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PhD in Mountain Environment and Agriculture (xIXth cycle)

PhD Dissertation

DEVELOPMENT OF NANOFIBROUS MEMBRANES FOR ENZYMATIC FOOD PROCESSING

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**ABSTRACT**

The main object of this work is to prepare nanofibrous membranes (NFM) for the development of novel Enzymatic Membrane Reactors (EMRs) to be used in food processing. EMRs are systems where a biochemical conversion occurs inside a compact and flexible membrane. Such membranes are of special interest in food processing as they can be used for a number of applications in food industries such as: production of electrochemical sensor for the detection of glucose in energy drink and during the beer processing and hydrolysis of sucrose in glucose and fructose for taste control and for the production of inverted sugar. However, conventional membranes reactors suffer from a number of drawbacks such as low enzyme loading, limited stability, loss of the biocatitytic agent, just to cite a few. These problems preclude their use in food processing. Thus, our working hypothesis is improve the performance of traditional membrane reactors thanks to the use of nanofibrous membranes with higher surface to volume ratio and higher surface availability.

The second part show other different applications of the nanofibrous membrane (NFM) in the food processing. Nanofibrous membranes, from Nylon 6, as used as a filter for bacteria and yeast suspension in water and beer. The results revealed that membranes are able to reduce the number of microorganisms in liquid food. The same NFM was also used for the adsorption of the Volatile Organic Compounds (VOCs) coming from coffee powder of six different geographical origin. The aroma of coffee in powder and after the adsorption on Nylon 6 NFM was used for the differential of the geographical origin of coffee. The last part of this work shows the successfully encapsulation and extension of the shelf-life of β-carotene when this bioactive compound was incorporated in PolyEthylene Oxide (PEO) nanofiber membrane.
RIASSUNTO

L’obiettivo principale di questo lavoro di tesi è quello di preparare delle membrane nanostrutturate costituite da nanofibre (NFM) che possano fare da supporto per sviluppare dei nuovi reattori di membrana enzimatici (EMRs) che saranno utilizzati nei processi alimentari. EMRs, sono dei sistemi dove la reazione biochimica, catalizzata da enzimi, avviene all’interno di una membrana compatta e flessibile. Queste membrane, sono di particolare interesse per l’industria alimentare perché esistono diversi utilizzi degli enzimi nei processi alimentari come: la produzione di sensori elettrochimici per la misura del glucosio contenuto in bevande energetiche e durante il processo di produzione di birra e l’idrolisi del zucchero in glucosio e fruttosio per controllare il gusto delle bevande e per la produzione di zucchero invertito. Comunque, i tradizionali reattori di membrane di solito hanno un sacco di svantaggi come una ridotta capacità di assorbire gli enzimi, una limitata stabilità, perdita dell’enzima, solo per citarne alcuni. Questi problemi precludono il loro uso nei processi alimentari. Per questo, la nostra ipotesi è quella di aumentare le performance dei tradizionali reattori di membrane grazie all’uso di membrane nanostrutturate costituite da nanofibre con un maggiore rapporto superficie volume e una maggiore superficie disponibile per l’immobilizzazione degli enzimi.

Nella seconda parte di questa tesi mostrano delle altre interessanti applicazioni delle membrane nanostrutturate costituite da nanofibre (NFM) nei processi alimentari. Queste membrane nanostrutturate, di Nylon 6, sono state utilizzate per filtrare batteri lieviti contenuti in acque e birra. I risultati ottenuti mostrano, che queste membrane grazie alla minima dimensione dei pori, sono capaci di ridurre il numero di microrganismi contenuti in bevande alimentari. Le stesse NFM dello stesso polimero, sono state utilizzate per adsorbire i composti organici volatili (VOCs) rilasciati da caffè in polvere provenienti da 6 differenti origini geografiche. Questi VOCs caratteristici, vengono utilizzati per differenziare questi caffè in base alla zona geografica di provenienza. L’ultima parte della tesi mostra come le membrane nanostrutturate di Ossido di PoliEtilene (PEO), un polimero non tossico che ha numerosi utilizzi in biologia e farmacologia, è stato utilizzato per incapsulare e aumentare la shelf-life del β-carotene.
ZUSAMMENFASSUNG


Chapter One

1. INTRODUCTION

Definition. Nanotechnology is an interdisciplinary field of research aimed at manipulating the matter at the atomic and molecular scale. Its first description was given by the American physicist Richard Feynman at an American Physical Society meeting at Caltech on December 29, 1959. During his lecture “There’s Plenty of Room at the Bottom”, where he described a process which could manipulate individual atoms and molecules (Feynman 1959). Later, the term “nanotechnology” was used again by Nario Taniguchi, a Japanese scientist, who defined it as follows: “Nanotechnology mainly consists of the processing of separation, consolidation and determination of materials by one atom or one molecule.” and put it into practice with the preparation of a novel semiconductor processes based on thin film deposition and ion beam milling. However, it was only until 1991 that, K. Eric Drexler concluded the first doctoral degree on the topic of molecular nanotechnology, in his thesis "Molecular Machinery and Manufacturing with Applications to Computation”.(Drexler 1991)

Scanning tunneling microscope. However, the real driver that determined the dramatic growth of nanotechnology came up in the early 1980s with the invention of the Scanning Tunneling Microscope (STM), an instrument for imaging surfaces at the atomic level that was developed at IBM Zurich Research Laboratory, and for which Gerd Binnig and Heinrich Rohrer received the Nobel Prize in Physics in 1986. (Ruska 1987)

The ability to observe the matter at the nanoscale level allowed, about ten years later, the development of nanostructures, such as the gold nanoparticles of Richard A. Zsigmondy or the fullerenes of Harry Kroto, Richard Smalley, and Robert Curl. (Curl 1996)

Early applications. After further 10 years, during the 2000s, research in nanotechnology led to the manufacture of commercial products. Relevant examples include titanium dioxide and zinc oxide nanoparticles in sunscreen, cosmetics and some food products (Lu et al. 2015; Weir et al. 2012; Smijs & Pavel 2011); silver nanoparticles in food packaging, clothing and disinfectants (Lu et al. 2015; Weir et al. 2012; Smijs & Pavel 2011), carbon nanotubes for stain-resistant textiles (Patra & Gouda 2013); and cerium oxide as a fuel catalyst. (Zhang et al. 2002)
Applications of nanotechnology. Today, nanotechnology has become a mature field of research, with utility in many fields:

1. **Nanomedicine.** The application of nanotechnology in medicine (nanomedicine) includes delivery of drugs to specific cells or tissues through nanoparticles that act as carriers (Wagner et al. 2006; Chalovich & Eisenberg 2005). Nanoelectronic biosensors (Pui et al. 2011; Huang & Chen 2010), lab-on-a-chip devices based on magnetic nanoparticles and antibody for the early sensing of antigens in blood (Holford et al. 2012)(Woolley et al. 1998), magnetic nanoparticles for blood purifications (Ito et al. 2005; Lee et al. 2009), and nanostructured scaffolds for tissue engineering and nanorobots. (Wen 2005; Cavalcanti 2002)

2. **Nanobiotechnology.** This discipline aims at using biological materials having at least one dimension within the nanoscale, such as DNA, RNA, peptides, antibodies or enzymes with nanostructures. Typical applications include nanorobots and nanomachines in medicine, where, for instance, nanorobots are supplied to patients through an injection that will seek for cancerous cells leaving the healthy ones untouched. (Tian et al. 2011)

3. **Green nanotechnology.** Research is underway to use nanomaterials for manufacturing products without harming the environment or human health. These include, for instance, the use of nanomaterials for building super capacitors in solar cells (Chen & Dai 2013), nanoremediation and water treatment (Bhattacharya et al. 2013), water filtration (Carpenter et al. 2015). Additionally, nanoparticles can be functionalized with enzymes for degrading biofilm (Baelo et al. 2015; Chen et al. 2013), water eutrophycation or water pollution. (Zhang et al. 2010)

4. **Agriculture applications of nanotechnology.** Nanotechnology allows the design of novel nanocapsules containing, for instance, herbicides that can effectively penetrate through cuticles and tissues, allowing the slow and constant release of active substances. This in turn would allow the reduction of fertilizer needs and minimize environmental pollution through precision farming. (Srilatha B. 2011)

5. **Carbon nanotubes.** Single Walled Carbon Nanotubes (CNTs) are cylinders with diameters between 0.8 and 2 nm and lengths below 100 nm to 0.5 m. Carbon nanotube production currently exceeds several thousand tons per year. Main applications include
energy storage (Frackowiak 2002), automotive parts (Breuer & Sundararaj 2004), boat hulls and sporting goods (De Volder M.F.L. 2013), water filters (Srivastava A. 2004), thin-film electronic, coatings, actuators and electromagnetic shield. (Avouris et al. 2003)

Food Nanomaterials. Although the food sector is typically very conservative in adapting new technology, nanotechnology has great potential for food applications. Further, nanotechnology is already in use in foods as they are naturally composed of biological matter that is already nanosized. Relevant examples include whey proteins in milk (Dickinson 2011), colloidal suspension in wine (Senkea et al. 1998) or damaged starch in flour (Le Corre et al. 2010). It is already known that the natural presence of nanocolloids, nanoemulsions or nanoparticles in food affect the resulting sensorial properties, such as color, texture or taste (Kumar R. & Lal 2015). Therefore, the food industry is continuously experimenting to find the potential applications of nanotechnology through two of the most traditional approaches, called “bottom-up” and “top-down”.

Bottom up approach. The bottom up approach is based on the self-assembly or self-organization of molecules into a nanomaterial (Balzani et al. 2005). One example can be amylose in starch. Especially alpha-amylase is an enzyme that can randomly break starch polymer into dextrin, a nano-sized oligosaccharide.

A further relevant example is the recrystallization of lipids. Lipids, liposomes or phospholipids can, in certain conditions, self-assemble into nano-sized structures, such as nano-tubes, nano-films or nano-shells (Thompson et al. 2010). This may lead to supra-molecular structures that can be used for encapsulation or bioactive delivery or for controlling the melting behavior of the resulting food. In principle, the self-assembly of lipid bi-layers may be used to develop food fat that is solid at room temperature but that is prepared from oil-in-water emulsions. Overall, this can be of great interest for the development of stable foods with lower fat content. (Wang & Marangoni 2016; Weiss et al. 2006; Calligaris et al. 2010)

Top down approach. The top-down approach is also of great interest as it is based on the reduction of the particle size through the use of grinding or milling technologies. A relevant example of this approach is the manufacture of nano-sized salt. As salt is reduced to the nanoscale, then there is an increased perception of saltiness. In principle, nano-sized salt would allow to prepare foods with less amount of salt. (Kesisoglou et al. 2007)
Limitations of nanotechnology in the food sector. The examples above show how nanotechnology can directly affect food structure and composition. These applications could impact the food industry but, at the moment, are not exploited worldwide due to the lack of legislation relative to their application and uncertainly on the perception of the consumer. Without the development of a legislative framework that clarifies the possible use of nanotechnology in food, the food industry not invest money on such research field due to the high risk of repeating the experience observed with Genetically Modified Organisms (GMO) in the past. (Fisher & Mahajan 2006)

Moreover, the consumers’ attitude towards the presence of nanomaterials in food is conservative. Food is perceived as a product where nanomaterials, even those naturally present, are artificial. Any change towards food based on nanotechnology may be regarded as suspicious. Therefore, any new food based on nanotechnology may become a commercial failure without specific programs that educate the consumer to understand what nanotechnology is, as well as, its potential benefits and its safety. (Amenta et al. 2015)

Nanotechnology for food processing. However, there are also a number of relevant applications where nanotechnology can lead to innovative solutions without affecting food composition. Such applications include, the generation of nanofibers and membranes with very high porous mesh for a number of different uses, such as filtration, volatile adsorption, membrane reactors, fining wine, filtrating beverages or the selective adsorption of bitter or undesired compounds (Ramakhrisna S. 2005). Moreover, the application of nanotechnology to the process would not affect the chemical composition of foods. Therefore, it can be applied by food industry regardless to the lack of the current legislation or to the perception of the consumer.

Electrospinning overview. Nanostructure membranes can be prepared by several methods, one of the most important is electrospinning. This technique is used to produce long nonwoven fibers with diameters within the nanometer scale. Electrospinning was first discovered and patented in 1934. However, it was only until the mid 1990’s that large scale nanofiber were implemented (Persano et al. 2013). The working principle of electrospinning is simple: a high-voltage is applied to a polymer fluid leading to the ejection of a fiber that is collected on a grounded target (Yuan et al. 2017). More precisely, the polymeric fluid is enclosed in a metallic
syringe needle, the fluid jet erupts from the droplet at the tip of the needle because of the large electric potential. The droplet is the result of the formation of a Taylor cone, followed by the ejection of a fiber toward the grounded collector. During the ejection, the fiber is stretched due to the electric forces and its diameter decreases. Finally, during the ejection, the solvent evaporates leading to a dry fiber mat that is continuously collected. (Konwarh et al. 2013)(Greiner & Wendorff 2007)(Ramakhrisna S. 2005)

Overall, electrospinning is a simple procedure that allows the rapid preparation of large amounts of nanostructured materials at low cost. In addition, a number of polymers can be used with electrospinning, from synthetic to natural origin. Relevant examples include nylon-6 (Fatarella et al. 2014a), polyethylene oxide (PEO) (Uyar & Besenbacher 2009), polyvinyl-alcohol (PVA) (Arecchi et al. 2010), cellulose acetate (CA) (Rodríguez et al. 2012) and polylactic acid (PLA) (Gómez-Pachón et al. 2014).

**Electrospinning process.** Figure 1 shows the basic electrospinning set-up. Three main components are required to obtain the desired nanofibers. First, a syringe pump that pushes the polymer solution or melt through a spinneret (a metallic needle) to the tip. Second, a high voltage power supply that gives a charge to the solution in the syringe needle, generating the driving force for the ejection of the fibers. Third, a grounded collector in which the ejected fibers are captured (Barhate & Ramakrishna 2007)
Figure 1. Setup for electrospinning. Adapted from (Bhardwaj & Kundu, 2010)

Effects of Operational Parameters. The electrospinning process can be manipulated by three types of parameters:

1. Solution properties (conductivity/solution charge density, surface tension, viscosity/concentration, polymer molecular weight, dielectric constant and dipole moment).
2. Machine parameters (field strength/voltage, distance between tip and collector, flow rate, needle tip design and placement, collector composition and geometry).
3. Ambient parameters (temperature, humidity and air velocity). (Ramakhrisna S. 2005)

Electrospinning for Biosensors. A biosensor is an analytical device, used for the detection of an analyte, that combines a biological component with a physicochemical detector (Turner et al. 1987). Immobilization of enzymes onto the electrode surface offers additional improvements compared to traditional first-generation biosensors. The advantage is that both enzyme and
mediator can be brought close to the electrode surface, increasing the efficiency of the electron transfer between the enzyme and the electrode. Several methods of immobilization have been tested such as hydrogel encapsulation (Martinkova & Pohanka 2015a; Bard 2003; Gonc et al. 2011), electro-polymerization (Gonçalves et al. 2011), or polymer film trapping (Goornavar et al. 2014; Kumar & Chen 2007; Tran & Balkus 2012; Homaei et al. 2013). These approaches require rigorous control of the positioning of the enzymes to minimize the loss of catalytic efficiency due to inadequate active site orientation. In this respect, recent research has pointed out the good performances of electro-spun nylon-6 nano-fibrous membranes (NFM) decorated with glucose oxidases for bio-sensing of glucose also in presence of interferents, showing good selectivity also in relatively complex matrices (Scampicchio, Arecchi, Bianco, et al. 2010).

**Electrospinning for Enzyme Reactors.** Although nanotechnology has become a mature field of research, there are still many unresolved problems related to the application of nanomaterials in food processing. One of these problems is how to immobilize enzymes onto nanofibrous membranes without losing their specific catalytic activity. (Xu 2009)

In general, the use of enzymes is of particular interest for many food processes. Enzymatic processes can be performed in batch reactors, continuous flow stirred tank reactors, fluidized bed reactors or immobilized bed reactors. Each of these processes are of potential interest for food industry because they can be used for preparing specific ingredients and bioactives in mild conditions of temperature and pressure with high yields. Relevant examples of traditional enzyme reactors are listed in the following table (Giorno & Drioli 2000):

Table 1. Some of the more important industrial uses of immobilised enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>EC number</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacylase</td>
<td>3.5.1.14</td>
<td>L-Amino acids</td>
</tr>
<tr>
<td>Aspartate ammonia-lyase</td>
<td>4.3.1.1</td>
<td>L-Aspartic acid</td>
</tr>
<tr>
<td>Aspartate 4-decarboxylase</td>
<td>4.1.1.12</td>
<td>L-Alanine</td>
</tr>
<tr>
<td>Cyanidase</td>
<td>3.5.5.x</td>
<td>Formic acid (from waste cyanide)</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td>3.2.1.3</td>
<td>D-Glucose</td>
</tr>
<tr>
<td>Glucose isomerase</td>
<td>5.3.1.5</td>
<td>High -fructose corn syrup</td>
</tr>
<tr>
<td>Histidine ammonia-lyase</td>
<td>4.3.1.3</td>
<td>Urocanic acid</td>
</tr>
<tr>
<td>Hydantoinase</td>
<td>3.5.2.2</td>
<td>D- and L-amino acids</td>
</tr>
</tbody>
</table>
The technology behind such reactors, either in batch or continuous, are relatively simple. However, the main limitation is their high cost and enzyme stability. (Giorno & Drioli 2000)

Immobilized enzyme bed reactors are an evolution of the traditional reactor and provide further advantages, such as small reactor volume, low enzyme consumption and low capital, labour and energy costs. The main requirements for membrane reactors is a semipermeable membrane with immobilized enzyme, which allows the free passage of the substrate and product molecules. A cheap example of such a membrane is the dialysis membrane used for removing low molecular weight species from protein preparations. (Bakajin & Noy 2009)

Nanotechnology can provide further advantages over the traditional membrane reactor. A nanostructured membrane reactor has a higher load of enzyme thanks to the high surface to volume ratio of nanomaterials. Further, the nanofibers have a lower barrier to diffusion, which allows the process to run at higher flow rate without the need of high pump power requirements. (Qin & Wang 2006)

In particular, nanofibrous membranes prepared by electrospinning could represent a possible solution to further enhance the performance of the current enzymatic treatments. These enzyme reactors are simple and cheap to manufacture (Bhardwaj & Subhas C Kundu 2010) and allow very large loading of the enzyme (Xu 2009) and also has the possibility to reuse the same reactor. A problem that is still unresolved is the possible denaturation of the enzyme after its immobilization on the three-dimensional network of the nanofibrous mat. It was reported that glucose oxidase lose its specificity toward glucose substrate (Senthamizhan et al. 2015). This may be a universal problem as polyphenol oxidase that become sensitive not only toward catechol but also towards reducing sugars (Liu et al. 2009). Apparently, the strong surface forces that are present inside the nanofibrous membranes may deform the three dimensional
structure of the enzymes, leading to a modification of the active site and, thus, to loss of specificity. (Hanefeld & Magner 2009)

**Electrospinning for encapsulation.** Encapsulation techniques can be quite useful to overcome the lack of stability of bioactives. Encapsulation consists on entrapping an active ingredient within a wall material (e.g. carbohydrate polymer, protein, lipids) (Bhushani 2014). Apart from traditional techniques, such as spray drying, liposome entrapment, coacervation, gelation, emulsion phase separation, etc. (Gibbs et al. 1999), nanoencapsulation was recently proposed as a new method for enhancing the storage stability of β-carotene (Guti et al. 2013). Relevant examples include the encapsulation β-carotene into self-assembled nanoparticles (Pan et al. 2007), solid lipid nanoparticles (Ecker et al. 2009), and nanoparticles based on supercritical fluids (Campardelli et al. 2012).

**Electrospinning for filtration of beverages.** Filtration is a unit operation commonly used in food processing aimed to remove suspended matter from food fluids. Generally, when a beverage is filtered through a porous membrane, solid particles accumulate on the filter and the fluid being filtered flows with a rate inversely proportional to the filter resistance (Foley, 2006; Mahdi & Holdich, 2013). Membrane filtration has been used extensively for complete or partial removal of microbes in beer, wine and juice to achieve the highest standards of food quality and safety. Recently, there is a trend to prepare membranes having smaller porous size, higher surface availability and working with higher flow rates. Among others, these characteristics are desirable to speed up filtration operation, reduce pressure drops and enhance selective adsorption toward specific molecules or biological matter. Nanofiber membranes have received a great attention for this purpose due to the inherent nanostructure achieved, as well as the simple and fast process and low cost, showing a promising potential for filtration applications (Fuenmayor et al., 2014; Daels et al., 2011; Li & Xia, 2004)

**Electrospinning for compounds adsorption.** Electrospun nanofibrous membranes (NFM) have been widely used to selectively capture molecules by binding their specific functional groups onto the membrane surface. NFM functionalized with amidino diethylenediamine were successfully applied for chelating metal ions (Kampalanonwat & Supaphol, 2010). NFM functionalized with laccase were used as bioreactor for the removal of chlorophenols in water (Dai, Niu, Yin, Xu, & Xu, 2013). Also, NFM were used as pre-filters for the removal of micro-
particles from waste-water (Bjorge et al., 2009; Gopal et al., 2007; Kaur, Rana, Matsuura, Sundararajan, & Ramakrishna, 2012). In addition, NFM were used to selectively adsorb hydrophobic molecules dissolved in water, such as quercetin (Scampicchio, Bulbarello, Arecchi, & Mannino, 2008), tannins (Fuenmayor et al., 2014), endocrine disrupters (F. Li, Scampicchio, & Mannino, 2011) and others persistent organic pollutants (Yue, Feng, Li, Jing, & Shao, 2012). Such applications have great promise as NFM have the advantage over conventional particle-based column-bed of reduced pressure drop, higher flow rate and higher surface availability.

2. OBJECTIVES

The aim of this work is to prepare nanofibrous membranes (NFM) for the development of novel enzymatic membrane reactors to be used in food processing in:

1. Biosensors for monitoring glucose during the brewing process.

2. Thermodynamically and kinetically characterizing invertase immobilized on nanostructured membranes by spectrophotometry and calorimetry (Isothermal Titration Calorimetry)

3. Application of nanofibrous membranes to remove bacterial and yeast from water and beer.

4. Application of nanofibrous membranes as scrubber to adsorb volatile organic compounds from coffee powder

5. Encapsulation of β-carotene in poly-ethylene oxide nanofibrous membranes to extend its stability toward oxidation.

3. RESEARCH QUESTION AND HYPOTHESIS

The aims of this work is to develop novel nanostructured materials that can be used in food processing. The main question that this work will try to answer is if the utilization of nanostructured materials in food processing can provide advantages respect the current state of the art.
In particular, to answer to this overall question, several specific aims will be derived.

➢ Determine if nanofibrous membranes prepared by electrospinning can enhance the performance of biosensors and bioreactors, in terms of enzyme loss, time stability and kinetic.

➢ Investigate if the performance of water and beverages filtration can be enhanced by the use of nanofibrous membranes.

➢ Use nanofibrous membranes to adsorb specific volatile organic compounds of coffee powdered samples.

➢ Test the performance of these membranes to encapsulate bioactives, such as beta-carotene

Overall, all these specific aims will allow to provide evidence to resolve the initial questions and, in detail determine if nanostructured materials can enhance the performance of standard food processes.

To test this hypothesis, the experimental plan will be based on three main activities:

➢ Preparation of nanofibrous membranes by electrospinning.

➢ Functionalization of nanofibrous membranes with enzymes, volatile organic compounds and carotene.

➢ Characterization of the performance of the biocatalytic membranes by chemical and physical methodologies such as thermal analysis, calorimetry, electrochemistry and microscopy.

4. STRUCTURE OF THE THESIS

Chapter one discusses the use of nanofibers membrane (NFM) in combination with multiwalled carbon nanotubes for the production of a second-generation enzymatic electrochemical biosensor. This part will determine glucose in commercial energy drink and during the beer production, from malt to beer. Chapter two presents the activity of the enzyme invertase before and after its immobilization on Nylon 6 nanofibrous membranes. Chapter three presents the
application of nanofibrous membranes to remove bacteria and yeasts from water, apple juices and beer. Chapter four shows the performance of nanostructurated membranes to adsorbed volatile organic compounds from coffee powders samples. Finally, chapter five shoes the possibility to preserve the stability of β-carotene by encapsulation into the nanofibrous membranes made by polyethylene oxide.
REFERENCE


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Thompson, M.P. et al., 2010. Smart Lipids for Programmable. , pp.2690–2693.


Chapter Two

2. Monitoring of Glucose in Beer Brewing by a Carbon Nanotubes based Nylon Nanofibrous Biosensor

Abstract

This work presents the design, preparation and characterization of a novel glucose electrochemical biosensor based on the immobilization of glucose oxidase (GOX) into a nylon nanofibrous membrane (NFM) prepared by electrospinning and functionalized with multiwalled carbon nanotubes (CNT). A disc of such GOX/CNT/NFM membrane (40 µm thickness) was used for coating the surface of a glassy carbon electrode. The resulting biosensor was characterized by cyclic voltammetry and chronoamperometry, with ferrocene methanol as mediator. The binding of GOX around the CNT/NFM greatly enhances the electron transfer, which results in a biosensor with a current five times higher than without CNT. The potential usefulness of the proposed biosensor was demonstrated with the analysis of glucose in commercial beverages and along the monitoring of the brewing process for making beer, from the mashing to the fermentation steps.

Keywords: glucose biosensor; beer brewing; electrospinning; carbon nanotubes; glucose oxidase
1. INTRODUCTION

Electrochemical biosensors represent a milestone in the field of analytical electrochemistry. High chemo- and stereo-selectivity, low manufacturing costs, thermal and chemical stability and simplicity of construction are some of the attractive features that drive the research on the development of new electrochemical biosensors (Bankar et al. 2009)(Wilson & Turner 1992). Among the different biosensors available, glucose biosensors have been one of the most successful examples due to the biomedical applications for diabetic patients (Wang 2008)(Martinkova & Pohanka 2015b). So far, researches have focused on the development of biosensors with enhanced signal transduction, larger linearity range and higher sensitivity (Devasenathipathy et al. 2015; Wang 2008). The approach used has always been based on the improvement of signal transduction between flavin adenine dinucleotide (FAD), the redox specie involved in the GOX kinetic, and an opportune transducer (Bourdillon et al. 1993). Overall, the process is a two electrons mechanism in which the reduction of FAD to FADH2 takes place at the expenses of glucose as the electrons donor (Vogt et al. 2014). Notably, in absence of oxygen only direct electrode oxidation of FADH2 can occur. The direct electron transfer kinetic of the oxidation of FADH2 by the electrode is hampered since FAD is buried 25 Å deep from the outer protein surface, preventing the direct electron transfer with the electrode (Wilson & Turner 1992)(Harper & Anderson 2010). To circumvent this drawback, first generation biosensors were based on the direct detection of the generated hydrogen peroxide (as FADH2 is re-oxidized at the expenses of oxygen). Although the direct measurement of peroxide formation is attractive because simple and fast, the amperometric measurement requires large over-potentials. When biological samples are analyzed, the resulting response current may suffer from the interferences of coexisting electroactive species.

Second generation biosensors overcame such limitations by replacing the oxygen with a reversible redox mediator that are able to shuttle the electrons from the redox center of the enzyme to the electrode surface (Figure 1).
A suitable mediator for the GOX enzyme is required to have a higher redox potential than the flavin redox center, so that it acts as the electron acceptor. For its fast reversibility and diffusion into interface layers, ferrocenemethanol has been widely employed as the redox mediator of choice \((E^0_{\text{vs Ag/AgCl}} = 0.216 \text{ V}, k^0 = 0.2 \text{ cm}\cdot\text{s}^{-1})\).\(^{(Cannes et al. 2003)}\)

Immobilization of the enzyme onto the electrode surface offers additional improvements. The advantage is that both enzyme and mediator can be brought close to the electrode surface, so increasing the efficiency of the electron transfer between the enzyme and the electrode. Several methods of immobilization have been tested such as hydrogel encapsulation \((\text{Martinkova \\& Pohanka 2015a; Bard 2003; Gonç et al. 2011})\), electro-polymerization \((\text{Gonçalves et al. 2011})\), or polymer film trapping \((\text{Goornavar et al. 2014; Kumar \\& Chen 2007; Tran \\& Balkus 2012; Homaei et al. 2013})\). Several improvements have also been devised in order to prevent unfavorable random orientations of the active site of the protein, such as templates or orienting functional groups \((\text{Rekuć et al. 2008; Sassolas et al. 2012; Feng et al. 2012; Y. Wang et al. 2015; Upadhyay et al. 2009; Tran \\& Balkus 2012; Homaei et al. 2013})\). However, these approaches involve a certain effort to control the enzymes positioning to minimize the partial loss of catalytic efficiency due to active site wrong orientations.

A way to enhance further the electrochemical response has been to incorporate conductive elements between the electrode surface and the flavin redox center to enhance the heterogeneous electron transfer process. Carbon nanotubes (SWCNT or MWCNT) have been tested within biosensors, allowing both faster heterogeneous electron transfer and rigid scaffold.
orientation locking, for more efficient biosensors (Scampicchio, Arecchi, Lawrence, et al. 2010), (Tijing et al. 2013; Lawal 2016). Some carbon nanostructures in particular show exceptional conductive properties. Moreover, polymer-CNT composites are easy to prepare and effectively displayed enhanced conductivity properties, along with better mechanical and thermal resistance (Yan et al. 2016; Agnihotri et al. 2011; Jose et al. 2007; Tijing et al. 2013). Electro-spinning (ES) has been recently applied as an intriguing way to prepare nano-fibrous (NF) polymers with high surface-to-volume ratio. ES has gained attention in engineering and material science for its exceptional simplicity, low cost and industrial applications (Tran & Balkus 2012) (Huang et al. 2003). Several reviews have been presented on this topic describing details on how this technique can be performed in house by any laboratory (A. Camposeo, M. Moffa 2015; Subbiah et al. 2005; Bhardwaj & Subhas C. Kundu 2010; Huang et al. 2003). Recent works in particular pointed out the good performances of electro-spun Nylon-6 nano-fibrous membranes (NFM) decorated with GOX for bio-sensing of glucose also in presence of interferents, showing good selectivity also in relatively complex matrices (Scampicchio, Arecchi, Bianco, et al. 2010).

Glucose biosensors have a number of well-developed applications, from the biomedical (i.e. glucose monitoring for diabetic patients) to the pharmaceutical field. Also in food technology there are several potential application where the features of glucose biosensors could provide a benefit for the producers. A part from the classical analysis of glucose in fruit beverages, a very intriguing application of glucose biosensors could be the monitoring of glucose evolution and/or consumption during the production of alcoholic beverages, such as wine or beer. In particular during beer brewing, the use of glucose biosensor could be of special help because it would provide information on the extent of the mashing process, where polysaccharides are progressively hydrolyzed into fermentable sugars, or during fermentation, where sugars are transformed into alcohol and carbon dioxide (Monošík et al. 2013). Thus, the simple and rapid monitoring of glucose could be of practical importance for the brew maker. In Table 1 some published results from glucose monitoring during several beer brewing steps and beer samples are reported.
<table>
<thead>
<tr>
<th>ref.</th>
<th>STEP MONITORED</th>
<th>TARGET</th>
<th>LINEAR RANGE</th>
<th>LOD</th>
<th>Sensitivity</th>
<th>RSD</th>
<th>METHOD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Monošk et al. 2013)</td>
<td>fermentation</td>
<td>fermentable sugars</td>
<td>50-960 µM (glucose)*</td>
<td>0.26 mg dm$^{-3}$*</td>
<td>62.78 nA µM$^{-1}$ cm$^{-2}$ (2.05 nA M$^{-1}$)*</td>
<td>NR</td>
<td>GDH–FAD and other specific oxidoreductases</td>
</tr>
<tr>
<td>(Tohill et al. 1997)</td>
<td>fermentation</td>
<td>glucose</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>amperometry</td>
</tr>
<tr>
<td>(Favier et al. 1997)</td>
<td>brewing</td>
<td>glucose</td>
<td>NR</td>
<td>0.14 g dm$^{-3}$</td>
<td>NR</td>
<td>NR</td>
<td>optothermal window</td>
</tr>
<tr>
<td>(Mauri et al. 2002)</td>
<td>beer</td>
<td>saccharides</td>
<td>5 - 100 µM</td>
<td>2 µM</td>
<td>NR</td>
<td>NR</td>
<td>flow injection, ESI MS</td>
</tr>
<tr>
<td>(Lapa et al. 2003)</td>
<td>fermentation</td>
<td>glucose and EtOH quantification</td>
<td>0.03 – 34.5 mg dm$^{-3}$ (H$_2$O$_2$)</td>
<td>0.015 mg dm$^{-3}$</td>
<td>Not reported</td>
<td>1%</td>
<td>Immobilized GOX, amperometry</td>
</tr>
<tr>
<td>(Nogueira et al. 2005)</td>
<td>beer</td>
<td>sugars/saccharides quantification</td>
<td>0.05 – 5.0 g dm$^{-3}$</td>
<td>0.008 g dm$^{-3}$</td>
<td>2514.9 area counts g$^{-1}$</td>
<td>NR</td>
<td>HPLC</td>
</tr>
<tr>
<td>(Li et al. 2007)</td>
<td>beer sample</td>
<td>glucose</td>
<td>0.1 - 1 mM</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Direct voltammetry of GOX</td>
</tr>
<tr>
<td>(Clowers et al. 2008)</td>
<td>beer</td>
<td>saccharides (for calibration of the mass spectrometer)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>HPLC or DIMS. ESI and MALDI MS</td>
</tr>
<tr>
<td>(Lehnert et al. 2009)</td>
<td>brewing</td>
<td>glucose</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>HPLC</td>
</tr>
<tr>
<td>(Piermarini et al. 2011)</td>
<td>fermentation (must, wine)</td>
<td>glucose</td>
<td>0.02 – 0.7 mM</td>
<td>NR</td>
<td>NR</td>
<td>&lt; 5%</td>
<td>immobilized enzyme, voltammetry</td>
</tr>
<tr>
<td>Study</td>
<td>Sample</td>
<td>Analyte</td>
<td>Limits</td>
<td>Limit of quantitation</td>
<td>Methodology</td>
<td></td>
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<td>---------------------</td>
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<tr>
<td>Tamura et al. 2010</td>
<td>beer</td>
<td>glucose and other six carbohydrates</td>
<td>5-10000 µg dm(^{-3})</td>
<td>0.9-1.4 µg dm(^{-3}) (LOQ)</td>
<td>NR</td>
<td>0.4-4.5% HPLC</td>
<td></td>
</tr>
<tr>
<td>Soldatkin et al. 2013</td>
<td>beer</td>
<td>maltose, lactose, sucrose and glucose</td>
<td>0.25-1.5 mM</td>
<td>0.001 mM</td>
<td>NR</td>
<td>&lt; 5% conductimetry, biosensor with immobilized specific enzymes</td>
<td></td>
</tr>
<tr>
<td>Rakete &amp; Glomb 2013</td>
<td>beer, wort, malt</td>
<td>reducing saccharides quantification</td>
<td>NR</td>
<td>1 µM RP-HPLC/MRM / FACE 100 µM</td>
<td>NR</td>
<td>NR RP-HPLC/MRM (HPLC separation and analyte derivatization) vs FACE electropherogram</td>
<td></td>
</tr>
<tr>
<td>Das et al. 2014</td>
<td>beer</td>
<td>many classes of compounds monitored</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>highest ≥ 3% HPLC-MS and GC-MS</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. List of some recent published works on glucose quantitation in several beer brewing steps. NR = not reported on the article. (*) data reported from ref. [53]
Apart from brewing studies, a wide investigation of immobilized GOX for glucose sensing has been done (Wilson & Turner 1992)(Harper & Anderson 2010)(Prodromidis & Karayannis 2002). Accordingly, this work aims at investigating the application of nylon-6 nanofibrous electrospun membranes (NFM), functionalized with multiwall carbon nanotubes (CNT) and loaded with glucose oxidase (GOX) for the analysis of glucose content in fruit beverages and for the monitoring of glucose evolution in the beer brewing process.

2. MATERIALS AND METHODS

GOX (from Aspergillus Niger Type XS, lyophilized powder, 100000-250000 units/g), salts, formic acid and Nylon 6 beads were purchased from Sigma-Aldrich and used as delivered. MWCNT were bought by Nano-Lab (Waltham, MA, US).

2.1 Carbon nanotubes preparation

MWCNT as delivered were treated in HNO₃ 6M (60ºC for 16 h) and HCl 8M (80ºC for 5 h) for activation. After pre-treatment, MWCNT were recovered by centrifugation at 6000 rpm for 15 minutes, washed in ethanol, ultra-sonicated for 2 hours and dried at 40ºC for 5 to 16 hours until no weight loss was observed.

2.2 Electrospinning

Electrospinning (ES) was performed with an in house apparatus based on a Spellman® CZE 1000R potentiostat equipped with a syringe pump. All NFM were electrospun at a flux of 0.02 mL/min onto an aluminium foil that acted as a grounded collector, when a +25 kV potential was applied on the flux exit needle located at 13 cm distance. Humidity in the environment was kept below 40% throughout the procedure. The NMF were functionalized with MWCNT by in-solution dipping. The resulting composite was analyzed by SEM (Figure 2). Co-mixing the polymer precursor solution with MWCNT was also undertaken but it did not yield a viable way in terms of NFM strength and membrane conductivity therefore this approach is not discussed any further herein. Immobilization of
the enzyme was achieved by means of glutaraldehyde as the cross-linker, in presence of bovine serum albumin. This approach was adapted from a published methodology. (Scampicchio, Arecchi, Bianco, et al. 2010)

2.3 Functionalization of NFM with MWCNT coating

A weighed amount of NFM 3.8 mg (2 sides of 9.62 cm²) with a thickness of 0.04 ± 0.01 mm was dipped into a 50 mM Triton™ X-100 solution containing a pre-treated and sonicated MWCNT dispersion with concentration of 0.5 mg mL⁻¹. The mixture was ultrasonicated for 10 hours a day for one week (50 W, 40 kHz at 25°C), then the membrane was removed and dried at 50°C for 20 minutes, carefully plunged in milliQ water until surfactant rinsing and dried again at 50°C for 30 minutes. After re-weighing the membrane coated with MWCNT, the final amount deposited on the membrane was 4.5% of initial weight. The final membrane presented a homogeneous black coating.

2.4 Scanning electron microscopy

NFM scanning electron micrographs were collected on a Phenom™ ProX SEM instrument (70-20000x digital zoom) with 30 nm max resolution. Samples were mounted on aluminium stubs and images were collected at 5 or 10 kV accelerating voltage. All samples were mounted on bi-adhesive tape and purged with a gentle air flow prior to measurement. Fibers diameter dispersion were evaluated with the provided PhenomPro Suite using the Fibermetric tool. Average diameter distributions were evaluated by fitting with a Gaussian function (Figure 2).
2.5 Electrode coating

A bare glassy carbon working electrode (BAS) was first polished with 0.3 and 0.05 μm alumina slurry, then rinsed with water and sonicated in EtOH, till a mirror-like surface was obtained. A piece of Nylon 6 NFM prepared as described before was placed on top of the electrode surface and fixed with two O-rings (all membrane exceeding parts were carefully cut off).

2.6 Enzyme immobilization

An enzyme solution was prepared in two steps: first, 40 mg of BSA and 11.4 mg of GOX were dissolved in 1 mL of Britton-Robinson pH 5. Then glutaraldehyde (5 μL, 2.5%) in water was added to 15 μL of this solution. The coating solution was freshly prepared before electrode coating. 5 μL were dropped twice to the center of the membrane and left drying for 10 minutes at room temperature. After the immobilization, the biosensor was placed in Britton-Robinson pH 5 and stored at 4°C.
2.7 Electrochemical characterization

CV experiments were performed using an AUTOLAB 128N potentiostat. A 0.07 cm² glassy carbon working electrode, Ag/AgCl reference and 1 cm² Pt counter electrode (BAS) were used in Britton-Robinson buffer 0.4 M at room temperature. Cyclic voltammetry was performed in the range from -0.2 V to +0.6 V. Ferrocene methanol 100 µM was used as mediator, with or without the presence of glucose. Chrono-amperometric measurements were performed under stirring at an applied potential of +0.5V with a 0.5 s per point acquisition time.

2.8 Determination of glucose with the dinitrosalicylic acid (DNS) reagent

The reagent was prepared by adding 5 g of DNS salt in 200 mL of distilled water containing 150 g of potassium sodium tartrate tetrahydrate and 8 g of sodium hydroxide. Care was taken by first mixing the tartrate and NaOH solutions, then adding the DNS salt. The solution was left overnight under stirring. The following day, a final volume of 500 mL was reached by adding distilled water (Miller 1959). An aliquot of standard or sample was diluted to 2 mL with deionized water and mixed with 2 mL of DNS reagent. Before UV-VIS measurement, the solution was heated in boiling water for 3 minutes. The determination was performed on an Agilent Cary 100 spectrophotometer provided with a Peltier thermostated 12 cells unit at 25°C. The spectra were collected between 500 and 650 nm (2 nm bandwidth, 1 point per sec integration time). The measurements of each standard or sample were repeated in triplicate. Sample concentrations were calculated by taking into account dilution factors.

2.9 Beer preparation and glucose analysis

The beer making process has been monitored throughout the brewing steps. The samples have been collected at the following steps: 1) Malt (500 g) was milled (Mill rollers knurled stainless steel AISI 316) and then added to warm water (1.5 L, 52°C). 2) The temperature was kept at 52°C for 15 minutes. In this step, the proteases contained in the malt provide a source of free amminoacids, which are being used later by the yeast during fermentation. 3) The temperature was raised to 62°C and kept for 10 minutes (maltose
production). 4) This was followed by a further raise at 68°C that was kept for 30 minutes (saccharification). In the step 3) and 4) the α- and β- amilases break down starch molecules into fermentable sugars (i.e. glucose and maltose). 5) The temperature was further raised at 78°C and kept for 15 minutes to inactivate the enzymes. 6) Then, the wort was filtered and washed with water (about 500 mL). 7) The sweet wort was then boiled for 1 h, during which 2.8 g of hops were added (Hallertau Hersbrucker). After boiling, the hops were removed and the wort rapidly cooled down to room temperature thanks to a heat exchanger. Then 1 g of yeast (Danstar Nottingham British Ale) was added for starting the fermentation (fermentation day 0). The fermentation was followed for an additional 4 days (steps 8 and 11). The evolution of glucose during the fermentation was monitored. Wort samples were filtered with a 0.2 µm CA membrane. 1 mL or 1.5 mL of the filtered solution were then added into the biosensor measurement cell prepared with 10 mL of 100 µM ferrocene methanol solution. The response current was read after at least 200 s of plateau equilibration.

3. RESULTS AND DISCUSSION

3.1 Characterization of the GOX-NFM/MWCNT by cyclic voltammetry

The sensing mechanism for this second generation GOX biosensor is schematically described in Figure 3. Briefly, glucose is converted to gluconate by GOX (A) and the produced protons and electrons are used by FAD to convert into FADH2 reduced form. This is then able to be oxidized back by the mediator (B) which converts its oxidized form to the reduced one (C). The mediator is free in solution therefore exchange electrons with the electrode directly and through mediation of the carbon nanotubes (D). The overall effect provided by carbon nanotubes in ET enhancement has been elucidated and consist in an better current transduction (Montanari et al. 1999; Patil et al. 2012a).
To show the effect of added MWCNT on our biosensor, Figure 4 compares the effect of CNT on the resulting mediated detection of glucose by ferrocene methanol. In details, when the electrode is coated with NFM and loaded with GOX, the resulting voltammogram reflects the typical response of a mediated biocatalytic reaction, where the reversible voltammetry of the mediator turns irreversible with the anodic current reaching a plateau at potential higher than +0.2 V vs Ag/AgCl. Upon reversing the direction of potential scan, no reduction peak is observed, as expected when the oxidized form of the mediator is rapidly consumed by the continuous oxidation of FADH2 (Figure 4-a). When the NFM was functionalized with CNT and the enzyme was free in the bulk solution, the result is similar but the background current is increased. The prominent background current is a consequence of the large, catalytically active surface area of the modified electrode. A part from the higher capacitive current, the signal enhancement is negligible (Figure 4-b). However, when the glassy carbon electrode is coated with NFM functionalized with CNT and loaded with glucose oxidase, a great enhancement on the current response is observed (Figure 4-c). Since such higher current density is observed only when the enzyme is immobilized on the conductive surface of the NFM functionalized
with CNT, we conclude that such enhancement is due to the electron shutteling of CNT from the active site of GOX toward the glassy carbon transducer. (Guiseppi-Elie et al. 2002)

Figure 4. Cyclic voltammetry of glucose at a glassy carbon electrode coated with (a) NFM with GOX immobilized; (b) NFM/CNT with GOX free in solution; (c) NFM/CNT with GOX immobilized. Experimental: 25.2 mM D-glucose, 100 µM ferrocene methanol; Britton-Robinson buffer, 400 mM, pH 5. Detection was performed at 100 mV s⁻¹ scan rate.

3.2 Analytical Performance of the Biosensor by Chronoamperometry

Chronoamperometry is a very sensitive technique for electrochemical biosensors characterization. In order to evaluate the analytical performance of the glassy carbon electrode coated with NFM/CNT/GOX, Figure 5 shows the chronoamperometric calibration of glucose in presence of 100 µM ferrocene methanol.
Although the GOX enzyme amount was the same both with loaded CNT and without, the effect of the biosensor design on the resulting signal intensity was huge. With respect both to the system with no immobilized enzyme and the one with no MWCNT loaded, NY CNT + imm GOX displayed a much higher response to glucose additions. Notably, all points correspond to 10 µL additions. As we discussed before, the addition of nanotubes (SWCNT or MWCNT) enhances the efficiency of the biosensor by increasing the electron transfer. This is consistent with our data. In fact, also with free GOX (purple curve) nanotubes induce a higher response. This effect can be seen by the higher plateau currents at the highest concentrations. On the contrary, the only immobilization of the
enzyme has a little effect on the overall current even if it can extend the linearity range against glucose addition (Scampicchio, Arecchi, Lawrence, et al. 2010). Under optimized conditions, the glassy carbon electrode coated with NFM/CNT/GOX showed a linearity range from 1 to 3 mM ($R^2 = 0.98$). In such range the sensitivity was 1.2 µA mM$^{-1}$ ($RSD\% = 2\%$) with a limit of quantitation of 20 µM (S/N = 10). Although the linear range was smaller than the one reported with a similar biosensor, (Scampicchio, Arecchi, Bianco, et al. 2010) the sensitivity was four times higher. Such higher sensitivity is a direct consequence of the loading of CNT on the surface of the nanofibers.

3.3 Kinetic behavior of GOX-NFM/MWCNT, effect of pH, interferents and ageing

The kinetic of the enzyme before and after its immobilization on the NFM/CNT was studied by chronoamperometry for increasing concentration of glucose (0 – 12 mM) and at several pH value. As an example, Figure 6 shows the resulting current vs concentration plots at pH 5. The experimental points were initially fitted with a classical Michaelis Menten model. Unfortunately, only when the enzyme was free in solution, the resulting current response obeyed to this model. Conversely, when GOX was immobilized on the NFM, the current response showed a sigmoidal trend. The trend is fitted instead very well with the Hill equation, a generalized Michaelis Menten model. The effect of pH on immobilized GOX kinetic was investigated between 5 and 7. For the free GOX, the optimum has been found between these values, and for immobilized GOX the values have been found below 6 and above 7, depending on the preparation (Bankar et al. 2009)(Monošík et al. 2012)(Arslan et al. 2011). In our case the optimum was found between 5 and 6 and the minimum value at 6.5. The Michaelis-Menten model did not fit the whole dataset properly. In fact, the current response for glucose concentrations below 1 mM deviates from linearity. Therefore, the Hill equation was alternatively applied to evaluate the asymptotes of the curves, namely the $V^{h}_{\text{MAX}}$ values ($V_{\text{MAX}}$ of Hill model): 4.96 ± 0.20 µA, 3.17 ± 0.04 µA, 8.05 ± 0.35 µA, 1.86 ± 0.03 µA and 3.84 ± 0.05 µA at pH 5, 5.5, 6, 6.5 and 7 respectively (data collected at the same ageing time on the same system equilibrated at the pH required). The reason for analyzing $V^{h}_{\text{MAX}}$ is that $k^{\text{app}}_{M}$ corresponds to one-half the concentration required to the enzyme to reach the conversion speed limit.
(the asymptote or \( V_{\text{MAX}} \)). Therefore, \( V^{h}_{\text{MAX}} \) (the actual experimental limit) can account qualitatively for the efficiency of the enzyme.

![Figure 6. Chronoamperometric current responses of the biosensor with the GOX free or immobilized on the NFM/CNT, for increasing concentration of glucose. In the case of the GOX free in solution (NY CNT + free GOX), experimental points were fitted with Michaelis Menten equation: \( y = V_{\text{MAX}} \frac{x}{K_{\text{appM}} + x} \), where: \( V_{\text{MAX}} = (3.8 \pm 0.1) \times 10^{-6} \) and \( K_{\text{appM}} = 2.53 \pm 0.23 \). In the case of GOX immobilized on the NFM/CNT, the points were fitted with a Hill type equation: \( y = \text{START} + (\text{END} - \text{START}) \frac{x^n}{k^n + x^n} \), where: \( \text{START} = (-4.1 \pm 1.7) \times 10^{-7} \), \( \text{END} = (1.52 \pm 0.03) \times 10^{-5} \), \( K_H = 3.64 \pm 0.1 \), \( n = 1.6 \pm 0.1 \). In both cases, \( r^2 \) was 0.998. For the Michaelis Menten fitting of NY CNT + imm GOX \( K_{\text{appM}} = 6.7 \pm 0.6 \text{ mM} \), \( V_{\text{MAX}} = 23.04 \pm 1.62 \mu\text{A} \) (fitting was bad therefore is not shown).

This trend is not trivial to discuss. More investigations will be required to elucidate the lack of a precise trend in efficiency moving away from pH 6. We carried out the analyses at pH 5 instead of 6 because of the wider linear range. For the linear fitting of the 1 - 4 mM range at pH 5 \( R^2 \) was 0.98 and at pH 6 was 0.94. At pH 6, the \( R^2 \) was near 0.98 only in the 1 -2 mM range. Just for a comparison with other published results, we calculated the apparent Michaelis Menten \( k_{\text{appM}} \) constant at pH 5 that was \( 6.7 \pm 0.6 \text{ mM} \). This constant accounts for the enzyme efficiency and the found value is in good agreement with others reported in literature (H. Wang et al. 2015). However, the immobilization
strategy and the biosensor design can affect the enzyme efficiency so that much higher \( k_{\text{app}} \) values are possible (e.g. 44 mM). (Patil et al. 2012b) Since the M.M. model did not fit the data and a generalized model (i.e. Hill equation) was used instead, we hypothesized the presence of a barrier-to-diffusion stage that the substrate has to overcome at lower concentrations. When the concentration of glucose is low, the substrate diffusion toward the “pseudo” pores of NFM is very limited. Only when glucose is higher than 1 mM, the transduction efficiency of the enzyme becomes linearly related with its concentration. Such biased response – low signal intensity for low concentration of the substrate and high sensitivity for concentrations higher than about 1 mM – was invariant for the amount of substrate added. Figure 7 shows the current response of the electrode coated with NFM/CNT/GOX for different initial (up to 1.5 mM) single spikes of glucose 0.5 M. The resulting calibration plot was the same when the spikes were done every 20 or 10 µL of glucose 0.5 M, and the plateau currents (above 20 µL) show a good agreement with the linear trend (see Figure 8 where the same data in the whole linear range are shown).

Figure 7. Three repetitions (black, red and blue) of glucose calibration performed with a membrane stored for 45 days, using different initial additions volumes (µL). Numbers represent volumes (µL) of 0.5 M glucose added. Data not corrected for 100 µM ferrocene methanol current (which account for the initial higher 0 µL point current in run 3).
The biased response observed for lower concentration of glucose was not ascribable to the loss of enzyme morphology. Although the immobilization process is known to cause shrinkage of the protein and possible modifications of the active site shape and dimension, the proposed biosensor did not show any loss of specificity. Figure 9 shows the current response of the electrode coated with NFM/CNT/GOX after the addition of (1) glucose, followed by sequential addition of other sugars, such as (2) sucrose, (3) maltose and (4) fructose (followed by a second addition of glucose).

The resulting signal of these interferents is limited to a negligible current decrease, which is consistent with the dilution factor consequent to the addition of a volume of solvent. After a further addition of glucose (point 5), the signal raised again consistently as the first spike, demonstrating that the presence of other sugars does not affect the current response to glucose. The maintenance of the initial specificity of GOX toward common sugars gives proof that the enzyme has not changed morphology during the immobilization procedure.

Figure 8. Linear region for replicates of the calibration of the biosensor at day 45. Biosensor was kept in a buffer solution at 4°C while not used.
Figure 9. Chronoamperometry analysis of possible interferents. The numbers indicate addition points where: (1) 50 µL glucose 0.5 M addition (2.5 mM final concentration); (2) 101.5 µL saccarose 0.5 M (5 mM final concentration); (3) 101.5 µL fructose 0.5 mM (5 mM final concentration); (4) 101.5 µL maltose 0.5 mM (5 mM final concentration); (5) 50 µL glucose 0.5 M addition (5 mM final concentration). Measurements were taken at day 45 in ferrocene methanol 100 µM in BR buffer pH 5.0.

We considered other possible interferents, namely phenolic acids and ascorbic acid. Both can theoretically contribute to the signal by direct oxidation at the anode at potential below 0.5 V. We measured a contribution of 2.8 µA mM⁻¹ for gallic acid spiked in the experimental conditions (immobilized GOX, 100 µM Ferrocenemethanol, Britton-Robinson buffer pH 5). However, the reported concentrations of this class of antioxidants in beer are some order of magnitude smaller (Montanari et al. 1999). For ascorbic acid instead, the response of spiked amounts in the set conditions was slightly higher (3 µA mM⁻¹) we found few references on its presence in beer. For example, in commercial beers the ascorbic acid concentration allowed are < 10 mg kg⁻¹ (58 µM) (Food & Authority 2015). Moreover, beer the aliquot of beer sample was diluted in the measurement cell by a 1:100
dilution factor. As a further precaution during beer samples analyses we left the sample to equilibrate at room temperature before and after filtration, to equilibrate oxygen content that acts as oxidizing agent for ascorbic acid.

The stability of the biosensor over time was monitored by repeating the glucose calibration, in the same condition, within 30 days from the first test. The results are reported in Figure 10.

![Figure 10. Main graph: plateau END values vs time/day, obtained by Hill fitting (day 0 to 26). Inset: correspondent chronoamperometry plateau currents at each addition. Modified GC working electrode was stored in a pH 5 buffered solution at 4°C over 26 days. Conditions used: 100 µM ferrocene methanol in 400 mM Britton-Robinson buffer pH 5. + 0.5 V applied potential, 1 point/sec, constant 10 µL additions.]

The loss of efficiency of the biosensor has been monitored over one month and the results of the first 26 days period are reported as overall loss of plateau END currents (analogue to M.M. \( V_{\text{MAX}} \), evaluated by Hill model fitting). Over one month it has been the 19 %.
Notably, the change is almost completely concentrated in the first 15 days and was almost negligible for the next 11 days (day 15 to 26).

3.4 Bio-sensing of glucose in commercial beverage and beer production monitoring

The proposed biosensor was firstly tested on a commercial beverage and then to monitoring a brewing process. In both cases, an aliquot of the selected beverage or wort (at each preparation stage) was added into 10 mL of Britton-Robinson buffer solution (0.4 M, pH 5), where ferrocene methanol (100 µM) had been dissolved. For the commercial energy drink (Energade Orange flavor), the results where comparable with those obtained by the dinitro salycilic acid assay. DNS test indicated a glucose concentration value of 0.3 +/- 0.1 g·100 mL\(^{-1}\) which is in perfect agreement with the result obtained with the biosensor of 0.297 +/- 0.007 g·100 mL\(^{-1}\). Notably, the measurements were performed in three replicates both for DNS and with the biosensor. The latter gave a much higher precision in terms percentage error, namely 33% for DNS and 2.3% for the biosensor. Then we applied the prepared biosensor to the analysis of the brewing process.
Figure 11. Chronoamperometric analysis performed on filtered (0.2 µm filter) wort samples taken at different process times. 1000 or 1500 µL were added to 10 mL 400 mM Britton-Robinson buffer pH 5. Dilution factors were taken into account for concentration calculation. Measurements were repeated in triplicate. Summary of analyzed steps: 1) Addition of the malt in water (52°C); 2) Protease rest (52°C for 15 min); 3) Maltose production (62°C for 10 min); 4) Saccharification (68°C for 30 min); 5) Deactivation of the enzymes (78°C for 15 min); 6) Filtration of the wort and washing with water (about 500 ml); 7) Addition of the hops and boiling (for 1 h) followed by removing the hops and cooling down to room temperature at which the yeast was added; 8) Fermentation: day 1; 9) Fermentation: day 2; 10) Fermentation: day 3; 11) Fermentation: day 4.

Figure 11 shows the current response obtained with the NFM/CNT/GOX biosensor during the beer brewing process. At the early steps of mashing (from 1 to 5), the concentration of glucose sharply increased, reaching a plateau value at about 0.3 g·100mL⁻¹. In step 6, the wort was washed to recover the leftover sugars (500 mL). The data were corrected to account for this dilution. During the fermentation the concentration of glucose dropped significantly, until the fourth day of fermentation, when the yeast has consumed almost all the glucose present in the wort.
4. CONCLUSIONS

GOX-NFM/MWCNT showed a good reproducibility over more than one month of utilization. This system is endowed with qualities such as a very low cost of production, storage resistance and fast response time in the linear range (10-20 s). Moreover, it displays a complementarity with other more classical analytical methods such as DNS and Brix. Together, they allow to investigate the evolution of glucose with respects to other sugars. Still, both DNS and Brix are not sufficient to account on the evolution of specific sugar in matrices as complex as beer. Conversely, the biosensor shows a complete insensitivity towards other mono- and oligo-saccharides even at concentration equal or higher than glucose. The sensitivity of the biosensor in the linear range was evaluated to be 12 µA·mM\(^{-1}\) with an evaluated limit of quantitation of 20 µM. Moreover, the kinetic of the immobilized GOX was evaluated with respect to free GOX. Michaelis Menten model failed to actually fit the data therefore the more general Hill equation was employed. Notably, the same effect was not observed with free GOX, where M.M. could be successfully applied. As future developments, the enzyme life and the enzymatic performances could be extended by preventing detachment from the nylon NMF. This research has demonstrated the suitability of the proposed biosensor for the monitoring of very complex fluids such as those deriving from the brewing process. Accordingly, the findings of this study may pave the way to a number of important applications for future practice. For instance, the proposed biosensor will be useful in the quantification of glucose in fruit juice, (Scampicchio, Arecchi, Lawrence, et al. 2010) fermentation process of fruit must, (Piermarini et al. 2011) or even in the development of electronic tongue method for the objective evaluation of sweetness in beverages. (Bulbarello et al. 2012) The possible applicability of the biosensor seems limited only by the presence of other redox species, such as ascorbic acid, that, when present at high concentrations, may interfere with the mediated biosensing mechanism. However, even in such unfavorable circumstance, the detection of glucose is still possible, provided that the sample is thoroughly aerated before the analysis, as ascorbic acid reacts quickly and irreversibly with oxygen.
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Chapter Three

3. Kinetics of sucrose hydrolysis by invertase immobilized on Nylon-6 nanofiber membrane measured by Isothermal Titration Calorimetry (ITC)

Abstract

Enzymes are biocatalysts that have high selectivity, can function at neutral pH, are food safe, and can be utilized for green chemistry. However, enzymes are not widely used commercially because of cost and thermodynamic instability. The aim of this work is to 1) immobilize invertase on a nylon-6 nanofiber membrane (NFM), 2) characterize the activity and stability of NFM immobilized invertase relative to free invertase, and 3) determine the utility of isothermal titration calorimetry (ITC) for measuring the activity of immobilized invertase. After immobilizing invertase with an efficacy factor of 83%, specific activity of free and immobilized invertase was determined by spectrophotometry and ITC at pH 4-6 and temperature 25-65°C. This work is the first to demonstrate that ITC can be used to determine the enzyme activity and stability of an enzyme immobilized on a membrane. The results from the ITC and spectrophotometric assays demonstrated that the optimal pH and temperature for invertase activity were 4.5 and 45-55°C, respectively. The immobilized enzyme is more thermodynamically stable and retains more enzymatic activity than free enzymes in solution.

Keywords: electrospinning, enzyme immobilization, invertase, Isothermal Titration Calorimetry (ITC), invert sugar
1. INTRODUCTION

Use of immobilized enzymes in the food industry is not a recent innovation (Hanefeld & Magner 2009; Homaei et al. 2013; Sheldon 2007; Sassolas et al. 2012), but enzyme immobilization on nanomaterials (Persano et al. 2013) has recently attracted many researchers (Tran & Balkus 2012; Rios et al. 2004; Giorno & Drioli 2000; Chakraborty et al. 2016; Ansari & Husain 2012). NanoFiber Membranes (NFM), with very large surface-to-volume ratios and high porosity, obtained by electrospinning, have many applications in food science and technology, i.e. filtrations (Lemma et al. 2015), sensors (Scampicchio et al. 2012), nutraceutical delivery matrices (Ghorani & Tucker 2015), and solid phase extraction of volatile organic compounds (Chu et al. 2015). NFMs are also a potentially good support for enzymes immobilized on a solid support called Enzyme Membrane Reactors (EMRs) (Zhang et al. 2012). EMRs are used to produce a wide array of products including detergents, food (wine, fruit juice, oils and fats, starch and sugar), animal feed, alcoholic beverages, fine chemicals, leather, textiles, pulp and paper (Giorno & Drioli 2000). There are also many applications in pharmaceuticals, environmental treatments (Chakraborty et al. 2016) and potential applications in green chemistry (Jochems et al. 2011).

The main advantage of EMRs is the ability to separate and reuse active enzyme, thus minimizing the amount of costly enzyme used per reaction (Jochems et al. 2011). Additionally, EMRs can be implemented in continuous processes to increase productivity, integrate into system operations, and conduct multiphase reactions. However, current EMR technology is limited by insufficient immobilization or absorption of the enzyme onto the NFM, product/substrate inhibition, leakage and deactivation of the enzyme, and loss
of enzyme activators or cofactors (Chakraborty et al. 2016). Major problems in industrial application of enzymes include instability against temperature, pH, mechanical stress, salt concentration, and surfactants.

Invertase catalyzes the hydrolysis of sucrose into an equimolar mixture of glucose and fructose called inverted sugar. The food industry uses invert sugar to increase sweetness and extend the shelf-life of food products (Kotwal & Shankar 2009). Inverted sugar is currently synthesized on an industrial scale using acid hydrolysis, which creates a syrup with a high mineral content by an inefficient process (Akgöl et al. 2001). An enzyme-catalyzed reaction will decrease the mineral content and could increase the efficiency of the reaction. However, enzyme-based reactions are often expensive due to the cost of the enzyme. This work demonstrates that invertase can be immobilized onto nylon-6 NFM and that the immobilized enzyme is more stable than free enzyme. Additionally, immobilized inveratase can be reused, but with the loss of activity over time. The aims of this work are to immobilize invertase on nylon-6 NFM, then characterize the activity and stability of NFM immobilized invertase relative to free invertase, and finally determine the utility of isothermal titration calorimetry (ITC) for measuring the activity of immobilized invertase.

Determination of enzyme kinetic parameters is fundamental for use of immobilized enzymes. The most common method for determining enzyme kinetics is spectrophotometry (UV-VIS or fluorimetry), which often requires a modified substrate or secondary reaction and cannot be used in opaque media, which can limit its utility (Ellis 2001; Rand et al. 1993; Dzingeleski & Wolfenden 1993). Alternately, isothermal titration calorimetry (ITC) is a universal and direct method for measuring the rates of enzyme-
catalyzed processes. ITC measures the heat rate from the enzyme-catalyzed reaction and ITC can be used with suspensions (Loh et al. 2016; Garidel & Hildebrand 2005; Fotticchia et al. 2014), in opaque media, or with a solids substance to determine enzyme kinetics. Additionally, because ITC directly measures the heat rate of the reaction, an output of nearly every chemical reaction, it can be used to quantify the activity of many enzymes whose activity cannot be determined by other means. This work is the first to report the use of ITC as a method of determining the kinetics of an enzyme immobilized on a membrane.

2. MATERIALS AND METHODS

2.1 Enzyme and chemicals

Invertase (EC 3.2.1.26; β-D-fructofuranosidase) from baker's yeast (S. cerevisiae) grade VII, > 300 units/mg solid (Sigma-Aldrich, Saint Louis, USA) was used without any additional purification. Nylon-6 polymer pellets and formic acid 98% were purchased from Sigma-Aldrich, Italy. Sodium tetraborate (99%), p-hydroxybenzoic acid hydrazide (PAHBAH) (97%) and polyethyleneimine (PEI), branched average MW ~25,000 by LS, and average MN ~10,000 by GPC were purchased from Sigma-Aldrich, USA. Glutaraldehyde 25% solution was purchased from Calbiochem, USA, and sucrose crystalline, ACS grade, was purchased from Fisher chemical, USA.

2.2 Electrospinning Nylon-6 for nanofiber membrane

Electrospinning was done at room temperature in a lab hood (Lemma et al. 2015). A polymer solution of nylon-6 (23% w/w) in 95% formic acid/water in a 5 ml syringe with a 0.5 mm internal diameter stainless steel needle was pumped at 0.02 ml/min (KDS Legato 111, KD Scientific, Italy). A 22kV voltage (Spellman CZE SL150) was applied between
the needle and a collector plate, causing a taylor cone to form by inducing repulsive electrostatic forces that exceeded surface tension. The polymer solution elongated and the fiber diameter decreased as the solvent evaporated. The nanofibers obtained were collected on a stationary collector 11 cm from the needle tip. The nanofiber membrane was composed of randomly distributed long nanofibers with diameters ranging from 100 nm to 100 µm.

2.3 Enzyme immobilization

The procedure used for enzyme immobilization was previously described by Amaya-Delgado et al. (L. Amaya-Delgado, M.E. Hidalgo-Lara 2006). This protocol was adapted for nylon-6 NFM by decreasing the concentration of the enzyme to 0.09 mg/cm².

2.4 Invertase activity assays

Invertase activity was measured with a spectrophotometric method (M. Lever 1972) in which p-hydroxybenzoic acid hydrazide (PAHBAH) reacted in an alkaline solution with the products of the invertase reaction, glucose and fructose. This produced yellow anions, which were then measured spectrophotometrically at 380 nm with a microplate reader (Fluo Star Optima from BMG Labtech Inc., Cary, USA).

ITC measurements were made with a NanoITC LV (TA Instruments Lindon, UT, USA). Four consecutive single injections of 3.98 µL of sucrose (1.2M, concentration in the syringe and 28 mM in the cell) was added into a 170 µL cell of the calorimetry (Figure 1). The concentration of the free Invertase in the cell was 0.00815 mg/ml. These data were analyzed by the method described in Hansen, et. al. (Hansen et al. 2016; Demarse et al. n.d.; Transtrum et al. 2015) to determine enzyme activity. The nylon 6 NFM was added and removed from the ITC using a 500 µL Hamilton syringe to pick up the NFM. The
activity was calculated by determining the rate of product formation based on the empirical kinetic model that will be more fully described in a future publication.

2.5 Differential Scanning Calorimetry (DSC)

For commercial applications, immobilized invertase must be thermodynamically stable through multiple uses. The relative stabilities of immobilized and free invertase were determined by analysis with a 10 mg/ml run in duplicate from 15 to 150°C at 2°C/min in a Multi-Cell DSC (TA Instruments, Lindon, UT, USA) in 0.1 M acetate buffer at pH 4.5.

2.6 Effectiveness factor ($F_\varepsilon$)

The effectiveness factor ($F_\varepsilon$) (Ahmad et al. 2001) (Fatarella et al. 2014) is a measure of the efficiency of immobilization and represents the retained activity after immobilization.

$$F_\varepsilon(\%) = 100 \times \frac{A_i}{(A_0 - A_e)}$$

Equation 1

$A_i$ is the activity of the immobilized enzyme; $A_0$ is the activity of the free enzyme solution added for immobilization, and $A_e$ is the activity remaining in the free enzyme solution after immobilization. $A_i$, $A_0$, and $A_e$ were determined by a spectrophotometric method (M. Lever 1972).
3. RESULTS

3.1 Measurement of immobilized invertase activity using ITC

Invertase was covalently, immobilized onto nylon-6 with an effectiveness factor ($F_\varepsilon$) of 83%, which is in agreement with the work of Ahmad et al. (Ahmad et al. 2001) for invertase immobilized on lectin (82%). Enzyme activity has been measured using ITC for decades, but it has not been used to determine the activity an immobilized enzyme. We therefore set out to determine if ITC could be used to determine the activity of immobilized invertase. Previous studies have demonstrated that there are two methods for determining enzyme activity using ITC, multiple injections (Mazzei et al. 2014; Todd & Gomez 2001) and single injection (Hansen et al. 2016; Demarse et al. n.d.; Transtrum et al. 2015). The single injection method was chosen for this study because it is can be performed faster and requires less enzyme (Hansen et al. 2016; Demarse et al. n.d.; Transtrum et al. 2015). To determine the activity for both the immobilized and free invertase, the invertase was loaded into the ITC and injected with 3.98µL of a 1.2M solution of sucrose for a final concentration of 28mM sucrose in the reaction vial. A buffer-only sample was also used to determine the heat of mixing for the sucrose into the buffer. Figure 1 demonstrates the change in heat rate following the injection of sucrose. For each reaction there were four sequential injections that were independent of the previous injection.
Figure 1. ITC heat rate signal of free invertase (0.0815 mg/ml) (black line), invertase immobilized on nylon-6 NFM (dark gray line), and blank titration of 1.2M sucrose into 0.1 M sodium acetate buffer (gray line) at pH 4.5 at 55°C

3.2 Measurement of the effects of temperature and pH on immobilized invertase activity using ITC.

The activity of free and immobilized invertase was measured with a well-established colorimetric assay and with the novel ITC method at temperatures from 25-65°C in increments of 10°C. In both the colorimetric and the ITC method the invertase specific activity is normalized to the peak enzymatic activity at 55°C and a pH of 4.5. The effect of pH on the activity was measured at 55°C at pH 4-6 in 0.5 pH unit increments using ITC in Figure 2A. Maximum activity of free enzyme (black bars) was at pH 4.5 to 5.5, whereas the immobilized enzyme (gray bars) had only a single pH where activity is detected at pH 4.5. The temperature dependence of the enzyme activity was then measured at a pH of 4.5 (Figure 2B). The maximal activity for both the immobilized and free invertase was 45-55°C as determined by ITC. The traditional colorimetric assay recapitulated the optimal
conditions for invertase activity determined by spectrophotometric method (Figure 2C-D) indicating that ITC is a useful and viable substitute for colorimetric assays. Additionally, these values agree with the work of Kotval et. al. and Ahmad et al. (Kotwal & Shankar 2009; Ahmad et al. 2001). The differences in the specific activity measurements using the colorimetric assay and ITC likely occurred because reactions in ITC assays require approximately 40 minutes to stabilize before they can begin, whereas the spectrophotometric assays begin immediately. This lag time occurs at the temperature and pH indicated, so if the enzyme is unstable under those conditions it will likely denature during the equilibration period.

Figure 2. (A). pH profiles of free (dark grey) and immobilized invertase (clear grey) in 0.1 M sodium acetate buffer from pH 4 to 6 with the use of ITC method (calorimetric) (C) with the traditional method (spectrophotometric). (B). Temperature profiles of free (dark grey) and immobilized invertase (clear grey) in 0.1 M sodium acetate buffer from Temperature 25 to 65°C with the use of ITC method (calorimetric) (D) with the traditional method (spectrophotometric).
3.3 Stability of free and immobilized invertase on Nylon-6 NFM

Differential scanning calorimetry (DSC) was used to determine the thermal stability of immobilized and free inveratase. In Figure 3, immobilization of the invertase shifts the value for denaturation of the enzyme from a $T_{\text{max}}$ of $67.3 \pm 0.5$ to $68.8 \pm 0.6^\circ$C for free and immobilized invertase, respectively. This increase in enzyme thermal stability agrees with the work of Ansari S.A. et. al. (Ansari & Husain 2012).

![Graph showing corrected heat rate vs. temperature](image)

*Figure 3. Thermograms of 10 mg/mL invertase immobilized in Nylon 6 NFM in 0.1 M sodium acetate buffer at pH 4.5 (black line) and 10 mg/ml of free invertase (gray line). Both samples were run in duplicate from 60 to 80 °C at 2°C/min on a Multi-Cell DSC (MC DSC)*

The DSC measurement demonstrates the increased thermodynamic stability; however, it does not prove the increased thermal stability translates into an increase in stability of enzymatic activity at lower temperatures. To determine the stability of enzymatic activity, four consecutive single injections of sucrose into buffer solution containing free and immobilized invertase were measured with ITC. The peak height is proportional to
maximal enzymatic activity and the slope of the four peaks at each temperature is indicated in Figure 4. The decreasing slope demonstrates a loss of enzymatic activity during the 8h assay. Figure 4 shows that the immobilized enzyme is less temperature sensitive at 25, 35, and 45°C. However, at 55°C the free enzyme is more stable than the immobilized enzyme. At 65°C, it was not possible to observe a significant signal from either the free or immobilized enzyme because this temperature was able to denature the enzyme only during the instrument stabilization signal (about 40 minutes).

![Figure 4. Thermal stability comparison of free invertase (Δ) and immobilized invertase (■) at 25, 35, 45 and 55 °C](image)

3.4 Reusability of invertase immobilized on nylon-6 membrane

Figure 5 shows the activity of the same immobilized invertase at 55°C and pH 4.5. During the first week, the immobilized invertase into nylon-6 NFM were used twice a day, but only once every other day thereafter. Between uses, the immobilized invertase reactor
were stored at 4°C in a solution of sodium acetate buffer (0.05M) pH 5.5. After one week (14 uses) the invertase activity decreased 70% and by 25 days and 25 uses, the enzyme activity decreased 90%.

![Activity of immobilized invertase over a 25 day period and 25 uses.](image)

**Figure 5.** Activity of immobilized invertase over a 25 day period and 25 uses.

### 4. DISCUSSION

The data demonstrate that immobilized invertase has increased enzymatic and thermal stability compared with the free enzyme in solution. However, the increase in thermal stability only results in a modest increase in the stability of the enzyme activity at 25°C. The immobilized enzyme can be reused (unlike free invertase); however, there is a loss in activity over time. There are several possible additional modifications that could be used in future studies to further stabilize the enzymatic activity of invertase such as PEGylation (Diwan & Park 2001). Ultimately, with additional modifications the invertase could be a viable EMR. We also established for the first time, to our knowledge, that ITC and DSC can be used to characterize immobilized enzyme activity.
Although, ITC can be used to measure enzymatic activity, this has been a relatively minor focus of its capabilities. The advantage of ITC is that it is not dependent on a secondary reaction or on the transparency of the media. Therefore, reactions using enzymes that are immobilized and/or novel, which can be difficult to characterize because they are opaque or no standard reaction has been developed, respectively, are can be measured by ITC. As an aside, we discovered how sensitive immobilized invertase is in suboptimal conditions because of 40-minute equilibration time for ITC experiments.

In summary, we have immobilized invertase and demonstrated that with further modification it could be useful in industrial applications. We have also developed a new tool for measuring the enzymatic activity of immobilized enzymes; namely ITC.
REFERENCE


Chapter Four

4. Removal of Bacteria and Yeast by Nylon Nanofibrous Membranes

Abstract

This work explores the capability of nylon-6 nanofibrous membranes (NFM) to remove bacteria and yeast cells. The membranes were prepared by electrospinning and used to filter water and beer samples fortified with bacteria and yeast cells (*Flavobacterium johnsoniae*, *Iodobacter fluviatilis* and *Saccharomyces cerevisiae*). NFM resistance (Rm) and cake resistance (Rc) were determined by a dead-end filtration system working under constant flow-rate. NFM resulted able to completely remove *Saccharomyces cerevisiae*, more than 5-log cycles for *Flavobacterium johnsoniae*. *Iodobacter fluviatilis* removal was limited to 3-log cycles. However, when we filtered a suspension containing a mixture of the bacteria, then the removal was complete. NFM showed promising capacity to remove microbial cells from food beverages.

**Keywords:** Dead-end filtration; Bacteria; Yeast; Electrospinning; Nanofiber

My contribution in this work was to help Solomon Mengistu Lemma (the first name of the article) to the use of Microcalorimetry instruments.
1. INTRODUCTION

Filtration is a unit operation commonly used in food processing aimed to remove suspended matter from food fluids. Generally, when a beverage is filtered through a porous membrane, solid particles accumulate on the filter as a cake, whereas the fluid being filtered flows with a rate inversely proportional to the filter resistance (Foley, 2006; Mahdi & Holdich, 2013). Membrane filtrations have been used extensively for complete or partial removal of microbes in beer, wine and juice to achieve the highest standards of food quality and safety.

Recently, there is a trend to prepare membranes having smaller porous size, higher surface availability and working with higher flow rates. Among others, these characteristics are desirable to speed up filtration operation, reduce pressure drops and enhance selective adsorption toward specific molecules or biological matter. Membranes prepared by electrospinning have received a great attention for this purpose as their inherent nanostructure, simple and fast processing, low cost, show a promising potential for filtration applications (Fuenmayor et al., 2014; Daels et al., 2011; Li & Xia, 2004)

The working principle of electrospinning is straightforward. Briefly, a polymer solution is continuously pumped through a metal syringe needle. When a high voltage is applied, an electrostatic repulsion between the polymer and the metal needle causes the instantaneous ejection of the polymer, which forms nanofibers collected as nonwoven membrane. The resulting morphology exhibits various useful characteristics for filtration applications (Barhate, Loong, & Ramakrishna, 2006; Li & Xia, 2004; Fuenmayor et al., 2014; Gopal et al., 2007).

Electrospun nanofibrous membranes (NFM) have been widely used as affinity membranes to selectively capture molecules by binding their specific functional groups onto the membrane surface. NFM functionalized with amidino diethylenediamine were successfully applied for chelating metal ions (Kampalanonwat & Supaphol 2010). NFM functionalized with laccase were used as bioreactor for the removal of chlorophenols in water (Dai et al. 2013). Also, NFM were used as pre-filters for the removal of micro-particles from waste-water (Kaur et al. 2012; Bjorge et al. 2009; Gopal et al. 2007). In addition, NFM were used to selectively adsorb hydrophobic molecules dissolved in water, such as quercetin (Scampicchio et al. 2008), tannins (Fuenmayor et al., 2014), endocrine
disrupters (Li et al. 2011) and others persistent organic pollutants (Yue et al. 2012). Such applications are of great premise as NFM have the advantage over conventional particle-based column-bed of reduced pressure drop, higher flow rate and higher surface availability.

However, NFM have not yet been applied for the removal of microbial cells in food beverages. Indeed, such application is of great importance as it may serve to remove specific pathogen bacteria responsible for safety concerns, remove bacteria responsible for changes in the food flavor, taste and appearance and, finally, reduce the overall food microbial concentration to enhance the stability of the final product. Furthermore, NFM could be used to remove yeast cells in fermented broth or must (such as wine, beer and cider processing) for their recovery and later reutilization (Chupakhina & Kottke 2008; Fillaudeau & Carrère 2002)

To test the suitability of NFM for the removal of microbial cells in food beverages, NFM made by nylon-6 were prepared and applied for the removal of bacteria and yeast from drinking water and beer beverages.

2. MATERIALS AND METHODS

2.1 Chemicals

Nylon-6 polymer and LB broth were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (98%) was purchased from Fluka, Sigma-Aldrich (Steinheim, Germany).

2.2 Microbial cultures

Bacterial strains were selected from the strain library of the Faculty of Science and Technology of the Free University of Bozen-Bolzano, Italy. The strains of bacteria (Flavobacterium johnsoniae and Iodobacter fluviatilis) were isolated from the Alpine environment and stored as glycerol stocks. These microbes are known as ubiquitary in freshwater and known as common contaminants in beer production (Bokulich & Bamforth 2013). The yeasts (Saccharomyces cerevisiae) were obtained from Lallemand Inc. All the suspensions of microbes were prepared using UV distilled deionized water obtained with a Milli-Q system (Millipore, Bedford, MA).
Beer samples were purchased from local supermarket of Bozen-Bolzano, Italy.

2.3 Microbial suspensions preparation

Glycerol stocks of the bacterial strains *Flavobacterium johnsoniae*, *Iodobacter fluviatilis* were thawed and an aliquot of 500 µL was inoculated into 200 mL liquid Luria-Bertrani (LB) media and incubated at 37 °C in a shaker (120 rpm) for 72 hours. Similarly, 3 mg of lyophilized *Saccharomyces cerevisiae* was incubated for 24 hours. The cultures were then centrifuged at 4600 rpm for 5 minutes (Thermo Science SL 16R centrifuge, German). The wet mass (386 ± 27) mg of cells was suspended in 100 mL of physiological solution (NaCl in water, 0.9 g/L) and beer for filtration. The densities of suspensions were measured.

2.4 Membrane production

Nylon-6 membranes were prepared by electrospinning as described by Scampicchio et al. (2010) with modifications. The Nylon-6 solution of 23% (w/w) was prepared in formic acid. This solution was placed in 5 mL plastic syringes fitted with a metallic needle (Hamilton). The solution in the syringe was pumped at 200 µL/h horizontally by KDS100 syringe pump (KD-Scientific, New Hope, PA). A 22 kV positive potential difference was generated by a Spellman SL150 high voltage power supply. Then, the electrospun nanofibers were deposited on grounded stationary metal collector covered with aluminum foil at 11 cm from the syringe nozzle to the tip. The electrospinning experiments were carried out at room temperature in an enclosed lab hood box.

2.5 Physical characterization

2.5.1 Morphology

The surface morphology of electrospun nanofiberous membranes was observed using a field emission scanning electron microscope (ZEISS SUPRA 40 VP SEM, German). The membranes samples were coated with 15 nm of Au (EMITECH K975X) prior to SEM imaging. The average fiber diameters (AFD) of the samples were determined by analysis of 100 fibers from SEM image.
2.5.2 Thickness and density

Thickness was measured by a micrometer. Apparent density was measured by weighting a unit area (cm$^2$) of each membrane. Porosity ($\phi$) was calculated from the density of the nylon-6 polymer (1.084 g/cm$^3$) at 25 °C and the filter media using the following equation (Ma et al. 2005):

$$\Phi = (1-\rho_m/\rho_b)$$  

Equation 1

where $\rho_m$ is the apparent density of the NFM and $\rho_b$ is the nylon-6 bulk density.

2.5.3 Turbidity and total soluble solid

Before and after filtration, UV–VIS spectrophotometer (Agilent Technologies G9820A, Malaysia) measurements were taken to express the turbidity as percentage of transmittance at 430 nm for beer suspensions, 630 nm wavelengths for water suspensions of bacteria and 600 nm Saccharomyces cerevisiae. The change of soluble solids in beer suspensions were measured with a Brix-meter (N-1 alpha, ATAGO, Japan).

2.5.4 Microbial counts

Microbial counts for yeast and bacteria before and after filtration was performed with the Koch (1883) standard dilution method as cited in (Albert & Ransangan 2013). Colony forming units (CFU) were estimated based on the plate showing the larger number of countable colonies (Sutton 2011). The log reduction value of the microbial by the filtration was calculated as the difference of CFU before and after filtration.

2.5.5 Isothermal microbial activity

Thermal activity of the cells suspensions was measured in a Thermal Activity Monitor-TAM III (TA Instruments) multichannel calorimetric system to determine variation in the heat flow of the microbes in isothermal conditions. The bacteria and yeast cells suspensions in LB broth at a concentration of 400 mg/100 mL were filtered at a rate of 1 mL/min for determination of the microbial activity. The samples before and after filtrations were placed in 4.0 mL of glass ampoules, which were hermetically sealed to control evaporation and energy loss. All experiments were carried out in ampoules containing 0.5
mL of cells suspensions at 30 °C. The broth in the ampoules was used as nutrition for microorganism growth. The thermal effect associated with microbial activity was recorded as a function of time. The final value was calculated from the subtraction of heat flow after filtration from before filtration for each microbe.

**2.6 Dead-end filtration (DEF)**

The cells suspension was pumped using laboratory scale FilterTec system (SciLog Inc, USA) Tandem 1081 peristaltic pump head driven by a 160-RPM motor connected with Tygon tubing (#16). Suspensions with the same concentrations (400 mg wet cells/100 mL) were prepared and agitated using a magnetic mixer at 30 rpm to prevent cells sedimentation in the course of filtration. Dead end filtration (DEF) of bacteria or yeast cells suspensions were typically carried out on a total volume of 100 mL at a constant flow rate (1 mL/min). A new loaded electrospun nylon-6 nanofibrous filter media with an area of 96.0 cm² were used in each filtration experiments. The detail of filtration setup is shown in Fig. 1. Before filtration the membranes, connecting tubes, filter cells holders and collector bottle were subjected to UV treatments (Asalair 700, Italy) in order to sterilize them for 30 minute. The pressure drop in the system was measured by a connected sensor throughout the experiments. The filtrate was collected and the weight monitored in a sterile bottle placed on an electronic balance (Adventurer Pro AV2102). Others related data were collected automatically as a function of time using a computer interfaced with Winwedge software (TAL Technologies, Inc., PA, USA) to the systems when the filtration starts to pump the suspensions. After each run, the apparatus were carefully cleaned to remove cross contamination by sterile milli-q water. All experiments were carried out at room temperature.
2.7 Membrane resistance a constant flow-rate

2.7.1 Specific membrane resistance

The membrane resistance ($R_m$) was measured by filtering the milli-q-water at constant flow rate. The pressure exerted by the membrane provided a direct measure of the membrane resistance value, according to the Darcy’s equation (Foley, 2006; Mahdi & Holdich, 2013):

$$R_m = \frac{A \cdot \Delta P}{q \cdot \mu}$$

Equation 2

where $A$ is the membrane area (typically, $9.6 \cdot 10^{-3} \text{ m}^2$), $\Delta P$ is the pressure exerted by the membrane (Pa), $q$ is the flow-rate (typically, $1.67 \cdot 10^{-8} \text{ m}^3/\text{s}$) and $\mu$ is the viscosity of water (1.00 Pa·s).

2.7.2 Cake resistance

The cake resistance was measured by filtering microbial cell suspension (40 mg/L) at a constant flow rate of 1 mL/min ($1.67 \cdot 10^{-8} \text{ m}^3/\text{s}$) and calculated according to the following equation:
\[ R_v = \frac{m \cdot A}{q \cdot \mu} \]

where \( m \) is the slope obtained by plotting the pressure exerted by the membranes as a function of the volume of filtrate; \( A \), \( q \) and \( \mu \) (1.00 mPa.s for water and 1.50 mPa·s for beer at 20°C) have the usual meaning.

### 3. RESULTS AND DISCUSSION

#### 3.1 Nanofibrous membrane morphology

We studied the morphological feature of NFM by scanning electron microscopy. Figure 2-a shows the microstructure of just-prepared membrane. The structure appears as a mesh of randomly oriented thread of fibers, having uniform diameter (197 ± 31 nm). The resulting morphology is typical of non-woven mesh structures, consistent with previously published works (Fuenmayor et al., 2014). Figure 2-b shows the NFM after filtration of bacteria suspensions (\textit{Flavobacterium johnsoniae}). The picture highlights the bacteria entrapped into the membrane structure. The small size of bacteria allows their entrance into the NFM mesh. For comparison, Figure 2-c shows the NFM after filtration of yeast suspensions. Here, the deposit appeared denser, wrapping the whole surface of the NFM. Apparently, the yeasts size is too large to enter into the NFM structure. As a result, a deposit over the membrane is formed.

![Figure 3. SEM images of the membrane surface. (a) NFM Filter media, (b) bacteria cells; (c) Saccharomyces cerevisiae](image)

Figure 3. The SEM images of the membrane surface. (a) NFM Filter media, (b) bacteria cells; (c) \textit{Saccharomyces cerevisiae}
3.2. Nanofibrous membrane resistance

The membrane resistance ($R_m$) of NFM was measured with the filtration of distilled water. The NFM had an average thickness of $(120 \pm 11) \mu\text{m}$, density of $(106 \pm 18) \text{kg.m}^{-3}$, area of $9.6 \cdot 10^{-3} \text{ m}^2$ and porosity of $90\%$. Filtration experiments conducted with such NFM resulted in a $R_m$ of $4.2 \cdot 10^{10} \text{ m}^{-1} \pm 0.3 \text{ m}^{-1}$. This value is in agreement with previous reports (Fuenmayor et al., 2014) and comparable with that of commercial membranes (Alhadidi et al., 2011).

When a suspension of microorganisms was used in place of water, then, the main resistance to flow became the filter cake ($R_c$) (Mahesh Kumar & Roy 2008). Figure 3 shows the dependency of the pressure resistance of NFM as a function of the collected volume of filtrate. During the collection of the filtrate, the differential pressure across the filter increased proportionally with the growth of the “filter cake” (Iritani et al. 2012; Mahdi & Holdich 2013).

We determined the filter cake resistance from the slope of the linear portion of the pressure-vs-volume curve. The slopes obtained followed the trend: *Saccharomyces cerevisiae* ($9.0 \times 10^7 \text{ kPa/mL}, R^2 = 0.99$) < *Flavobacterium johnsoniae* ($2.0 \times 10^8 \text{ kPa/mL}, R^2 = 0.97$) *Iodobacter fluvialis* < ($2.1 \times 10^8 \text{ kPa/mL}, R^2 = 0.98$) < mixed bacteria strains ($4.0 \times 10^8 \text{ kPa/mL}, R^2 = 0.98$). The magnitude of the slopes is proportional to the resistance offered by the cake, where bigger slopes mean a higher resistance to flow. Table 1 reports the cake resistances ($\Box$), as calculated with eq-2. These values can be explained in terms of the NFM interstitial volume left open during the packing of the cells. Cells with bigger dimensions formed cakes with higher interstitial space or lower $\Box$ value. Conversely, smaller cells formed cakes with a close-pack arrangements resulting in cakes with higher density, smaller interstitial space and, thus, higher resistance to flow. Similar results were obtained by (Nimhurchu & Foley 2006) for dead-end filtration of yeast suspensions, (Tanny et al. 1980) in cross-flow capsule for harvesting of bacteria and (Hwang et al. 2012) in the microfiltration of yeast cells in dextran suspensions.
Figure 4. Dead end filtration of microbial cells suspensions in water (bacteria: F. johnsoniae (5.1 x 10^8 CFU/mL), I. fluviatilis (1.0 x 10^4 CFU/mL), a mix of these two bacteria (8.0 x 10^8 CFU/mL) and Saccharomyces cerevisiae (2.9 x 10^8 CFU/mL)).

Table 2. Values of specific cake resistance ($\alpha$) and turbidity reduction in water and beer microbial cells suspensions filtration and isothermal calorimetry measurement difference of total heat produced during the microbial growth in LB broth at 30 °C before and after filtration.

<table>
<thead>
<tr>
<th>Strain suspension</th>
<th>Cake resistance</th>
<th>Turbidity reduction$^a$</th>
<th>Heat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha \times 10^{13}$ (m/Kg)</td>
<td>%</td>
<td>J/g</td>
</tr>
<tr>
<td>water beer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flavobacterium johnsoniae</td>
<td>29.90 23.00</td>
<td>95.0±1.2 99.6±0.1</td>
<td>28.86</td>
</tr>
<tr>
<td>Iodobacter fluviatilis</td>
<td>31.80 9.05</td>
<td>96.7±1.7 95.6±1.6</td>
<td>2.86</td>
</tr>
<tr>
<td>Mixed bacteria</td>
<td>53.30 45.30</td>
<td>97.2±1.5 98.4±0.2</td>
<td>22.22</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>9.66 3.86</td>
<td>99.9±0.1 99.6±2.6</td>
<td>24.66</td>
</tr>
</tbody>
</table>

The values of ($\alpha$) were determined by taking the linear portion in the region from 0 to 50 mL filtrate for water and 0 to 25 mL filtrate for beer suspensions.

$^a$Bacterial cells suspensions transmittance at 600 nm and yeast cells suspension at 630 nm.
3.3. Microbial removal capacity

The capability of NFM to remove microbial cells was measured by microcalorimetry. Figure 4 shows the thermal power generated by *Saccharomyces cerevisiae* suspension (2.1 × 10⁷ CFU/mL). The suspension generated a positive thermal power, whose area (24.7 ± 0.2 J/g) represented the heat of the yeast metabolism. After filtration, the heat dropped to 0.0 ± 0.2 J/g. The negligible heat measured after filtration was consistent with the substantial removal of the yeasts cells. Traditional plate-counts experiments performed on suspensions after filtration could count no yeasts cells.

![Figure 5. Isothermal calorimetry measurement of heat produced during the yeast growth in LB broth at 30 °C. BF: is the yeast cells suspension before filtration and AF: the yeast cells suspension after filtration.](image)

We repeated the experiment for suspensions of (a) *Flavobacterium johnsoniae*, (b) *Iodobacter fluviatilis* and (c) a mix of these two bacteria. Figure 5 (a-c) shows the resulting thermal power achieved before and after filtration of the suspensions. *Iodobacter fluviatilis* generated a heat of 27.3 ± 0.2 J/g which dropped to 24.5 ± 0.2 J/g after filtration. Similarly, *Flavobacterium johnsoniae* and mix of the bacteria generated a heat of 29.7 ± 0.2 J/g and
87 ± 0.2 J/g which dropped to 0.9 ± 0.2 J/g and 0.1 ± 0.2 J/g after filtration, respectively. Plate-counts experiments resulted in a 5.1 and 3.1 log-reduction for *Flavobacterium johnsoniae* and *Iodobacter fluviatilis*, respectively. The lower capability of NFM to remove bacteria than yeast is consistent with their smaller dimension and easier penetration through the NFM mesh. On the other side, the differences between the two tested bacterial strains can be explained by the respective bacterial cells shape: while *F. johnsoniae* is a long rod-shaped bacterium (from 8-12 μm), *I. fluviatilis* is characterized by short rod cells (3 μm) that easier can pass through the filter (Stanier 1947; Logan 1989). Figure 5-c also shows the thermal power signal generated by mixture of bacteria, before and after filtration. Before filtration, the thermal power curve showed two peaks, resulting from the sum of the metabolism of the two single strains. After filtration, the thermal power was negligible because of the complete microbial removal. This result is comparable with the one obtained by (Daels et al. 2011) and (Bjorge et al. 2009) during filtration of water by nanofiber functionalized membranes.

![Graphs showing heat flow over time](image)

*Figure 6. Isothermal calorimetry measurement of heat produced during the bacterial growth in LB broth at 30 °C. The letter ‘A’ refers the bacteria Flavobacterium johnsoniae ; ‘B’ is Iodobacter fluviatilis and ‘C’ is mixed bacterial strain of (Flavobacterium johnsoniae and Iodobacter fluviatilis). ‘BF’ and ‘AF’ has the same meaning as figure 4.*

### 3.4 Application on beer samples

Beer samples were inoculated with *Saccharomyces cerevisiae* (2.9 × 10^8 CFU/mL), *Flavobacterium johnsoniae* (5.1 × 10^8 CFU/mL), *Iodobacter fluviatilis* (1.0 × 10^4 CFU/mL) and with a mix of bacteria strains (8.0 × 10^8 CFU/mL) and then filtered with NFM. Figure 6 shows the results of the filtration experiments by plotting the differential pressure as a function of time. The curves showed a trend similar to those observed for the filtration of water suspensions (Figure 3). The beer inoculated with *Saccharomyces cerevisiae*
offered the lowest resistance to flow (Table 1). Plate count experiments resulted in 4.6 log-reductions, confirming what observed during the filtration of water suspensions. The filtration of *Iodobacter fluviatilis* and *Flavobacterium johnsoniae* resulted in 6.6 log-reductions. Turbidity experiments performed with the beer samples before and after filtration resulted in a reduction of more than 95%, regardless to the bacteria or yeast used. Interestingly, either the total soluble solid content (5.33 ± 0.05) °Brix or the pH (4.3 ± 0.1) of beer samples did not change during filtration (t-Test, p > 0.1), indicating that NFM minimally affected the nutritional composition of the samples.

![Graph](image.png)

*Figure 7. Dead end filtration of microbial cells suspensions in beer.*

### 4. CONCLUSION

The study presented the capability of selected bacteria and yeast cells removal in lab scale dead end constant rate filtration using produced electrospun NFMs. The results obtained show that these porous filter membranes played an important role for the removal of microbial cells from water/broth and beer suspensions.
REFERENCE


Chapter Five

5. Nanofibrous membrane for aroma compound extraction and release: a case study on coffee

Abstract

The consumer are most critical and want to know a lot of different characteristics of foods. Geographical origin is one essential characteristic to differentiate the same foods come from a different part of the world. Nowadays, there are a lot of different analytic method for the determination of the geographical origin of a product: the analysis of the Volatile Organic Compounds (VOCs) is an example. Coffee is one of the typical foods essentially consumed for the flavour and aroma.

Electrospun nanofiber (NFM) of Nylon 6 was successfully used to make a Solid Phase Extraction (SPE) of the aroma compounds release from 6 different coffee powders coming from Colombia (COL), India (IND), Costa Rica (CRC), Brazil (BRA), Ethiopia (ETH) and Guatemala (GUA). The VOCs release from the coffee powder was compared with the aroma compounds release after the adsorption on Nylon 6 NFM and a homemade film of Nylon 6. The geographical origin differentiation, the characterization of the different chemical class that make the classical coffee aroma was done by the use of PTR-QMS. Scanning Electron Microscope (SEM) was also used for the characterization of the NFM and the film of Nylon 6.

Keywords: electrospinning, coffee, Volatile Organic Compounds (VOCs), PTR-MS, Scanning Electron Microscope (SEM), Solid Phase Extraction (SPE)
1. INTRODUCTION

Electrospun nanofibers, with us resulting morphology exhibits various useful characteristics for extraction of volatile compounds: high surface-to-volume ratio, high specific surface and number of potential active sites enable more efficient adsorption and make these nanofibrous material advantageous in this area of application (Zhang et al. 2012). Nanofibers membrane was recently use as adsorbents of volatile organic compounds (VOCs) similar to Solid Phase Extraction (SPE) application, this was development in different field: human plasma analysis (Kang et al. 2007) but also in food analysis there are a lot of examples: pesticides, drugs and mycotoxins (Chigome & Torto 2012; Baggiani et al. 2007; Newsome et al. 2012).

Quality of foods became important for the consumer, especially in the last few years. In addition to food authenticity and product specification, geographical and/or botanical origin is nowadays required for most products. There are a lot of foods classification by the geographical origin by volatile organic compounds: honey (Schuhfried et al. 2016; Karabagias et al. 2014; Luykx & Ruth 2008), oil (Tres et al. 2013; Araghipour et al. 2008; Ruiz-samblás et al. 2013) and chocolate (Acierno et al. 2016).

Coffee is a good example of food consumed essentially for its characteristic aroma and flavor. Flavor compounds are formed in coffee during the roasting processing. The mechanisms of formation of coffee aroma (volatile and non-volatile substances) are extremely complex and there is clearly a wide range of interactions between these compounds (Özdestan et al. 2013). In coffee, about 900 volatile compounds have been identified, although fewer have been found to be relevant to coffee aroma (Buffo & Cardelli-Freire 2004).

This work explores the capability of Nylon-6 nanofibrous membranes (NFM), produced with an electrospinning home-made apparatus, for the extraction of the characteristic coffee aroma compounds.

The applicability of nanofibrous membranes for aroma compound extraction and release was compared on coffee powders and also with Nylon film. Coffee aroma is an extremely complex and diverse mixture of aroma compounds and its determination represents an analytical challenge. The traditional method of analysis of volatile compounds (VOCs) is
2. MATERIALS AND METHODS

2.1 Chemicals

Nylon-6 polymer was purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (98%) was purchased from Fluka, Sigma-Aldrich (Steinheim, Germany).

2.2 Coffee samples

Six coffees from different geographic origins, Brazil (BRA), Colombia (COL), Costa Rica (CRC), Ethiopia (ETH), Guatemala (GUA) and India (IND), were supplied by Illy caffè S.p.A, (Trieste, Italy) in commercially available forms (Monoarabica™). The coffee beans, C. arabica, were medium roasted (total weight loss: 15-18% w/w) at temperatures up to 220°C, coarsely ground to powder for stove-top coffee maker (moka) and packed in inert atmosphere under pressure in 125 g stainless steel cans.

2.3 Coffee powder

For coffee powder headspace measurements two different jars were taken from one batch. After opening the jars, 200 mg coffee powder was weighed into 40-ml glass vials (Supelco, Bellefonte, PA), suited for volatile compound analysis. Six analytical replicates were prepared for each jar. Four empty vials were employed as blanks.

2.4 Membrane and film production

Nylon-6 membranes were prepared by electrospinning as described by (Scampicchio, Arecchi, Bianco, et al. 2010) with modifications. The Nylon-6 solution of 23% (w/w) was prepared in formic acid. This solution was placed in 5 mL plastic syringes fitted with a metallic needle (Hamilton). The solution in the syringe was pumped at 200 μL/h horizontally by KDS100 syringe pump (KD-Scientific, New Hope, PA). A 22 kV positive
potential difference was generated by a Spellman SL150 high voltage power supply. Then, the electrospun nanofibers were deposited on grounded stationary metal collector covered with aluminium foil at 11 cm from the syringe nozzle to the tip. The electrospinning experiments were carried out at room temperature in an enclosed lab hood box. The same solution used for the electrospinning was also used for obtained a homemade film of Nylon 6. A portion of Nylon 6 (23%) in formic acid was added in the middle of 2 microscope glass slide. These two slides was manually pressed for obtained a similar thickness of the NFM (~ 100 μm) and leave under a chemical hood till the formic acid was completely evaporated.

2.5 Solid-phase microextraction (SPME) of volatile compounds of coffee

In the extraction phase, solid nanofibrous membranes and a homemade film of Nylon 6 were briefly exposed to the headspace of the powders (30 minutes at 40°C). The membranes were then transferred to separate vials and, after an adsorption step (30 minutes at 40°C), the VOCs released in the headspace, from the NFM and film of Nylon 6, was analysed by means of Proton Transfer Reaction Mass Spectrometry (PTR-MS). A schematic representation of the experimental set up was represent in figure 1.

Figure 1. Experiment design for the analysis with PTR-MS
2.6 PTR-QMS: description and advantage respect to traditional method

The IONICON PTR-QMS instrument is a real-time monitoring system for volatile organic compounds (VOCs) that allows for continuous VOC quantification at very low concentrations. The PTR-QMS series is based on quadrupole mass spectrometry and combines market-leading low online detection limits with high selectivity and a very fast response time. Direct injection of sample gases without preparation contributes to the speed and simplicity that is common to all our instruments.

PTR-MS (Lindinger, Fall, & Karl, 2001) is based on a novel design for the chemical ionisation cell (Field, 1966), which was developed out of the swarm technique. The sample gas is continuously introduced through a Ventury type inlet system into the chemical ionisation (CI) cell. Volatiles that have proton affinities higher than water (proton affinity of H$_2$O: 166.5 kcal/mol) are ionised by proton transfer from H$_3$O$^+$ and mass analysed in a quadrupole MS.

The CI-source was designed to achieve high sensitivity, low fragmentation, and allows for a rough quantification of VOCs. To achieve these targeted specifications, generation of the primary H$_3$O$^+$-ions and the CI process are spatially and temporally separated and individually controlled. This allows (i) maximising signal intensity by increasing the generation of primary reactant ions, H$_3$O$^+$, (ii) reducing fragmentation and clustering by optimising the conditions for proton transfer in the CI cell, and (iii) quantifying VOCs. Consequently, the key features of PTR-MS can be summarised as follows. First, it is fast. Time-dependent variations of the HS can be monitored with a sub-seconds time-resolution. Second, VOCs are not subjected to work-up with solvents, trapping and desorption or thermal stress and little fragmentation is induced in the ionisation step. Hence, mass spectral profiles closely reflect genuine HS distributions. Third, mass spectral intensities can be transformed into absolute HS concentrations. Finally, it is not invasive.
The great advantage of PTR-MS, besides giving immediately absolute concentrations, is that fragmentation of the analyte molecule is very much reduced so the mass spectra produced are much easier to interpret and are more straightforward to quantify (Lindinger & Jordan 1998). This rather novel technique enables a variety of organic species (such as alkenes, alcohols, aldehydes, aromatics, ketones, nitriles, sulphides and many others) in complex matrices to be monitored in real-time (within seconds), with detection limits as low as a few parts per trillion, volume (pptv).

GC-MS is an instrumental technique, it is a combination of a gas chromatograph coupled to a mass spectrometer (Gohlke & McLafferty 1993). GC separates the volatile compounds injected in a mixture and MS characterizes each of the components individually. In this way, one can both qualitatively and quantitatively analyse complex mixtures containing numerous compounds. In order for a compound to be analysed by GC-MS it must be sufficiently volatile and thermally stable. In GC-MS, the ions required for mass analysis are generally formed by electron impact ionisation. Gas molecules exiting the GC are bombarded by a high-energy electron beam (70 eV). GC-MS is one of the most widely used technique represents the method of choice for the analysis of food volatiles because of its high reproducibility (Pillonel et al. 2007). However, this technique is rather expensive and time-consuming.

2.7 PTR-QMS analysis

The headspace measurements were performed by using a commercial high-sensitivity PTR-QMS instrument (Ionicon Analytik GmbH, Innsbruck, Austria). The instrumental
conditions in the drift tube were: drift voltage 550 V, drift temperature 80°C, drift pressure 2.01 mbar, affording an E/N value of 140 Td (1 Td = 10^{-17} \text{cm}^2/\text{V} \cdot \text{s}). Sampling was performed with a flow rate of 80 sscm. The measurement order was randomized to avoid possible systematic memory effects. All the vials were incubated at 40 °C for 30 min before PTR-QMS analysis. Each sample was measured for 1 min, an acquisition rate of one spectrum each 20 seconds. One sample was analysed at every 5 min.

2.8 Data analysis

Peak intensities (expressed in cps, or counts per second) were converted into volatile compound concentrations, expressed in ppbv (parts per billion in volume) levels employing the formulas described by (Lindinger & Jordan 1998). A relative transmission curve was calculated employing a standard gas mixture (Restek, Bellefonte, PA) and following a previously published procedure (Taipale et al. 2008). A constant reaction rate coefficient ($k_r = 2 \times 10^{-9} \text{cm}^3/\text{s}$) was assumed for H$_3$O$^+$ as a primary ion.

2.9 SEM analysis of the morphology of Nylon 6 NFM and film

A desktop Scanning Electron Microscope (SEM) (Phenom Pro X, Germany) was used to characterize the morphology, the average diameter of the fibers and the average dimension of the pores in the NFM and film of Nylon 6.

3. RESULTS AND DISCUSSION

3.1 Characterization with SEM of morphology of Nylon 6 NFM and film

The morphology, the average fibers diameter and the average dimension the pores present in the NFM and film of Nylon 6 was characterized by three different software of Phenom World. 3D Roughness Reconstruction was used for take a pictures of the surface of the two different materials (NFM and film of Nylon 6) (Figure 3). With this picture is it possible to see the different surface conformation of the 2 materials, the NFM had a more uniform and planar surface, instead the homemade film had a more roughness surface.
With the fibermetric software also the average diameter of the fibers (for the NFM) was evaluated (Figure 3a). The average diameter of the fiber evaluated by the mean value of 300 nanofiber was included from 86.16 nm to 489.75 nm. With the poremetric analysis the average value of the pores area found was 4137.37 nm² for the NFM, while for the homemade film was 1.29 nm². This bigger dimension of the pores is a typical characteristic of the NFM structure (Zhang et al. 2012).

3.2 Total VOCs emission of coffee powder, after the adsorption on Nylon 6 NFM and film

After the analysis of VOCs by PTR-QMS emitted from the coffee powder, Nylon 6 NFM and film of Nylon 6 (after the adsorption and released step) are showed in the Figure 4. In Figure 4 can be observed the difference in the total VOCs intensity: coffee > NFM > Film. This figure shows the capability of the NFM respect to film of the same material to adsorbed and released a bigger amount of the VOCs of the same sample of coffee.
3.3 Principal chemical class present in the VOCs emission coffee powder, after the adsorption on Nylon 6 NFM and film

When you go more inside of the characterization of the single mass relived from the PTR-QMS is it possible to differentiate the volatile compounds in 6 principal chemical class of compounds (Ivon Flament 2002; Buffo & Cardelli-Freire 2004): Alcohols, Carbonyls, Ester/acids, Furans/pyrans, N-heterocycles and other compounds with the presence moreover of phenol and sulfur functional groups (Figure 5). In Figure 5 shown the relative intensity of the 6 different class of chemical compounds characteristic of the coffee aroma powders and when these compounds was released from the Nylon 6 NFM. As view, in figure 4 the coffee powder had the bigger emission but for a certain class of compounds (ester/acids, furans/pyrans and other compounds) the bigger emission is obtained from the sample after the adsorption on Nylon 6 NFM, probably the Nylon 6 membrane don’t have a big affinity with the low molecule substance, especially for the carbonyls this group of compounds are the biggest emission for the coffee powder.
3.4 Classification of coffee according to geographical origin after absorption on Nylon-6 NFM and coffee powder

However, after the PCA analysis was made the tentative identification of the major chemical class compounds present in the coffee aroma, also the geographical classification of the coffee powders and also when these compounds was adsorbed on Nylon 6 NFM. The results of this analysis are shown in Figure 5 (Nylon 6 nanofibers membrane) and Figure 6 (coffee powder).

The geographical origin coffee powders was analyze by head-space PTR-QMS and the results obtained with the PCA was employed to visualize the score and loading plots obtained using the first two principal components explaining more than 66% of the total variance in H₂O⁺ data, Fig. 5 and 6 show it is possible to differentiate samples taking into...
account geographical origin because of changes of m/z profile. Separation of coffee, after the adsorption on Nylon 6 nanofibers membrane, according to origin was also clear for Brazil and India, for Colombia and Ethiopia there is a little similarity only form 1 sample and the same result, for the analysis only with the coffee powder, is it possible to see for Costa Rica and Guatemala (Figure 5).

Principal Component Analysis (PCA) was employed to visualize all geographical origin coffee powder samples data. The score and loading plots obtained using the first two principal components explaining more than 72% of the total variance in H$_3$O$^+$ data, Figure 6 show it is possible to differentiate samples taking into account geographical origin because of changes of m/z profile. Separation of coffee powder according to origin was clear except to Guatemala and Costa Rica. For the other coffees powder origin with the head-space analysis with PTR-QMS there are a good separation (Figure 6).
4. CONCLUSION

The rapid analysis of volatile compounds in the headspace of six Arabica coffees powder, was performed with PTR-QMS, Nylon 6 NFM are used for explores the capability of electrospinning nanostructures, characterized by a very high surface to volume ratio and high porosity (Zhang et al. 2012), to extract the aroma compounds from the samples. Principal component analysis allowed visualising a separation according to geographic origin in both case. Overall, these results demonstrate the capability of nylon nanofibrous membranes to extract and release aroma compounds under mild conditions and in sample-specific fashion. In perspective, these materials could be employed in the manufacture of active packaging materials or analytical cartridges.
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Chapter Six

6. Stability of β-carotene in PolyEthylene Oxide Electrospun Nanofibers

Abstract

β-carotene (βc) was successfully incorporated into electrospun nanofibers of poly-(ethylene oxide) (PEO) with the aim of prolonging its shelf life and thermal stability. The physical and thermal properties of the βc-PEO-nanofibers were determined by scanning electron microscope (SEM), color analysis and differential scanning calorimetry (DSC). The nanofibers of PEO and βc-PEO exhibited average fiber diameter of 320 ± 46 and 230 ± 21 nm, with colorimetric coordinates L*=95.7 ± 2.4 and 89.4 ± 4.6 and b*= -0.5 ± 0.1 and 6.2 ± 3.0 respectively. Thermogravimetric analysis coupled with Proton Transfer-Mass Spectrometry (TGA/PTR-MS) demonstrated that coated βc inside PEO nanofibers increased thermal stability when compared to standard βc in powder form. In addition, β-carotene in the membranes showed higher stability during storage when compared with β-carotene in solution with a decrease in concentration of 57 ± 4% and 70 ± 2% respectively, thus should extend the shelf life of this compounds. Also, TGA coupled with PTR-MS resulted in a promising technique to online-monitoring thermal degradation.

**Keywords**: Electrospinning, β-carotene, Nanofibers, Poly-(Ethylene Oxide), Thermogravimetric analysis (TGA), Proton Transfer – Mass Spectrometry (PTR-MS)

My contribution in this work was to help Irene Peinado Pardo (the first name of the article) of the use of TGA and DSC instruments. I did part of the experiments.
1. INTRODUCTION

Carotenoids are a family of fat-soluble pigments naturally occurring in plants and some other photosynthetic organisms. Besides their use as food colorants, they have been also widely used as nutritional supplement because of their health related benefits, such as the reduction of the risk of chronic diseases such as cancer, ischemia, diabetes type 2, hypertension and cataract (Guti et al. 2013; Kriegel et al. 2016; Zeb 2012). Furthermore, carotenoids are also widely used as dietary supplements because of their strong antioxidant activity and capacity to protect against free radicals chain reactions. Despite these attractive properties, carotenoids are sensitive to light exposure, presence of oxygen and thermal treatments. All these events cause the irreversible oxidation of the molecule and, as a result, reduce its antioxidant and biochemical activity (Zheng et al. 2007; Wicklund et al. 2005; Arranz et al. 2008; Zhu et al. 2011; Betoret et al. 2012).

Suitable encapsulation techniques can therefore be quite useful to overcome the lack of stability of β-carotene. Encapsulation consists on entrapping an active ingredient (i.e. carotenoids) within a wall material (e.g. carbohydrate polymer, protein, lipids) (Bhushani 2014). Apart from traditional techniques, such as spray drying, liposome entrapment, coacervation, gelation, emulsion phase separation, etc. (Gibbs et al. 1999), nanoencapsulation was recently proposed as a new method for enhancing the storage stability of β-carotene (Guti et al. 2013). Relevant examples include the encapsulation β-carotene into self assembled nanoparticles (Pan et al. 2007), solid lipid nanoparticles (Ecker et al. 2009), and nanoparticles based on supercritical fluids (Campardelli et al. 2012).

In addition to the above, electrospinning is a further encapsulation technique which has gained a large interest in recent years due to its simplicity, capacity to produce non-woven nanofibrous mats with high surface to volume ratio, low cost and scale-up potential. These features have driven the development of a number of applications for encapsulation purpose (Kriegel et al. 2016).

In particular, electrospun nanofibrous membranes are an attractive technique to encapsulate sensitive materials such as carotenoids, because it allows preparing even fibers with excellent homogeneity of the bioactive along the fibers. These attractive
features were recently showed in connection with the encapsulation of retynil acetate (Mascheroni et al. 2013; Mascheroni et al. 2015; Solomon M Lemma et al. 2015) and β-carotene (Fernandez et al. 2009). Both these studies showed the potential of the electrospinning technique for encapsulating bioactive compounds inside biopolymer nanofibrous mats.

In this work, we extend the use of electrospinning for the encapsulation and enhanced stability of β-carotene in nanofibrous membrane made by PolyEthylene Oxide (PEO). PEO is widely used polymer because of its semi-crystalline state, bio-compatibility, biodegradability and water-solubility (Pielichowski & Flejtuch 2005; Erceg 2010). In the recent years, PEO gained interest in the pharmaceutical field for the controlled-release of solid-dose matrix system, transdermal drug delivery systems and mucosal bio-adhesives (Bhushani 2014; Erceg 2010). Thus, this work will show for the first time the potential of PEO-nanofibers to encapsulate β-carotene. Analysis of the surface of the membrane by attenuated total reflectance infrared spectroscopy will be used to infer about where β-carotene is located within the fibers. Also, the thermal stability of β-carotene will be evaluated by thermo-gravimetric (TGA), differential scan calorimetry (DSC), spectrophotometer UV-VIS, and by Evolved Gas Analysis (EGA) based on a novel hyphenation technique based on the connection of Proton Transfer Mass Spectrometry (PTR-MS) with the outlet of the thermogravimetric furnace. The results of this work will provide new insights into the use of PEO nanofibrous membranes for encapsulation purpouses.

2. MATERIALS AND METHODS

2.1 Reagents

PolyEthylene Oxide (PEO) (Mw: 400000), ethanol (100% proof), and hexane (99%) were purchased from Sigma-Aldrich (Via Gallarate 154-20151, Milano). β-carotene was received from DSM (DSM Nutritional products AG, Basel, Switzerland). The reagents were analytical grade and used as received. Milli-Q water was used throughout the experiments.
2.2 Electrospinning

The prepared polymer solutions were placed in a 5 ml syringe connected with a stainless steel needle having a inner diameter of 0.5mm. The solution/dispersion in the syringe was pumped at 500µL/h horizontally (KDS legato 111, KD scientific, Italy) and the solutions/dispersions were electrospun with an applied voltage of 25 kV using an high voltage power supply (Spellman 150, Hauppauge, NY). Then, the nanofibers were deposited on a grounded stationary metal collector covered with aluminum foil at 13 cm from the syringe nozzle to the tip. The electrospinning experiments were carried out at room temperature in an enclosed fume hood.

2.3 Measurements and characterization

Ionic conductivities of the polymer solutions were measured using a portable conductivity meter (WTW 3410, WTW GmbH, Germany) with a precision of ± 0.1 µS cm⁻¹, which was calibrated with 1413 µS cm⁻¹ standard conductivity solution at 25°C temperature. The viscosity of the solutions was measured with a rheometer (HR-2, TA Instruments, New Castle, DE, USA) using a parallel plate system (4 cm diameter) with a gap of 500 µm at 25°C with a shear rate from 1 up to 600 s⁻¹.

Color was measured at 25°C with a portable Minolta spectrophotometer, model-CM 5 (Konica Minolta Sensing Europe B.V. Italy Branch Office) using a black plate as the background to standardize the measurements. Visible absorption spectrum was recorded between 380 and 700 nm by reflectance to obtain tris-timulus values of CIEL*a*b*, using illuminant D65 and standard observer (10° visual field) as references.

The morphology and the fiber diameter of the electrospun nanofibers were analyzed by Scanning Electron Microscope (SEM) (Phenom ProX, SEM, Germany). The average fiber diameter (AFD) for the samples was calculated by analysis of 300 fibers from the SEM images. Attenuated Total Reflectance Fourier Transform Infrared spectroscopy (ATR-FTIR) was used to characterize the surface of the nanofibers (Tensor 27 FTIR, Bruker, Ettlingen, Germany). Spectra were recorded from 600 to 4000 cm⁻¹ with a resolution of 8 cm⁻¹ by taking 32 scans for each sample.
Thermal properties of the nanofibers were investigated after 24h of storage (25°C in the dark) by using Differential Scanning Calorimetry (DSC, 200 Maja, Netzsch, Selb, Germany) and thermogravimetical analysis (TGA, STA 449 F3 Jupiter, Netzsch, Selb, Germany). For DSC analyses, the sealed 4 mg samples were initially equilibrated at 25°C and then heated to 600°C at heating rate 5°C/min using nitrogen as a purge gas. For TGA, data from sample masses (6-10 mg) were recorded from 40 to 600°C at a heating rate of 10°C/min, under a flow rate of nitrogen and air of 100 ml/min.

The analysis of total volatile organic compounds (VOCs) was performed by coupling Proton Transfer – Mass Spectrometry (PTR-MS) (PTR-QMS 500, Ionicon Analytic GmbH, Innsbruck, Austria) with the gas exit of the TGA furnace. Prior to analysis, the gas exiting the furnace was submitted to a dilution with clean air in a 1:40 ratio. Drift chamber temperature, pressure and voltage were 80°C, 2.01 mbar and 550V respectively, resulting in a E/N value of 140 Td (1 Td = 10^{-17} \text{cm}^2 \text{V s}^{-1}). Measurement were carried out in the “mass scan” mode, whereby a complete mass spectrum in the range of 20-200 amu, at a mass detection rate of 0.1 s mass^{-1}, was gathered in approximately 18 sec.

2.4 Stability

Stability of β-carotene was estimated by storing the membranes in the dark at 25°C and controlled humidity (50%). At a different predetermined time (24h, 48h, 96h, 1, 2,3 and 4 weeks), samples were analyzed in terms of color and β-carotene content. Color measurements were carried out using a Minolta spectrophotometer, model CM-5. β-carotene content was spectrophotometrically determined on hexane extracts by using Cary 100 UV-visible spectrophotometer (Agilent Technologies Italia). In brief, 0.025g of NFM was accurately weighted and mixed with 2 ml of deionized Milli-Q water and 1 ml of hexane, vortexed for 1 min and centrifuged (15 min, 2800 x g at 20°C). measurements were immediately performed in a 0.6 ml aliquot taken from the upper phase at 641 nm. A calibration curve of β-carotene (0.2-1 mmol/L) was prepared and results were expressed as mg/g sample. For comparison, the same stability study was performed also on a β-carotene dispersion (2.5 g/L in ethanol) that was stored under the same conditions as for the NFM samples.
3. RESULTS AND DISCUSSION

3.1 β-carotene dispersion in polyethylene oxide

Preliminary experiments evaluated the effect of β-carotene on the solution used for electrospinning. When β-carotene was dispersed in PEO solution, we observed a change in the resulting viscosity, electrical conductivity and color coordinates (Table 1).

Table 1. Viscosity, electrical conductivity, β-carotene content, average electrospin fibre diameter (davg) and colour of nanofiber membrane and solutions

<table>
<thead>
<tr>
<th></th>
<th>NFM</th>
<th>NFM_βc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viscosity (Pa s)</td>
<td>190 ± 1</td>
<td>195 ± 1</td>
</tr>
<tr>
<td>Conductivity (μS/m)</td>
<td>39.6 ± 1.7</td>
<td>90.0 ± 1.2</td>
</tr>
<tr>
<td>β-carotene (g/L)</td>
<td>-</td>
<td>2.57 ± 0.2</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L*</td>
<td>23.17 ± 0.26</td>
<td>31.39 ± 0.55</td>
</tr>
<tr>
<td>a*</td>
<td>-0.07 ± 0.02</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>b*</td>
<td>-0.09 ± 0.03</td>
<td>8.06 ± 0.36</td>
</tr>
<tr>
<td><strong>Fibre</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Davg (nm)</td>
<td>320 ± 46</td>
<td>230 ± 21</td>
</tr>
<tr>
<td>β-carotene (mg/g)</td>
<td>-</td>
<td>40.2 ± 5.1</td>
</tr>
<tr>
<td>Colour</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L*</td>
<td>95.67 ± 2.4</td>
<td>89.35 ± 4.62</td>
</tr>
<tr>
<td>a*</td>
<td>0.07 ± 0.02</td>
<td>-0.60 ± 0.28</td>
</tr>
<tr>
<td>b*</td>
<td>-0.47 ± 0.09</td>
<td>6.19 ± 3.02</td>
</tr>
</tbody>
</table>

After the addition of β-carotene, the conductivity of PEO solution significantly increased from 39 to 90 μSm (p>0.05). Apparently, the presence of conjugated C-double bonds imparts a significant doping effect to the solution (Lotfy & Fawzy 2014). Such higher electrical conductivity of PEO solution reflects the mobility of β-carotene. Accordingly, a decrease in the viscosity would be expected. Instead, after the dispersion of β-carotene,
the viscosity of the solution slightly increased from 190 to 195 Pa s. As observed also with other organic compounds, when non polar species are mixed into protic solvents (i.e. water), the resulting dispersion or emulsion shows a greater viscosity (Mit-uppatham et al. 2004), likely because the viscoelastic forces of the solvent-particle interaction prevail to the coulombic forces.

3.2 Morphological characterization of NFM

PEO solution, with and without the dispersion of β-carotene particles, were next used to prepare nanofibrous membranes by electrospinning. The optimal applied potential was 25 kV, that allowed the collection of uniform nanofibers. The PEO solutions containing β-carotene particles yielded fibers with smaller mean diameter (230 ± 21 nm) compared to the fibers obtained with the control PEO solution (320 ± 46 nm). The smaller diameter was explained by the higher conductivity of the solution. The presence of β-carotene increased the charge repulsion and enhanced the stretching phenomena of the electrospinning process. As a result, smooth and thinner fibers were obtained. Finally, the presence of β-carotene into the NFM affected the color (Figure 1).

![Figure 8. Images of NFM (a) and NFM-β-carotene (b) after 24h hours of the electrospinning](image)

L* value decreased when β-carotene was incorporated in the fibers (Table 1), indicating a slight decrease in lightness. In addition, the significant increase in the b* chromatic coordinate, indicates a stronger yellow color of the membrane, while a* coordinate did not
show any significant change. Similar results were observed when zein fibers were used to encapsulate β-carotene (Fernandez et al. 2009).

3.3 Content of β-carotene encapsulated into NFM

Next experiments aimed at characterizing the content of the β-carotene on the surface of NFM. At this purpose, infrared spectroscopy (ATR-FTIR) was used to characterize the NFM before and after the encapsulation of β-carotene (Neo et al. 2013; Fernandez et al. 2009; Pielichowski & Flejtuch 2005; Kriegel et al. 2016). Without β-carotene, the resulting absorption spectra shows the typical stretching bands of PEO, such as C-O (from 800 to 1400 cm\(^{-1}\)), C-C (1400-1600 cm\(^{-1}\)) and C-H (2800-3200 cm\(^{-1}\)) (Figure 2).

Instead, when β-carotene was encapsulated, a negligible increase of the absorption intensity appeared in the regions from 500 to 750 cm\(^{-1}\) and from 1500 to 1850 cm\(^{-1}\). The low absorption intensity of β-carotene suggests that only a slight amount of carotenoid is located on the surface of NFM.

![Figure 2. FTIR spectra for the PEO-fibres (1), PEO-β-carotene-fibres (2) and β-carotene (3). 500-750 cm\(^{-1}\) bands (a), 800-1,400 cm\(^{-1}\) bands (b), 1,400-1,600 cm\(^{-1}\) bands (c), 1,500 to 1,850 cm\(^{-1}\) (d), and 2,800-3,200 cm\(^{-1}\) bands (e)](image-url)
However, the amount of β-carotene encapsulated inside the NFM resulted in a concentration of 40.2 ± 5.1 mg/g of fiber (Table 1). The large loading of β-carotene inside the NFM together with the low surface absorption intensity observed by FT-IR suggests a centripetal distribution of β-carotene, where the highest concentration is in the center of the fiber. Such confinement, likely due to the hydrophobicity of β-carotene, is desired, as it would create a barrier against oxygen and a protection against thermal decomposition processes.

3.4 Stability of β-carotene encapsulated into NFM by DSC and TGA

Thermal analysis was used to investigate the stability of β-carotene and the thermal behavior of PEO. Figure 3a shows that the melting temperature of NFM/β-carotene is higher (68°C) than the one observed for NFM alone (62°C). The effect of β-carotene on the melting point of NFM seems analogous to the observed increase in viscosity of PEO solution. Apparently, β-carotene reduces the mobility inside the resulting PEO process, resulting in a higher melting.

The thermograms of NFM/β-carotene proceed flat until a further endothermic process (400°C) appears, corresponding to a thermal decomposition reaction, the DSC of β-carotene in powder form shows a large endothermic process at about 180°C which was not visible when β-carotene was encapsulated inside the NFM.

Since decomposition reactions are generally accompanied by loss of weight, a thermogravimetric analysis (TGA) was next performed to describe the decomposition of the sample. NFM and NFM/β-carotene shows a unique weight loss step at about 400°C (Figure 3b), associated with the decomposition of PEO (Pielichowski & Flejtuch 2005; Erceg 2010). Instead, β-carotene (in powder form) started losing weight at about 120°C and proceeds towards a series of consecutive and overlapping decomposition processes. However, such decomposition steps are not visible when β-carotene is encapsulated in NFM. Tentatively, this supports the thermal protective effect of NFM on β-carotene.

The same analysis was repeated under oxidative atmosphere (air flow 100 ml/min). As expected, the onset of the mass loss appeared earlier (~200°C) because of the rise of
oxidative degradation reactions. Again, the contribution of β-carotene to these traces was not distinguished from that of NFM of NFM/β-carotene.

Figure 3. Thermograms DSC and TGA. 3a: DSC thermograms under inert atmosphere (N2) β-carotene (a), PEO-fibres (b) and PEO-β-carotene-fibres (c). 3b: TGA thermograms under inert atmosphere (N2) (grey lines: β-carotene (a), PEO-fibres (b) and PEO-β-carotene-fibres (c)), and air, (black lines: β-carotene (d), PEO-fibres (e) and PEO-β-carotene-fibres (f)) in a temperature range of 40°C up to 600°C at 10 °C/min
3.5 Stability of β-carotene encapsulated into NFM by evolved gas analysis

The weight losses observed in the previous experiments should be the consequence of the emission of the some volatile compound leaving the sample. From the analysis of the gas evolved from the TGA experiment, it would be possible to determine if a degradation product of β-carotene is produced and at what temperature.

Accordingly, the total volatile organic volatile compounds (VOCs) evolved during the TGA experiment were next analyzed by connecting the outlet of the TGA furnace with the injection port of a mass spectrometer (PTR-MS).

Figure 4 shows the resulting profile of the total VOCs emission per mg of sample as a function of time. When the powder forms of β-carotene were analyzed, a maximum of VOC emission was absorbed at about 180°C. This temperature corresponds to that observed by DSC for the thermal decomposition of β-carotene in powder form (see Figure 3). On the other hand, when β-carotene was encapsulated into NFM, the emission of volatiles was observed only at higher temperatures, not before than 350°C for experiments performed in air atmosphere or 395°C for inert atmosphere.

![Figure 4. Volatile Organic Compounds (VOC) emissions during thermal degradation in a temperature range of 40°C up to 600°C at 10 °C/min detected by PTR-MS: volatiles detected during β-carotene degradation under air and inert atmosphere (1) and (2); volatiles detected during degradation of the β-carotene encapsulated in the membranes under air and inert atmosphere (3) and (4). TGA Thermograms under air and inert atmosphere (β-carotene (5) and (6); PEO-βc-fibres (7) and (8))](image-url)
A more detailed analysis of the fragments detected by PTR-MS allowed monitoring the time-evolution of individual fragments, such as m/z 127 and m/z 153. Previous works showed that during the thermal degradation of β-carotene, the fragment m/z 127 was related to 2-methyl-2-hepten-6-one and 2-methyl-2-hepten-4-one (boiling point 178°C), whereas the fragment m/z 153 was related to β-cyclocitral (boiling point 212°C) (Berset 1990; Onyewu et al. 1982; Crouzet 1990).

![Graph](image)

Figure 5. m.z. 127 and m.z.153 emissions during thermal degradation in a temperature range of 40°C up to 600°C at 10 °C/min detected by PTR-MS: emissions detected during β-carotene degradation under air and inert atmosphere (1) and (2); emissions detected during β-carotene encapsulated in the membranes degradation under air and inert atmosphere (3) and (4). Thermograms under air and inert atmosphere (β-carotene (5) and (6); PEO-βc-fibers (7) and (8)).
Figure 5 shows their specific evolution during the TGA analysis of powder of β-carotene. The fragment m/z 127 was released at 178°C, followed by the fragment m/z 153 that was released later (~210°C). However, when β-carotene was encapsulated inside the nanofibers, the emission of those two ions was detected only later (at 390°C for both ions under oxidative atmosphere and after 410°C for both ions under inert atmosphere). This experiment confirms that β-carotene is more stable when encapsulated in NFM than as a powder.

3.6 Stability of encapsulated β-carotene by UV-VIS

The storage stability of a β-carotene solution was compared with the stability achieved by β-carotene once encapsulated into the NFM. The results are shown in Figure 6. The solution of β-carotene lost about 70% of β-carotene in less than 96-h of storage. Instead, when β-carotene was encapsulated inside the NFM, the loss was limited to 20%. The enhanced stability of β-carotene once encapsulated inside NFM reflects the capacity of the polymer to limit the diffusion of oxygen and reduce the light exposure (Fernandez et al. 2009; W. von E. Doering 1995). This result confirms that the encapsulation of β-carotene slows down the degradation process and supports the previous results obtained with DSC, TGA and total VOC.

Figure 6. Stability of β-carotene during 4 weeks (w) of storage. Evolution of β-carotene encapsulated in the membrane expressed as ΔC (%). Evolution of the absorbance at 461 nm of a β-carotene dispersion (2.5 g/L in ethanol) stored under the same conditions as nanofibers. Evolution of colorimetric coordinate b* of the membranes.
4. CONCLUSION

Thermogravimmetrical analysis coupled with PTR-MS as well as color and stability analysis in this work, demonstrated that β-carotene can be encapsulated by electrospinning as a method to fabricate nanofibrous structure that will prolong shelf life and thermal stability. Electrospinning is thus reported here as a technology suitable for producing added-value PolyEthylene Oxide micro and nanofibers with good potential in food packaging and food processing industries. As a novelty, TGA coupled with PTR-MS was proved to be a suitable and promising technique to monitor the thermal degradation processes in real time. Further research in this field might involve encapsulation of other bioactive compounds with thermal degradation produces specific volatiles in contrast with those produced by the encapsulation material.
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GENERAL CONCLUSION

The first part of the research was focused on the development of a novel Enzymatic Membrane Reactors (EMRs), by the use of a nanofibrous membrane as a support, for using in food processing.

In particular, the immobilization of Glucose oxidase (GOX) in a Nylon 6 nanofibres membrane (NFM) in addition of carbon multiwalled nanotubes (MWCNT), showed a good reproducibility over more than one month of utilization. This system is endowed with qualities such as a very low cost of production, storage resistance and fast response time in the linear range (10-20 s). Moreover, it displays a complementarity with other more classical analytical methods such as DNS and Brix. Together, they allow to investigate the evolution of glucose with respect to other sugars. Still, both DNS and Brix are not sufficient to account on the evolution of specific sugar in matrices as complex as beer. Conversely, the biosensor shows a complete insensitivity towards other mono- and oligo-saccharides even at concentration equal or higher than glucose. The sensitivity of the biosensor in the linear range was evaluated to be 12 µA.mM-1 with an evaluated limit of quantitation of 20 µM. Moreover, the kinetic of the immobilized GOX was evaluated with respect to free GOX. Michaelis Menten model (M.M.) failed to actually fit the data therefore the more general Hill equation was employed. Notably, the same effect was not observed with free GOX, where M.M. could be successfully applied. As future developments, the enzyme life and the enzymatic performances could be extended by preventing detachment from the nylon NMF. This research has demonstrated the suitability of the proposed biosensor for the monitoring of very complex fluids such as those deriving from the brewing process. Accordingly, the findings of this study may pave the way to a number of important applications for future practice. For instance, the proposed biosensor will be useful in the quantification of glucose in fruit juice,(Scampicchio, Arecchi, Lawrence, et al. 2010) fermentation process of fruit must,(Piermarini et al. 2011) or even in the development of electronic tongue method for the objective evaluation of sweetness in beverages.(Bulbarello et al. 2012) The possible applicability of the biosensor seems limited only by the presence of other redox species, such as ascorbic acid, that, when present at high concentrations, may interfere with the
mediated biosensing mechanism. However, even in such unfavorable circumstance, the
detection of glucose is still possible, provided that the sample is thoroughly aerated before
the analysis, as ascorbic acid reacts quickly and irreversibly with oxygen.

The Nylon 6 NFM was also used for the covalent immobilization of the invertase, probably
the mayor enzymes used in the food processing for the production of inverted sugar, after
the activation with an alkaline Glutaraldehyde solution (GA) and the use of
PolyEthylениmine (PEI) as used as a spacer. The data demonstrate that immobilized
invertase has increased enzymatic and thermal stability compared with the free enzyme
in solution. However, the increase in thermal stability only results in a modest increase in
the stability of the enzyme activity at 25°C. The immobilized enzyme can be reused
(unlike free invertase); however, there is a loss in activity over time. We also established
for the first time, to our knowledge, that ITC and DSC can be used to characterize
immobilized enzyme activity.

Although, ITC can be used to measure enzymatic activity, this has been a relatively minor
focus of its capabilities. The advantage of ITC is that it is not dependent on a secondary
reaction or on the transparency of the media. Therefore, reactions using enzymes that
are immobilized and/or novel, which can be difficult to characterize because they are
opaque or no standard reaction has been developed, respectively, are can be measured
by ITC. As an aside, we discovered how sensitive immobilized invertase is in suboptimal
conditions because of 40-minute equilibration time for ITC experiments.

In summary, we have immobilized invertase and demonstrated that with further
modification it could be useful in industrial applications. We have also developed a new
tool for measuring the enzymatic activity of immobilized enzymes; namely ITC.

The second part of this work shows other applications of the NFM in the food processing.

In this study was presented the capability of filtration using produced electrospun NFMs
of bacteria and yeast cells removal in lab scale dead end constant rate filtration (DEF).
The DEF of suspended cells in beer arrive a complete removal of bacteria F. johnsoniae,
6.6 log reduction of mixed bacteria strain (F. johnsoniae and L. fluviatilis) and 4.6 log
reduction value of yeast cells. The results obtained show that these porous filter
membranes played an important role for the removal of microbial cells from water/broth and beer suspensions.

The rapid analysis of volatile compounds in the headspace of six Arabica coffees powder, was performed with PTR-QMS, Nylon 6 NFM are used for explores the capability of electrospinning nanostructures, characterized by a very high surface to volume ratio and high porosity (Zhang et al. 2012), to extract the aroma compounds from the samples. Principal component analysis allowed visualizing a separation according to geographic origin in both case. Overall, these results demonstrate the capability of nylon nanofibrous membranes to extract and release aroma compounds under mild conditions and in sample-specific fashion. In perspective, these materials could be employed in the manufacture of active packaging materials or analytical cartridges.

Finally, Thermogravimetrical analysis coupled with PTR-MS as well as color and stability analysis, demonstrated that β-carotene can be encapsulated by electrospinning as a method to fabricate nanofibrous structure that will prolong shelf life and thermal stability. Electrospinning is thus reported here as a technology suitable for producing added-value PolyEthylene Oxide micro and nanofibers with good potential in food packaging and food processing industries. As a novelty, TGA coupled with PTR-MS was proved to be a suitable and promising technique to monitor the thermal degradation processes in real time. Further research in this field might involve encapsulation of other bioactive compounds with thermal degradation produces specific volatiles in contrast with those produced by the encapsulation material.

In conclusion, this thesis has investigated new way to use nanofibers membranes obtained by electrospinning in food processing. In particular, this inert support was used for the immobilization of enzymes for the production of biosensor and new enzyme membrane reactors. The same support was used for development a new filter membrane for the reduction of microorganisms, adsorption of the Volatile Organic Compounds (VOCs) of the coffee aroma and for encapsulation and increase the shelf-life of bioactives.
List of publications

Peer-reviewed journals papers included in the thesis:


Peer-reviewed journals papers not - included in the thesis:


- Valentinuzzi Fabio, **Mason Marco**, Scampicchio Matteo, Andreotti Carlo, Cesco Stefano, Mimmo Tanja. “*Enhancement of the bioactive compound content in strawberry fruits grown under iron and phosphorus deficiency*”. *Journal of the Science of Food and Agriculture* (2014). DOI: 10.1002/jsfa.6924

- Martha Cuenca, Benjamin Romen, Giacomo Gatti, **Marco Mason**, Matteo Scampicchio. “*Microcalorimetry as a Tool for Monitoring Food*
Fermentations”. Chemical Engineering Transactions, Vol. 57, 2017. DOI: 0.3303/CET1757327

Submitted papers:

- Irene Peinado Pardo, Marco Mason, Franco Biasioli and Matteo Scampicchio. “Hyphenation of Proton Transfer Reaction Mass Spectrometry with Thermal Analysis (TG/PTR-MS) for Monitoring the Thermal Degradation of Retinyl Acetate”. Submitted to Talanta in April 2017

Conference presentations

Oral presentations:

- Marco Mason, S.M. Kamrul Hasan, Lara Manzocco, Alexandra Ignat, Stefano Cesco, Tanja Mimmo, Matteo Scampicchio, Maria Cristina Nicoli. Comparison of dipping treatments and pulsed light on fresh cut apples by microcalorimetry. European Society of New Methods in Agricultural Research, ESNA 2014, 3 – 6 September 2014, Bolzano (Italy)

- Marco Mason, Andrea Romano, Luciano Navarini, Franco Biasioli and Matteo Scampicchio. “Nanofibrous membrane for aroma compound extraction and release: a case study on coffee”. 2° International conference on Food and Biosystem Engineering, FABE 2015, 28-31 May 2015, Mykonos island (Greece)

Poster presentations:

• Marco Mason, Tanja Mimmo and Matteo Scampicchio. “Monitoring of fermentation metabolism of different commercial yeast in apricot must by microcalorimetry”. TA Instruments 2015 Users Meeting and Symposium, 15-18 February 2015, San Antonio, TX (USA)

Other activities:

• Summer school of Calorimetry, Calorimetry and thermal methods in catalysis, IRCELYON, 22 – 27 June 2014, Lyon (France)

• Workshop on the developments in the Italian PhD Research on Food Science, Technology and Biotechnology, 24 – 26 September 2014, Bari (Italy)

• 8th International Advanced School, Reaction kinetics in food science, The Graduate school VLAG, 20 – 24 October 2014, Wageningen (the Netherlands)

• Workshop on the developments in the Italian PhD Research on Food Science, Technology and Biotechnology, 23 – 25 September 2014, Perugia (Italy)

• Convegno “Le nuove sfide della sicurezza alimentare: gestione del rischio microbiologico lungo la filiera in un mercato globalizzato” organized by bioMérieux Industry and Istituto Zooprofilattico Sperimentale Lombardia e Emilia Romagna

• Workshop on ITC at The Seventy First Calorimetry Conference (CALCON 2016), 30-31 July 2016, Turtle Bay, Oahu, Hawaii, USA

• The Seventy First Calorimetry Conference (CALCON 2016), 1 - 4 August 2016, Turtle Bay, Oahu, Hawaii, USA
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**Critica personale al mondo accademico**

Vorrei aggiungere alla mia tesi, come espressione del mio pensiero, una particolare critica (in italiano perché’ non vorrei essere mal interpretato) al mondo accademico, soprattutto italiano, che ho avuto il piacere di conoscere in questi anni. La mia polemica è rivolta soprattutto al sistema delle pubblicazioni che viene usato per valutare sia i professori e le università a livello mondiale. Io non penso che questo sistema sia assolutamente il più adeguato e che porti alla riduzione della qualità della ricerca. Penso che degli emeriti professori con molti anni di esperienza e pubblicazioni alle spalle dovrebbero trovare il coraggio di ricercare metodi alternativi (per esempio: valutando quanti studenti sono in grado di trovare un lavoro stabile dopo la laurea, quante collaborazioni hanno i professori con aziende o enti locali, come vengono valutati i professori dagli stessi studenti, ecc.) per valutare i ricercatori e gli stessi professori.

Vorrei riportare la vostra attenzione, su una cosa che penso sia capitata a tutti, a chi non è mai capitato di replicare quello che ha trovato scritto in un articolo e che questa cosa non funziona. Non pensate forse che questo sia una perdita di tempo e soldi, la maggior parte delle volte, pubblici?

Non credo sia possibile pubblicare molto spesso, dei buoni dati scientifici, senza che questi a volte vengano inesorabilmente falsificati per dimostrare qualsiasi cosa che passa in mente ai professori e ricercatori di tutto il mondo e che il più delle volte non hanno nessuna relazione con la realtà. Non credo più, quando le persone mi dicono, che l’università deve pensare a quello che succederà tra 10 o 20 anni.
Io penso che questo sistema non rappresenti l’espressione del pensiero scientifico originale ma è dapprima un business per le case editrici di questi giornali e penso che la cosa peggiore che questo venga usato come metro di giudizio fondamentale per selezionare i professori e i ricercatori di tutto il mondo. Non penso che questo sistema sia molto obiettivo e valuti veramente le capacità personali ma di quanto a volte la persona è in grado di dimostrare (a volte solo a parole, quello che è capace di fare). Io penso che sia arrivato il momento per dire basta a questo schifo che ha rovinato la ricerca scientifica negli ultimi anni e penso che oggigiorno il mondo abbia dei problemi molto più seri e che la ricerca scientifica ha i mezzi e le conoscenze per aiutare a risolverli.

Questo è il mio pensiero che ha preso ormai forma in questi 4 anni passati a lavorare in università e che mi hanno definitivamente convinto di non voler più continuare su questa strada, fino a quando questo sistema non venga sostituito da qualcosa di più onesto che possa veramente classificare le persone in base a quello che sanno fare e non per quello che sanno dire.