $10^5$ CFU/mL sample, bar = 5 µm, 5,000X. D) Cellulose membrane without sampling (control), bar = 100 µm, 150X

Fig. 3.1 Microfluidic concentrator design A) 2D front view design of concentrator. B) Exploded view of assembled concentrator which contains: 1) top concentrator card, 2) nylon membrane, 3) support part, 4) pectin-based hydrogel film, 5) bottom card.

Fig. 3.2 Operation principle of the hydrogel film based concentrator

Fig. 3.3 Swelling degree of pectin-based hydrogel used in the concentrator. ■ = Swelling degree

Fig. 3.4 Swelling ratio of pectin-based hydrogel used in the concentrator. ■ = Swelling ratio.

Fig. 3.5 Concentration of fluorescent beads in the concentrator. Fluorescence images of the channels over time

Fig. 3.6 Fluorescence intensity trough the channel over concentration time

Fig. 3.7 Picture of microfluidic concentrator before running it with bacteria sample.

Fig. 3.8 Recovery efficiency after concentration from different bacterial concentrations. Values expressed as mean ± standard deviation of three determinations. A-D Means with different letters are significantly different (p<0.05).

Fig. 3.9 Bacteria counts after concentration with microfluidic concentrator device. Values expressed as mean ± standard deviation of three determinations. ± Means in columns with different letters are significantly different (p<0.05). □ = L. innocua counts before concentration. ■ = L. innocua counts after concentration

Fig. 4.1 SWCNTs oxidation reaction

Fig. 4.2 SWCNTs functionalization scheme

Fig. 4.3 Electrochemical measurement scheme with potentiometric biosensor
Fig. 4.4 Process for detection of bacteria from food samples using the biosensor. A) Steps for detection from meat sample; 1) inoculation of bacteria on meat surface, 2) sampling from meat surface using cellulose membrane; 3) recovery, separation and concentration of collected bacteria prior to detection using concentrator device, 4) detection of bacteria with potentiometric biosensor. B) Steps for detection of milk sample; 1) inoculation of milk sample with bacteria, 2) separation and concentration of bacteria, 3) detection with potentiometric biosensor. 136

Fig. 4.5 FTIR spectra of raw (untreated) and functionalized (acid treated) SWCNTs. 137

Fig. 4.6 Scanning electron micrographs of the SWCNTs modified electrode. A) Micrograph of electrode’s surface (bar = 100 µm, 150X). B) Close-up of the deposited layer of SWCNTs on the GCE (bar = 5 µm, 3000X). 139

Fig. 4.7 TEM images showing the antibody functionalization of the SWCNTs. A) Close-up image showing the antibody conjugated SWCNTs. B-C) Images showing random attachment of antibodies to the walls of SWCNTs. D) Aggregates of conjugated antibodies in the suspension. Arrows indicate the antibodies in the figures. 141

Fig. 4.8 TEM images showing unconjugated SWCNTs (control). (a) Suspended SWCNTs showing the presence of shorter and larger sizes of CNTs. (b) Close-up image of unconjugated SWCNTs showing the presence of the surfactant in the solution. 143

Fig. 4.9 Standard curve for EMF values vs concentrations of Listeria innocua. EMF = electromotive force. 144

Fig. 4.10 Biosensor real-time response when subjected to stepwise increases of concentrations of L. innocua. 147

Fig. 4.11 Biosensor response vs time for different concentrations of bacteria (CFU/mL) for selectivity test. A) EMF response for biosensor tested with Lactobacillus plantarum; B) EMF response for biosensor tested with Lactobacillus acidophilus; C) EMF response for biosensor tested with generic E. coli (non-pathogenic). EMF = electromotive force. 149

Fig. 4.12 Biosensor response with meat samples EMF values versus concentrations of inoculated L. innocua in meat. EMF = electromotive force. 150

Fig. 4.13 Biosensor response for milk samples. EMF values versus concentrations of inoculated L. innocua in milk. EMF = electromotive force. 152

Fig. 4.14 Pictures of the potentiometric carbon nanotube based biosensor. 154
Fig. 4.15 Biosensor system design. (a) Exploded view of biosensor system design. (b) Scheme of assembled biosensor system. (c) Picture of fabricated biosensor system.
ABSTRACT

The development of biosensors may assist for the on-site detection of foodborne pathogens. The overall goal of this study was to develop a biosensor system for detecting *Listeria innocua* (non-pathogenic surrogate bacteria used as a model for pathogenic *Listeria monocytogenes*) in food systems. The study was divided into three main parts: (1) development of a sample collection and interface system for *Listeria innocua* from food samples, (2) development of a sample concentration system for the collected bacteria prior detection, and (3) development of a detection system based on a carbon nanotube potentiometric biosensor for a quantitative detection of *Listeria innocua*.

In the second chapter, we discussed a sample collection protocol and delivery system developed for bacteria from food surfaces. *Listeria innocua* was used for testing and illustration. For this purpose, the surface of meat samples was inoculated with *Listeria innocua* at different concentrations from $10^1$-10$^5$ CFU/mL. Then, cellulose membranes were applied to the surface of products for different times: 5, 10, 15, 20, 25, and 30 min sampling. The cellulose membranes were analyzed for their suitability for bacteria enumeration using a plating method for *Listeria innocua*. It was observed that sampling times between 5-10 min were the best and collection of $>80\%$ of bacteria from the food’s surface was achieved.

In the third chapter we discussed a microfluidic device for concentration of biological samples based on removal of liquids by hydrogel films. The performance of the device was demonstrated by concentrating 1-5 µm fluorescent beads followed by concentration of bacteria samples such as *Listeria innocua*. Results showed that fluorescence intensity of the beads was increased by 10 times at the end of concentration. Recovery efficiencies of 85.60 and 91.75 % were obtained for
initial bacteria concentrations of 1x10¹ and 1x10² CFU/mL. Moreover, cell counts were observed to increase by up to 10 times at the end of concentration. This study showed that the concentrator device successfully concentrated the samples and no significant loss of living cells was observed for most of the bacteria concentrations.

A carbon nanotube potentiometric biosensor for the detection of bacteria from food samples was demonstrated in the fourth chapter. The biosensor was constructed by depositing carboxylic acid (–COOH) functionalized single walled carbon nanotubes (SWCNTs) on a glassy carbon electrode (GCE), followed by the attachment of anti-Listeria antibodies to the SWCNTs between the amine groups and the –COOH by covalent functionalization using EDC/Sulfo-NHS chemistry.

The performance of the biosensor was evaluated at various concentrations of L. innocua, for factors such as limit of detection, sensitivity, response time, linearity, and selectivity. In addition, the application of the complete detection system based on sample collection, concentration and detection of bacteria from food samples such as meat and milk was evaluated. Results showed that the system could successfully detect L. innocua with a linear response between electromotive force (EMF/voltage) and bacteria concentrations and a lower limit of detection of 11 CFU/mL. Additionally, similar results were obtained from the biosensor system for L. innocua from food samples.
CHAPTER 1

LITERATURE REVIEW
1.1 Sample collection

The detection of pathogens usually requires the use of different steps that include: sample collection, enrichment with selective media, culturing in specific agar or media for the organism, isolation, and biochemical tests. Sample collection is the first step on the list, therefore it is crucial that the sample is collected appropriately. (Fig. 1.1) (Brehm-Stecher, Young, Jaykus, & Tortorello, 2009). Most research has been focused on the improvement of the detection of pathogens and the part of the process that deals with sample collection and preparation is often disregarded. According to the International Commission on Microbiological Specifications for Foods (ICMSF), a sampling plan is defined as a statement of the criteria of acceptance to be applied to a lot, based on examination of a required number of sample units by defined analytical methods (Thatcher, 1974). The ICMSF has established and written standard procedures for the development of sampling plans for a variety of food products (Staff, 2012; Thatcher, 1974). As Brehm-Stecher et al. (2009) points out, sampling involves considerations of unit size, it depends on the purpose of the analysis, and also the need for knowledge of the targeted microbial load and its distribution in the sample. Therefore, the sampling step is critical in the design of any detection method.

Fig. 1.1 Functional components of food microbiological analysis (Brehm-Stecher et al., 2009).
Some approaches for sampling surfaces include wet vacuum-based surface sampling, which is based on the use of high-efficient particulate arrestance (HEPA) socks assembled in a vacuum filtration system (Sanderson et al., 2002), recirculating immunocapture (Morales-Rayas, Wolffs, & Griffiths, 2008; Warren, Yuk, & Schneider, 2007), and continuous flow centrifugation (Ágoston et al., 2009). The drawback of these methods is that they require specialized instrumentation, which is usually expensive. The use of simpler and less expensive methods for sampling has been evaluated. For example, Bisha and Brehm-Stecher (2009) showed the use of adhesive tape for sampling tomato surfaces combined with rapid fluorescence for Salmonella detection. They observed that the tape-based sampling accomplished both removal of attached organisms from the surface and also two-dimensional presentation of the cells on an optically clear film, which helps further processing like staining and direct microscopy observation. In addition, Fung, Thompson, Crozier-Dodson, and Kastner (2000) demonstrated the use of pop-up adhesive tape for microbial sampling of meat surfaces, allowing measurement of microbial loads up to 2.2 log CFU/cm² on the meat surfaces. However, this method was still not efficient compared to the conventional swab/rinse method that can measure up to 8.3 log CFU/cm² of inoculated E. coli. Vorst, Todd, and Ryser (2004) investigated the use of Kimwipe® absorbent tissues for recovery of Listeria monocytogenes from stainless steel surfaces. They obtained a 2.70 log greater recovery for the absorbent tissues compared to other sampling methods evaluated. Johnston, Elhanafi, Drake, and Jaykus (2005) used centrifugation for PCR-based detection of Salmonella or E. coli O157:H7 from alfalfa sprouts and sprout irrigation waters. In addition, Wolffs, Glencross, Thibaudeau, and Griffiths (2006) used simple filtration for PCR assay for the detection of Salmonella in chicken rinse, and spent irrigation water samples.
1.1.1 Use of cellulose membranes for sample collection

1.1.1.1 Cellulose structure

Cellulose is an organic polysaccharide consisting of a linear chain of several units of $\beta(1\rightarrow 4)$ linked D-glucose units. The cellulose structure has multiple hydroxyl groups on the glucose from one chain and form hydrogen bonds with oxygen atoms on the same or on a neighbor chain, holding the chains firmly together side-by-side and forming microfibrils with high tensile strength (Fig. 1.2) (Purves, 1954). Cellulose is one of the most abundant biopolymer on earth and it is the main component of plants since it helps to maintain their structure. Also cellulose is present in bacteria, fungi, algae, and in animals (O'Sullivan, 1997). Cellulose has been used for many applications such as paper products, fibers including textiles made of cotton, linen and other plant fibers. Other applications include biofuels and constructing material. Regarding to paper products, cellulose is the main constituent of paper, paperboard, and card stock. Currently there is an array of paper products in the market, making cellulose-based materials available everywhere.

Fig. 1.2 Repeating units of a cellulose chain (O'Sullivan, 1997).
1.1.1.2 Morphology of cellulose

The cellulose’s fiber morphology plays a major role in its biological functions and applications. Cellulose morphology is based on different parts such as elementary fibrils, microfibrils, and microfibrillar bands (Fengel & Wegener, 1984). The dimensions of these structural parts are in the range of 1.5-3.5 nm for elementary fibrils, 10-30 nm for microfibrils, and 100 nm for microfibrillar bands (Klemm, Heublein, Fink, & Bohn, 2005). An electron micrograph of the fibrillar structure of cellulose is shown in Fig. 1.3.

![Electron micrograph of cellulosic microfibrils.](image)

Fig. 1.3 Electron micrographs of cellulosic microfibrils of different origins (Kennedy, Phillips, & Williams, 1993).

1.1.1.3 Cellulose membranes for collection of bacteria

The detection of bacteria requires the food samples to be processed to remove the foodborne bacteria from the food matrix, sample the bacteria, and recover the cells for its further analysis.
There are various studies that report the use of membrane filters to concentrate or collect bacteria for enumeration and/or identification purposes (Anany, Chen, Pelton, & Griffiths, 2011; W. T. Chen et al., 2005; Crow, Ahearn, Cook, & Bourquin, 1975; Griffin et al., 2011; Hobbie, Daley, & Jasper, 1977; Lundholm, 1982; Lye & Dufour, 1991; Vasconcelos & Swartz, 1976; Zierdt, 1979).

Cellulose has been used for the production of absorbent pads or membranes. Cellulose and its derivatives is gaining attention for production of high absorbent polymers due to their biodegradable characteristics, their high natural abundance, and their high absorption capacity (A. Esposito et al., 2005; F. Esposito, Del Nobile, Mensitieri, & Nicolais, 1996; Lim, Yoon, & Kim, 2003; Lionetto, Sannino, & Maffezzoli, 2005; Sannino, Maffezzoli, & Nicolais, 2003). W. T. Chen et al. (2005) studied the concentration and recovery of bacterial cells from different membrane types including nylon, mixed cellulose esters, polyvinylidene fluoride (PDVF), and polycarbonate. This study reported that entrapment of the microorganisms was better for the mixed cellulose membranes due to its higher size distribution compared to the polycarbonate membranes (Fig. 1.4). Also, they indicated that the recovery of the cells is influenced by factors such as weak hydrophobic, van der Waals forces and/or physical interaction with the membrane’s surfaces.

Fig. 1.4 Comparison of electron micrographs at 7000 magnification (scale bars = 2 µm), for the different membranes (A) PVDF, 0.45 µm; (B) mixed cellulose, 0.45 µm; (C) polycarbonate, 0.4 µm. Arrow indicates where the bacteria are buried inside tortuous and non-uniform structure of
(A) PVDF or (B) cellulose, but not (C) polycarbonate (Pettipher, Mansell, McKinnon, & Cousins, 1980).

Pettipher et al. (1980) used membrane filtration and epifluorescent microscopy for the direct enumeration of bacteria in raw milk samples. A nucleopore polycarbonate membrane filter (pore size 0.6 µm) was connected to a vacuum line at 100 kPa for filtration and the membrane was mixed with 2 mL of stain for analysis under a microscope for epifluorescence detection. The method was found to be suitable for milks containing $5 \times 10^3$ to $5 \times 10^8$ CFU/mL.

1.2 Bacteria concentration and immobilization for detection

One of the critical aspects for the detection of bacteria is the direct detection from complex systems such as food matrices or clinical samples. A preparation step such as separation, concentration, or enrichment is often required previous to the detection of the bacteria using any biosensor or microdevice. Concentration is easily accomplished in laboratory with the use of equipment such as centrifuges. However, the use of centrifuges is limited by the size of the pathogens, becoming less effective as the pathogens are smaller. Therefore there is a need for solutions for sample preparation in order to decrease the time needed for concentration into small volumes for detection. Current methods for concentration of bacteria include dielectrophoresis (DEP) and field-flow fractionation (FFF).
1.2.1 Dielectrophoresis

Dielectrophoresis (DEP), as first adopted by Pohl (1951), refers to the motion of particles caused as a result of its dielectric properties (Pethig, 2010). DEP can be classified as positive or negative; where positive DEP is when a particle is attracted to a region of high electric field strength and negative DEP is when it is repelled from it. The use of dielectrophoresis for the separation and concentration of bacteria in BioMEMS and biosensors has been demonstrated in the recent years. Gomez-Sjoberg, Morisette, and Bashir (2005) introduced an impedance detection microdevice called “Impedance Lab-on-a-chip” which consisted in the development of a microfluidic device with the objective to concentrate the bacterial cells in orders of $10^4$~$10^5$ and measure their metabolic activity by means of impedance measurements. A silicon based microfluidic chip was built containing a channel for concentration of the bacterial cells by DEP forces into a main chamber with a volume of 400 pL for incubation and impedance measurement. Fig. 1.5 shows a diagram of the DEP mechanism for deviation and capture of the bacterial cells.

Fig. 1.5 Principle and operation of the dielectrophoresis-based deviation and capture of cells in the bioprocessor (Gomez-Sjoberg et al., 2005).
Besides having the advantage of reducing time for detecting the presence of bacteria by concentrating diluted bacteria suspensions, this system have some limitations. One of the problems found in the concentration and capture process was the loss of cells caused by instabilities in the flow rate in the detection chamber since it was controlled by applying a back-pressure at the chamber output using pressurized nitrogen. This problem could be improved by a better flow control system such as a syringe pump for example. Another limitation was the sample dilution was fixed and no more diluted cell suspensions could be tested since it was impossible to control the flow rates through the device. This problem also could be solved using a better flow control system. Another problem found was that the impedance was temperature sensitive since changes in temperature affected the conductance of the samples and a temperature correction factor was needed as a shortcoming procedure. Therefore there is need for a temperature control system able to maintain a standard an accurate temperature during the time of analysis.

Another example on the use of DEP was showed by Dastider, Barizuddin, Dweik, and Almasri (2013). In this case a micromachined impedance biosensor based on interdigitated electrode (IDE) arrays was fabricated for the detection of *Escherichia coli* O157:H7. Positive DEP (p-DEP) was used to concentrate the *E. coli* in the center of the microchannel and direct it towards the detection zone microchannel which has dimensions of a third of the first channel (Fig. 1.6).
Fig. 1.6 3-D schematic of (a) the impedance based biosensor for pathogens detection. (b) Magnified view of the focusing region, and detection region (Dastider et al., 2013).

Another example of the use of DEP for the assistance of the immuno-capture and detection of pathogens was introduced by Yang (2009). In this project, DEP was integrated with a non-flow through biochip in order to assist the immune-capture of bacteria coupled with absorbance measurement (Fig. 1.7). The use of this technology improve the detection by concentrating the bacterial cells from the suspension to different locations of the chip which is very useful for manipulation purposes, also by making the bacterial cells to have a close contact with the immobilized antibodies, therefore the immune-capture process is improved.
Fig. 1.7 (A) The non-flowthrough chip used in this study for DEP enhanced immunocapture of Salmonella cells, which consists of a chamber formed by PDMS and a set of interdigitated microelectrodes (IMEs) on a glass substrate and (B) the experiment setup (Yang, 2009).

In this study, the immune-capture efficiency was improved by 56–64% with a considerable short reaction time (15–30 min). One of the problems encountered in this device was that the absorbance signals had a decrease with the increase in the cell concentrations in the ELISA detection on the chip. This was due to aggregation of cells on the chip surface during the DEP capture, which resulted in a less signal since not all the cells had a reaction with the antibodies on the chip. This problem can be overcome by increasing the antibodies concentration on the chip surface. The detection limit of the system was 5x10³ cells/20µL (2.5x10⁵ CFU/mL), this detection limit can be improved by incrementing the antibodies concentration as well.
1.2.2 Field-flow fractionation

Field-flow fractionation (FFF) is one of the most suitable techniques for the selection of bacteria since it is able to separate analytes on a wide range, from very small biomolecules to cells from complex samples (Reschiglian et al., 2002). FFF is a technique in which separation is achieved within a capillary, empty channel in which a laminar flow of a mobile phase sweeps the sample components down the channel (Roda et al., 2009). Several studies have shown the use of FFF to separate bacteria according to differences in shape and morphology from environmental, clinical, and food samples. This method also is recognized as a soft separation technique since bacteria are separated without modification of their native properties (Rambaldi, Reschiglian, & Zattoni, 2011). A schematic representation of the FFF separation principle is shown in Fig. 1.8.

Fig. 1.8 Schematic representation of the FFF separation principle. Separation depends on the combined action of an axial flow and a field applied in a perpendicular direction. The axial flow is governed by an ideally parabolic flow profile along the channel thickness. Separation depends on interaction of the sample components with an externally generated field, which is applied perpendicularly to the direction of the longitudinal flow. The role of the field is to drive different
species into different regions of the parabolic flow profile, from where they are eluted at different velocities (Rambaldi et al., 2011).

Reschiglian et al. (2004) introduced for the first time the use of hollow fiber field-flow fractionation (HF FFF) for the separation of whole bacteria prior to analysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOFMS). HF FFF is a variant of FFF based on the use of hollow fibers of sub millimeter diameter as cylindrical, micro column fractionation chambers. In this study, HF FFF was used for the fractionation of a mixture of two bacteria (Bacillus subtilis and Escherichia coli) and results showed that each bacteria species was separated and preserved their most intrinsic ion characteristics signals without the signals of the other species. This paper shows that HF FFF has advantages such as miniaturization, simplicity, and low costs. Therefore, there is possibility to use HF FFF in BioMEMS, also for disposable usage, thus eliminating the risk of run-to-run contamination.

A FFF assisted noncompetitive chemiluminiscent (CL) immunoassay for detection of pathogenic bacteria was presented by Magliulo et al. (2006). In this case, gravitational FFF (GrFFF), which is one of the simplest FFF variant and consists in using gravity as the applied field was used in conjunction with a whole-cell CL immunoassay for detection of pathogenic bacteria. Fig. 1.9 shows a schematic representation of the GrFFF-CL method. The system could be able to recognize all of the wild strains of the target bacteria without cross reactivity with other species. This method also showed that whole cell, noncompetitive immunoassays can be performed and therefore can be suitable for automation. Also, it can be used for simultaneous analysis of bacterial mixtures since they can be fractionated by GrFFF efficiently. Despite all the advantages of this method, one
limitation was the limit of detection (LOD) of the system. As shown in Fig. 1.9B, the detection limit was of $10^6$ CFU/mL, therefore modifications of the system would be needed in order to improve this LOD. Another disadvantage is that a cleaning procedure is needed after using the system each time in order to avoid cross-contamination.

Fig. 1.9 Schematic of GrFFF-CL immunometric method for determination of Y. enterocolitica. B) Presentative calibration curve, obtained by averaging 10 calibration curves from different days. RLU=relative light units (Magliulo et al., 2006).
1.2.3 Dielectrophoresis Field-flow fractionation (DEP-FFF)

DEP-FFF utilizes the DEP force to separate particles or cells in a continuous flow (Lewpiriyawong & Yang, 2014). In general, DEP-FFF can be classified in two operation modes based on how the particles interact with the DEP force. The first one occurs when two different particle groups experience nDEP of difference in magnitude. Therefore, the particles at the higher equilibrium height having a higher velocity in the pressure-driven flow will be separated prior to the others. The second operation mode separate particles undergoing different polarities of DEP forces, called pDEP and nDEP. DEP-FFF devices have been used for particle separation, immobilization of probe beads for affinity assay, cell genetic detection, and analyses of cellular properties such as dielectric, density, and deformability (Auerswald et al., 2005; P. R. Gascoyne, 2009; Lagally, Lee, & Soh, 2005; Ramadan et al., 2006). Some limitations of DEP-FFF include an overlapping of sample zones which is caused by similar displacement velocity and dispersion of individual subpopulations during separation. This problem can be overcome by adjusting the chamber dimensions, applied electric field strength, and the flow rate. Fig. 1.10 shows the working principle of DEP-FFF.
Fig. 1.10 Separation principle of DEP-FFF. a) A conventional DEP-FFF device with interdigitated electrodes levitating particles by nDEP force. b) Combined pre-focusing and fractionation functions using both nDEP and pDEP (P. Gascoyne, Satayavivad, & Ruchirawat, 2004; Holmes, Green, & Morgan, 2003).

1.2.4 Other concentration methods

1.2.4.1 Microfluidics and meniscus dragging effect

Microfluidics are techniques that deal with the behavior, precise control, and manipulation of fluids that are confined into small structures in the sub-millimeter scale (Yoon & Kim, 2012). Microfluidics can be a suitable and low cost method to concentrate into small volumes for detection in biosensors. Meniscus dragging consists in the use of evaporation microfluidics. The principle of the method is based on the combination of glass flow, a partial pressure gradient, mass transfer, and capillary surface tension effects in order to evaporate liquid from the sample and
deliver a very small volume of the concentrated analyte for its detection (Zhang, Do, Premasiri, Ziegler, & Klapperich, 2010). The working principle of the device is shown in Fig. 1.11. The evaporation causes a depleted liquid over the sample, and as a result, the liquid forms a meniscus which moves in the top liquid channel towards the sealed outlet, and the analyte is collected and concentrated in the liquid meniscus, and moved as the evaporation continues.

![Fig. 1.11 Working principle for the meniscus dragging effect (Zhang et al., 2010).](image)

Besides the advantages of this method there are some limitations. One problem is that there is no way to control the movement of all of the bacteria through the device, some bacteria can adhere in the channel walls and left apart from the rest of the concentrated sample. Therefore is necessary to modify the design of the system to minimize the loss of sample during concentration. The use of hydrophobic materials (e.g. PDMS, Teflon) for the construction of the channel is recommended to prevent this losses due to sample adhesion in the walls of the microchannel.
1.2.4.2 Nanoimprinted holes

Kano, Inaba, and Miki (2013) developed a device containing nanoimprinted holes for the immobilization of bacteria. In this case nanoimprinting enables the fabrication of micro holes with diameter size smaller than 2 µm, thus overcoming the limited size obtained with photolithography (3 µm). The fabrication process for the nanoimprinting is presented in Fig. 1.12. In general, a SU-8 with thickness of 3 µm is spin-coated, baked, and nanoimprinted with applied pressure of 12.5 MPa. Then, the resulting thin film of SU-8 is plasma etched with oxygen. With this process the new thickness of the SU-8 is 1 µm. The challenge in this system is to observe immobilized bacteria after the rinsing process. The results of this study showed that the density of the immobilized bacteria increased as the hole diameter decreased. However, a diameter of 2 µm was more effective for the immobilization of bacteria compared to the ones with 1.5 µm.

![Fabrication process of nanoimprinted holes](image)

Fig. 1.12 (a) Fabrication process of nanoimprinted holes. (a-i) Photoresist coating on ITO glass slide. (a-ii) development. (b-i) SU-8 coating. (b-ii) Micro holes patterning by nanoimprint. (b-iii) Plasma etching. (iv) Fluidchannel patterning. (v) Thermo compression bonding. (vi) Completion (Kano et al., 2013).
1.3 Sample detection

1.3.1 Biosensors for food safety

In the food industry, quality and safety assurance is the main issue in order to produce food products acceptable for the consumer. Quality and safety assurance include the evaluation of microbiological, toxicological, physical, chemical, and organoleptic characteristics of the food products (Holleran, Bredahl, & Zaibet, 1999). The Center of Disease Control (CDC) estimates that more than 36 million cases of illness occur annually because of food- and waterborne pathogens with more than 5000 deaths a year (Mead et al., 1999). Several pathogenic bacteria including \textit{E. coli} O157:H7, \textit{Staphylococcus aureus}, \textit{Listeria monocytogenes}, \textit{Clostridium botulinum}, \textit{Campylobacter jejuni}, \textit{Pseudomonas aeroginosa}, \textit{Salmonella sp.}, and \textit{Bacillus cereus} are of great interest due to their potential of causing outbreaks.

Quality control from raw to fork is also a concern for the food industry. Also, the competitiveness in producing better and novel food products is increasing year by year. Thus, there is a need for determination of food quality characteristics not only microbiological but other including flavor compounds, organic volatiles, phenolic compounds, proteins, sugars, moisture, fermentation processes, etc. Moreover, the government agencies enforce the food industry to include food labeling of the major and minor components. For example, artificial flavors, antimicrobial agents, allergens, % alcohol in alcoholic beverages, to mention a few.

Being those concerns mentioned there is a need for analytical methods for the detection, identification, and quantification of the compounds/microorganisms of interest. Conventional methods usually are time consuming, costly, and require trained personnel. During the last two
decades, an increase in the technological evolution has been observed, especially in the area of sensors.

1.3.2 Definitions and classification of biosensors

A biosensor is a device that contains a biological sensing element either intrinsically connected or integrated within a transducer. This device will respond to the concentration or activity of the species or compounds in the biological samples (Mutlu, 2010). There is a rapid growth in the development of biosensors in order to provide automation, easy assay performances, and lower cost of analyses in order to replace the old conventional methods. Table 1.1 presents a comparison between biosensors and traditional analytical techniques.

Table 1.1 Comparison of the characteristics between traditional techniques and biosensors (Van Dorst et al., 2010)

<table>
<thead>
<tr>
<th>Traditional analytical techniques</th>
<th>Biosensors</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-) Time consuming</td>
<td>(+) Rapid, real time detection</td>
</tr>
<tr>
<td>(-) Expensive</td>
<td>(+) Cost-effective</td>
</tr>
<tr>
<td>(-) Laboratory monitoring</td>
<td>(+) Portable (in-situ monitoring)</td>
</tr>
<tr>
<td>(-) Trained laboratory personnel</td>
<td>(+) Simple use</td>
</tr>
<tr>
<td>(-) High tech equipment</td>
<td>(+) Simple apparatus</td>
</tr>
<tr>
<td>(-) Extensive sample preparation</td>
<td>(+) Limited sample preparation</td>
</tr>
<tr>
<td>(-) More organic solvent consumption</td>
<td>(+) Less organic solvent consumption</td>
</tr>
<tr>
<td>(+) Multianalyte detection possible</td>
<td>(-) Single analyte detection</td>
</tr>
<tr>
<td>(+) Commercial availability</td>
<td>(-) Limited commercial availability</td>
</tr>
<tr>
<td>(+) Standardized</td>
<td>(-) Non standardized</td>
</tr>
<tr>
<td>(+) Sensitive</td>
<td>(+) Sensitive</td>
</tr>
<tr>
<td>(+) Specific</td>
<td>(+) Specific</td>
</tr>
<tr>
<td>(+) Reusable</td>
<td>(+) Reusable</td>
</tr>
</tbody>
</table>
There are different types of biosensors including electrochemical, optical, mechanical, thermal, and magnetic. Among, these, electrochemical biosensors have been of the most used due to their simplicity and availability, easy integration and miniaturization. Each sensing device has its own sensing principles, and their use will depend on the particular compound to be measured. The present article presents a review of the development of different types of biosensors and includes a discussion of the different problems found in applying these biosensor technologies for the monitoring of quality and safety of food products.

1.3.3 Biorecognition elements in biosensors

1.3.3.1 Antibodies

Antibodies, also called immunoglobulins (Ig), are soluble proteins produced by the plasma cells in response to stimulation by an antigen. Ig’s molecular weight is around 150,000 Da, and its structure is composed of four polypeptide chains in a Y-shaped form (Fig. 1.13). Two of them are heavy chains (H), with molecular weights of ca. 55,000 Da, and have held together by disulfide bonds (hinge region). The two light chains (L) have molecular weights of 22,000-23,000 Da, and are bound with the H chains by noncovalent bonds. Also the antibody can be divided into three fragments. Two of the fragments are called the Fab (fragment antigen binding), and have the ability to bind a specific antigen. The third fragment is called the Fc (fragment-crystallizable), which cannot bind to the antigen but it has biological functions after binding with the antigen (Benjamini, Coico, & Sunshine, 2000). Antibodies have the ability to recognize a unique molecule (called antigen) of the foreign agent (bacteria, virus), thus antibodies are widely used in different applications including biosensors.
1.3.3.2 Enzymes

Enzymes are proteins that act as biological catalysts by converting specific substrates into products. Enzymes bind their substrates by using their binding pockets having a complementary shape, charge and hydrophobic/hydrophilic characteristics specific to the substrate. In biosensors, redox enzymes are used since they catalyze reactions that produce or consume electrons, and such electrons are detected. The limitations of using enzymes is that they are more expensive to produce due to increased costs of isolation from the source, also enzymes are often unstable when isolated, limiting the shelf life as well the operational stability of biosensors, and last, many enzymes need cofactors for the detection of substances (Mulchandani, 1998; Zhao & Jiang, 2010).

1.3.3.3 DNA based recognition elements
Other recognition elements used in biosensing include biomolecules based on DNA, such as aptamers. Aptamers are biomolecules constructed from specific nucleic acid sequences that bind to a target molecule with high affinity and specificity (Fig. 1.14). The word aptamer is derived from the Latin word “aptus”, which means “to fit” (Ellington & Szostak, 1990). Therefore, aptamers are emerging compounds which are suitable for many applications based on molecular recognition including biosensing. However, aptamer research is in still on the way and will require time before examples of aptamer products are available (Jayasena, 1999).

![Aptamer-target binding mechanism](image)

**Fig. 1.14** Aptamer-target binding mechanism (Bio-Resource, 2016).

### 1.3.4 Electrochemical biosensors

Electrochemical biosensors are based on the measurement of a current, impedance, or potential generated by the oxidation/reduction reaction of the electroactive analyte species, and these current, impedance, and potential is proportional to the concentration of the analyte species. Electrochemical biosensors are classified into amperometric, impedimetric, and potentiometric
biosensors. Electrochemical biosensors are widely used due to their good linearity and sensitivity over a wide range of analyte concentration (Wang, 1999).

Electrochemical biosensors are one of the most used for the detection of bacteria and quality control in foods. Stobiecka, Radecka, and Radecki (2007) developed a voltammetric biosensor for the determination of acrylamide in food samples. Acrylamide is a compound of particular interest in the food industry due to its relation with possible carcinogenetic effects in humans, and it is produced by heat treatments in fried foods containing reducing sugars. The recognition element of the biosensor was based in the interaction between acrylamide and hemoglobin (Hb). That interaction can lead to an acrylamide-Hb adduct, which could alter the electroactivity of Hb. Therefore, as there is an increase of concentration of adduct acrylamide-Hb, the current peak of cyclic voltammogram (CV) decreases. In this study, one of the challenges was the immobilization of the Hb, for which a carbon-paste electrode (CPE) was built for this purpose. However, the interaction of acrylamide with the CPE was irreversible, which means that the CPE should be removed and the electrodes prepared again. This eliminates the possibility of reusing the biosensor every time, which makes the system more time consuming due to preparation of the biosensor. Therefore, it is suggested to test another means of immobilization of the Hb, such another types of ligands or crosslinking polymers that do not react irreversible with the acrylamide and thus make the biosensor reusable.

Javier Manso, Mena, Yanez-Sedeno, and Pingarrón (2008) proposed an amperometric biosensor for the detection of inulin in foods. Inulin determination is of great interest in the food industry since this compound has functionality as dietary fiber in food products. This biosensor was based on the use of two enzymes such as fructose dehydrogenase (FDH) and inulinase (INU). The main challenge of this study was the immobilization of the two enzymes in a gold nanoparticle-
cysteamine self-assembled monolayer-modified gold electrode (Au\textsubscript{col}–Cyst–AuE). Also there was immobilization of a Tetrathiafulvalene (TTF) used as mediator. The enzymes and the mediator were immobilized by crosslinking using glutaraldehyde. The transduction process consisted in the hydrolysis of inulin catalyzed by inulinase. The product of the reaction, fructose, is detected by the electrochemical oxidation of TTF at the electrode (Fig. 1.15). The biosensor showed high selectivity with respect to other carbohydrates. However, an error close to 10% was observed when saccharose was present, and this was due to weak catalytic activity of the enzyme INU in the hydrolysis of the O-glycosidic bond of saccharose. In this case, it is suggested to treat the samples in order to remove saccharose (if present) for the correct functioning of the biosensor.

![Diagram of the functioning of a biosensor for detection of inulin in foods (Javier Manso et al., 2008).](image)

Electrochemical biosensors can also be used to determine quality of packaged food such as fruits. An enzyme-conjugated electrochemical biosensor was studied for the determination of amines in fresh and modified atmosphere packaged fruits (Esti et al., 1998). The development of amines in plants is indicator of physiological process such as fruit maturation and senescence. Also, they are indicator of microbial contamination as an observed increase of biogenic amine content in the fruits or vegetables. For this purpose, enzymes such as diamine oxidase (DAO) and polyamine
oxidase (PAO) were immobilized in glutaraldehyde in a nylon membrane and a hydrophilic poly(vinylidene difluoride) (PVDF) membrane by covalent protein immobilization of ε-amino groups of lysines for DAO and PAO respectively. Both enzymes produce $\text{H}_2\text{O}_2$ in the presence of amines, which can be oxidized at the electrode and the current produced is related to the amine content of the product (Fig. 1.16). The main issue to improve in this study was the optimization of the selectivity of the biosensor. An inner cellulose acetate membrane with cutoff of 100 Da was used for $\text{H}_2\text{O}_2$ since signal interferences from ascorbic acid and phenolic compounds were observed when no membrane was used.

![Fig. 1.16 DAO and PAO enzyme reactions with amines (Esti et al., 1998).](image)

The detection of foodborne pathogens is of extremely importance in the food sector. The main food pathogens in concern include *Salmonella, E. Coli O157:H7, Listeria monocytogenes, Staphylococcus aureus, Campylobacter, Cryptosporidium*, and viruses like the Norwalk virus (Palchetti & Mascini, 2008). Susmel, Guilbault, and O'sullivan (2003) developed an electrochemical-immunological biosensor for detection of *Listeria monocytogenes* and *Bacillus cereus* using a redox probe and screen printed gold electrodes. Antibody immobilization is one of
the important factors to take into account in the manufacturing of immunological biosensors. In this investigation the antibodies were immobilized in the electrode’s surface through a thiol based self-assembled monolayer (SAM). After immobilization, a 3% of powder milk solution was used as blocking agent to avoid foreign particles from taking place into the electrode surface. The measurement of the analytes was based on the change in the diffusion coefficient (D) of the redox probe (potassium hexacyanoferrate (II)) due to the immunocomplex formation between the bacteria and the antibodies. Table 1.2 shows the results for the D values obtained for *L. monocytogenes*:

Table 1.2 Table of diffusion coefficient (D) of the redox probe for *L. monocytogenes* (Susmel et al., 2003)

<table>
<thead>
<tr>
<th>Surface</th>
<th>Value of diffusion coefficient of redox probe (cm²/s)</th>
<th>R.S.D. % (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare electrode (no SAM)</td>
<td>5.26x10⁻⁶</td>
<td>8</td>
</tr>
<tr>
<td>Electrode + MPA-based SAM</td>
<td>3.08x10⁻⁶</td>
<td>8</td>
</tr>
<tr>
<td>Electrode + MPA-SAM + Ab (no <em>L. monocytogenes</em>)</td>
<td>2.54x10⁻⁷</td>
<td>10</td>
</tr>
<tr>
<td>Electrode + MPA-SAM + Ab + <em>L. monocytogenes</em> (1x10⁷ cells/mL)</td>
<td>8.12x10⁻⁸</td>
<td>6</td>
</tr>
<tr>
<td>Electrode + MPA-SAM + <em>L. monocytogenes</em> (no Ab)</td>
<td>2.78x10⁻⁶</td>
<td>12</td>
</tr>
<tr>
<td>Electrode + MPA-SAM + Ab + <em>B. cereus</em> (1x10⁷ cells/mL)</td>
<td>2.23x10⁻⁷</td>
<td>4</td>
</tr>
</tbody>
</table>

The results indicated that both bacteria showed similar trend. Also, the biosensor showed good selectivity and specificity, also a detection limit of 1000 cells was observed. Non-specific bonding is one of the concerns in developing immunobased sensors. In this case, the non-specific binding was controlled by immobilizing a mouse IgG (antibody with no affinity for target analytes) in the place of the specific antibodies.
An amperometric-immunoassay biosensor using highly-dispersed carbon particles and horseradish peroxidase (HRP) conjugated antibodies were used for the detection and quantification of *Escherichia coli*, *Listeria monocytogenes*, and *Campylobacter jejuni* was demonstrated by Chemburu, Wilkins, and Abdel-Hamid (2005). In this case, the amperometric signal produced by the flow of peroxidase substrate is measured, and the signal is proportional to the number of bacterial cells. In order to develop the biosensor, different factors were optimized such as the immunosorbent quantity, concentration of primary antibodies, and HRP-labeled antibody concentration.

The immunosorbent (antibody-modified dispersed carbon nanoparticles) and primary antibody quantity was optimized since problems such as clogging, increased of back pressures, and flow pulsations were observed when the immunosorbent concentration was too high (>6 mg). Moreover, electrical discontinuity and incomplete capture of the antigen cells were observed due to void spaces in the filter when too small immunosorbent quantities were used (<1 mg). In the case of primary antibody, optimal concentration was found between 20-30 µg/mL. An optimal concentration of immunosorbent and antibodies is an important factor in order to avoid problems such as the ones found in this study, also because they are the recognition element of the biosensor and play an important role to improve performance factors such as selectivity, linearity, limit of detection, sensitivity, dynamic response, and others. Fig. 1.17 shows results for the calibration curves developed for *Escherichia coli*, *Listeria monocytogenes*, and *Campylobacter jejuni*. A maximum limit of 1000 cells/mL and not higher could be measured due to a “hook effect” (a decrease in the immunological capture, and hence sensor signal at high concentrations of analytes) was observed. Therefore, the range can be increased by increasing the concentration of antibodies in the sensing device.
Some problems were found when the biosensor was tested in raw samples like milk. The authors observed a clogging in the filter membrane caused by the fat content of the milk. Also, deposition of particulates into the dispersed carbon layer was observed. To overcome this problem, the raw milk was diluted previously the analysis. Similar problems were found in the analysis of chicken samples. Therefore, it is recommended to add a sample preparation step such as diluting and filtering in order to avoid those problems.

![Fig. 1.17 calibration curves for amperometric detection of Escherichia coli (a), Listeria monocytogenes (b), and Campylobacter jejuni (c) (Chemburu et al., 2005).](image-url)
1.3.5 Optical biosensors

Optical biosensors are biosensor based on the measurement of the target analytes by optical means, and different optical properties such as absorbance, emission, reflectance, fluorescence, refractive index, refraction, and light scattering are measured. Optical biosensors offer different advantages such as high sensitivity, direct and indirect detection, multiple analyte detection (using labels), real time detection in some cases, and avoid electromagnetic interferences found in most electrochemical biosensors.

An optical biosensor based on the use of fluorescence labels and dendrimers was developed by Chang, Gillespie, and Tabacco (2001), and the detection of pathogenic bacteria such as *Pseudomonas aeruginosa* was evaluated. The label used was SYTOX Green nucleic acid stain (S7575) and hydroxy-terminated polyamidoamine (PAMAM) as dendrimer. Dendrimers are monodisperse, hyperbranched polymers and their use in biosensors is increasing (e.g. DNA biosensors) (Zeng & Zimmerman, 1997).

A sensing film was used as the recognition element, which was placed on top of the reflection probe of the biosensor (Fig. 1.18).

Fig. 1.18 Fiber optic spectrometer based biosensor (Chang et al., 2001).
SYTOX Green nucleic acid was used to analyze dead cells, since it cannot cross the membrane of live bacterial cells (Chang et al., 2001). The amount of fluorescence produced is dependent on the concentration of bacteria and the concentration of the dye. In the case of this biosensor in particular, the fluorescence increased in a 26-fold as the concentration of bacteria increased from $2.7 \times 10^7$ to $5.4 \times 10^8$ cells/mL. On the other side, the amount of fluorescence decreased as the dye concentration increased, this was result of self-quenching of the dye according to the authors. Moreover, the use of dendrimer PAMAM-OH improved the fluorescence as the bacteria concentration increased from $2.7 \times 10^7$ to $5.4 \times 10^8$ cells/mL. One of the challenges found in this study was the stability of the sensing reagents and the sensitivity compared to the controls. The authors found that the nucleic acids (dyes) tended to aggregate in a dry film and lose the ability to stain bacteria; however that problem was overcome by the use of the dendrimer PAMAM-OH coupled to SYTOX Green dye since the cell membrane of live bacteria becomes more permeable. Other challenge found in this study was the stability of the sensing reagents while maintaining the sensitivity of the biosensor.

A fluorescence resonance energy transfer (FRET) fiber optical biosensor for detection of *Salmonella typhimurium* in ground pork samples was developed by Ko and Grant (2006). The FRET principle is based on the non-radioactive energy transfer from a fluorescence donor dye to an acceptor molecule (Lakowicz, 1999). For the recognition element, labeled antibody-protein G complexes were formed with anti-Salmonella antibodies labeled with FRET donor Alexa Fluor 546 and protein G (PG) labeled with FRET acceptor Alexa Fluor 594. Then, the labeled antibodies-PG complexes were immobilized on tapered silica fiber cores in order to produce the evanescence wave region. For the transducer, a ratiometric-based fluorometer system was built. A diagram of the recognition and transduction element of the biosensor is shown in Fig. 1.19.
Fig. 1.19 Diagram of the FRET immuno sensor. Left: recognition element. Right: transduction element (Ko & Grant, 2006).

One of the important factors in developing a FRET biosensor is the packing density, which is the concentration of antibody/PG immobilized on the tip of the recognition element. For this biosensor, the packing density was optimized by using different concentrations of labeled antibody/PG complexes. This optimization is important since the higher energy transference between donor and acceptor fluorophores, the better the sensitivity of the biosensor. The biosensor was tested in pork samples by inoculating them with \( S. \text{typhimurium} \) at different concentrations \((10^2-10^7 \text{ CFU/g})\). The biosensor responded to concentrations of \( 10^5 \text{ CFU/g} \) in 5 minutes, which indicated a rapid response but a low limit of detection. The authors recommended different options to improve the limit of detection: a) fabrication of the biosensor with longer crosslinkers to increase the interaction of the antibodies with the analyte, b) optimization of the detection system and signal processing to reduce noise signal, c) performing additional packing density tests to increase the FRET energy transfer. It is suggested is to include a filtering step in order to reduce the effect of
the food matrix in the analysis and thus reduce the complexity of the raw food samples and improve the limit of detection.

Other optical biosensors include the use of fiber optics. Fiber optics is an optical technology based on the evanescence waves (EW) which uses attenuated total reflection spectroscopy principles (Geng, Uknalis, Tu, & Bhunia, 2006). One of the benefits of fiber optical sensors is the real-time analysis of biomolecule’s interaction. Geng et al. (2006) developed a fiber optic biosensor conjugated with antibodies for detection of *Escherichia coli* O157: H7 from ground beef samples. Detection antibodies were conjugated with Alexa Fluor dye for fluorescent measurements of the reactions occurring on the surface of the optical biosensor.

As mentioned before, one of the factors of concern in immunological based detection is the concentration of capture and detection antibodies. In this case, optimal concentration for both was 50 µg/mL and 25 µg/mL for capture and detection antibodies respectively. Detection limit and specificity of the biosensor were optimized as well (Fig. 1.20). The optical biosensor was tested to determine if it could detect *Escherichia coli* O157: H over time. Results showed that with continuing incubation, both signals and concentrations increased gradually up to 3578 pA and 6.8x10⁹ CFU/mL after 8 h, separately. However, cells in EC had poor growth (3.4x10³ CFU/mL) after 4 h and the signal (239 pA) from the biosensor was not significantly higher than that of background.
Fig. 1.20 Optimization of detection limit (a) and specificity (b) of the fiber optic biosensor (Geng et al., 2006).

An optical biosensor for analysis of folate-binding protein (FBP) in milk was presented by Indyk and Filonzi (2004). FPBs are molecules that have high affinity for binding folates (i.e. vitamins) in milk, and their detection and quantification is of great importance for quality purposes after thermal processing in milk. This type of biosensor used the Surface Plasmon Resonance (SPR) principle, which is based in the resonance or oscillation of electrons induced by incident light. The biosensor required a surface preparation, which consisted in the immobilization of folic acid into a chip (CM5) of the sensor (Biacore Q) via amine coupling. The immobilization of folic acid
provided a high density FBP specific surface, which was appropriate for the analysis. The authors evaluated the capability of the biosensor to respond to non-specific binding since this type of analyses (immuno assay or binding assay) are prone to have interferents. Other factors evaluated were ligand immobilization, flow rate, contact time, and regeneration. The biosensor was tested in retail milks containing different fat contents, and FBP content between 4.6 and 8.0 µg/mL was detected.

1.3.6 Surface plasmon resonance (SPR) based biosensors

Surface Plasmon Resonance is a type of optical biosensing technique. SPR technology is based on the detection of changes in the incident angle or shift in the wavelength of the absorbed light, resonant intensity change, or polarization caused by the absorption of the energy from the incident light to the metal surface. These changes can be expressed as a change in the SPR signal (Mutlu, 2010). One of the biggest advantages of SPR biosensors is real time analysis. A SPR biosensor in conjunction with alkane monothiol and dithiol dendritic tether based self-assembled monolayers (SAM) for the detection of *Staphylococcus aureus* was developed by Subramanian, Irudayaraj, and Ryan (2006). The biosensor consisted of a flow cell for the flow of reagents and samples (Fig. 1.21).

The recognition element of the biosensor consisted in antibodies immobilized into the SAM layers of monothiols and dithiols by covalent amide bonds. One factor determined was the sensitivity of the biosensor, which was determined by passing different antigen concentrations against primary and secondary *S. aureus* antibodies. The sensitivity was fixed since at the beginning a direct immunoassay was used and a number of 10⁷ CFU/mL was detected. Then, a sandwich assay was used and sensitivity was improved with a detection of 10⁵ CFU/mL (Fig. 1.22). The specificity
was determined by testing the biosensor against cocktails of *S. aureus* and *E. coli* O157:H7. The results showed that the response reduced when the concentration of *E. coli* O157:H7 was >10^2 CFU/mL. This was attributed to a decrease of the accessibility of epitopes by the antibodies. Therefore, our suggestion is to increase the antibody concentration in the recognition element of the biosensor.

![Experimental setup for SPR biosensor](image)

**Fig. 1.21** Experimental setup for SPR biosensor (Subramanian et al., 2006).
Fig. 1.22 Sensitivity of detection of *S. aureus* on alkane monothiol surface (Subramanian et al., 2006).

An SPR biosensor to detect veterinary drug residues in milk was developed (Keegan et al., 2009). Drugs such as benzimidazole carbamate (BZT) are commonly used in veterinary to treat infections (helminth) in animals such as cows. There is a concern for the residually of these drugs in milk due to their teratogenic and embryotoxic properties (D. Chen et al., 2010). The preparation of the biosensor consisted in modifying the surface of a CM5 chip (general purpose sensor chip) with an HBS-EP buffer (10mM HEPES pH 7.4 with 0.05M NaCl ) and 50 mM NHS(100mMN-hydroxysuccinimide in water):200 mM EDC (400mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodimide hydrochloride in water) and other reactants were added. Antibodies against methyl 5(6)-[carboxypentyl]-thio]-2-benzimidazolecarbamate derivative (CMB) were bound into the chip surface so reaction could be measured. The binding of the antibodies into the chip was measured as a change in the SPR signal occur (RU). A competitive immunoassay was used to detect inhibition of antibody binding into the surface.

The biosensor was calibrated against milk samples mixed with ABZ-SO2 at concentrations of 0, 2.5, 5, 10, 15, 25, and 50 µg kg⁻¹. The calibration curves are presented in Fig. 1.23. The efficiency of the biosensor was determined by examining contaminated milk samples, and results were compared to UPLC-MS/MS. Results showed that the SPR biosensor identified residues at higher values than UPLC-MS/MS at the 63 and 72 h of sampling. Sample preparation and sample size was one of the main concerns in this study. Losses in the recovery of the samples were found when a spiking method was tested. This was due to the difficulty of resuspending residues, and this was due to adsorption of residues into the glassware surface or due to binding by the milk proteins. Therefore, the authors suggested using other alternate suspension solvents such as MeOH and
water mixtures. The optimum sample size was 12 g in order to find detection of benzimidazoles to be <5µg kg\(^{-1}\) in milk. Similarly, the working antibody dilution of 1/1200 (v/v), flow rate of 10 µL min\(^{-1}\), a contact time of 1 min, and antibody: extract mix ratio of 1:3 (v/v) were optimized and a response of 380 RU was obtained.

Fig. 1.23 Calibration curves for 11 benzimidazole carbamates in bovine milk matrix (Keegan et al., 2009).

A SPR immunoassay biosensor for determination of Ractopamine (Rac) residues in pork was developed by Lu et al. (2012). Ractopamine is a compound used in veterinary medicine, and their residually in food is a danger for consumer health (Elliott et al., 1998). One of the challenges in producing an immuno-based SPR biosensor is the immobilization of the reagents in the chip of the sensor. In this study, the surface of the sensor was modified with a biochemical sensitive membrane made with carboxymethylated dextran matrix. The immobilization of the Rac was done
by injecting the Rac at a flow rate of 10 µL/min on the chip surface. For the assay, samples were mixed with Rac antibody in a 10:90 (v:v) ratio and diluted 1:200 with PBS buffer. This mixture was then injected into the chip’s surface. 0.1 m hydrochloric acid was added for 3 min at a flow rate of 20 µL/min. The assay was compared to UPLC-MS/MS assay in order to compare the results.

The quantification accuracy of the biosensor was improved by making pure and matrix-matched solutions to make calibration curves. In this study, the standard curve was affected by factors such as the antibody, Rac derivative, flow rates, and reaction time. One of the issues found in the development of this biosensor was the reaction time, which was optimized for 5 min; otherwise the standard curve would have been in an “S” shape instead of linear. In addition, a limit of detection of 0.6 µg/kg was determined by analyzing six controlled negative pork samples and detecting them with the SPR biosensor. The biosensor analytical method was corrected since two reaction methods (direct reaction and inhibition reaction) were attempted. In the direct method, the Rac standard solution was injected into the chip surface; however the baseline did not change before and after the Rac standards passed through, this might be due that Rac molecule was too small to produce a change in the SPR when it bound to the antibody. Therefore, an inhibition reaction method was used. In this case, the SPR response was increased significantly after injection of the Rac standard containing the Rac antibody (Fig. 1.24).
Fig. 1.24 The immobilization of Rac derivatives and inhibition response SPR curve (1) activation; (2) immobilization or Rac derivatives; (3) inactivation; (4) Rac containing Rac antibody injected on the chip surface (Lu et al., 2012).

Another factor of concern in the development of this biosensor was that false negatives could be shown in inhibition response of the sensor since Rac antibody may be coupled with pork matrix such as fat and protein in the chip surface besides the Rac derivatives. As mentioned before, our suggestion is to include a mechanism to filter the samples in order to improve problems such as false negatives due to the food matrix. The reuse of the chip was another factor to determine. The bonds between the antibody and the Rac derivatives can be broken by applying acid, base, or organic solvents into the chip surface. In this case, hydrochloric acid was effective since it didn’t damage the chip surface and the sensor could work effectively again (similar response to the initial assays). Yet, the baseline response increased gradually with the increase of injection. In this case it was recommended to regenerate the sensor after more uses in order to reduce the accumulation
of antibodies on the surface. Table 1.3 presents a comparison between the data generated by the SPR sensor and the UPLC-MS/MS assay.

Table 1.3 Concentrations of Rac (ng g⁻¹) detected by SPR biosensor and UPLC-MS/MS (Lu et al., 2012)

<table>
<thead>
<tr>
<th>Spiked (ng g⁻¹)</th>
<th>SPR</th>
<th>UPLC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.37</td>
<td>1.10</td>
</tr>
<tr>
<td>1.0</td>
<td>0.91</td>
<td>0.90</td>
</tr>
<tr>
<td>1.0</td>
<td>0.97</td>
<td>0.80</td>
</tr>
<tr>
<td>2.0</td>
<td>2.38</td>
<td>2.10</td>
</tr>
<tr>
<td>2.0</td>
<td>1.85</td>
<td>1.70</td>
</tr>
<tr>
<td>2.0</td>
<td>1.61</td>
<td>1.8</td>
</tr>
</tbody>
</table>

The accuracy and precision of the biosensor method was tested and compared to the UPLC-MS/MS method using a mean recovery of 2.0 µg/kg and the biosensor showed a good accuracy but lower precision compared to UPLC-MS/MS method (99% and 89%, 4.5% and 5.7%, respectively). A limit of detection of 0.6 µg/kg for the SPR biosensor was calculated by using a value three times the background noise obtained for blank samples and 0.1 µg/kg of the UPLC-MS/MS method. Therefore, the limit of detection could be improved by increasing the signals from background noise in the pork sample. To achieve this, the pork samples could be prepared in a better way in order to reduce noise in the analysis. In this context, the sample matrix effect is one of the main concerns when doing assays with immunobased SPR biosensors, and this factor should be considered before their development.

1.3.7 Enzyme based biosensors
Enzyme based biosensors are based on the immobilization of specific enzymes in the sensing device in order to analyze specific compounds or the product of the reaction of these with the enzyme. Different problems are found when it becomes to development of enzyme based biosensors. For example, loss of the enzyme with time, maintenance of the enzyme stability, the shelf life of the biosensor, and the time for the enzyme response to the analyte (Amine, Mohammadi, Bourais, & Palleschi, 2006).

Enzyme based biosensors are being broadly used for analyses in the food industry (e.g. fermentation processes). In fermentation, compounds of interest are glucose and sucrose, and several biosensors have been developed for this purpose. Gouda, Kumar, Thakur, and Karanth (2002) worked in the enhancement of an enzyme based biosensor for glucose and sucrose determination using protein based stabilizing agents. In this biosensor, the enzymes were immobilized by crosslinking on a cellophane membrane. The enzyme electrode construction was done by using a detachable membrane unit (DMU) consisting of a Teflon membrane and a cellophane membrane with the enzyme layer.

The stability of the biosensor’s enzyme system was tested by addition of an inert protein such as BSA, since the material used for the crosslinking (glutaraldehyde) is detriment for the enzyme stability. It is known that this material produces conformational changes in the enzymes, leading to loss of activity (Broun et al., 1973). A DMU containing the immobilized enzyme membrane system was fixed to the electrode in order to quantify the operational stability. The change in the response in % dissolved oxygen was measured by injecting 50 µl of 10% glucose and 100 µl of 10% sucrose.
The operational stability of the electrode containing immobilized GOD for glucose with different PBSAs was determined (Fig. 1.25). Among the proteins, lysozyme was found to be the best in stabilizing the GOD, followed by BSA and gelatin. Therefore, from these results it could be observed that the biosensor had good reproducibility. Fig. 1.26 shows the operational stability of the sensing for sucrose containing the different PBSAs. Again, lysozyme was found to be effective in protecting the enzyme and thus a providing a good reproducibility of the biosensor.

Fig. 1.25 Residual activity after repeated glucose analysis with biosensor using GOD immobilized with different PBSA (Gouda et al., 2002).
An acetylcholinesterase (AChE) enzymatic biosensor as a rapid method for detection of neurotoxic insecticides in food was developed by Schulze, Schmid, and Bachmann (2002). The analysis of pesticides in foods is of great interest for the regulatory agencies of the government and for the food industry. Therefore, there is a need for rapid and sensitive detection systems for these pesticides, since the current used methods such as gas chromatography (GC) or high performance liquid chromatography (HPLC) take long time for the analyses and also the detection of pesticides is limited. In this study, a disposable biosensor with immobilized AChE was produced by the screen-printing method. In addition, enzyme activity was determined by monitoring thiocholine formed by enzymatic hydrolysis of acetylcholine chloride. Also, the biosensor was tested for inhibition by incubation with a sample for 30 min in a non-stirred solution. Percentage of inhibition was calculated after measurement of residual activity.

For the preparation of the samples for analysis, food samples (50 g aliquot) such as apple, peach, and orange were extracted using isooctane solvent. Aliquots of the extracted solution (after centrifugation) were incubated with the AChE biosensor. The recovery rates of spiked food samples were calculated using inhibition values from phosphate buffered saline (PBS). The biosensor was tested to analyze insecticide paraoxon. Fig. 1.27 presents a standard curve for AChE inhibition by different concentrations of paraoxon insecticide. A working range between 1 and 60 µg/L paraoxon was determined for the system, according to the authors, this working range is enough for the maximum residue limits in food (between 10 and 10,000 µg/L) depending on the type of food. Also a detection limit of 1 µg/L was observed.
A direct measurement of the food matrix is desired in order to make the assay simple. However, it should be considered that the matrix of the food can interfere with the analysis. Therefore false positives or false negatives can be obtained. In this study, a direct measurement of the food samples was attempted. However, high AChE inhibition yields were obtained for the different samples (Table 1.4). According to the authors, one reason for this high inhibition was due to the low natural pH of the fruits. Therefore, this could cause false negatives since the inhibition could not be caused by the presence of organophosphates or carbamates (pesticides) in the food samples. Also, other problems resulted due to the tick-film of the electrodes took longer equilibration for the measurements after the incubation of the samples.

![Calibration curve for AChE inhibition caused by different paraoxon concentrations](Schulze et al., 2002)

Fig. 1.27 Calibration curve for AChE inhibition caused by different paraoxon concentrations (Schulze et al., 2002).
Table 1.4 Effect of untreated food samples on the activity of AChE in comparison to isooctane extracts of corresponding foodstuff (unspecific inhibition) (Schulze et al., 2002)

<table>
<thead>
<tr>
<th></th>
<th>AChE-inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(%)</td>
</tr>
<tr>
<td></td>
<td>pH 4</td>
</tr>
<tr>
<td>Orange juice</td>
<td>56</td>
</tr>
<tr>
<td>Peach baby food</td>
<td>21</td>
</tr>
<tr>
<td>Apple baby food</td>
<td>19</td>
</tr>
</tbody>
</table>

In order to overcome the problems mentioned above, the authors suggested an extraction of the samples with solvents. The effect of the presence of the solvent (isooctane) on the activity of the immobilized enzyme was tested, since it could give problems. No significant reduction of the enzyme activity was found. In this case, the solvent extracts gave better results compared to doing the assay in the direct sample (Table 1.4). Also, the equilibration time was improved with a time of 15 min, which is within the normal range acceptable. The accuracy of the sensor was tested by spiking food samples with paraoxon.

Besides orange juice, the biosensor was tested with apple and peach in solid forms (not juice). A paraoxon concentration of 10 µg/kg in apple baby food caused an AChE inhibition of 12%. Results are observed in Fig. 1.28. In the case of peach, the same amount of paraoxon produced an inhibition of 14% of AChE. In general, this biosensor was very useful to analyze foods with high water content (or liquid food products). However, it was not suitable for the analysis of food products with low water content. Therefore, we suggest that it should be adapted to measure food products with low moisture. Also the accuracy and speed of analysis should be improved when analyzing this type of foods.
Mechanical biosensors are based on the detection of mass or stress changes in a material due to the reaction of the molecules or analytes of interest. Mechanical detection offer advantages such as a label free detection, which make the assays simple. Mechanical biosensors are divided into mass sensitive detection (using piezoelectric materials), Quartz Crystal Microbalance (QCM), and Surface Acoustic Wave (SAW). Stress sensitive detection includes the use of microcantilevers.

Different studies refer to mechanical biosensors for the detection of bacteria. A piezoelectric immunosensor was developed for the detection of *Escherichia coli* O157:H7 (Su & Li, 2004). When producing immunobased detection, the antibody immobilization is very important for the correct functioning of the biosensor. The piezoelectric material used was 8MHz AT-cut quartz crystals coated with polished Au electrodes. The affinity-purified antibodies were immobilized
onto a monolayer of 16-mercaptohexadecanoic acid (MHDA) on the AT-cut quartz crystals Au electrode surface using N-hydroxysuccinimide (NHS) ester as a reactive intermediate. The antibody-modified crystals were treated with 1% BSA-PBS in order to block any non-reacting and non-specific sites (noise). The QCM and electrochemical measurements were done in a commercial electrochemical QCM system (EQCM).

Fig. 1.29 presents a schematic demonstration of the bacterial binding into the biosensor. First, an oriented monolayer of MHDA is formed via the strong Au-thiolate bond with the tail carboxylic group exposed at the monolayer-air or monolayer-liquid interface (Bain et al., 1989). The second step is the activation of the monolayer, which involves a stepwise formation and replacement of terminal EDC and NHS in sequence to form an NHS ester. In the third step, the active NHS ester is replaced by the primary amines of the antibody, and the antibody is thus immobilized through the amide bond. Finally, in detection of the target bacteria, a specific binding event occurs between the immobilized antibodies and the antigens on the cell surface (Su & Li, 2004).
The electrochemical measurements showed that the Au electrodes surface was successful to give good signal since it was cleaned properly by the cleaning procedure. This was supported by a reversible and clear cyclic voltammogram obtained (Fig. 1.30) (Su & Li, 2004).

From the cyclic voltammetry study it was found that the formation of MHDA-monolayer resulted in insulation of the surface and this blocked most of the faradic currents. In order to correct this problem, the MHDA was activated with EDC/NHS and the surface became less insulated (Fig.
1.30, c). However, the binding of the target cells resulted in a good current measurement, and insulation was not an issue in this case.

The EQCM biosensor was tested by three different procedures: immersion, dip-and-dry, and flow-through procedure in order to optimize for the best applicability of the biosensor. According to the results, the immersion method had poor reproducibility since the behavior of the piezoelectric sensor in solution gave nonlinear response with changes in concentration of the bacteria cells. According to Martin, Granstaff, and Frye (1991), there can be variation in solution applications of piezoelectric biosensors related to surface mass changes due to non-specific adsorption, and also the solution density and viscosity can result in frequency changes in the quartz crystal. In the case of the dip and dry method, the binding of the bacterial cells resulted in a frequency decrease as the concentration of bacterial cells increased, giving a sigmoidal curve with a detection range of $10^3$ to $10^5$ CFU/mL. Therefore, the dip and dry method was better than the immersion method, with a limit of detection of $10^3$ CFU/mL. In the case of the flow-through method, the sensitivity was increased by lowering the flow rate and increasing the sample running time. However, the time for the analysis is affected by doing this. Regarding to interference, the dip-and-dry and flow-through methods did not present any interference. The immersion method presented interference by the matrices (liquid media).

In the case of piezoelectric immunosensors, one of the main factors of concern is the biomolecule immobilization. The principal methods used for the antibody immobilization at the solid surface (piezoelectric crystal) of the sensor include covalent binding, entrapment within a membrane, surfactant matrix, polymer or microcapsules, and cross-linking between molecules (Collings & Caruso, 1997). More recent methods for immobilization include molecular imprinted sol-gel film entrapment, Langmuir-Blodgett deposition, and electropolymerization (Bidan et al., 2000). A
piezoelectric immunosensor for detection of *Pseudomonas aeruginosa* was developed by Kim, Park, and Kim (2004). The anti-*P. aeruginosa* antibody was individually chemisorbed onto one-side gold electrodes of piezoelectric quartz crystals according to a thiolated antibody coupling procedure initiated with a thiol-cleavable heterobifunctional cross-linker (sulfosuccinimidyl-6-[3-(2-pyridyldithio) propionamido]hexanoate). Therefore, self-assembled monolayers (SAM) were formed. Fig. 1.31 depicts the response of the piezoelectric biosensor to different concentrations of *Pseudomonas aeruginosa*. From the graph it is observed that a limit of detection of $1.3 \times 10^7$ CFU/mL was obtained, and this showed that the immobilization of the antibody was effective for the biosensor.

One of the problems found in this type of biosensor was the selectivity. Since other types of bacteria can bind the surface of the piezoelectric sensor and signal can be produced. The piezoelectric sensor was tested against bacteria with genetic similarity of *Pseudomonas aeruginosa* such as *X. campestris*, *Xanthomonas* sp., *E. coli* and *S. typhimurium*. Results showed that the biosensor had signal response to those bacteria as well. Therefore it is suggested to include antibodies strictly specific to the bacteria of interest.
Fig. 1.31 Response of the biosensor to different concentrations of *Pseudomonas aeruginosa*. (a) $1.3 \times 10^7$, (b) $2.0 \times 10^7$, (c) $3.1 \times 10^7$, (d) $6.3 \times 10^7$, and (e) $1.3 \times 10^8$ CFU/mL (Kim et al., 2004).

Surface Acoustic Wave (SAW) biosensors are based on the production of a SAW when the material or molecule of interest binds on a piezoelectric material. In the food industry, SAW biosensors are used to detect vapors and organic volatiles due to their high sensitivity and fast response for these compounds. A SAW array biosensor for wine discrimination was proposed by Santos et al. (2005). The SAW sensor used consisted of delay lines (DL) with different polymer thin films of various thicknesses. The polymer films were deposited by spray-coating using an airbrush with a solution of the polymer and solvent. Three polymers were used: polyepichlorohydrin (PECH), polyetherurethane (PEUT) and polydimethylsiloxane (PDMS). The sensor array was tested with six different wines, which were injected in the measurement chamber for 10 min, and the response was calculated as the difference between the baseline frequency and the minimum frequency for each sensor. The SAW biosensor showed fast response, however, it
did not detected properly for octane and toluene compounds with the PDMS polymer coated sensor. The biosensor showed good results in separating the components of the wines in the principal component analysis (PCA) (Fig. 1.32). However, some overlapping is observed in the figure. In order to overcome this, the authors suggested optimizing the coating thicknesses, composition, and measurement parameters such as operation temperature and vapor generation times.

Fig. 1.32 Principal component analysis (PCA) plot for the six wines (Santos et al., 2005).

1.3.9 Other novel sensors – nanobiosensors

The use of nanotechnology is in a fast growth in the last years. The use of nanomaterials such as magnetic nanoparticles (MNPs), carbon nanotubes (CNTs), nanorods (NRs), quantum dots (QDs), nanowires (NWs), and nanochannels (NCs) aid in the improvement of the sensing capacity and other performance factors of the biosensors (Pérez-López & Merkoçi, 2011). Varshney, Li, Srinivasan, and Tung (2007) developed a microfluidic flow cell with embedded gold interdigitated
array microelectrode (IDAM). The IDAM was integrated with nanoparticle-antibody conjugates (MNAC) into an impedance biosensor for the rapid detection of *Escherichia coli* O157:H7 in ground beef samples. The concentration of the target bacteria was done by conjugating streptavidin-coated magnetic nanoparticles with biotin-labeled polyclonal anti-*E. coli* antibodies. The IDAM chip, a microchannel, and the assembled microfluidic flow cell are shown in Fig. 1.33.

Fig. 1.33 a) IDAM chip with gold microelectrodes on a glass wafer, b) microchannel with detection microchamber, and inlet and outlet channels, c) assembled microfluidic flow cell with embedded IDAM and connection wires (Varshney et al., 2007).
A novel biosensor for electrochemical detection based on rigid conducting gold nanocomposite (nano-AuGEC) was presented by Brasil de Oliveira Marques et al. (2009). This biosensor was used for the rapid detection of *Salmonella sp.* Results showed that a concentration range from 250 fmol to 10 pmol for the PCR amplicon concentration. Also, a low limit of detection of 200 fmol could be achieved, with an electrochemical signal of ~3 µA. The biosensor also showed good reproducibility, and a signal to noise ratio of about 15 for 200 fmol of amplicon was obtained (Fig. 1.34).

![Fig. 1.34 A) gel electrophoresis for the DNA double-tagged amplicon. B) Rapid electrochemical verification of PCR amplification of *Salmonella enterica* serovar *Typhimurium* (Brasil de Oliveira Marques et al., 2009).](image)

1.4 Point-of-care (POC) detection and recent developments in POC

Point of care testing is defined as any testing performed near the patient (Li et al., 2011). Currently, there is a need for POC assays for fast, on-site, and easy-to-perform detection of pathogens for
environment monitoring, clinical diagnosis, and food industry as well. The use of immunosensors incorporated to lateral flow devices is very popular in POC since they are cost effective, simple, portable, and therefore easy to incorporate in POC detection. Recent developments of POC assays include multiplexing in order to detect different pathogens at once. An enzyme-linked immunosorbent assay (ELISA) was developed on an immunochromatographic strip for the multiplex detection of *Salmonella typhimunium*, *Staphylococcus aureus*, *Legionella pneumophila*, and *Escherichia coli* O157:H7 (Park, Park, & Kim, 2010). For the fabrication of the immunochromatographic strip, four different functional membranes were used (Fig. 1.35). As shown in the figure, the immunochromatographic strip has 4 independent dots to generate detection signals and 1 dot control to confirm the working mechanism of the strip. The colorimetric signals were processed using an in-house-developed image analysis software program for quantification and development of standard curves. One of the limitations found in this type of assay is the ability for development of signals that are strong enough for detection. In this case, results showed that the signal intensity decreased at bacterial concentrations of $10^4$~$10^5$ CFU/mL and had a gradual decrease at lower concentrations. Another problem was the development of spot background signals, which are undesired for the correct performance of the assay. These background signals could be a result of cross-reactivity between the bacteria. Therefore, optimizations should be done in this sensor in order to get better signal to noise ratio of the detection. One suggestion can be the optimization of the antibody concentration in order to increment the sensitivity and avoid cross reactivity of the bacteria.
Fig. 1.35 Schematic illustration of a fabricated multiplex immunochromatographic strip. The strip is composed of four different functional membranes: (A) sample-application pad, (B) conjugate-release pad containing the reporter probes, (C) signal generation pad, and (D) absorption pad. The signal-generation pad contains four dots to detect (a) Salmonella typhimurium, (b) Staphylococcus aureus, (c) L. pneumophila, and (d) E. coli O157:H7, and one dot for control signal (e). The arrow indicates vertical flow of the immunoreaction (Park et al., 2010).

Recently, efforts have been done in order to achieve better detection limits in lateral flow devices as well development of multiplexing detection as mentioned above. Hossain et al. (2012) demonstrated a multiplexed paper strip for quantitative detection of Escherichia coli pathogenic and non-pathogenic. A LOD of 5 CFU/mL of E. coli O157:H7 and 20 CFU/mL of E. coli BL21 was achieved by using immunomagnetic nanoparticles for selective preconcentration within 30 min without any cell culturing. The working principle of the paper-based sensor is shown in Fig. 1.36. Besides the low LOD achieved, one limitation of this system is the need of trained personnel to perform the complex filtration steps, therefore, efforts should be done to substitute the filtration steps in order to have a more simplistic use of this sensor.
Fig. 1.36 Schematic diagram of the paper-based bacteria sensor. A) Sensing strategy for detection of single bacterial species, in which either XG or CPRG (color reaction or CR zone) and FeCl₃ (Fe zone) are entrapped within sol–gel-derived silica materials in the two dashed regions on a Whatman no. 1 paper strip (0.5×8 cm) via inkjet printing. A hydrophobic barrier (HB zone) composed of MSQ is layered at the top of the sensing zone to prevent leaching of color and thus increase signal intensity. The sensor is dipped into a cell lysate until the liquid front reaches the substrate/sensing region. Color appearance in the CR zone is indicative of the presence of bacteria.
B) Bidirectional multiplexed sensing strategy in which XG, CPRG, and FeCl3 are entrapped within sol–gel-derived silica materials in separate regions on a paper strip. A HB barrier is introduced between two sensing zones. The bottom of the paper strip is first placed into a cell lysate (E. coli BL21 in this case) and allowed to flow. The sensor is then inverted, immersed into the same lysate, and allowed to flow. The color intensity is monitored after 30 min at room temperature, unless otherwise stated. C) Optional preconcentration step in which cells are first isolated from samples via magnetic pulldown, resuspended in a minimal volume of a lysing reagent (10–100-fold less volume than initial sample) and then tested (Hossain et al., 2012).

A paper based POC testing immuno-disc for multiplex detection of *Pseudomonas aeruginosa* and *Staphylococcus aureus* was developed by Li et al. (2011). This sensing device provide the advantage of not requiring any preprocessing of the sample and the capability for whole cell bacteria detection. Antibody conjugated gold nanoparticles were used as the signaling agents of the sensor. An optimization of the shape of the lateral flow device was done in order to achieve multiplex detection, and the results showed that a shape of “peace-sign” performed better since it worked sufficiently with volumes as small as 15 µL (Fig. 1.37). This device demonstrated good multiplexing detection, however the results showed that the visible detection was limited to concentrations higher than 500 CFU/mL. It is suggested to optimize different factors in the sensing device such as the volume of the conjugated gold nanoparticles, the volume of antibodies, and the volume of the sample to be tested in order to obtain better LOD in this type of system.
Fig. 1.37 Specificity testing using samples of (a) *S. aureus*, (b) *P. aeruginosa*, (c) *S. aureus + P. aeruginosa*, (d) *E. coli*, (e) *S. aureus + E. coli*, and (f) *P. aeruginosa + E. coli*. Left: the testing lines of the strips were modified by anti-*S. aureus* antibody. Right: the testing lines of trips were modified by anti-*P. aeruginosa* antibody. Sample (d) served as a negative control (Li et al., 2011).

### 1.5 *Listeria monocytogenes*

*Listeria monocytogenes* is a gram positive non-spore forming pathogenic bacteria, it has a rod shape and it’s motile at room temperature with characteristic tumbling motility. It is a facultative anaerobe and grows better with 5% O₂, and 5-10% CO₂. *Listeria monocytogenes* can grow at low temperatures such as 4 °C and can withstand freezing, also it is salt tolerant up to 25% at 4 °C. There are other five species of the genus *Listeria* that include: *L. grayi, L. innocua, L. ivanovii, L. seeligeri*, and *L. welshimeri*, from this five species only *L. ivanovii* is considered pathogenic, mainly in ruminants rather than humans (Microorganisms & Toxins, 2005).

*Listeria monocytogenes* is an important food pathogen since it has been responsible for many outbreaks of human illnesses. Approximately 1600 illnesses and 260 deaths are caused by
listeriosis (infection caused by *L. monocytogenes*) occur per year in the United States (Scallan et al., 2011). In addition, the Center for Disease Control (CDC) reported that the average annual incidence of listeriosis in the US was 0.26 cases per 100,000 individuals in 2013 (CDC, 2014). The latest reported outbreaks related with *L. monocytogenes* were in 2012, in which 4 confirmed outbreaks and 1 suspected outbreak was reported in the US (CDC, 2014). High risk foods related to *L. monocytogenes* include: ready to eat (RTE), foods stored at refrigeration, and raw produce. Sources in food processing environments include: floors, coolers, freezers, processing rooms, wet, damp areas, and ventilation systems. There are three social patterns has increased the emergence of *L. monocytogenes*: 1) an increased number of elderly and immunocompromised patients, 2) increased production of minimally processed foods, and 3) the development of cold storage systems. The illness from *L. monocytogenes* generally occurs by ingestion of the organism and its passage through the G.I.-tract. Transmission can occur by secretions such as fecal, blood, neonatal fluids, etc. The infective dose of *L. monocytogenes* ranges from 100-1000 cells.

**1.6 Teichoic acids in cell wall of Gram-positive bacteria**

Teichoic acids are anionic carbohydrate-containing polymers present in the cell wall of many Gram-positive bacteria, and are divided into wall teichoic acids (WTAs) and lipoteichoic acids (LTAs), where WTAs can account for up to 60% of the total cell wall mass in Gram-positive bacteria (Eugster & Loessner, 2011). WTAs composition can be described mainly by two components, a disaccharide linkage unit and a main chain polymer composed of phosphodiester-linked polyol repeat units (Fig. 1.38) (Neuhaus & Baddiley, 2003). The WTAs are covalently bound to the peptidoglycan by phosphodiester bonds between N-acetylmuramic acid and a special
linkage unit, whereas LTAs are amphipathic molecules tethered to the cytoplasmic membrane via a glycolipid moiety (Fig. 1.39) (Araki & Ito, 1989; Baddiley, 1972; Neuhaus & Baddiley, 2003).

There is evidence that the cell wall of gram positive bacteria is protonated during respiration (Calamita, Ehringer, Koch, & Doyle, 2001), therefore suggesting that the cell wall of bacteria cells could have a relatively low pH environment. Previous studies have demonstrated that the polyanionic properties of WTA may contribute to the binding of protons, thereby affecting the local pH in the cell wall as a defensive mechanism to prevent autolysin (enzyme) activity (Fig. 1.40) (Biswas et al., 2012; Kemper, Urrutia, Beveridge, Koch, & Doyle, 1993). In addition, other negatively charged residues in LTA, peptidoglycan, phospholipid head groups, and surface associated proteins in the Gram-positive bacteria cell envelopes may assist to proton-binding capacity (Biswas et al., 2012).
Fig. 1.38 Wall teichoic acid structure (Neuhaus & Baddiley, 2003).

Fig. 1.39 Schematic of the gram-positive cell wall showing the wall teichoic acids (Brown, Santa Maria Jr, & Walker, 2013).
Fig. 1.40 Model for the role of WTA in proton binding and control of autolysin activity. (A) The negatively charged WTA phosphate groups retain protons in the cell wall, which creates an acidic environment keeping the activity of the major autolysin AtlA low. (B) In the absence of WTA protons are not retained, which avoids local acidification and leads to higher activity of AtlA (Biswas et al., 2012).

1.7 Carbon nanotubes

1.7.1 Definitions and electrical properties of carbon nanotubes

Carbon nanotubes (CNTs) are stable carbon structures made when a sheet of hexagonal carbon atom rings, called the graphene sheet, is rolled into a tube. This CNT structure is mostly described as a single wall carbon nanotube (SWCNT) (Fig. 1.41), while multiple-wall carbon nanotubes (MWCNTs) (Fig. 1.42) consists of many concentric rolled graphene sheets (Binns, 2010). SWCNTs have typical diameters between 1.2-5 nm, while MWCNTs have diameters between 10-50 nm.

CNTs were discovered by Sumio Iijima in 1991 by adding an Fe catalyst to one electrode in an arc-discharge apparatus (Iijima, 1991), and represent an important class of nanomaterials that has been used from basic research to advanced materials science. Nowadays, the three most common methods to produce CNTs are the carbon arc method, laser ablation, and plasma-enhanced chemical vapor deposition (PECVD).
CNTs can have different structures depending on the direction that the graphene sheets are rolled. This is known as the chirality of the tubes, and it affects the CNTs properties, including electrical properties. There are three basic known chiralities for CNTs and are classified as armchair, zig-zag, and chiral (Binns, 2010). There are some rules to define the electrical properties of CNTs according to their chiralities; all \((n,n)\) or armchair tubes are conducting, while \((n,m)\) tubes with n-
m=3i (i=integer) are almost metallic. Any CNT for which n-m≠3i is semiconducting (Osawa, 1970). Fig. 1.43 shows the pattern for the formation of these chiralities in the CNTs.

The type of conductance in a CNT is known as “ballistic”, meaning that an electron injected into one end propagates through the tube at ~10^6 m/s without dissipating heat, also, resistance is only encountered at the contacts at the ends and in any defects in the material (Binns, 2010).

Fig. 1.43 System for specifying nanotube chiralities. (a) The tube is specified by the circumferential vector \( n\mathbf{a}_1 + m\mathbf{a}_2 \) denoting a vector joining two equivalent points on the graphene lattice in terms of the unit vectors \( \mathbf{a}_1 \) and \( \mathbf{a}_2 \). The vector notation is simplified to \((m, n)\). The tube is generated by rolling the graphene lattice so that the vector lies on a circumference, as shown by
the arrows, and joining the start and end points. (b) An example of an (8,8) tube, also called an armchair tube because of the pattern of carbon atoms at the end. Any (n, n) tube will have an armchair configuration. (c) An example of a (12,0) tube, also called a zigzag tube because of the pattern of carbon atoms at the end. Any (n, 0) tube will have a zigzag configuration (Binns, 2010).

1.7.2 Use of carbon nanotubes in electrochemical biosensors

Electrochemical biosensors are defined by the Union of Pure and Applied Chemistry (IUPAC) as self-contained integrated devices, which are capable of providing specific quantitative or semi-quantitative information using a biological recognition element (biochemical receptor) which is retained in direct spatial contact with an electrochemical transduction element (Wilson, Thévenot, Durst, & Toth, 1999). As mentioned before, electrochemical biosensors are based on the measurement of an electrical signal (current, impedance, or potential) generated by the reaction of the electroactive analyte species with the biorecognition element of the biosensor, and the signal is proportional to the concentration of the analyte species (Wang, 1999). Electrochemical biosensors offer advantages such as good specificity, good selectivity, low detection limits, and easy integration and miniaturization, which makes this type of biosensors able to be produced at low cost and with automation. With regards to miniaturization, the use of nanomaterials can aid in the development of miniaturized and low cost instruments that require smaller sample volumes, decreased energy consumption, and improved performance. CNTs have shown excellent electron transport capacity with high current density, making them an efficient nanomaterial for electrochemical applications. Moreover, CNTs have a high surface-to-volume ratio, which makes them a promising material for biological sensing applications (Balasubramanian & Burghard,
Hence, the use of CNTs for electrochemical biosensors have been exploited in the recent years (Wang, 2005). There are there main approaches in developing CNT-based biosensors which include: 1) casting of CNT thin films from suspensions of CNTs in solvents on electrode surfaces (Huang, Niu, Xie, & Wang, 2010; Kang, Mai, Zou, Cai, & Mo, 2007; Musameh, Wang, Merkoci, & Lin, 2002; Yu, Mai, Xiao, & Zou, 2008), 2) using CNTs as paste electrodes or electrode composites (Dhand, Arya, Datta, & Malhotra, 2008; J Manso, Mena, Yanez-Sedeno, & Pingarron, 2007), and 3) using aligned CNTs as substrates for immobilization of analytes (Lin, Lu, Tu, & Ren, 2004; Tu, Lin, & Ren, 2003; Yeh, Lazareck, Kim, Xu, & Du, 2007).

1.7.3 Incorporation of carbon nanotubes as transduction elements in potentiometric biosensors

Potentiometric sensors are based in the determination of the potential difference between an indicator and a reference electrode, or two reference electrodes separated by a semipermeable membrane, with no significant current flowing between (Wilson et al., 1999). The transducer in a potentiometric sensor is usually an ion-selective electrode (ISE), which is based on selective membranes as recognition elements for different ions. The most popular potentiometric sensor is the pH electrode (H⁺), however ISE can also be designed for other ions including F⁻, I⁻, CN⁻, Na⁺, K⁺, Ca²⁺, NH₄⁺, and the measured potential differences are linearly dependent on the logarithm of the activity of the ion in solution (Buck & Lindner, 1994). The reference electrode is based on an electrode with a known potential, and the relationship between the analyte concentration and the potential can be explained by the Nernst equation (Eq. 1.1):

\[ E_{cell} = E_{cell}^0 - \left( \frac{RT}{nF} \right) \ln \left( \frac{\alpha_{red}}{\alpha_{ox}} \right) \]  

(1.1)
Where $E_{\text{cell}}$ is the cell potential at zero current, which is also known as the electromotive force (EMF), $E^0_{\text{cell}}$ is the standard cell potential, $R$ is the gas constant (8.314 J/K-mol), $T$ is the absolute temperature in K, $n$ is the number of electrons transferred in the cell reaction, $F$ is the Faraday constant (96487 C/mol) (Stock & Orna, 1989).

As mentioned before, the indicator electrode (ISE) is designed to respond to a particular ion or charged species in solution by incorporating a selective membrane (recognition element) at the transducer/solution interface. This selective membrane will develop a potential if there is a concentration difference across the membrane of the ion that is being tested. On the other hand, the indicator electrode can be custom made by adding other recognition elements such as antibodies or aptamers in their development, replacing the selective membranes in the ISE. These new types of indicator electrodes are termed solid-contact ion-selective electrodes (SC-ISE), and nowadays are getting the attention of researches as a tool for the detection of chemical or biological species (Bakker & Pretsch, 2002; Zhu, Li, Qin, & Zhang, 2010).

Carbon nanotubes provide a good option for the construction of SC-ISE due to their outstanding properties such as high surface-to-volume ratio, excellent electrical properties including high charge transfer capacity. The incorporation of CNTs as transduction elements in potentiometric biosensors offer the advantages such as enhanced electron transfer, rapid electrode kinetics, and an increased accumulation of biomolecules. Thus, by using CNTs as nanomaterials in potentiometry, there is a possibility of manufacturing high sensitive and selective biosensors for the detection of biomolecules. The transduction process of the CNTs relies on the high double layer capacitance that results from the large interface between the nanomaterial and the analytes in the solution (Fig. 1.44) (G. A. Crespo, Macho, & Rius, 2008; G. n. A. Crespo, Macho, Bobacka, & Rius, 2008).
1.8 Objectives

The main objective of this Doctoral Dissertation was to design, develop, and characterize a biosensor system for detection of bacteria in food based on electrochemical detection using solid-contact indicator electrode based on single wall carbon nanotubes. The specific objectives in order to achieve the general objective were:

1) To develop a sample collection and recovery protocol for *Listeria innocua* (non-pathogenic) from food samples.
2) To design, develop and characterize a sample concentration system based on volume reduction by hydrogels for manipulation of the sampled bacteria prior to detection.

3) To develop a detection system based on a carbon nanotube potentiometric biosensor for a quantitative detection of *Listeria innocua* and to demonstrate the application of the complete detection system based on sample collection, concentration, and detection of bacteria from food samples such as meat and milk.
1.9 References


Staff, I. C. M. S. F. (2012). Microbiological Testing in Food Safety Management: Springer US.


CHAPTER 2

DEVELOPMENT OF A SAMPLE COLLECTION PROTOCOL AND RECOVERY SYSTEM FOR *LISTERIA INNOCUA* FROM FOOD SURFACES
Abstract

A study of sample collection from the surface of food products was performed in this chapter. The sample collection was based on the use of a cellulose membrane to sample the food. The cellulose membrane was evaluated for bacteria recovery efficiency. For this purpose, surfaces of pre-cut meat pieces were inoculated with *Listeria innocua* at concentrations ranging from $10^1$-$10^5$ CFU/mL. Then, sample collection membranes were applied to the meat surfaces for different times such as 5, 10, 15, 20, 25, and 30 min and bacteria was enumerated with a standard plating method. Additionally, the cellulose membranes were observed under a scanning electron microscope (SEM) in order to observe the distribution of the sampled bacteria. Results showed that sampling durations between 5-10 min were the best for sample collection, with efficiencies >80% for some bacteria concentrations. Furthermore, SEM analysis showed that the bacteria cells were distributed at the surface of the cellulose membranes with no entrapment inside the membranes.

2.1 Introduction

The detection of pathogens requires the implementation of different steps in consecutive order that typically include sample collection, enrichment or concentration, culturing in specific agar or media for the organism, isolation, and biochemical tests (or functionally equivalent steps). Sample collection, the first step on the list is crucial since the results of the following steps depend upon it. Therefore, it is critical that the sample is collected in an appropriate manner (Brehm-Stecher, Young, Jaykus, & Tortorello, 2009). Most of the research efforts have been focused on the improvement of the detection of the pathogens, and the part of the process that deals with sample preparation is often disregarded. The International Commission on Microbiological Specifications for Foods (ICMSF) has established and written standard procedures for the development of
sampling plans for a variety of food products (Staff, 2012; Thatcher, 1974). As Brehm-Stecher et al. (2009) points out, sampling involves considerations of unit size, it depends on the purpose of the analysis and also needs of knowledge of the microbial load and distribution of the target in the sample. Therefore, this step is critical in the design of any detection method.

Some approaches for sampling surfaces include wet vacuum-based surface sampling, which is based on the use of high-efficiently particulate arrestance (HEPA) socks assembled in a vacuum filtration system (Sanderson et al., 2002), recirculating immunocapture (Morales-Rayas, Wolffs, & Griffiths, 2008; Warren, Yuk, & Schneider, 2007), and continuous flow centrifugation (Ágoston et al., 2009). The drawback of these methods is the requirement of specialized instrumentation, which is usually expensive. The use of simpler and less expensive methods for sampling has been evaluated. For example, Bisha and Brehm-Stecher (2009) showed the use of adhesive tape for sampling tomato surfaces combined with rapid fluorescence for Salmonella detection. They observed that the tape-based sampling accomplished both removal of attached organisms from the surface and also two-dimensional presentation of the cells on an optically clear film, which helps further processing like staining and direct microscopy observation. In addition, Fung, Thompson, Crozier-Dodson, and Kastner (2000) demonstrated the use of pop-up adhesive tape for microbial sampling of meat surfaces allowing to measure microbial loads up to 2.2 log CFU/cm² on the meat surfaces. However, this method was still not efficient compared to conventional swab/rinse method that could measure up to 8.3 log CFU/cm² of inoculated E. coli. Vorst, Todd, and Ryser (2004) showed the use of Kimwipe® absorbent tissues for recovery of Listeria monocytogenes from stainless steel surfaces, with a 2.70 log greater recovery for the absorbent tissues compared to other sampling methods. Johnston, Elhanafi, Drake, and Jaykus (2005) used centrifugation steps for PCR-based detection of Salmonella or E. coli O157:H7 from alfalfa sprouts and sprout irrigation
waters. In addition, Wolffs, Glencross, Thibaudeau, and Griffiths (2006) used simple filtration for PCR assay for the detection of *Salmonella* in chicken rinse, and spent irrigation water samples. Cellulose has been used for the production of absorbent pads or membranes. Cellulose and its derivatives is gaining attention for production of high absorbent polymers due to their biodegradable characteristics, their high natural abundance, and their high absorption capacity (A. Esposito et al., 2005; F. Esposito, Del Nobile, Mensitieri, & Nicolais, 1996; Lim, Yoon, & Kim, 2003; Lionetto, Sannino, & Maffezzoli, 2005; Sannino, Maffezzoli, & Nicolais, 2003). The objective of the present study is to develop a sample collection protocol and interface system for the detection of *Listeria innocua* from food samples using cellulose-based membranes.

### 2.2 Materials and methods

#### 2.2.1 Inoculum and inoculation

*L. innocua* strain (B-33016) was obtained from the ARS Culture (NRRL) Collection (Peoria, IL, USA), this strain was used since it is a non-pathogenic strain and could be used as model for the pathogenic bacteria *Listeria monocytogenes*. The strain (1 mL) was cultured in 9 mL of PALCAM enrichment broth (Neogen Corp., Lansing, MI, USA) with added selective supplement at 37 °C for 24 h. Then, a refreshed inoculation was done by transferring 1 mL of the culture into 9 mL of new PALCAM broth (Neogen Corp., Lansing, MI, USA) and incubated at 37 °C for another 24 h. Afterwards, serial dilutions of the bacteria were made by placing 1 mL of the culture into 9 mL of sterile 0.1% peptone water to obtain concentrations of $10^1$, $10^2$, $10^3$, $10^4$, and $10^5$ CFU/mL. The standard plate count technique was used to enumerate the concentrations of bacteria.
For inoculation, meat (pork) samples of 1 cm thickness were cut into pieces of 5 x 5 cm for length and width aseptically under a laminar flow hood. The surface of the meat was inoculated with 0.5 mL of the of *L. innocua* using a sterile glass spreader. This amount of inoculation was the amount that was determined to be optimum in the laboratory. After inoculation, the tissue was left undisturbed for 15 min at 25 °C under the hood (Cutter, 1999). The process was repeated for each of the bacteria concentrations.

### 2.2.2 Sampling protocol and optimization of sample collection with cellulose membrane

A study of sample collection from the surface of the food samples was performed. The sample collection was based on the use of a cellulose membrane (AP1004700, Merck Millipore Ltd., Billerica, MA, USA) to sample the surface of the previously inoculated food, then, the cellulose membrane can be introduced into the biosensor for subsequent detection of bacteria. Prior to sampling, the membrane was moistened with 1.5 mL of sterile deionized water in accordance with the amount previously determined in our laboratory to be optimum. Then, sample collection membranes were applied to the surface of the meat for different times such as 5, 10, 15, 20, 25, and 30 min in order to determine the optimum sampling time for the cellulose membrane. After sampling, the membranes were placed into stomacher bags (Whirl-Pak® 165, 118 mL, Nasco Corp., Fort Atkinson, WI, USA) containing 8.13 mL of 0.1% sterile peptone water. The stomacher bags containing the sampling membranes were homogenized for 2 min using a stomacher homogenizer (Stomacher® 80 Lab System, Seward Laboratory Systems Inc., FL, USA). Then, the sampling membranes were analyzed for bacteria using the standard plating method for *L. innocua*. Briefly, 0.1 mL of the liquid from the stomacher bags containing the homogenized membranes
was placed onto PALCAM agar (Neogen Corp., Lansing, MI, USA) petri dishes and spread into the surface using a sterile glass spreader. Afterwards, the plates were incubated for 48 h at 37 °C for bacteria enumeration.

The bacteria recovery efficiency of the sampling membranes was determined in order to know the percentage of the bacteria that was collected and recovered by the sampling method and it was calculated by using Eq. 2.1:

\[
Recovery\ efficiency\ (\%) = \frac{\text{Bacterial counts after sampling}}{\text{Initial inoculated bacterial counts}} \times 100 \tag{2.1}
\]

2.2.3 Scanning electron microscopy of cellulose membranes

Scanning electron microscopy (SEM) images of the cellulose membranes was used to observe the membrane’s surface after sampling for bacteria. For SEM characterization, the cellulose membranes were fixed with a formaldehyde glacial acetic acid (FAA) solution [consisting of ethanol (95% purity), glacial acetic acid, formaldehyde (40% purity), and water] overnight. Then, samples were dehydrated in ethanol series (50%, 70%, 80%, 90%, and 100%, respectively) followed by drying with liquid CO\textsubscript{2} in a Denton Critical Point Drier (CPD-1, Denton Vacuum LLC, NJ, USA). Samples were mounted on aluminum SEM stubs and coated with palladium in a sputter coater (EMS550X, Electron Microscopy Sciences, PA, USA), and imaged with a SEM (JSM-6610 JEOL Inc., MA, USA) system under high vacuum mode.

2.2.4 Statistical analysis
The statistical significance of differences observed among sample means was evaluated by analysis of variance (ANOVA) (SAS Version 9.2, SAS Institute Inc., Cary, NC, USA), followed by post hoc Tukey’s studentized range test.

2.3 Results and discussion

2.3.1 Optimization of sampling time with cellulose membrane

The cellulose membrane’s absorption capacity was evaluated by enumerating the number of bacteria that was collected from the meat surface and results for the optimum sampling time with different concentrations of inoculated bacteria are presented in Fig. 2.1.
Fig. 2.1 *Listeria innocua* counts for different sampling times with cellulose sampling membrane. Values expressed as mean ± standard deviation of three determinations. ABC Means within columns with different letters are significantly different (p<0.05).

The results showed that the bacterial counts decreased with increased sampling time for all concentrations of bacteria. For example, from Fig. 2.1 it could be observed that for a bacteria concentration of $10^1$ CFU/mL, bacterial counts were 45 CFU/mL for 5 min sampling, which decreased to 30 CFU/mL, 25 CFU/mL, 10 CFU/mL, 10 CFU/mL, and 2.5 CFU/mL for sampling times of 10 min, 15 min, 20 min, 25 min, and 30 min respectively. In other words, the bacteria counts were 52.63%, 68.42%, 73.68%, 89.47%, 89.47%, and 97.37% lower than the control counts (95 CFU/mL) for 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min sampling times respectively. A similar trend was observed for $10^2$ CFU/mL bacterial concentration. This could be explained as resulting from bacteria that could be migrating and adhering back to the food’s surface with longer sampling times.

The adherence of bacteria to meat and other surfaces has been studied previously (Benito et al., 1997; Chae, Schraft, Hansen, & Mackereth, 2006; Dickson, 1991; Dickson & Koohmaraie, 1989; Dickson & Siragusa, 1994; FIRSTENBERG-EDEN, Notermans, & Schothorst, 1978; Frank, 2001; Mafu, Roy, Goulet, & Savoie, 1991; Narendran, 2003; Piette & Idziak, 1989; Schwach & Zottola, 1982; Selgas, Marin, Pin, & Casas, 1993; Silva, Teixeira, Oliveira, & Azeredo, 2008). According to Frank (2001), the adherence of bacteria to surfaces is explained by a combination of hydrophobic bonding and electrostatic attraction/repulsion, being hydrophobic interactions a dominant parameter in bacterial adhesion. Benito et al. (1997) studied the effect of cell hydrophobicity on the attachment to meat surfaces of pathogenic bacteria including *L.*
monocytogenes, E. coli, S. aureus, Cl. perfringens, and E. aerogenes and reported that there was an apparent linear relationship between hydrophobicity and attachment. In the case of L. monocytogenes, it has been reported that it has low hydrophobic nature (Mafu et al., 1991), and other factor besides surface hydrophobicity, such as surface charges and presence of exopolymer, could have more importance in the adhesion of this bacteria to surfaces (Mafu, Roy, Goulet, & Magny, 1990). In our study we demonstrated that L. innocua could be recovered from the food surface with short sampling times, therefore suggesting that the bacteria could be establishing greater electrostatic interactions with the food surface with longer sampling times and, as a result, becoming more difficult to remove from the food’s surface.

Regarding higher concentrations in the range of $10^3$-10$^5$ CFU/mL, the bacterial counts did not significantly change ($p>0.05$). This could be due to the higher bacterial population inoculated into the product’s surface, therefore the sampling time didn’t significantly affect these concentrations. In general, it was observed that sampling times between 5 to 10 minutes were the best for lower concentrations of bacteria to ensure higher collection of the bacteria present in the surface of the food product.

2.3.2 Sample recovery efficiency of cellulose membrane

Results for recovery efficiency % are reported in Fig. 2.2.
Fig. 2.2 Absorption efficiency (%) of cellulose membrane at different sampling times from inoculated meat samples with *Listeria innocua*. Values expressed as mean ± standard deviation of three determinations. 

**Means within columns with different letters are significantly different (p<0.05).**

Results showed that the recovery efficiency of the sampling membrane decreased with increasing sampling times. From Fig. 2.2 it can be observed that a $10^1$ CFU/mL concentration of bacteria had recovery efficiencies of 47.37%, 31.58%, 26.32%, 10.53%, 10.53%, and 2.63% for 5 min, 10 min, 15 min, 20 min, 25 min, and 30 min sampling time, respectively. Similar results were observed for a $10^2$ concentration of bacteria. Higher recovery efficiencies were observed for higher bacteria concentrations including $10^3$-$10^5$ CFU/mL in the range of 80-89% for 5 min sampling time, and it
decreased with increasing sampling time. This analysis provided important information for further steps in the process for the detection of bacteria. For example, as mentioned above, for low concentrations of bacteria present on the surface of the product, the membrane’s recovery efficiencies were in the range of 2.63-57.80%, which indicates the need for further processing steps such as concentration or enrichment of the sample prior to analysis or detection using techniques including bio-sensing.

Recently, Panpradist et al. (2014) studied the efficiency of seven commercially-available clinical swabs for sample transfer for POC diagnostics including rayon, cotton, mid-turbinate (MT) nylon, regular-tip nylon, polyester (PES) foam, polyurethane (PUR) foam, and calcium alginate. They reported that PUR foam had the highest organism recovery of 79%-98%, and MT nylon and PES had intermediate organism recovery of 51-70% and 21-65% respectively. In the case of biosensors, the limit of detection is one of the most important reasons for the need of processing steps such as enrichment or concentration. However, if the biosensor is able to detect 10 cells or less, the need for such steps could be avoided. From the results of this study, it was concluded that 5 min sampling time were sufficient to recover more than 80% of the bacteria present in the food’ surface for some concentrations of bacteria, however, for concentrations <10³ CFU/mL the sampling did not recover more than 60% of the bacteria.

Fig. 2.3 presents a diagram of the sample collection and handling steps in the process for bacteria detection using the biosensor. After sampling the food, the cellulose membrane containing the bacteria is placed in the biosensor’s sampling introduction area. Then, the bacteria is transported from the membrane using water or any liquid needed for the detection of the sample in the biosensor. In this regard, a method using forced fluid flow based on the use of a syringe was developed to increase the organism recovery from the sampling membrane.
Fig. 2.3 Scheme of sample introduction after sampling with cellulose membrane. (A) Cellulose membrane containing sampled bacteria before detection. (B) Sampling membrane is placed into biosensor and bacteria is carried out from membrane into the detection zone by forced fluid flow.
2.3.3 Characterization of cellulose membranes by SEM

Scanning electron micrographs of the cellulose membranes after sampling bacteria from the food samples are shown in Fig. 2.4.

Fig. 2.4 Scanning electron micrographs of cellulose membranes after sample collection. A) Cellulose membrane after sampling *L. innocua* $10^1$ CFU/mL concentration, bar = 10 µm, 2,000X. B) Cellulose membrane after sampling *L. innocua* $10^5$ CFU/mL concentration, bar = 10 µm,
The SEM showed the sampled *L. innocua* cells which had been inoculated in the meat samples at different concentrations. From the images it was observed that the cells were retained at the surface of the membranes, with no entrapment between the pores of the cellulose structure. A few cells were located at the membrane’s surface after sampling a concentration of $10^1$ CFU/mL (Fig. 2.4A). Similarly, sampling the bacteria at $10^5$ CFU/mL showed some cells randomly distributed around the surface of the cellulose membrane (Fig. 2.4B). Fig. 2.4C shows a close-up of a cell of *L. innocua*, showing sizes between 1-2 µm, which were similar to the sizes reported in literature (CDC, 2005). Previous studies suggest that entrapment of the bacteria cells is not desired since entrapped cells could be difficult to recover to analyze (Chen et al., 2005). In our case, as seen in the SEM micrographs, no entrapment was observed in any sample, which is favorable for their recovery and analysis.

### 2.4 Conclusion

In this chapter, a sample collection and interface using a cellulose membrane was evaluated in terms of the number of bacteria that could be recovered from the food’s surface. This information was presented as sample recovery efficiency (%). Results demonstrated that 5-10 min sampling durations were the best for sampling the surface of the food samples (meat), with more than 80% of sample recovery efficiency for bacteria concentrations >$10^3$ CFU/mL. However, the cellulose membrane had lower recovery efficiencies (<57.80%) for bacteria concentrations between $10^1$ and
$10^3$ CFU/mL, which indicated the need for extra steps such as enrichment or concentration in the detection of low initial bacteria concentrations prior to detection or enumeration using any analytical technique. SEM analysis showed that cells were retained at the cellulose membrane’s surfaces and not entrapped in the membrane’s structure, which is desirable for their recovery and detection.
2.5 References


Frank, J. F. (2001). Microbial attachment to food and food contact surfaces. Advances in food and nutrition research, 43, 320-357.


CHAPTER 3

DEVELOPMENT OF A MICROFLUIDIC DEVICE FOR CONCENTRATION OF BACTERIA BASED ON VOLUME REDUCTION BY HYDROGELS
Abstract

The objective of this study was to design a microfluidic device for concentration of gram positive bacteria. The microfluidic device consisted of five parts: (1) top concentrator card containing a serpentine channel, (2) a negatively charged nylon membrane, (3) a support part, (4) a pectin-based hydrogel film, and (5) a bottom card. The performance of the microfluidic device was demonstrated by concentrating 1-5 µm fluorescent beads followed by concentration of bacterial samples such as *Listeria innocua*. The concentrator was operated by introducing a total volume of 1 mL of suspended bacteria sample in stepwise additions of 0.3 mL in the inlet port of the microfluidic device. Then, sample was allowed to dry for 30 min and a final sample volume of 100 µL was collected from the output port. Results showed that fluorescence intensity of the concentrated beads was increased by 10 times at the end of concentration. Recovery efficiencies of 85.60 and 91.75 % were obtained for initial bacteria concentrations of $1 \times 10^1$ and $1 \times 10^2$ CFU/mL respectively. Moreover, final cell counts were observed to increase by 10 times at the end of concentration. This study demonstrated that the microfluidic concentrator device successfully concentrated the bacteria sample and no significant loss of living cells was observed for most of the bacteria concentrations.

3.1 Introduction

In the field of food safety, there is an urgent need for quick food pathogen diagnostics that satisfies factors such as rapid, low cost, sensitive, accurate, specific, and robust detection (Mabey, Peeling, Ustianowski, & Perkins, 2004). Most hand-held analytical devices are usually small to provide portability and handle small sample volumes (e.g. 100 µL). One of the critical aspects for the detection of bacteria is the direct detection from complex systems such as food matrices or clinical
samples, and preparation steps such as separation, concentration, or enrichment are often required for detection.

Disposable concentration equipment and/or devices are needed for rapid pathogen detection. Enrichment of the bacteria of interest usually requires one or two days since the bacteria is allowed to multiply in a specific enrichment media. Concentration is easily accomplished in a laboratory with the use of equipment such as centrifuges. However, the use of centrifuges is limited by the size of the pathogens, becoming less effective as the pathogens are smaller, also it is not a suitable method for on-site detection. There is a need for solutions in order to concentrate samples into small volumes for detection in a rapid, portable, and cost effective approach that satisfies on-site detection. Currently available methods for concentration of bacteria include dielectrophoresis (DEP), field-flow fractionation (FFF), evaporation based concentrators, and microfiltration (Pethig, 2010; Reschiglian et al., 2002; Zhang, Do, Premasiri, Ziegler, & Klapperich, 2010; Zhu et al., 2004).

DEP refers to the motion of particles caused as a result of its dielectric properties (Pethig, 2010). The use of DEP for the separation and concentration of bacteria in biosensors and biological microelectromechanical systems (BioMEMS) has been demonstrated in the recent years. Gomez-Sjoberg, Morisette, and Bashir (2005) showed the development of a DEP based microfluidic device used to concentrate the bacterial cells in orders of $10^4$ to $10^5$ CFU/mL and measure their metabolic activity by means of impedance measurements. A silicon based microfluidic chip was built containing a channel for concentration of the bacterial cells by DEP forces into a main chamber with a volume of 400 pL for incubation and impedance measurements. Besides having the advantage of reducing time for detecting the presence of bacteria by concentrating diluted bacteria suspensions, this system has some limitations. One of the problems found in the
concentration and capture process was the loss of cells caused by instabilities in the flow rate in the detection chamber. Another limitation was that the sample dilution is fixed and no more diluted cell suspensions could be tested since it is impossible to control the flow rates through the device.

Field Flow Fractionation (FFF) is a separation technique achieved within a capillary, empty channel in which a laminar flow of a mobile phase sweeps the sample components down the channel (Roda et al., 2009). Several studies have shown the use of FFF to separate bacteria according to differences in shape and morphology from environmental, clinical, and food samples. This method also is recognized as a soft separation technique since bacteria are separated without modification of their native properties (Rambaldi, Reschiglian, & Zattoni, 2011). Some limitations of FFF include an overlapping of sample zones caused by similar displacement velocity and dispersion of individual subpopulations during separation.

Other studies have demonstrated the concentration of bacteria samples based on evaporation in microfluidic devices. Sharma, Lukyanov, Bardell, Seifried, and Shen (2008) showed the fabrication and testing of an evaporation-based microfluidic sample concentrator. The sample concentration was achieved using isothermal evaporation, and the concentrator’s performance was evaluated using computer simulations. The results for the simulations indicated that the concentrator would be able to remove water at rates of 0.6-1.8 mL/min. However, one drawback from this work is that no test with real samples was done, therefore it didn’t show how the device would perform concentrating bacteria samples. In addition, the device uses convective heat in order to evaporate the liquids, hence this could affect heat sensitive samples such as proteins, viruses or bacteria.

Zhang et al. (2010) developed a concentration system based on volume reduction by evaporation and concentration of bacteria by meniscus dragging. The working principle of this device is based
on the use of a combination of convective gas flow, partial pressure gradient, mass transfer, and capillary surface tension to evaporate liquid and to concentrate the sample into a small volume. This device was able to concentrate bacteria from $10^3$ to $10^6$ CFU/mL. However, the use of complicated settings makes this system difficult to operate for on-site detection.

The use of microfilters inside of microfluidic channels to trap microbes and parasites has been demonstrated (Zhu et al., 2004). The microfilters are constructed on silicon chip with gaps from 1-4 µm, and can trap samples of bacteria and protozoa that cannot pass the filters. This device therefore can be effectively used for concentration of biological samples. The possible limitation of this device is clogging of the pores as the samples are trapped, and adjustments in the design should be made such as incorporation of bypass microfilters in the micro-fabricated device.

Most of the concentration methods mentioned above use complicated settings, only can be used inside a laboratory, and are generally costly to fabricate, making them difficult to use. Hydrogels have been used in medicine, pharmacy, and life sciences. Their physicochemical properties make them suitable for an array of applications, especially, their high water affinity makes them suitable for swelling when exposed to liquids (Iijima, Hatakeyama, & Hatakeyama, 2005). Therefore, in this study, we introduce a microfluidic device for concentration of samples based on volume reduction of liquids by hydrogels. This device can be fabricated to be used as a suitable method to concentrate samples into small volumes for detection.

3.2 Materials and methods

3.2.1 Microfluidic device fabrication
The microfluidic device consisted of (1) a top concentrator card; which contains a serpentine channel in which the volume of liquid is reduced and the sample is concentrated at the end of the channel, (2) a nylon membrane (negatively charged, hydrophilic, pore size 0.43 µm) (Nylon 6, 6, Pall Corp., Port Washington, NY, USA) used as a direct contact surface for the bacteria sample to be concentrated, (3) a support card used as flat perforated surface for the nylon membrane, (4) a pectin-based hydrogel film used to reduce the sample volume, and (5) a bottom card used to give support for assembling the different parts of the concentrator (Fig. 3.1).

![Fig. 3.1 Microfluidic concentrator design A) 2D front view design of concentrator. B) Exploded view of assembled concentrator which contains: 1) top concentrator card, 2) nylon membrane, 3) support part, 4) pectin-based hydrogel film, 5) bottom card.](image)

The pectin-based hydrogel film is one of the most important parts of the concentrator since it is used to swell and reduce the sample volume. As the sample is introduced into the concentrator, the liquid is absorbed by the hydrogel film, thus decreasing the sample and concentrating the bacteria at the end of the channels (Fig. 3.2).
The channel dimensions were 77 mm length, 0.8 mm wide, and 0.5 mm depth. The top and bottom layers were designed in AutoCAD® software (AutoCAD® 2016, Autodesk, San Rafael, CA, USA) and patterned in Poly-(methyl methacrylate) using a computer numeric control (CNC) milling machine (KERN MMP 451, KERN Micro & Feinwektechnik GmbH & Co. KG, Germany). The layers were aligned and sealed by applying pressure to the assembly.

3.2.2 Fabrication and characterization of hydrogel films

A pectin-based hydrogel film was prepared as described by da Silva, Bierhalz, and Kieckbusch (2009). The pectin hydrogel film was fabricated by casting in a two-stage crosslinking process. For the first stage, 4 g of pectin (Pectin LM 32 Powder, Tic Gums, Belcamp, MD, USA) was dissolved in 266.67 g of deionized water containing 2.4 g glycerol (Fisher Scientific, Fair Lawn, NJ, USA). The mix containing pectin was completely dissolved by stirring at 1000 rpm using a RCT Basic S1 magnetic stirrer (IKA Labrotechnik, Janke & Kunkel GmbH & Co., Staufen,
Germany) for 1 h at room temperature. Then, the solution was heated to 70 °C and a calcium chloride (Fisher Scientific, Fair Lawn, NJ, USA) solution (0.16 g CaCl₂ in 30 mL H₂O) was added using a syringe pump (Harvard apparatus pump 11, Holliston, MA, USA) at a flow rate of 1 mL/min. The high temperature, low flow rate, and the agitation were required to avoid instant gelation. Then, 70 g of the solution were poured into polystyrene Petri dishes (diameter = 15 cm). The solution in the Petri dishes was dried in a convection oven (VWR 1330FM, Cornelius, OR, USA) at 40 °C for 20 h. Then, films were detached from the Petri dishes and the crosslinking was complemented in a second stage, which was done by immersing the films in 50 mL of a calcium chloride solution (4% w/v) containing 5% glycerol for 30 min. Then, the excess surface liquid was removed and films were placed over Petri dishes that were previously inverted and conditioned at room temperature for 6 h.

The hydrogel films were characterized for swelling degree (SD) as described by Xu, Bartley, and Johnson (2003). The initial mass (m₀) of a circular cut (d = 2.5 cm) of the film was determined using an analytical balance (Sartorius ED224S, Sartorius Corporation, Bohemia, NY, USA). Then, the sample was immersed in 100 mL of deionized water at 25 °C and kept immersed for different periods of time. The sample was removed at different time intervals and the mass was taken (mₜ) after carefully blotting and removing excess surface water. The SD was calculated by Eq. 3.1, which was used to determine the amount of water absorbed relative to the initial mass of the sample (m₀).

\[
SD = \frac{(mₜ-m₀)}{m₀} \times 100
\]  

(3.1)

The swelling ratio (SR) of the films was determined with Eq. 3.2, SR is the ratio of the final sample mass (after water absorption) to the initial mass of the sample.
\[ SR = \frac{m_w}{m_o} \]  

(3.2)

3.2.3 Concentration visualization with fluorescent beads

The device was characterized for concentration using fluorescent beads prior to testing with bacteria. Fluorescent orange-yellow microspheres (FMOY-1.3 1-5 µm, Cospheric LLC, Santa Barbara, CA, USA) were used to mimic concentration of bacteria in the concentrator device. One milligram of fluorescent beads was suspended in 1 mL of deionized water and applied into the concentrator using a syringe (Luer-Lok™ tip, BD Co., Franklin Lakes, NJ, USA) that was connected into the concentrator inlet. The syringe was also used to pump the liquid through the device channels during concentration without the need of an external pumping device. The concentrator was run and a fluorescent image was taken every 5 min using a Lumar fluorescence stereomicroscope (Zeiss SteREO Lumar.V12, Carl Zeiss Co., Oberkochen, Germany) to monitor the progress of the concentration. Background intensity was removed from the images using ZEN imaging software (ZEN lite, Carl Zeiss Co., Oberkochen, Germany). The concentrator was run for 30 min and a final sample volume of 100 µL was collected from the output port.

3.2.4 Bacteria preparation

Listeria innocua strain B-33016 was obtained from the ARS Culture (NRRL) Collection (Peoria, IL, USA). This is a non-pathogenic strain and can be used as model for pathogenic bacteria Listeria monocytogenes. One milliliter of the strain was cultured in 9 mL of PALCAM enrichment broth with PALCAM selective supplement at 37 °C for 24 h. Then, a fresh inoculation was done by
transferring 1 mL of the culture into 9 mL of broth and incubated at 37 °C for another 24 h. Serial dilutions of the bacteria were made by placing 1 mL of the culture into 9 mL of sterilized 0.1% peptone water to obtain concentrations ranging from $10^1$-10$^7$ CFU/mL. Standard plate count technique was used to enumerate the concentrations of bacteria.

### 3.2.5 Concentration of bacteria and recovery efficiency determination

Microfluidic concentration was performed using *Listeria innocua* samples at concentrations ranging from $10^1$-10$^7$ CFU/mL. To operate the concentrator, 1 mL of the bacteria was introduced into the inlet port of the device using a syringe by stepwise additions of every 0.3 mL. The sample was allowed to dry and another 0.3 mL were pipetted until completing the total sample volume. The concentrator was run for 30 min and a final sample volume of 100 µL was collected from the output port. At the end of concentration, the bacteria in the collected sample was enumerated using standard plate count technique. The recovery efficiency (RE %) was determined as the ratio of bacteria concentrated into the final volume (100 µL) divided by the initial number of bacteria in 1 mL of starting sample (Eq. 3.3) according to Zhang et al. (2010).

$$Recovery\ efficiency\ (RE\%) = \frac{Number\ of\ bacteria\ in\ final\ volume}{Initial\ number\ of\ bacteria\ in\ sample} \times 100 \quad (3.3)$$

### 3.2.6 Statistical analysis

The statistical significance of differences observed among sample means was evaluated by analysis of variance (ANOVA) (SAS Version 9.2, SAS Institute Inc., Cary, NC, USA), followed by post hoc Tukey’s studentized range test.
3.3 Results and discussion

3.3.1 Hydrogel films characterization

The produced pectin-based hydrogel films were characterized for water uptake and swelling in contact with distilled water at 25 °C. The swelling kinetics obtained from this study are presented in Fig. 3.3 and Fig. 3.4 respectively.

Fig. 3.3 Swelling degree of pectin-based hydrogel used in the concentrator. ■ = Swelling degree.
Results showed that after 5 min, samples had a swelling degree (SD) of 109.98 ± 12.16%, which indicated that hydrogels had a water uptake of more than 100% their weight during that swelling time. A linear increase in SD was observed from min 0 until 30 min of swelling. Afterwards, equilibrium was reached after 30 min of immersion in water with a SD of 150.12 ± 16.97 %, and the water uptake was stable for the rest of the time until 60 min. Similar equilibrium times were reported by da Silva et al. (2009), in which pure pectin films reached an equilibrium SD after more than 20 min of immersion in water. The high SD found in our hydrogel films also was due to the low concentration of plasticizer (5% glycerol) used in the formulation. da Silva et al. (2009) found that the swelling degree had a tendency to decrease with an increase in the glycerol concentration.
in the crosslinking solution. This can be due to weakening of the crosslinking between the pectin and the calcium chloride caused by the glycerol, as a result, having a decrease of water uptake equilibration time with increasing the plasticizer concentration.

Fig. 3.4 shows the swelling ratio (SR) of the hydrogel films during 60 min of immersion in water. The SR indicates how many times the hydrogel films increased in weight with respect to their initial weight. After 5 min of immersion, it was observed that the hydrogel films increased more than 2 times their weight, indicating that the sample could absorb more than 2 times their weight in water. A linear increase in swelling ratio was observed until 30 min of immersion in water, where it reached equilibrium with a swelling ratio of 2.50 ± 0.17. After 30 min the SR of the films was stable until 60 min of immersion in water.

3.3.2 Fluorescent beads concentration and quantification

Fluorescent beads of diameters ranging between 1-5 µm where used to visualize the concentration inside the channels of the concentrator and to simulate the movement of particles with similar size of the bacteria of interest (2-5 µm size range). Fig. 3.5 shows an increase in intensity that indicated increased concentration of the beads during time. From min 5 to min 10 of concentration the intensity of the channels had some increase. Furthermore, from min 15 to min 20, the channels showed a significantly increase in the intensity, which means the particles were concentrated more by this time. At the end of 30 min the particles were agglomerated together and a meniscus is shown at the end of the channel, with a significant increase in fluorescent intensity detected by the fluorescence microscope. Some migration of particles out of the channels can be seen from the pictures, which was due to movement of smaller particles (<2 µm) outside the channels since beads
in the size from 1-5 μm were used. Therefore, particles smaller than 2 μm can get diffused outside the channels carried by some leakage of the liquid. However, this is less likely to happen when concentrating bacteria since the average size of bacteria is higher than 2 μm generally (3-5 μm).

At the end of 30 min of concentration the sample volume was approximately 100 μL, which indicated that the sample was effectively reduced 10 times in volume, while concentrating the sample 10 times as well. Zhang et al. (2013) reported similar results, in which the intensity of fluorescent beads (100 nm) increased over time in a concentration device based on evaporation and interfacial tension. However, the device developed by Zhang et al. (2013) showed loss of particles (up to 15%) that were due to photobleaching and particle retention on the channel walls. In the case of our device, no big loss of particles was observed.
Fig. 3.5 Concentration of fluorescent beads in the concentrator. Fluorescence images of the channels over time.
Fig. 3.6 Fluorescence intensity through the channel over concentration time. ● = Fluorescence.

Fig. 3.6 shows a graph of quantification of fluorescence intensities over the channel during concentration time. The fluorescence intensity increased linearly through time until the end of concentration. For example, at min 5, the intensity mean value was of $98.61 \pm 13.17$ a.u., then it increased to $1235.11 \pm 40.65$ a.u at the end of 30 min.

3.3.3 Concentration of bacteria samples

The concentration of bacteria was achieved by lowering the volume of the suspended sample from 1 mL to 0.1 mL in 30 min of concentration time. A picture of the concentrator device is shown in Fig. 3.7.
Fig. 3.7 Picture of microfluidic concentrator before running it with bacteria sample.

One important factor is to achieve concentration but also preserve as many living bacteria cells as possible. The recovery efficiencies (%) of the bacteria after concentration are presented in Fig 3.8.
Fig. 3.8 Recovery efficiency after concentration from different bacterial concentrations. Values expressed as mean ± standard deviation of three determinations. A-D Means with different letters are significantly different (p<0.05).

Results showed that, for concentrations in the range from $10^3$-$10^6$ CFU/mL bacteria adhered more to the channels, therefore less recovery efficiency was obtained for these concentrations. The device developed by Zhang, Do, Premasiri, Ziegler, and Klapperich (2010) showed similar results, in which loss of bacteria samples was observed for high initial concentrations due to adhesion of bacteria into the channel walls. In general, bacteria left in the channels was considered as loss in the concentration process. On the other hand, it was observed that for concentrations of bacteria
between $10^1$-$10^2$ CFU/mL recovery efficiencies of 85.60 ± 12.92% and 91.75 ± 2.61% were obtained, respectively. This indicated that bacteria in these concentrations traveled easier inside the channels without adhering to the walls, therefore no significant loss of the samples was observed.

It has been reported that bacterial attachment is influenced by cell surface charge, hydrophobicity, and structures like extracellular polysaccharides and flagella (Dahlbäck, Hermansson, Kjelleberg, & Norkrans, 1981; Fletcher & Floodgate, 1973; Fletcher & Loeb, 1979; Notermans & Kampelmacher, 1974). It is important to mention that the design of the device included a negatively charged membrane which help in the movement of the samples suspended in the liquid by electrostatic repulsion between the membrane and the bacterial cells having a relative negative charge (Dickson & Koohmaraie, 1989).

In a clinical or a food safety point of view, it is known that high initial concentration samples are not relevant, while very low sample concentrations are more important to determine any diagnostic for illness in its early stage, or the quick detection of a contaminated food product in the case of a food sample. In the case of concentrations of $10^7$ CFU/mL, it was observed that the recovery efficiency increased (81.29 ± 0.96%). This was due to the high initial numbers or bacteria, hence, even if some of them could adhere to the channels, the process didn’t significantly lower the numbers of bacteria after concentration.

Fig. 3.9 shows the results for the concentration of *Listeria innocua* in the sample concentrator. As mentioned before, the concentration was done by lowering the volume of suspended sample from 1 mL to 100 µL final volume.
Fig. 3.9 Bacteria counts after concentration with microfluidic concentrator device. Values expressed as mean ± standard deviation of three determinations. AB Means in columns with different letters are significantly different (p<0.05). □ = L. innocua counts before concentration. □ = L. innocua counts after concentration.

Results showed that despite some loses discussed previously, the numbers of bacteria in the final concentrated samples were significantly higher (p<0.05) compared to the initial bacteria concentrations in the samples. It was observed that for all samples, bacteria was concentrated 10 times compared to initial values, showing the functionality of the device. As seen in Fig. 3.9, a 1 mL initial volume of bacteria sample with a concentration of 9x10^1 CFU/mL was concentrated
into 100 µL and a final cell count of 7.75x10² CFU/mL was obtained after enumeration. In the case of 10² CFU/mL bacteria concentration, the bacteria sample was concentrated to a final cell counts of 2.97x10³ CFU/mL obtained by enumeration after the concentration process. A similar trend was observed for the rest of the samples, with an increase in the cell counts in the final concentrated samples.

3.4 Conclusion

A sample concentrator device was presented in this study. Its applicability to a wide variety of biological molecules makes it a good option for sample concentration without the use of heat or convective gas such as nitrogen. Coupled with passive pumping (e.g. using syringe pumps) this device can be integrated into complex microfluidic systems to process small quantities of sample. The results for recovery efficiency and final bacteria cell counts after concentration demonstrated that biological samples could be concentrated in 30 min without significant loss of living cells. The current design allows for concentration of sample into a final volume of 100 µL, however, the design can be modified to accomplish lower final volumes of sample such as 500 nL or less, depending on the number of hydrogel film layers included into the concentrator.
3.5 References


CHAPTER 4

DEVELOPMENT OF A CARBON NANOTUBE POTENTIOMETRIC BIOSENSOR FOR A QUANTITATIVE DETECTION OF LISTERIA INOCUA FROM FOOD SAMPLES
Abstract

In this chapter, a carbon nanotube potentiometric biosensor for the detection of bacteria from food samples was demonstrated. The biosensor was constructed by depositing carboxylic acid (–COOH) functionalized single walled carbon nanotubes (SWCNTs) on a glassy carbon electrode (GCE), followed by the attachment of antibodies to the SWCNTs between the amine groups and the –COOH by covalent functionalization using EDC/Sulfo-NHS chemistry. The performance of the biosensor was evaluated by using concentrations of *L. innocua* in the range from $1.1 \times 10^1$ to $1.36 \times 10^8$ CFU/mL, and other factors such as limit of detection, sensitivity, response time, linearity, and selectivity were also determined. The selectivity was evaluated by testing the biosensor against other bacteria than *L. innocua* such as *Lactobacillus plantarum*, *Lactobacillus acidophilus*, and *Escherichia coli*. In addition, the biosensor was tested with food samples inoculated with *L. innocua* including meat and milk. Results showed a linear response for all *L. innocua* concentrations with a sensitivity of $-0.8708 \pm 0.25$ mV per decade and a lower limit of detection of 11 CFU/mL. The results from selectivity test showed that the biosensor did not respond for other bacteria than *L. innocua* indicating that the biosensor was highly selective. The biosensor could successfully detect bacteria inoculated in the food samples with a limit of detection of 25 CFU/g and 27 CFU/mL for meat and milk samples respectively. In addition, a biosensor system containing sampling, concentration, and detection was proposed. This system could be used for on-site detection of food pathogens.

4.1 Introduction

Food safety is a main concern for the food industry. It is estimated that each year 31 major pathogens cause 9.4 million episodes of foodborne illnesses, 55,961 hospitalizations, and 1,351
deaths in the United States. Most illnesses are caused by norovirus, followed by *Salmonella* spp., *Clostridium perfringens*, and *Campylobacter* spp., the leading causes of hospitalization are *Salmonella* spp., norovirus, *Campylobacter* spp., and *Toxoplasma gondii*, and leading causes of death are *Salmonella* spp., *T. gondii*, *Listeria monocytogenes*, and norovirus (Scallan et al., 2011). There are three main conventional detection methods for pathogens: (1) conventional cell culturing and plate colony counting, (2) enzyme-linked immunosorbent assay (ELISA), and (3) polymerase chain reaction (PCR) (Arora, Sindhu, Dilbaghi, & Chaudhury, 2011). However, these methods are labor intensive, time consuming, require skilled personnel to operate laboratories with expensive equipment, and mostly do not offer a real-time detection. The lack of portable, real-time biosensors for detection of these pathogens results in a significant time lag (a couple of days to a week) between an outbreak and its identification. In many cases, actions in response to pathogen contamination are not taken until there is an outbreak detected at the post-consumer level (Yoon & Kim, 2012). In this sense, there is need for the development of novel detection sensors that are capable of detecting pathogens faster with good sensitivity and reproducibility. In addition, there is a need for the implementation of different techniques and methodologies for the production of portable and miniaturized sensors.

A biosensor is a device that contains a biological sensing element either intrinsically connected or integrated within a transducer. This device responds to the concentration or activity of the species or compounds in the biological samples (Mutlu, 2010). There is a rapid growth in the development of biosensors in order to provide automation, easy assay performances, and lower cost of analyses. Teichoic acids are anionic carbohydrate-containing polymers present in the cell wall of many Gram-positive bacteria and are divided into wall teichoic acids (WTAs) and lipoteichoic acids (LTAs) (Eugster & Loessner, 2011). There is an evidence that the cell wall of gram positive
bacteria is protonated during respiration (Calamita, Ehringer, Koch, & Doyle, 2001), therefore suggesting that the cell wall of bacteria cells may have a relatively low pH environment. Biswas et al. (2012) have demonstrated that the polyanionic properties of WTA contribute to the binding of protons thereby affecting the local pH in the cell wall. By knowing that the WTAs in the bacterial cell wall are protonated (H\(^+\) ions), there is a possibility of detecting pathogens by electrochemical based detection using carbon nanotubes (CNTs). Previous studies showed that single-wall carbon nanotubes (SWCNTs) are efficient conductive transducers due to their high surface-to-volume ratio and high charge transfer characteristics (Balasubramanian & Burghard, 2006; G. A. Crespo, Macho, & Rius, 2008). Additionally, CNTs are easily deposited on surfaces, making them a good option for contact electrode designs (Huang, Niu, Xie, & Wang, 2010; Kang, Mai, Zou, Cai, & Mo, 2007; Musameh, Wang, Merkoci, & Lin, 2002; Yu, Mai, Xiao, & Zou, 2008).

Among electrochemical biosensors, potentiometric sensors provide outstanding sensitivity and selectivity due to the species-selective working electrode used in their design (Su, Jia, Hou, & Lei, 2011). Potentiometric detection is based on the determination of a potential difference between an indicator electrode (working electrode) and a reference electrode (Thévenot, Toth, Durst, & Wilson, 2001). The working electrode can be customized for detection of specific species by adding recognition elements such as antibodies or aptamers in order to produce solid-contact ion-selective electrodes (SC-ISE). The incorporation of CNTs as transduction elements in potentiometric biosensors offer the advantages such as enhanced electron transfer, rapid electrode kinetics, and an increased accumulation of biomolecules. The transduction process of the CNTs relies on the high double layer capacitance that results from the large interface between the nanomaterial and the analytes in the solution (G. A. Crespo et al., 2008; G. n. A. Crespo, Macho,
Thus there is a possibility of detecting the bacteria of interest by measuring the change in potential when the bacteria establish contact with the CNTs. The objective of this chapter was to develop and characterize a biosensor based on the potentiometric detection of Gram positive *Listeria innocua* from food samples.

### 4.2 Materials and methods

#### 4.2.1 SWCNTs oxidation

Single wall carbon nanotubes (SWCNTs) (>90% purity, 1.1 nm average diameter, length of 5-30 µm) were purchased from US Research Nanomaterials Inc., TX, USA. SWCNTs were functionalized in order to attach carboxylic groups in their walls by oxidation (Fig 4.1).

![SWCNT oxidation reaction](image)

Fig. 4.1 SWCNTs oxidation reaction.
The oxidation process was done using a modified method from Ciobotaru, Damian, and Iovu (2013). Briefly, 50 mg of SWCNTs were mixed with 30 mL of 70% nitric acid (Sigma Aldrich, MO, USA). The mixture was refluxed at 120 °C in a silicone oil bath and kept stirring at 400 rpm for 4 h. After refluxing, the mixture was cooled and 30 mL of deionized water were added in order to dilute the acid solution. Then, the mixture was filtered using a 0.22 µm pore polystyrene (PES) filter (Corning Inc., NY, USA) to remove the acid, and washed with deionized water until pH was ~6. After filtration, the sample was dried for 3 h in a convection oven at 105 °C.

4.2.2 Oxidation characterization by Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of the raw SWCNTs (used as control) and the oxidized SWCNTs was determined on a Bruker instrument (Bruker Alpha & Tensor 27, Bruker Inc., MA, USA) with 4 cm\(^{-1}\) resolution and 32 scans. Approximately 10 mg of the samples were dispersed between KBr plates (KBr windows, 25 mm x 4 mm, Pike Technologies, WI, USA) and analyzed at room temperature for the FTIR measurements. Measurements were taken under absorbance mode using a diamond cell (Pike Miracle Diamond ATR cell, Pike Technologies, WI, USA). Results of the spectra were recorded using Opus® software (Opus® version 7.2, 2012, Bruker Optics, Bruker Inc., MA, USA).

4.2.3 Preparation of biosensor

4.2.3.1 Glassy carbon electrode activation
The biosensor was prepared on a glassy carbon electrode (GCE) (Glassy carbon rod 42824, Alfa Aesar Inc., MA, USA) with an outside diameter (OD) of 3 mm and a length of 50 mm. The GCE was covered by a Teflon jacket of 6 mm diameter in order to give protection during handling of the electrode. The tip of the electrode was activated by sequential polishing using 1 µm, 0.3 µm, and 0.05 µm grain size alumina powder slurry (Electron Microscopy Sciences, PA, USA) in order to obtain a smooth surface. For the polishing process, approximately 2 mL of alumina slurry was placed on a clean ground glass plate. Then, GCE was polished with light pressure following a figure 8 pattern for approximately 30 seconds. Residual alumina were removed by streaming deionized water against the surface of the electrode. Excess water was removed with a soft tissue paper (Kimwipe™), and the electrode was dried with N₂ gas (ResearchGate, 2016).

4.2.3.2 SWCNTs deposition on GCE

A 2.5 mg/mL solution of SWCNTs was prepared by dispersing 25 mg of previously functionalized SWCNTs in 10 mL of deionized water containing 100 mg of sodium dodecyl sulfate (SDS, Sigma Aldrich, MO, USA). Then, the mixture was sonicated in a waterbath sonicator (Bransonic® 1210R-MT, Branson Ultrasonics Corporation, CT, USA) for 2 h to obtain a stable dispersion of the CNTs. For the deposition of SWCNTs on the electrode, 20 µL of the solution was deposited on the electrode’s previously polished surface and allowed to dry for 45 min in a convection oven at 70 °C. The deposition was repeated six times, and the tip of the electrode was rinsed with deionized water at every third interval in order to remove the SDS. The CNT modified electrode was characterized using a scanning electron microscope (SEM) (JSM-6610 SEM, JEOL Inc., MA, USA) to observe the surface morphology. The thickness of the deposited CNTs layer on the
electrode’s tip was measured by a profilometer (Alpha-Step D-100 Stylus Profiler, KLA Tencor Corp., CA, USA) using a force of 0.05 mg and 1-mm length of scans.

4.2.3.3 Activation of CNT layer on GCE by EDC/Sulfo-NHS chemistry

Activation of the carboxylic acids present on the nanotube sidewalls was achieved by placing the electrode for 30 min at room temperature in an aqueous solution of 0.4 M N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) (Sigma Aldrich, St. Louis, MO, USA), and 0.1 M sulfo-N-Hydroxysulfosuccinimide (sulfo-NHS) (Sigma Aldrich, St. Louis MO, USA), prepared in 1 mL of 0.1 M 2-(N-morpholino)ethanesulfonic acid sodium salt (MES) (Sigma Aldrich, St. Louis, MO, USA), which was adjusted to pH 6.0 using 4 N HCl.

4.2.3.4 SWCNTs-antibody functionalization

Listeria sp. antibodies suspended in phosphate buffer saline (PBS), pH 7.2 containing 0.09% NaN₃ and sodium azide as preservative were obtained from Antibodies-online Inc., Atlanta, GA, USA. A 100 µg/mL solution of anti-Listeria antibodies was prepared by mixing 25 µL of the antibodies into 225 µL of 50 mM PBS of pH 7.4. Immediately after activation with the EDC/Sulfo-NHS chemistry, the modified electrode was placed in the anti-Listeria antibody solution for 5 h at room temperature. The EDC/Sulfo – NHS chemistry was used in order to enable covalent bonding between the carboxyl groups (-COOH) on the surface of the CNTs and the amine groups (-NH2) of the antibodies through amidation process (Fig. 4.2). The biosensor was kept stored in PBS 1.7 mM pH 7.4 in a fridge at 4 °C until use.
4.2.4 Characterization of SWCNT-antibody functionalization

The antibody conjugation efficiency was further characterized by transmission electron microscopy (TEM). For this purpose, 2 mL of carboxylic acid functionalized dispersed SWCNT solution (2.5 mg/mL) were centrifuged for 3 min in order to separate and discard nanotube aggregates. Then, coupling agents, EDC (0.4 M) and Sulfo-NHS (0.1 M), prepared in 1 mL of MES buffer (pH 6.0) were mixed with the modified SWCNT solution. The mixture was left to react for 30 min at room temperature as described previously. Then, excess surfactants and coupling agents were removed by centrifuging the mixture for 3 min at 13,000 rpm. Furthermore, 200 µL of the Listeria-antibody solution (100 µg/mL) were mixed with the SWCNT solution and the mixture was incubated for 5 h at room temperature. The centrifuging process was repeated to remove unbound antibodies, and the resulting solution was kept at 4 °C until use.

For characterization by TEM, 5 µL of SWCNT-antibody functionalized solution was placed on a gold coated carbon TEM grid (150 square mesh, Nickel grid, Electron Microscopy Sciences, PA, USA). Then, sample was stained with 2% Uranyl Acetate (UA), followed by glow discharging in a glow discharge instrument (Leica EM ACE600, IL, USA). Sample was observed in a
transmission electron microscope (JEM-1400, JEOL Inc., MA, USA). A solution of SWCNT without antibody functionalization was used as a control.

4.2.5 Electrochemical measurements

Potentiometric analysis was performed by real-time measurements of the electromotive force (EMF) between a two electrode system (Koryta, Dvorak, & Kavan, 1993). The two electrode system was based on the functionalized CNT-GCE (carbon nanotube biosensor) as the working electrode and a double-junction Ag/AgCl/KCl electrode as the reference electrode (Fig. 4.3).

Fig. 4.3 Electrochemical measurement scheme with potentiometric biosensor.
Potentiometric measurements were performed in a 5 mL buffer solution, which consisted in a low ionic strength Phosphate Buffer Saline (PBS) 1.7 mM, pH 7.4 at room temperature (23.0 ± 0.5 °C) under continuous stirring (300 rpm). Measurements were taken using a high-input impedance voltmeter (VirtualBench Digital Multimeter, National Instruments, TX, USA), and sample was added to the electrochemical cell with stepwise increase in concentrations from $10^1$-10$^8$ CFU/mL. The EMF values were recorded in real-time using LabView™ software (LabView™ 2014, Version 14.0.1f3, National Instruments, TX, USA). After use, the biosensor was regenerated by soaking the electrode in a 2 M NaCl solution for 30 min, and then conditioning with PBS to take new measurements. The electrode was stored in PBS 1.7 mM pH 7.4 at 4 °C when not in use.

4.2.6 Bacteria preparation

*Listeria innocua* strain B-33016 was obtained from the ARS Culture (NRRL) Collection (Peoria, IL, USA), (this strain was used since is a non-pathogenic strain and can be used as model for pathogenic bacteria *Listeria monocytogenes*). One milliliter of the strain was placed in 9 mL of PALCAM enrichment broth with PALCAM selective supplement and incubated at 37 °C for 24 h. Then, a fresh inoculation was done by transferring 1 mL of the culture into 9 mL of broth and incubated at 37 °C for another 24 h. The bacteria concentration was ~10$^8$ CFU/mL determined by standard count plate method in PALCAM agar plates. Following incubation, the bacteria was centrifuged at 7840 rpm for 15 min and broth was discarded. Then, bacteria was washed by centrifuging again and suspended in sterilized PBS 1.7 mM pH 7.4. Serial dilutions of the bacteria were made by placing 1 mL of the culture into 9 mL of PBS to obtain final concentrations ranging
from $10^1$-$10^8$ CFU/mL. Standard plate count technique was used to enumerate the concentrations of bacteria.

### 4.2.7 Characterization of the biosensor’s selectivity

The biosensor was characterized for selectivity by testing other bacteria in order to determine its capability of detecting only the bacteria of interest (L. innocua in our case). For that purpose, 2 strains of bacteria obtained from the ARS Culture (NRRL) Collection (Peoria, IL, USA) including *Lactobacillus plantarum* (Gram positive, NRRL-B4496), *Lactobacillus acidophilus* (Gram positive, NRRL-B4495) and 1 strain obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA) generic (non-pathogenic) *Escherichia coli* (Gram negative, ATTC 25922) were cultured by the following methods: For *L. plantarum* and *L. acidophilus*, 1 mL of the strain was placed in 9 mL of MRS broth and incubated at 37 °C for 24 h. Then, a fresh inoculation was done by transferring 10 mL of the culture into 15 mL of MRS broth and incubated at 37 °C for another 16 h in order to reach stationary phase. Bacteria was washed following method mentioned above and resuspended in PBS 1.7 mM pH 7.4. Serial dilutions were made in PBS as mentioned above in order to obtain concentrations from $10^1$-$10^8$ CFU/mL. Standard plate count technique was used to enumerate the concentrations of bacteria using MRS agar plates. For *E. coli*, 1 mL of the strain was cultured in 9 mL of Brain Hearth Infusion (BHI) broth, and incubated at 37 °C for 24 h. Bacteria was washed following method before mentioned and resuspended in PBS 1.7 mM pH 7.4. Serial dilutions were made in PBS as mentioned above in order to obtain concentrations from $10^1$-$10^8$ CFU/mL. For this experiment, the bacteria samples were added to the electrochemical cell with stepwise increase in concentrations from $10^1$-$10^8$ CFU/mL, and the EMF values were recorded in real-time using LabView™ software as mentioned above. The biosensor was
regenerated by soaking the electrode in a 2 M NaCl solution for 30 min, and then conditioning with PBS between measurements.

4.2.8 Testing of biosensor with food samples

For this study, the potentiometric biosensor was prepared as mentioned above (see preparation of biosensor). *Listeria innocua* B-33016 obtained from the ARS Culture (NRRL) Collection (Peoria, IL, USA) was cultivated under the same incubation time and temperature conditions mentioned before. Bacteria was resuspended in PBS 1.7 mM pH 7.4 and serial dilutions were made in PBS in order to obtain concentrations from $10^1$-$10^7$ CFU/mL. Fresh meat and whole milk samples were obtained from a local store. For inoculation of the meat samples, meat was cut into pieces of 5 x 5 cm for length and width aseptically under a laminar flow hood (Contamination Control 1140, Contamination Control Inc., PA, USA). The meat surfaces were inoculated with 0.5 mL of the different concentrations of *L. innocua* using a sterile glass spreader. After inoculation, samples were left undisturbed for 15 min at 25 °C under the hood (Cutter, 1999). Then, a cellulose membrane (AP1004700, Merck Millipore Ltd., Billerica, MA, USA) was used to sample the surface of the meat. Prior to sampling, the membrane was moistened with 1.5 mL of sterile water. The sample collection membrane was applied to the meat surface for 5 min. Following sampling, the cellulose membranes were placed into stomacher bags (Whirl-Pak® 165, 118 mL, Nasco Corp., Fort Atkinson, WI, USA) containing 8.13 mL of PBS. Then, 1 mL of sample was introduced into a concentrator device in order to remove the food matrix and concentrate the bacteria prior to detection. The concentration process was repeated by collecting the sample from the output port of the concentrator and resuspending it in 1 mL of PBS. Then the sample was run again in the concentrator until a clean concentrated sample of the bacteria was obtained.
A similar procedure was used for the milk samples. In this case, milk samples of 9 mL were placed into sterile stomacher bags and 1 mL of *L. innocua* at the different concentrations mentioned previously were mixed with the milk samples for inoculation. Samples were then homogenized for 2 min using a stomacher homogenizer (Stomacher® 80 Lab System, Seward Laboratory Systems Inc., FL, USA) and left for 15 min under a laminar flow hood. Then 1 mL of the inoculated milk sample was introduced into the sample concentrator device and the process was repeated until removing the food matrix and obtaining a clean sample for detection with the biosensor. Food samples that did not contain any microorganism were used as control. All samples were analyzed for bacteria enumeration using a standard plate count technique. For the measurements, samples were added to the electrochemical cell containing 5 mL of low ionic strength PBS (1.7 mM, pH 7.4), under stirring at 300 rpm and at room temperature (23.0 ± 0.5 °C). The EMF values were recorded when adding the samples in stepwise concentrations of the inoculated bacteria using LabView™ software as mentioned before. The biosensor was regenerated by soaking the electrode in a 2 M NaCl solution for 30 min, and then conditioning with PBS between measurements with the different food samples. A scheme of the sampling, concentration, and detection process is shown in Fig. 4.4.
Fig. 4.4 Process for detection of bacteria from food samples using the biosensor. A) Steps for detection from meat sample; 1) inoculation of bacteria on meat surface, 2) sampling from meat surface using cellulose membrane; 3) recovery, separation and concentration of collected bacteria prior to detection using concentrator device, 4) detection of bacteria with potentiometric biosensor. B) Steps for detection of milk sample; 1) inoculation of milk sample with bacteria, 2) separation and concentration of bacteria, 3) detection with potentiometric biosensor.

4.2.9 Statistical analysis
The statistical significance of differences observed among sample means was evaluated by analysis of variance (ANOVA) (SAS Version 9.2, SAS Institute Inc., Cary, NC, USA), followed by post hoc Tukey’s studentized range test.

4.3 Results and discussion

4.3.1 Characterization of oxidation of SWCNTs by FTIR

The FTIR spectra for the raw (untreated) SWCNTs and the oxidized SWCNTs (acid treated) is shown in Fig. 4.5 with the raw and treated sample data in the lower and upper spectrum respectively.

![FTIR spectra of raw (untreated) and functionalized (acid treated) SWCNTs.](image)

Fig. 4.5 FTIR spectra of raw (untreated) and functionalized (acid treated) SWCNTs.
From the spectrum of the raw sample, an absorption band was observed at 3405 cm\(^{-1}\). This band is assigned to vibration of \(-\text{OH}\) groups, indicating that the raw material also had some functional groups present. The bands at 2850 cm\(^{-1}\) and 2920 cm\(^{-1}\) correspond to C-H vibration from the carbon nanotube synthesis. Multiple bands around \(~1530\) cm\(^{-1}\) and \(~1710\) cm\(^{-1}\) are assigned to C=O stretching vibration (Ciobotaru et al., 2013). In the spectrum of the treated SWCNTs (acid treatment), it can be observed that most of the bands were enhanced, especially the absorption bands appearing at 1580 cm\(^{-1}\) and 1721 cm\(^{-1}\), that are related to C=O carboxylic group vibrations. This indicated that the SWCNTs were successfully oxidized by the acid treatment, which increased the functionalization of the CNTs. According to Barros et al. (2005), these functional groups usually get attached along the nanotube wall and at the ends of the nanotubes due to their high surface area. From our results it was also observed that the acid treated SWCNTs showed a spectrum with fewer peaks compared to the untreated SWCNTs, which indicated that the acid treatment purified the sample by removing impurities from the synthesis of the CNTs. Previous studies have shown that acid treatments can be used to purify and oxidize carbon nanotubes (Dillon et al., 1999; Li et al., 2004; Shao et al., 2007). Li et al. (2004) studied different purification methods using pure and mixed acids such as 6 M HNO\(_3\), 3:1 H\(_2\)SO\(_4\)/ HNO\(_3\), and also incorporating sonication and air oxidation procedures. They found that nitric acid pre-sonication and refluxing in the mixture of acids were the best methods of purification, reaching purities of 98% and also obtaining a peak at 1630 cm\(^{-1}\) which is also assigned to C=O vibration from COOH groups. In our case, the use of the pure acids mix of 3:1 H\(_2\)SO\(_4\)/ HNO\(_3\) was tried, however no sample was recovered, indicating that the pure acids mix melted or diluted completely the SWCNTs. We observed that the best treatment for oxidation was the use of pure HNO\(_3\) for 4 h, this optimum treatment was obtained in our laboratory.
4.3.2 Characterization of SWCNTs modified electrode by Scanning Electron Microscopy (SEM)

Fig. 4.6 shows the SEM micrographs of the SWCNTs modified glassy carbon electrode (GCE).

![SEM micrographs of the SWCNTs modified electrode](image)

Fig. 4.6 Scanning electron micrographs of the SWCNTs modified electrode. A) Micrograph of electrode’s surface (bar = 100 µm, 150X). B) Close-up of the deposited layer of SWCNTs on the GCE (bar = 5 µm, 3000X).

The micrographs revealed a uniform layer of the SWCNTs on the tip of the electrode (Fig. 4.6A) indicating that the deposition of the CNTs was successful. The active area of the modified SWCNT-GCE was 0.071 cm², calculated using the diameter of the electrode (Ø = 3 mm). In a close-up image of the sample it can be observed that the layer consisted of agglomerated SWCNTs with some void spaces along the surface (Fig. 4.6B). This can be attributed to the sample being dried as a required step for imaging with SEM. Also, SWCNTs were observed between the spaces in the CNTs layers. The modified SWCNT-GCE is intended to be used in liquid samples, which can help to de-agglomerate the CNTs and therefore would have a good exposure of the CNTs with the samples to be analyzed. The deposition method (drop casting method) of the CNTs also played
a major role in the structure of the layer. In drop casting, as mentioned before, the sample is deposited directly on the surface and dried in an oven. Therefore the CNTs can adhere to the surface by forming a self-assembly of layers of on top of each other. The deposited layers of CNTs had a thickness of 55.81 ± 1.83 µm as measured by using a profilometer.

### 4.3.3 SWCNT-antibody functionalization characterization by TEM

The antibodies were attached on the –COOH groups of the SWCNTs by EDC/Sulfo-NHS chemistry in a two-step process. First, the SWCNT-COOH were mixed with EDC/Sulfo-NHS solution in order to obtain a semi stable amine NHS ester attached to the –COOH groups. Then, the activated SWCNTs were incubated with the solution of anti-*Listeria* antibodies for 5 h to optimize the conjugation efficiency. The antibody functionalization was characterized by TEM by placing 5 µL of solution on a gold coated carbon TEM grid. Fig. 4.7A presents a close-up image of the SWCNT-antibody conjugate, showing that the antibodies were successfully attached at the surface of the SWCNTs.

In addition, the immobilization of the antibodies occurred at different sections of the SWCNTs since the carboxylic groups were located randomly in the CNTs surfaces as shown in Fig. 4.7B-C. Some aggregates of conjugated antibodies that resulted from the reaction of the EDC/Sulfo-NHS chemistry can also be seen in Fig. 4.7D. These aggregates could have had an effect by limiting the complete attachment of some antibodies to the SWCNTs. Similar results were observed by Abera (2010), in which the aggregates of the conjugated antibodies were attributed to the CNTs being folded during the conjugation process.
Fig. 4.7 TEM images showing the antibody functionalization of the SWCNTs. A) Close-up image showing the antibody conjugated SWCNTs. B-C) Images showing random attachment of antibodies to the walls of SWCNTS. D) Aggregates of conjugated antibodies in the suspension. Arrows indicate the antibodies in the figures.

Fig. 4.8 is an image of unconjugated SWCNTs (control). It can be observed that the SWCNTs are well dispersed in the solution. However, some short CNTs were observed in the solution, which could be due to shortening during the oxidation and sonication processes that the CNTs were subjected prior to functionalization with antibodies. Fig. 4.8b shows a larger and a shorter SWCNT
for comparison. The white snow-like spots observed in the close-up image in Fig. 4.8b are due to the presence of the surfactant (SDS) in the solution.
Fig. 4.8 TEM images showing unconjugated SWCNTs (control). (a) Suspended SWCNTs showing the presence of shorter and larger sizes of CNTs. (b) Close-up image of unconjugated SWCNTs showing the presence of the surfactant in the solution.

4.3.4 Detection of bacteria using the carbon nanotube based potentiometric biosensor

The developed carbon nanotube potentiometric biosensor was tested against concentrations of suspended bacteria (*L. innocua* B-33016) in PBS (1.7 mM, pH = 7.4). For this study, the different concentrations of bacteria were added to the electrochemical cell in a step-wise mode in order to record the changes in the electromotive force (EMF) and evaluate the response of the biosensor. The purpose of this study included the development a calibration curve (standard curve) for concentrations of pure bacteria vs EMF values of the biosensor. The biosensor’s response was evaluated with *L. innocua* concentrations from $10^1 – 10^8$ CFU/mL, with pure PBS as a base control (0 CFU/mL). Results for EMF values against concentrations of bacteria are presented in Fig. 4.9.

\[
y = -0.8708x + 149.03
\]

\[
R^2 = 0.9717
\]
Fig. 4.9 Standard curve for EMF values vs concentrations of *Listeria innocua*. EMF = electromotive force.

The initial EMF values of the biosensor were in the range of 150 – 160 mV, which corresponded to the measurements of the pure PBS before additions of bacteria. As shown in Fig. 4.9, a linear response was observed to the concentrations of the *L. innocua*, with a sensitivity (slope) of -0.8708 ± 0.25 mV per decade, and a regression coefficient of 0.97. A limit of detection of 11 CFU/mL was determined according to the final concentrations of bacteria in the electrochemical cell (Table 4.1), calculated using the initial volume of PBS (5 mL). Table 4.1 also shows a correction for the measured EMF values of each of the concentrations. The correction was done by subtraction of the EMF of the PBS solution from the EMF of each of the bacteria concentrations.

Table 4.1 EMF values for final concentrations* of *L. innocua*

<table>
<thead>
<tr>
<th>CFU/mL</th>
<th>EMF (mV)</th>
<th>Corrected EMF (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>149.72 ± 0.59a</td>
<td>0.00</td>
</tr>
<tr>
<td>1.10E+01</td>
<td>147.85 ± 0.14b</td>
<td>1.87</td>
</tr>
<tr>
<td>1.19E+02</td>
<td>147.26 ± 0.08b</td>
<td>2.46</td>
</tr>
<tr>
<td>1.91E+03</td>
<td>146.45 ± 0.55bc</td>
<td>3.27</td>
</tr>
<tr>
<td>1.00E+04</td>
<td>145.23 ± 0.90cd</td>
<td>4.49</td>
</tr>
<tr>
<td>1.64E+05</td>
<td>144.08 ± 0.12de</td>
<td>5.63</td>
</tr>
<tr>
<td>1.49E+06</td>
<td>143.73 ± 0.36de</td>
<td>5.99</td>
</tr>
<tr>
<td>1.34E+07</td>
<td>143.08 ± 0.26c</td>
<td>6.64</td>
</tr>
<tr>
<td>1.36E+08</td>
<td>142.58 ± 0.56e</td>
<td>7.13</td>
</tr>
</tbody>
</table>

CFU = Colony forming units, EMF = electromotive force. Data expressed as mean ± standard deviation of three determinations. *Means with different letters are significantly different (p<0.05). **Final concentrations calculated by stepwise additions of 0.5 mL of *L. innocua* concentrations from 10^1-10^8 CFU/mL to an initial volume of 5 mL PBS solution.
Fig. 4.10 presents the biosensor response versus time during the stepwise additions of the concentrations of bacteria. The biosensor’s response stability was tested by adding bacteria at every 5 min interval in order to see the biosensors response to the bacteria addition and to determine if the readings would be constant during that interval. As seen in Fig 4.10, after a rapid response to the addition of bacteria, the biosensor response remained stable during the 5 min intervals with drifts only at every inoculation of bacteria. In Fig. 4.10 it can be observed that the biosensor detected the bacteria immediately after each addition, with a response time of < 10 s, which indicates that the biorecognition element (antibodies) of the biosensor could recognize the analyte of interest and send the signal to the transduction part of the sensor.

The proposed mechanism of detection of the biosensor is explained by the formation of a double layer capacitor in the tip of the CGE. A double layer capacitor is formed by an increasing positive charge on the solution side of the electrode interface which forces accumulation of negative charges on the electrode surface. Therefore, the detection mechanism of the biosensor would be based on the accumulation of positively charged hydrogen ions present in the cell wall of Gram positive bacteria (L. innocua in our case) when they interact with the anti-Listeria antibodies (recognition element). These hydrogen ions then force accumulation of negative charge on the electrode surface, resulting in the formation of a double layer capacitor. As seen in Fig. 4.9, the biosensor response had a negative slope, with a decrease in potential as the bacteria concentration increased. This can be attributed to the formation of the double layer capacitor, since the higher the concentration of the analyte (having positive charges), the more negative the electrode side becomes, therefore, the decrease in potential is influenced by the concentration of positive charges being increased (Adenuga, 2013).
This phenomena can be explained by the equation of capacitance (Eq. 4.1) (Busch-Vishniac, 1998):

\[ C = \frac{q}{V} \quad (4.1) \]

Where \( q \) is the amount of charges (positive and negative) in each side of the capacitor, and \( V \) is the voltage. Capacitance is defined as the ability of a body to store electrical charge (Sadiku & Alexander, 2012). The capacitance is inversely proportional to the voltage. In other words, the higher the amount of charges (q) the higher the increase in capacitance (energy storage), therefore less voltage is measured in the system. Our results for are in agreement with the results presented by Adenuga (2013). In this study, a potentiometric biosensor was developed for the detection of positively charged thrombin (proteins) molecules. A linear response was observed for log thrombin concentrations from \( 5 \times 10^{-10} \) M to \( 7 \times 10^{-8} \) M with a sensitivity of \(-31.01 \pm 1.41 \) mV, suggesting that the biosensor had a decrease in potential with an increase of thrombin concentrations.
Fig. 4.10 Biosensor real-time response when subjected to stepwise increases of concentrations of *L. innocua*. 
4.3.5 Selectivity of the biosensor

The selectivity of a biosensor is an important parameter to analyze since it demonstrates the ability of the sensor to respond to the target analyte and differentiate from other like analytes. The selectivity was measured by subjecting the developed biosensor to other Gram positive bacteria such as *Lactobacillus plantarum* and *Lactobacillus acidophilus*, and a Gram negative bacteria such as generic *E. coli* (non-pathogenic), in concentrations ranging from $10^1$-$10^8$ CFU/mL. Results from the biosensor’s response to other bacteria are presented in Fig. 4.11. As shown in Fig. 4.11, the biosensor is highly selective since no significant change in signal was observed when subjected to either Gram positive bacteria *L. plantarum* and *L. acidophilus*, or Gram negative bacteria *E. coli*. For this test, the different bacteria concentrations were added to the electrochemical cell in a stepwise mode in each experiment run, and a stable signal response was observed during the 5 min of running for every concentration. With these results it could be confirmed that the biosensor’s response is caused by the binding of the specific analyte (*L. innocua*) with the antibodies, and the resulting transduction from the SWCNTs in the working electrode.
Fig. 4.11 Biosensor response vs time for different concentrations of bacteria (CFU/mL) for selectivity test. A) EMF response for biosensor tested with *Lactobacillus plantarum*; B) EMF response for biosensor tested with *Lactobacillus acidophilus*; C) EMF response for biosensor tested with generic *E. coli* (non-pathogenic). EMF = electromotive force.
4.3.6 Testing of biosensor with food samples

The purpose of this study was to determine the biosensor’s capability of detecting bacteria from inoculated food samples including meat and milk. Prior to detection, the food samples were inoculated with *L. innocua* at concentrations from $10^1$-$10^8$ CFU/mL, with no inoculated food product as a control (0 CFU/mL). As mentioned before, the collected bacteria from the food samples was placed in a sample concentrator device prior to detection in order to obtain a cleaner sample by removing possible interfering compounds in the food matrices, and the final samples were resuspended in PBS (1.7 mM, pH 7.4) for analysis. Results for the biosensor response to samples from inoculated meat are presented in Fig. 4.12.

![Graph showing biosensor response with meat samples EMF values versus concentrations of inoculated *L. innocua* in meat. EMF = electromotive force.]

**y** = -1.0291x + 151.34  
**R²** = 0.9934
It was observed that the sensor had a quick response evidenced by a change of signal after addition of the samples containing different concentrations of bacteria. As seen in Fig. 4.12, a similar trend was observed as with the results with *L. innocua*, with a linear response ($R^2 = 0.99$) for the biosensor’s signal versus concentrations of bacteria, and a slope of $-1.03 \pm 0.01$ mV per decade. A limit of detection of 25 CFU/g was obtained, which was based on the final concentration of *L. innocua* in the electrochemical cell (Table 4.2).

Table 4.2 EMF values for final concentrations* of *L. innocua* from meat samples

<table>
<thead>
<tr>
<th>CFU/g</th>
<th>EMF (mV)</th>
<th>Corrected EMF (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>151.29 ± 0.20$^a$</td>
<td>0.00</td>
</tr>
<tr>
<td>2.50E+01</td>
<td>150.25 ± 0.26$^{ab}$</td>
<td>1.04</td>
</tr>
<tr>
<td>2.24E+02</td>
<td>149.14 ± 0.17$^{ab}$</td>
<td>2.15</td>
</tr>
<tr>
<td>1.91E+03</td>
<td>148.49 ± 0.54$^{ab}$</td>
<td>2.79</td>
</tr>
<tr>
<td>1.66E+04</td>
<td>147.67 ± 0.47$^{ab}$</td>
<td>3.61</td>
</tr>
<tr>
<td>1.64E+05</td>
<td>145.92 ± 1.86$^{ab}$</td>
<td>5.36</td>
</tr>
<tr>
<td>1.49E+06</td>
<td>144.96 ± 2.37$^{ab}$</td>
<td>6.33</td>
</tr>
<tr>
<td>1.34E+07</td>
<td>144.06 ± 2.84$^{b}$</td>
<td>7.23</td>
</tr>
<tr>
<td>1.36E+08</td>
<td>143.23 ± 3.32$^{b}$</td>
<td>8.06</td>
</tr>
</tbody>
</table>

CFU = Colony forming units, EMF = electromotive force. Data expressed as mean ± standard deviation of three determinations. $^{ab}$ Means with different letters are significantly different (p<0.05). *Final concentrations calculated by stepwise additions of 0.5 mL of *L. innocua* concentrations from $10^1$-$10^8$ CFU/mL to an initial volume of 5 mL PBS solution.

Fig. 4.13 shows the biosensor response for milk samples. Similar pre-processing steps were carried out as done with the meat samples. The inoculated milk samples were concentrated and the final sample was resuspended in PBS prior to detection.
Fig. 4.13 Biosensor response for milk samples. EMF values versus concentrations of inoculated *L. innocua* in milk. EMF = electromotive force.

Similarly with results from the pure bacteria and the meat samples, the biosensor response was linear with a decrease in the EMF as the different concentrations of inoculated bacteria increased. A slope of $-0.73 \pm 0.08$ mV per decade was observed in the biosensor signal, which was similar to the slope of the pure bacteria and that obtained from the testing of meat samples. A detection limit of 27 CFU/mL was observed (Table 4.3), which was also similar to that obtained from the meat samples. The total calculated time for detection of the food samples with the biosensor was approximately 35 min, with 5 min required for sampling and 30 min for concentration in the concentrator device. The bacteria can be detected in the biosensor in close to real-time with a response in seconds (<10 s). In general, the total analysis time for food samples with the biosensor is lower than the time of analysis with other standard methods. For example, the time for analyzing a sample with PCR is usually 24-48 h due to the need of amplification of the sample (Rossmanith, Krassnig, Wagner, & Hein, 2006), while the standard colony count method can take from 5-14
days since it needs enrichment and incubation (Harbeck, Teague, Crossen, Maul, & Childers, 1993; Villari, Motti, Farullo, & Torre, 1998).

Table 4.3 EMF values for final concentrations* of *L. innocua* from milk samples

<table>
<thead>
<tr>
<th>CFU/mL</th>
<th>EMF (mV)</th>
<th>Corrected EMF (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>151.67a</td>
<td>0.00</td>
</tr>
<tr>
<td>2.70E+01</td>
<td>151.00ab</td>
<td>0.67</td>
</tr>
<tr>
<td>1.78E+02</td>
<td>150.50ab</td>
<td>1.17</td>
</tr>
<tr>
<td>1.89E+03</td>
<td>149.96abc</td>
<td>1.71</td>
</tr>
<tr>
<td>1.31E+04</td>
<td>149.22bcd</td>
<td>2.45</td>
</tr>
<tr>
<td>1.77E+05</td>
<td>148.41cde</td>
<td>3.26</td>
</tr>
<tr>
<td>1.49E+06</td>
<td>147.52def</td>
<td>4.14</td>
</tr>
<tr>
<td>1.23E+07</td>
<td>146.72ef</td>
<td>4.95</td>
</tr>
<tr>
<td>1.16E+08</td>
<td>145.80f</td>
<td>5.86</td>
</tr>
</tbody>
</table>

CFU = Colony forming units, EMF = electromotive force. Data expressed as mean ± standard deviation of three determinations. a-f Means with different letters are significantly different (p<0.05). *Final concentrations calculated by stepwise additions of 0.5 mL of *L. innocua* concentrations from $10^1$-$10^8$ CFU/mL to an initial volume of 5 mL PBS solution.

The low limits of detection observed for the meat and milk samples would allow for determination of compliance with established regulations. According to the Commission Regulation (EC) of the European Communities on microbiological criteria for food stuff, an upper microbiological limit of 100 CFU/g is established for *Listeria monocytogenes* in ready-to-eat (RTE) food products (Communities, 2005). The biosensor could be used in the field with a portable voltmeter since the EMF values measured in our results can be measured by these portable devices. A picture of the biosensor is shown in Fig. 4.14.
4.3.7 Design and fabrication of biosensor system

The biosensor system employed three different steps for the detection of the bacteria from food samples which included: 1) sampling, 2) separation and concentration, and 3) detection. The biosensor system combined all three parts in a lab-on-a-chip scheme. The device consisted of (1) a top card; which contained an adaptor for a syringe in the sample input area, a serpentine channel for concentration of the sample, and the output area with space for introducing the working electrode and reference electrode of the biosensor, (2) a nylon membrane (negatively charged, hydrophilic, pore size 0.43 µm) (Nylon 6, 6, Pall Corp., Port Washington, NY, USA), (3) a support card, (4) a hydrogel film, and (5) a bottom card; which consists on an input area for placing the sampling membrane, the sample concentration area, and the output area with a reservoir for the detection of the sample with the biosensor. A design of the proposed biosensor system is presented in Fig. 4.15.
The diameter of the input area of the system is 47 mm, which is also the size of the sampling membrane. The serpentine channel dimensions are 42 mm length, 0.8 mm wide, and 0.5 mm depth. The diameter of the output circles are 12 mm and 4 mm which correspond to the reference and working electrodes respectively. The top, bottom, and support cards were designed in AutoCAD® software (AutoCAD® 2016, Autodesk, San Rafael, CA, USA) and patterned in Poly-(methyl methacrylate) using a computer numeric control (CNC) milling machine (KERN MMP 451, KERN Micro & Feinwektechnik GmbH & Co. KG, Germany). The layers were aligned and sealed by applying pressure to the assembly. The fabrication of smaller reference electrodes (current commercial reference electrodes are 15-16 cm in length) would allow further miniaturization of the biosensor system.

4.4 Conclusion

The development and characterization of a carbon nanotube-based potentiometric biosensor was discussed in this chapter. It was shown that the biosensor could successfully detect the bacteria of interest (L. innocua) with high selectivity for specific bacteria. A calibration curve with standard concentrations of L. innocua was developed and a linear response was obtained with a lower limit of detection of 11 CFU/mL. The biosensor was able to detect food samples such as meat and milk with limits of detection of 25 CFU/g and 27 CFU/mL in meat and milk respectively. Finally, a biosensor system capable of performing sampling, concentration, and detection steps was proposed in order to follow a lab-on-a-chip scheme. In general, this study demonstrated that the biosensor
could be successfully constructed and the detection of *L. innocua* was achieved with high selectivity and a low limit of detection.
4.5 References


CHAPTER 5

GENERAL CONCLUSIONS
The purpose of this dissertation was to develop a biosensor system for detection of bacteria in food. A sample collection and interface protocol using a cellulose membrane was evaluated by sampling the surface of meat pieces previously inoculated with different concentrations of *L. innocua* (10\(^1\)-10\(^5\) CFU/mL) and the sampling time was optimized. Results showed that 5 and 10 min were the best sampling times with recoveries of >80% for some of the bacteria concentrations. The second study demonstrated the development and characterization of a bacteria concentrator device based on volume reduction by hydrogels. The results for recovery efficiency and bacterial cell counts showed that bacteria was concentrated in 30 min and without significant loss. The last study showed the development and characterization of a carbon nanotube-based biosensor for detection of *L. innocua* from food samples such as meat and milk. The results showed that the biosensor was able to detect *L. innocua* with high selectivity and a lower limit of detection of 11 CFU/mL. Additionally, the biosensor was performed for analysis of previously inoculated *L. innocua* in meat and milk samples and results demonstrated that the biosensors could detect *L. innocua* with lower limit of detection of 25 CFU/g and 27 CFU/mL, respectively. Furthermore, a biosensor system able to perform sampling, concentration, and bacteria detection was proposed, this biosensor system could be used for on-site detection of bacteria.
VITA

Luis Alonso Alfaro Sanabria was born in April 1985 in Tegucigalpa, Honduras. He earned his Bachelor of Science in Food Technology in Zamorano University, Honduras, in December 2007. After working for one year, he joined the Department of Food Science at Louisiana State University in May 2009 as intern in the food safety and food engineering areas. Luis got his Master of Science in Food Science in December 2012 and started his Ph.D. in Engineering Science in January 2013. Luis will receive his Ph.D. degree in December 2016.