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**Preparation of living bacteria containing biomaterials and their quality
analysis**

Bachelor's Thesis (12 ECTS)

Curriculum Science and Technology

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Tartu 2021

Preparation of living bacteria containing biomaterials and their quality analysis

Abstract:

The aim of the thesis was to encapsule living bacteria into functional biomaterials using two different methods: mono- (*Escherichia coli* Nissle, *Lactococcus lactis*) and coaxial (*Escherichia coli* with Timer plasmid) electrospinning (ES). The quality analysis of biomaterials was based on bacterial viability and fiber morphology and consequently, the most optimal parameters for these methods were established. The addition of lyoprotectant trehalose dihydrate or Casamino acids before ES increased fiber diameters and only slightly improved the bacterial viability. Notably, the encapsulation of bacterial cells in the solution into the fibers increased the fiber diameters and their concentration affected the electrospinnability of the solution.

Keywords: Electrospinning, encapsulation, probiotics, viability

CERCS: B230 Microbiology, bacteriology, virology, mycology; T490 Biotechnology

Elusaid baktereid sisaldavate biomaterjalide valmistamine ja nende kvaliteedi uurimine

Lühikokkuvõte:

Käesoleva töö eesmärk oli valmistada biomaterjal enkapsuleerides elusad bakterid kahel meetodil: mono- (*Escherichia coli* Nissle, *Lactococcus lactis*) ja koaksiaalne (*Escherichia coli* Timer plasmiidiga) elektrosppinnimine (ES). Biomaterjalide kvaliteedi määramine sõltus nii bakterite elujõulisusest kui ka fiibrite morfoloogiast. Vastavalt saadud tulemustele tuvastati kõige optimaalsemad ES parameetrid. Lüoprotektandi trehaloos dihidraadi ja aminohapete hüdroolüsaadi (CAA) lisamine enne ES suurendas fiibrite läbimõõtu ja ning veidi ka bakterite elulemust. Sellele vaatamata, kõige rohkem suurendas fiibri läbimõõtu enkapsuleerimine fiibrisse ning bakterite kontsentratsioon mõjutas oluliselt lahuste elektrosppinnitavust.

Võtmesõnad: elektrosppinnimine, enkapsuleerimine, probiootikumid, elulemus

CERCS: B230 Mikrobioloogia, bakterioloogia, viroloogia, mükoloogia; T490 Biotehnoloogia

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TERMS, ABBREVIATIONS, AND NOTATIONS

ES	electrospinning
PEO	polyethyleneoxide
PCL	polycaprolactone
PDLA	poly-D-lactide
DMSO	dimethylsulfoxide
LB	lysogenic broth
PBS	phosphate saline buffer
DDS	Drug Delivery System
SEM	scanning electron microscopy
FDA	U.S. Food and Drug Administration
EMA	European Medicines Agency
GFP	Green fluorescent protein

INTRODUCTION

There is an ample of different applications an electrospun biomaterial can have from tissue engineering scaffolds, drug delivery to wound dressings.

One of the methods for the production of functionalized and smart biomaterials is electrospinning. Electrospinning is a versatile method used in the field of biotechnology to produce fibers from micro- to nanoscale, which enables to not only encapsulate antimicrobial drugs but also microbes, like bacterial cells. It is said that electrospun nanofiber mats are akin to the natural extracellular matrix (ECM), having features such as high surface area to volume ratio, tunable porosity, sufficient gas-exchange which all refer to a viable treatment for wounds. (Pant *et al.*, 2019) If alive bacteria can be incorporated into nanofibers in tolerable and soft conditions, these bacterial cells can potentially produce locally relevant antimicrobial agents for the treatment of wound infections. (alleviating the issue of excessive drug residues and resistance to antibiotics)

Previous studies have shown that harsh conditions during traditional electrospinning, such as toxic organic solvents, results in poor bacterial viability during and after electrospinning. Hence, different approaches need to be investigated that should improve the viability and functionality of bacteria. For the preparation of electrospun functionalized biomaterials, careful selection of suitable materials, type of bacteria, electrospinning conditions and possible stabilization methods prior and/or during electrospinning needs to be performed in order to i) preserve the viability and functionality of the living cells and ii) and be biocompatible.

The first part of the study covers biomaterials, different methods of encapsulation of bacteria, several polymers tested for electrospinning and the description of electrospun biomaterials. The second part focuses on analyzing and discussion of experimental results.

The general aim of the research was to design and develop electrospun smart and functional biomaterials that contain living bacterial cells.

This research work is part of the Estonian Research Council funded research project PRG726.

1 LITERATURE REVIEW

1.1 Biomaterials

The word “biomaterial” has many definitions, the conventional meaning addresses the biomaterial as a non-living material implemented in medical devices (e.g. artificial ligaments and tendons). (Tatthe *et al.*, 2010) However, as the technology of biomaterials has progressed over time, the definition can also incorporate substances that control the biological environment of cells and tissues. (Naderi *et al.*, 2011) Biomaterial-based 3D systems have become a major application in cell-cell and cell-material communications. The design of these systems enables to provide quantitative adhesion, growth, and migration signals of a biological microenvironment, as shown in the field of myocardial tissue engineering. (Davis *et al.*, 2009)

The first biomaterials were applied in medicine in the 1940s and 1950s. In 1960, a seminal paper by Wichterle O, Lim D. entitled “Hydrophilic gels for biological use” published in Nature described the properties and chemical design that might be desirable for a biomaterial, accidentally launching hydrogels as we know them today. (Ratner, 2019)

1.2 Properties and applications of biomaterials

“Biocompatibility” is a crucial term in the field of biomaterials, as that is the first requirement a biomaterial should fulfill. This property first appeared in literature around 1970, when CA Homsy *et al.* first shed light on why a material should be biocompatible when used for implantation. (Ratner, 2011) Throughout the years, the definition has been tailored to suit a wider range of fields and is said to be the ability of a material to perform with an appropriate host response in a specific application. (Ratner, 2019)

In the 1960s and 1970s, biomaterials were largely inert and “nonresponsive”, and typically designed to be biostable. Polyethyleneoxides (PEO), silicones, polyurethanes, fluoropolymers, and inert metals and ceramics dominated the biomaterials literature. Towards the late 1970s, researchers began to understand the potential of designing biomaterials so that they perform their intended function and are then resorbed into the body. An early example of a biodegradable polymer in the clinical field was the DexonTM suture, which was made from poly(glycolic acid) (PGA) polyester. Alongside, other polymers, like poly(lactic acid)

(PLA), polycaprolactone (PCL), copolymers started to be developed for medical use. It should be highlighted that the aforementioned polymers degrade to small, non-toxic molecules by hydrolysis of ester bonds. The non-toxicity (and U.S. Food and Drug Administration (FDA) approval) of a biomaterial is another important property that is considered when using it in real-life applications. (Ratner, 2019)

In the 1980s, the design of biomaterials became more versatile, as focus shifted more towards degradability, microenvironmental responsiveness, and controlled biointeraction with cells and proteins. (Ratner, 2019)

Thus far, biomaterials have been applied in a vast proportion of the medical field. There are many examples of application, including, but not limited to, (surgical) adhesives, breast implants, ocular prosthesis (globe replacement), sutures, additionally in drug delivery systems (DDS), and burn/wound dressings. (Ratner, 2019; Tathe *et al.*, 2010)

1.2.1 Wound dressings

Wounds can be divided into many groups and each category has its own healing requirements. (Kujath and Michelsen, 2008) This insight has propelled the development of a variety of different wound dressings, each with specific features.

As seen in **Table 1**, wound dressings can be divided into three main categories based on their mechanism: inactive, interactive, and (bio)active wound dressings (graft materials). Furthermore, **Table 1** provides an overview of the description and expected mechanism of action of wound dressings.

Inactive or passive wound dressings are known for their high absorbency. They are comprised of cotton (gauze compresses), synthetic fibers (non-wovens), or multiple layers of material (wound gauzes). To maintain humid conditions, the compresses are saturated in a physiological saline (0.9% NaCl concentration) solution and covered with a waterproof film. The positive aspects of these conventional dressings are their absorbency and their low cost. Disadvantages are claimed to be the possible desiccation of the wound and adhesion to the wound bed. (Kujath and Michelsen, 2008)

Interactive wound dressings can be made of alginates, hydrofiber dressings, silver-containing dressings, hydrogels, hydrocolloids, hydropolymer dressings, foam dressings, impregnated gauzes. All the interactive wound dressings need to fulfill the following criteria:

maintenance of a moist environment, pH of 5.5, absorption of toxic components of bacterial degradation products, protection against secondary infection, gentle painless dressing change, and practicability. (Kujath and Michelsen, 2008)

Bioactive wound dressings also entail autologous skin, lyophilized porcine skin, autologous keratinocytes, collagen-based wound dressings, and use of growth factors. (Kujath and Michelsen, 2008) Akin to interactive dressings, bioactive dressings can modify the physiology of the wound environment and interacting with the surface to optimize healing, by promoting debridement, enhancing granulation, and re-epithelization. The main advantage is that these enable to maintain an optimal environment for wound repair. The downside of these products is the high cost and low stability (Preem and Kogermann, 2018) For chronic wounds, more complex wound dressings are usually required (Dhivya *et al.*, 2015) and thus, drug-loaded bioactive dressings have been created.

Table 1. Wound dressings with their description and mechanism of action [modified (Preem and Kogermann, 2018)]

Wound dressings	Materials	Description	Mechanism of action/application
Passive (inactive)	Cotton (gauze compresses), synthetic fibers	Non-occlusive. Covering the wound to restore its function.	No regulatory function, high absorbency. For superficial acute wounds.
Interactive	Hydrogels, hydrocolloids, semipermeable polymeric films and foams	Semiocclusive, highly elastic. Barrier against bacterial contamination, permeable to water vapor and oxygen.	Control of moisture level. For burn wounds, chronic wounds, superficial wounds and dry wounds.
Bioactive	Collagen, gelatin, chitosan, growth	Semiocclusive or occlusive highly elastic. Barrier	Delivering bioactive substances that assist wound healing,

	factors, enzymes, phages, skin grafts	against contamination.	the material has endogenous activity. Control of moisture level. Infected wounds, burns, chronic wounds (pressure/diabetic foot/venous ulcers)
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1.2.2 Polymers used for wound dressings

The European Wound Management Association has described a strategy called TIME:

T (tissue management) – wound cleaning, I (infection of inflammation) – decolonization and inhibition of infection, M (moisture imbalance) – humidification, E (edge of a wound, epithelium) – epithelization support. (Skórkowska-Telichowska *et al.*, 2013) These objectives are reachable with the appropriate wound dressings and hence polymers.

When a biomaterial is placed in a physiological environment, it will partake in different reactions (cell-material), thus one should select the right material which can create a suitable environment for the cell. This healing process is induced by providing protection from infection, thereby enhancing the activity of enzymes to promote epithelialization. (Mir *et al.*, 2018)

That being said, physical properties like thickness, porosity, density, and fluid absorption capacity of wound-healing materials, are of major importance in providing optimal parameters for the wound to heal. (Mir *et al.*, 2018)

Based on the experiment by Lee *et al.* in 2016 (Lee *et al.*, 2016), it can be concluded that small pore size with limited variability improves the fluid absorption capacity of the dressing – it inhibited the movement of phosphate buffered saline (PBS) through the material compared to the other tested materials.

Biopolymers, such as animal fats, animal fibers, and honey pastes were often used in the past. A couple of years later, materials like cotton wool, lint and gauzes started being used as dressings, and their main function was to keep the wound dry. (Thomas, 1990)

Winter's experiments (Winter, 1962) proved that epithelization happens twice as fast in young domestic pigs with moist wound dressings opposed to those with a dry wound dressing. The moist environment of open wounds induces cell growth, angiogenesis, and fibrinolysis. (Kujath and Michelsen, 2008)

Foam dressings became more popular in the 1970s, as compared to the traditional gauze dressings, they do not shed particles and can be left on the wound for several days without being touched. (Mir *et al.*, 2018)

Hydrocolloids are absorbent, semipermeable to vapor. They can be obtained synthetically or naturally. In practical applications polysaccharides are often used. They are known for their encapsulation properties which allows controlling of drug release. This can be done through optimization of the size of particles and the permeability of the gel membrane. Hydrocolloids are mostly utilized in healing diabetic foot ulcers. (Mir *et al.*, 2018)

Agar is one of the known hydrocolloid gels, which is gathered from special type of seaweeds, gelidium and gracilaria. The gelling of agar appears due to the double helices in the structure. Agarose and carrageenan are also hydrocolloids extracted from seaweed. Pectin is a hydrocolloid found in land based plants. The linear chains of galacturonic acid make up the basic chemical structure of pectin. Opposed to the aforementioned hydrocolloids, gelatin is obtained from collagen, an animal protein. (Mir *et al.*, 2018)

Hydrogels are made of crosslinked polymers, such as polyvinylpyrrolidone (PVP), polyacrylamide and polyethyleneoxide (PEO). Hydrogels do not dissolve in water and have the feature of swelling when in contact with water. (Mir *et al.*, 2018)

Poly(lactide-co-glycolide) (PLGA) is a blend of polylactic acid (PLA) and polyglycolic acid (PGA). This polymer is known for its biocompatibility, mechanical strength, and its ease to form it into desired shapes and sizes. (Mir *et al.*, 2018) When combined with collagen, it has been used for three-dimensional culture of human skin fibroblasts and ultimately in the process of wound healing. (Chen *et al.*, 2005)

PEO is a hydrophilic, biocompatible, nonimmunogenic, flexible ether-based polymer. (Mir *et al.*, 2018) During wound healing, a lot of cellular interactions between different cell types, such as fibroblasts, neutrophils, macrophages, take place and happens in the presence of mediators known as growth factors. (Kujath and Michelsen, 2008) One of them is an epidermal growth factor (EGF) and it has been discovered that EGF and PEG macromers

have a good affinity, thus they can be bounded together and targeted to the wound site. (Goh *et al.*, 2016)

Polyurethane (PU) consists of carbamate linkages. It acts as a semi-permeable membrane protecting the wound from its surrounding environment and bacterial entry. (Mir *et al.*, 2018)

PCL is obtained from degraded linear aliphatic polyester and autocatalyzed bulk hydrolysis. (Mir *et al.*, 2018) It is non-toxic and the electrospun PCL has features akin to the ECM and thus, can be suitable to treat acute and chronic wounds. As PCL material is not known for its antimicrobial properties, it is possible to incorporate different antimicrobial particles, for example, biosynthesized silver nanoparticles (AgNPs), into the fiber and hence, resist wound-associated infection. (Thomas *et al.*, 2015)

2 Encapsulation of living bacteria

Biodegradable microenvironments are defined as delivery systems for bioactive compounds, such as proteins (Meinel *et al.*, 2001), viruses, (Stureson *et al.*, 1999) and even bacteria. (Klein *et al.*, 2009) Encapsulation shell materials vary, depending on the core to be protected, they can be polymers, carbohydrates, fats and waxes. (Chavarri *et al.*, 2012) Microspheres provide a sustained and controlled release of the encapsulated bioactive compound while maintaining the non-released material from degradation. (Freitas *et al.*, 2005)

The employment of bacteria has great potentials to alleviate polluted water resources. The bioremediation methods vary, however, the most promising is based on immobilization of bacterial cells capable of removing specific contaminants. Encapsulation of bacterial cells has been found to improve the stability of cell enzymatic activities, the protection of biocatalysts from environmental stresses, toxic substances, and mechanical damage, and can sustain large bacterial populations over an extended period. (Klein *et al.*, 2009) In the case of storage, transportation, manipulation with encapsulated cells and separation of products, susceptibility to contamination is reduced, while plasmid stability is increased. The study of bacteria in bioremediation varies as it depends on the pollutants needed to react with – for 4-Nitrophenol (PNP) degradation, microorganisms, like different strains of *Pseudomonas*, *Arthrobacter* sp., and strain *Corynebacterium* sp. 8/3 have been an area of interest. (Kotoučková *et al.*, 1997)

For probiotics to entail a health benefit (the probiotic strains should be at suitable levels for a long period – from shelf life to consumption and digestion), the probiotic bacteria should provide physical barriers to resist harmful environmental conditions (i.e. low gastric pH). Additionally, encapsulation can promote controlled release and optimize delivery to the site of action, thereby enhancing the efficacy of the respective probiotic strain. As lactic acid bacteria (LAB) are the most important probiotics known to have beneficial effects on the human gastro-intestinal (GI) tract, the different strains of *Lactobacillus* are commonly studied and tested on. (Chavarri *et al.*, 2012)

2.1 Microencapsulation methods

There is a vast number of different methods for bacterial encapsulation, the following will introduce just a few.

2.1.1 Traditional methods

Most common encapsulation techniques include spray-drying, freeze-drying, or coacervation. (San Keskin *et al.*, 2018)

Spray-drying is most often considered as part of a dehydration process, however, it can also be used as an encapsulation method (**Figure 1**). As opposed to other conventional microencapsulation techniques, it has the attractive advantage of producing microcapsules in a simple continuous processing operation. The stages of spray drying are atomization, mixing of spray and air, evaporation, and product separation. (Kata *et al.*, 1983) In short, this method transforms liquid into dry powder in one step.

Despite the positive aspects of low cost, short cycle time, the probiotic cells encounter different stresses which impede their viability and functionality. The different stresses during spray-drying include thermal stress, dehydration, shear stress, osmotic and oxidative stress. (Broeckx *et al.*, 2016)

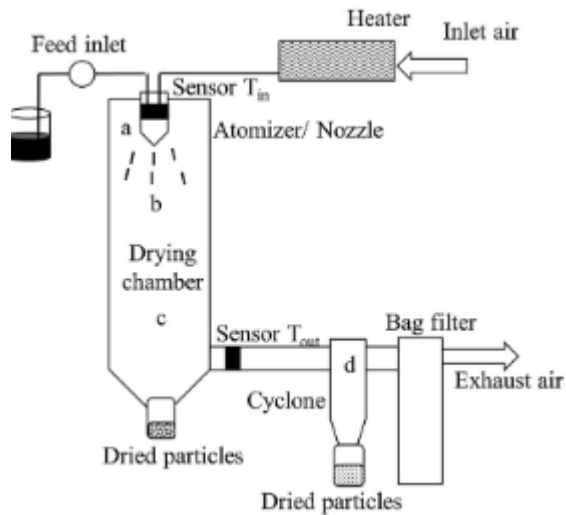


Figure 1. Spray drying process (Broeckx *et al.*, 2016)

Freeze-drying or lyophilization is the removal of water to enhance the storage stability of probiotics. During freezing, ice crystals form which can damage the probiotics. The growth of the ice crystals is dependent on the freezing rate and temperature. A high freezing rate is preferred, as it will lead to the formation of smaller ice crystals, avoiding extensive cellular damage. Next to ice crystals, osmotic and chemical damage is prevalent in this method. (Broeckx *et al.*, 2016)

Coacervation (also known as phase separation) is the precipitation of a polymer or several polymers by phase separation. It is induced by adding a non-solvent (coacervating agent), generally, salts, that have a higher affinity to water than the polymer. This is essentially a dehydration process, whereby polymer-rich coacervate droplets are separated from the polymer-poor region. This dispersion is then transferred into a hardening agent, where the solid microspheres are formed. The downside of this method is that most of the substances used as solvents are harmful and may cause toxicological risk in the microspheres. (Thomasin *et al.*, 1996)

However, these systems often output thermal and mechanical stresses, leading them to the potential destruction of encapsulated nutrients/organisms, hence these are not suitable for further mass production. (San Keskin *et al.*, 2018)

2.1.2 Electrospinning versus electrospraying

Although there are many other plausible methods for microencapsulation, electrospinning is often preferred, as it is a straightforward, cost-effective technique to create a fibrous

membrane. In short, it applies an electric field on a polymer solution, in which the jet is split into fibers upon electrostatic force and then assembled into a nonwoven membrane with fibers of micro- to nanometer range diameters. (Han *et al.*, 2018) Despite the process seeming simple, there is an ample of different parameters that can be modified – different materials for spinning, processing (voltage, flow, distance), and environmental parameters (temperature, humidity) (Preem and Kogermann, 2018)

Nanofibers have mostly been investigated for the delivery of small drug molecules – antibacterials or antifungal agents (Krogstad *et al.*, 2017) antioxidants, and anti-inflammatory agents (Khoshnevisan *et al.*, 2018). However, recently electrospinning has also become an alternative to integrate microbial cells into nanofibers. (Škrlec *et al.*, 2019)

A considerable alternative to the previous method is electrospraying. Electrospraying, also known as electrodynamic spraying is capable of producing droplets with submicron sizes utilizing an electric field. The common setup is rather identical to the setup for electrospinning - high-voltage power supply, syringe capped with a metallic needle to hold a polymer solution, a syringe pump to control the flow of the solutions, and a grounded collector. (Wang *et al.*, 2019)

As this method enables to produce small capsules or biocarriers, researchers have proposed this technique to protect the cell viability of different bacteria, like the probiotic *Bifidobacterium*. Compared to electrospinning, this method demonstrated a prolonged survival of storage at different temperatures and humidities. (López-Rubio *et al.*, 2012)

Compared to electrospinning, the viscosity of the electrosprayed polymer solution is less viscous, as electrospinning requires a rather polymer solution for fiber formation. It has been also argued that the organic solvents used for spraying may damage the bioactivity of cells, whereas in electrospinning the incorporation of bacteria would physically protect it from external threats. Additionally, it has been objected that electrospraying has some issues with upscaling the process and thereby having a commercial value. (Wang *et al.*, 2019)

2.1.2.1 Traditional electrospinning

For bacterial encapsulation, traditional electrospinning is generally succeeded via initially having two experiments in parallel.

One experiment deals with producing and culturing the chosen bacteria, harvesting them, thus obtaining a certain amount of colony-forming units (CFUs). The second workflow is

dedicated to selecting the suitable polymer and creating the polymer solution by dissolving the polymer in its respective solvent to a certain concentration. After the bacteria and polymer solution are ready, both are mixed and taken up with a syringe. (Heunies *et al.*, 2010)

The fundamental setup for monoaxial electrospinning (ES) consists of three main parts: high voltage source, capillary tube (pipette or syringe), electrically conductive collecting device. The schematic representation of the monoaxial ES setup is provided in **Figure 2**.

The suspension in the syringe is fixed on a pump and connected to the cathode of the circuit. The collecting device which lies a certain amount of distance from the syringe is covered with foil (or some other metallic component) and is connected to the anode/grounded. The collecting device is generally a plate, but depending on the application, it can also be a rotating collector or a frame for individual fiber analysis. (Huang *et al.*, 2003)

With the input of a constant electric field of high voltage (ranges from 1 kV to 20kV), the surface of the liquid in the syringe is subject to electric charge. As the intensity of the electric field is increased, the surface of the fluid elongates to form a Taylor cone. With further increase in voltage, the electrostatic force overcomes the force of surface tension that holds the liquid inside the capillary tube, thereafter, the jet is ejected from the tip of the Taylor cone. The emitted solution undergoes a phase of instability and elongation through which the jet becomes very long and thin and the solvent of the polymer solution evaporates simultaneously. (Huang *et al.*, 2003) Finally, the electrospun fibers land on the grounded collector plate with bacterial cells encapsulated in the fibers. (Heunies *et al.*, 2010)

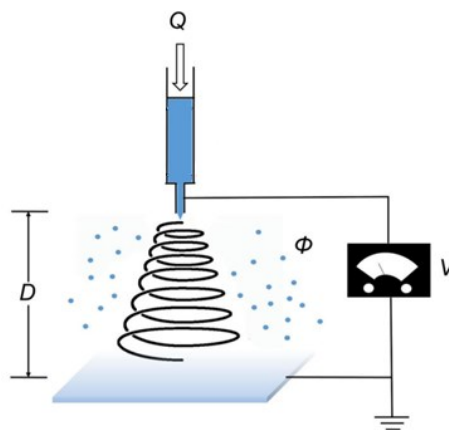


Figure 2. Schematic representation of the experimental setup and main adjustable parameters of monoaxial electrospinning. Q: flow rate, V: high voltage, D: distance from the syringe nozzle to the collector, Φ : relative humidity [modified(Weng and Xie, 2015)]

2.1.2.2 Coaxial electrospinning

Coaxial ES was first introduced by Loscertales *et al.* in 2002. (Loscertales *et al.*, 2002) This method is said to provide more stability than other electrospinning methods due to its multi-channel approach (see **Figure 3**): core/outer liquid and sheath/inner liquid channel which results in fibers that have a core-sheath structure. (Qu *et al.*, 2013) The coaxial method enables to create a more natural environment for the immobilized cells, due to the water-soluble core polymer. (Letnik *et al.*, 2015) It can also be said that coaxial electrospinning can overcome issues with fast drug release or problems with incompatibilities between drug(s), polymer(s), and solvent(s).

The general setup is conceptually similar to the traditional electrospinning introduced in chapter 2.2.2. One of the peculiarities in coaxial electrospinning is that the spinneret/nozzle is two-piece, where a smaller capillary is inserted into the bigger capillary (interpretation visible in **Figure 3**). As the name of the technique suggests, it creates a co-axial configuration, where the outer needle contains a sheath solution and the inner needle contains a core solution. When the polymer solutions are charged with a high voltage, the accumulation of the charge takes place on the sheath solution, inducing it to exit the capillary. As a result of solution elongation and charge-charge repulsion, the jet ejects the tip and lands onto the electrode. (Pant *et al.*, 2019)

If the core-shell structured fibers have the drug only in the inner core of the fiber, they are prolonging the release compared to simple (blend or copolymer) electrospinning. Another option to avoid rapid drug release is to encapsulate it prior to electrospinning into nanoparticles. (Preem and Kogermann, 2018)

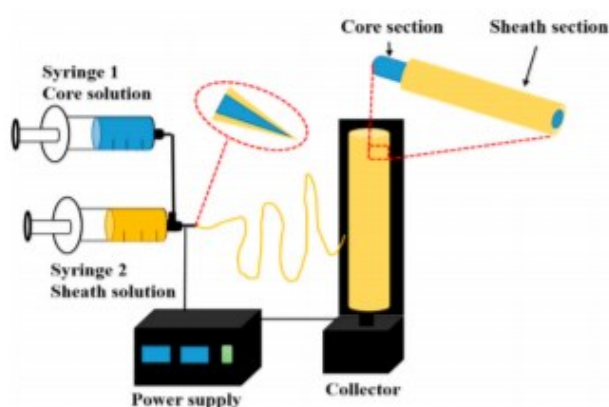


Figure 3. Schematic representation of the coaxial electrospinning system. (Pant *et al.*, 2019) Alternatively, the collector can be a grounded plate where the core-sheath fibers land.

3 Suitable materials for electrospinning

Various polymers with compatible biological, chemical, mechanical, and electrical properties have been electrospun into nanofibers. These electrospun fibers are architecturally akin to the natural ECM which can potentially provide support in wound healing. Therefore, the interest in discovering and employing suitable polymers has increased. Additionally, as the structure of the electrospun fiber changes structurally over time and degrades at some point, it allows the wound to recover fully and produce its own ECM. (Thomas *et al.*, 2015) In general, the materials for wound dressing preparation should always be non-toxic, with good mechanical properties, biodegradable and biocompatible and their safety is certified by FDA/European Medicines Agency (EMA).

For the successful creation of electrospun nanofibers, past studies have shown that one of the most important parameters to consider is the molecular weight of the polymer. Higher molecular weight is usually preferred as more effective topological entanglements between polymer chains occur which is the cornerstone for the formation of continuous fibers in ES. In contrast, the lower molecular weight polymer solution may break up into droplets or form beads. (Huang *et al.*, 2003)

Although more than fifty different polymers (Huang *et al.*, 2003) have been successfully electrospun, the materials selected for making the mats enable to tune the features of the fiber matrices which ultimately support wound healing. Most of the polymers introduced below will be used in the experimental part of this work. (Preem and Kogermann, 2018)

3.1 Hydrophilic polymers

Hydrophilic polymers have shown better wound healing properties - as they are biocompatible with the wound, allowing the removal of excessive wound exudate when required. (Preem and Kogermann, 2018)

PEO (linear formula $(\text{CH}_2\text{CH}_2\text{O})_n$, chemical structure is shown in **Figure 4**) is a white substance that is often used as a binding agent in pill manufacturing. Due to the wide range of possible molecular weights (100000 – 7000000 g/mol), PEO entails the opportunity to involve in different forms of drugs.

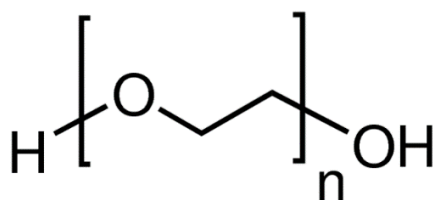


Figure 4. Chemical structure of PEO (<https://www.sigmaaldrich.com>)

Poly(vinyl alcohol) (PVA, linear formula $(\text{C}_4\text{H}_6\text{O}_2)_n$, molecular weight $\sim 31\,000$ g/mol, chemical structure is shown in **Figure 5**.

PVA is a highly hydrophilic, non-toxic and biocompatible semicrystalline polymer with excellent properties such as strength, water-solubility, gas permeability, and thermal characteristics. (Park *et al.*, 2010) The basic properties of PVA are dependent on the degree of polymerization or the degree of hydrolysis. It has been widely used for enzyme immobilization where mechanical, thermal, and chemical stability is crucial. Applications of PVA can be limited due to its hydrophilicity, however, chemical cross-linking by glutaraldehyde (GA) guarantees its stability in aqueous media. Cross-linking is useful as it enables the insolubility of nanofibers in all solvents and increases the mechanical properties of the membranes. (desired in filtration applications) (Abd El-aziz *et al.*, 2017)

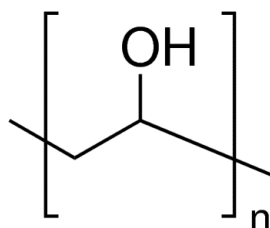


Figure 5. Chemical structure of PVA (<https://www.sigmaaldrich.com>)

3.2 Hydrophobic polymers

Hydrophobic polymers are said to have better control over drug release due to surface erosion. (Nazila *et al.*, 2016)

To increase the potential applicability of the encased living cell, fibers, that are insoluble in water and biodegradable for a predetermined amount of time, are of bigger interest. (Klein *et al.*, 2009)

Polycaprolactone (PCL, linear formula $\text{CH}_3(\text{C}_6\text{H}_{10}\text{O}_2)_n\text{CH}_3$, chemical structure shown in **Figure 6**) is a white solid substance, that is biodegradable and non-toxic. PCL has been utilized a lot for biomaterial and DDS applications. Due to the fact that PCL degrades slowly, it is often preferred in applications that provide a long-term effect. The upside of PCL is that it combines well with other polymers. It is possible to make copolymer systems with other polymers. (Woodruff and Hutmacher, 2010) Next to wound dressings, PCL is used to engage different medications. (Huang *et al.*, 2006) Due to the hydrophobicity, it is important to identify other potential alternatives. It has been proven that PCL dissolves well in dichloromethane (DCM), chloroform (CF) and tetrahydrofuran (THF). As more porous fibers enable better drug release (Lanno *et al.*, 2019), the use of a solvent that evaporates quicker, is preferred. (Qin *et al.*, 2012)

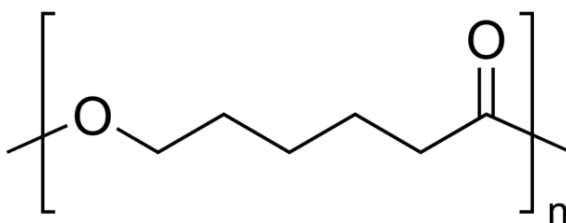


Figure 6. Chemical structure of PCL (<https://www.sigmaaldrich.com>)

Poly(lactide) (PLA) is a polymer obtained by the ring-opening polymerization of lactide (lactic dimer of lactic acid) as a monomer. Since lactide has three optical isomers – L-lactide, D-lactide, and DL-lactide, the polymers obtained are PLLA, PDLA, PDLLA, respectively. (Nazila *et al.*, 2016)

Poly(D, L-lactic acid)/Poly-D-lactide (PDLA, linear formula $(\text{C}_3\text{H}_4\text{O}_2)_n$, chemical structure shown in **Figure 7**) is a ubiquitous biodegradable polymer. Also, because the melting temperature of the analogous PLLA alone is not enough in some applications, it is often mixed with PDLA, creating a stereocomplex-type PLA (SCPLA). Since nanofibers can undergo a lot of stress and strain, the mechanical properties are always subject to improvement and more testing. (Tanaka *et al.*, 2010)

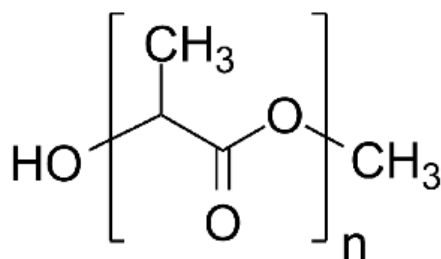


Figure 7. Chemical structure of PDLA (<https://www.polysciences.com>)

4 Characterization of electrospun biomaterials

4.1 Morphology

The key points for the morphological analysis of fibers are fiber size (diameter and diameter distribution), fiber orientation/alignment/arrangement, pore size, surface topography/features.

Structural properties of the fibers affect the wound healing process. It is now known that the high porosity of the nanofiber matrix enables oxygen and water permeability, nutrient exchange, and the removal of metabolic waste preventing fluid accumulation at the wound site. Additionally, the high surface area to volume ratio of the fibers enables cell attachment and growth, and differentiation during tissue regeneration. (Kamble *et al.*, 2017)

The morphology of the electrospun fibers depends on the strength of the electric field, solution viscosity, charge density, and the solution feeding rate. The fiber diameter is tuned through properties like solution conductivity and polymer concentration (usually fiber diameter increases together with the solution concentration). (Preem and Kogermann, 2018) Research has also shown that the strength of fibers increases with the decrease of their diameter due to improved chain orientation and crystallinity. (Yao *et al.*, 2014)

As discussed earlier, electrospun matrices manifest structural similarities with dermal ECM and are especially attractive for wound care applications. (Preem and Kogermann, 2018)

When the diameters of electrospun fibers are decreased down to the micro- and nanometer scale, the electrospun material entails an ample of benevolent properties for wound dressings. A few of them is a very large surface area to volume ratio, flexibility in surface functionalities, and superior mechanical performance (stiffness and tensile strength).

4.2 Stability and viability of the cells

4.2.1 Before encapsulation

Stability and viability of the cells before encapsulation is ensured by providing the bacteria its specific media for culturing. This can be done by adding components to the culture plate media. Additionally, lyoprotectants can be used to the bacterial suspension just before electrospinning to improve the viability post-electrospinning and during storage. (Škrlec *et al.*, 2019)

4.2.2 After encapsulation

After electrospinning, the number of bacterial cells is generally smaller than before by many folds. In the work of Heunis *et al.*, (Heunis *et al.*, 2010) the cell numbers of *L. plantarum* 423 decreased from 2.3×10^{10} cfu/ml before electrospinning to 4.7×10^8 cfu/ml. After further investigation, only 2% of the *L. plantarum* cells remained viable 48 hours post-electrospinning. There can be many reasons for why this is so: high voltage, suitable selection of polymers, number of bacterial cells pre-encapsulation.

Additionally, something to consider is the type of bacteria used for electrospinning, although *L. plantarum* showed low viability in the aforementioned work, *M. Luterus* used in the work by Gensheimer *et al.*, (Gensheimer *et al.*, 2007) resulted in viability of 74% and remained so when stored at 4 °C in dry conditions.

4.3 Functional testing

Usually, the drop-plate method is used to determine the number of CFUs and hence the viability of the bacteria in polymer solutions and fibers. (Nagy *et al.*, 2014)

Next to the drop-plate method, bacteria are often engineered beforehand to comprehend the viability of the post-spun bacteria. The bacteria are usually modified by including a fluorescent protein plasmid (like mCherry or green fluorescent protein (GFP)) via electroporation. This way the plasmid provides a continuous expression of the protein and thus, fluorescence detection of the bacterial cells. (Škrlec *et al.*, 2019)

However, the expression of fluorescence does not indicate bacterial activity and growth, it is solely showing if the bacteria can express the plasmid. Alternatively, a TIMER plasmid

can be implemented – this is a constitutively expressed protein that shows rapid emergence of green fluorophores and delayed formation of red fluorophores. The dividing cells have more green fluorophores and the non-dividing, dormant cells have both fluorophores. (Claudi *et al.*, 2014)

Additionally, in order to determine the long-term viability, fiber with encapsulated cells are stored in fridge or freezer temperatures and the CFU is investigated after a certain amount of time (can be weeks to months to years). (Nagy *et al.*, 2014)

5 AIMS OF THE STUDY

The overall aim of the research is to design and develop electrospun smart and functional biomaterials that contain living bacterial cells.

In order to achieve the overall aim, the following specific tasks were set:

- To select the electrospinning method (choice between traditional or coaxial electrospinning) for the encapsulation of bacteria into fibers;
- To select the type of bacteria, the concentration of polymers and electrospinning conditions for the encapsulation of bacteria into fibers;
- To test bacterial viability and metabolic activity using different preparation methods and pre-growth conditions;
- To understand the effect of bacteria on the electrospinnability, fiber diameter;
- To understand the effect of additives on the electrospinnability, fiber diameter and viability of bacteria during electrospinning.

6 EXPERIMENTAL PART

6.1 MATERIALS

6.1.1 Materials for electrospinning, bacterial strains, culture conditions

In the following experiments, monoaxial electrospinning was tested with PEO (*molecular mass: 900000 g/mol, Sigma-Aldrich, China*), poly-D-lactide (PDLA) (*molecular mass: 200 000 g/mol, Purac Corbion*) and coaxial electrospinning was experimented with PDLA and hydrophobic polymer X (*molecular mass: 200 000 g/mol, Purac Corbion*) as the shell polymer and a hydrophilic polymer X for the core. The used solvents for the polymer solutions with PEO (6% w/w), PDLA (15%, 10% w/w), hydrophobic polymer X (15%, 12.5%, 10% w/w) were distilled water (H₂O) (for PEO) and organic solvent X (99%, *Thermo Fisher, Germany*) (for others). All solvents used in the present thesis (organic solvent X, dimethyl sulfoxide (DMSO)) were of pharmacopoeia grade and used as received from the supplier.

The encapsulated organisms in this work were probiotic bacteria *Escherichia coli* Nissle 1917 (ECN), *Lactococcus lactis* IL1403 and laboratory *Escherichia coli* with an electroporated plasmid pSC101-Timer-TetR-PtetA-BFP-noDeg (EC101) (see map of plasmid in **Figure 8**). All of the aforementioned bacterial strains were provided by the supervisors (G.-M.Lanno). Plasmids were prepared by a PhD student of the Institute of Technology M.Hinno. Pure cultures of ECN were prepared and stored by the thesis author in 8% DMSO at -80 °C. The procedure included taking an ECN colony from a freshly grown (24-48h) plate, suspending the colony in lysogeny broth (LB), thereafter adding DMSO. The cultures of *L.lactis* and EC101 were stored in a 20% glycerol stock at -80 °C.

Additionally, trehalose dihydrate (*Sigma-Aldrich, China*), hydrophilic polymer X (*AppliChem, Germany*) Casamino acids (cAA, *VWR Chemicals, LLC, USA*), phosphate-buffered saline (1xPBS, with adjusted pH 7.4), M9 (components provided by M.Hinno), M17 (*BD DifcoTM, US*) stocks were used for improving the bacterial viability during and after the electrospinning process.

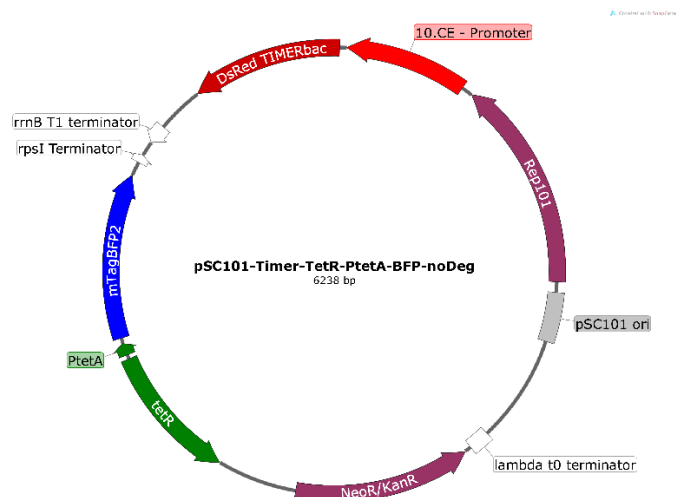


Figure 8. Map of pSC101-Timer-TetR-PtetA-BFP-noDeg

6.1.2 Preparation and selection of growth media

LB was used to traditionally grow bacteria, whereas, M9 was implemented when bacteria (in particular EC101) were analyzed in confocal fluorescence microscopy. The reason for using different media lies in the fact that, unlike M9, LB is autofluorescent. To prepare the M9 growth medium, the following components were used 0.1 mM 50 μ L CaCl_2 , 1X 10 ml M9 salt solution ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, KH_2PO_4 , NaCl , NH_4Cl), 2mM 100 μ L MgSO_4 , 0.2% 500 μ L glucose, and dH_2O up to 50 mL. The components were pipetted into a 50 mL flask. The solution was filtered with a 0.22 μm syringe filter into a separate falcon which was stored at refrigerator temperatures.

For the experiments where the pre-growth conditions were modified, different additives, namely sucrose, polymer polyethylene glycol (PEG), and NaCl , were added to the growth media during the bacterial overnight growth at 37°C. The additives were dissolved together with LB agar, autoclaved and poured into Petri dishes. ECN was grown on 4 different culture plates in standard conditions – LB (control), LB with sucrose (15% w/w), LB plates with PEG (5% w/w) and LB with NaCl (100 mM). The colonies were inoculated on the specific plates (after 24 h incubation) and proceeded according to the steps described below.

6.2 METHODS

6.2.1 Preparation for electrospinning (ES)

The preparation for ES can generally be divided into separate working threads.

Polymer solution preparation. For monoaxial ES, hydrophobic polymer PEO (6% w/w) was used. The solution was prepared by adding distilled water to pre-weighed PEO granules and thereafter the solution was left on a magnetic stirrer until PEO was dissolved completely. All ingredients (polymer, solvent) were added by weight.

For coaxial ES, two different polymer solutions were prepared: core-solution and shell solution. The concentration of the core solution for both tested (see chapter 6.3.1.3) polymers hydrophobic polymer X and PDLA were 10% w/w. The solution was prepared by adding the organic solvent X (to PDLA or polymer X) to the pre-weighed polymer granules. The solution was left on a magnetic stirrer until polymers were dissolved in their appointed solvents. All ingredients (polymer, solvent) were added by weight.

For the preparation of hydrophilic polymer X 0.625% w/w solution the polymer was weighed together with the distilled water. A magnetic stirrer was added and the solution was heated on a heater plate until the polymer was fully dissolved.

Bacterial dispersion preparation. The conditions and solutions for the preparation were bacteria-specific (see **Table 2**). First, a colony was picked from a relatively fresh (48 - 72h) plate which was stored at refrigerator temperatures (+2..+6 °C). The bacterial colony was inoculated into either 10 mL (*E.coli*) or 5 mL (*L.lactis*) of its entitled growth medium and incubated for 24 h in a OrbiSafe incubator (220 rpm, *Sanyo, Japan*). The optical density (OD) of overnight cultures was measured with Ultrospec 700 (*Biochrom, USA*) at 600nm to ensure that the bacteria were in their exponential phase and the number of bacterial cells was growing. As the bacterial concentrations varied for ES experiments, it enabled to comprehend how it might affect the bacterial viability post-ES. Thereafter, all of the overnight culture was poured into a falcon tube and centrifuged for 10 min at 5000 rpm-s. After discarding the medium, suspending the pellet with 1mL of water, the suspension was placed into an Eppendorf and was ready for the final step. It should be pointed out that in the case of coaxial ES, the EC101 pellet was not suspended with 1mL of distilled water but 1mL of hydrophilic polymer solution. (core solution)

Table 2. Bacteria-specific conditions for the preparation of ES

Bacteria used for monoaxial ES	<i>Escherichia coli</i> Nissle or EC101	<i>Lactococcus lactis</i>
Growth plate/medium	LB or M9	M17
Volume of medium for overnight culture	10 mL	5 mL
Any possible additions to the overnight culture	Pre: 100 μ L Casamino acids (cAA) Post: 4% trehalose dihydrate	Post: 4% trehalose dihydrate
Temperature for overnight growth	37 °C	30 °C

Key: pre- means the additive was included to the medium together with bacterial colony before incubation in a shaker for 24 h; post- means the additive was combined with the overnight culture prior to ES (after 24h of incubation)

Mixing of polymer solution and bacterial dispersion. During the final step the polymer solution and bacterial suspension were mixed in 1:1 (V/V), which was then left stirring for at least 30 min.

Preparation of the solutions with additives. Trehalose dihydrate, Casamino acids were used as additives for the preparation of ES fibers (see **Table 2**). The concentration of trehalose dihydrate was 4%, which was weighed and added to the bacteria-polymer solution. 100 μ L of cAA was pipetted into the LB medium and ECN colony for overnight incubation.

6.2.2 Electrospinning (ES)

The bacteria were ES into fibers using the ESR200RD (*NanoNC, South-Korea*) automatized ES device. The setup consisted of a high-voltage input, syringe pump(monoaxial ES) or 2 syringe pumps (coaxial ES, additionally Chemyx Fusion 400 pump was used) together with plastic syringes and metal needles and a grounded metal plate which was fixed horizontally to the syringe(s). For monoaxial ES, 3 mL syringes (diameter of 9.52 mm) and a metal needle

of size 0.52 mm (25G) were used. The sizes for the concentric nozzle for coaxial ES were 0.63 mm (23G) and 1.47 mm (17G) for the inner and outer needle, respectively.

As monoaxial ES has been a method of research of many works at the Institute of Pharmacy and with the comprehensive experience of both supervisors, the parameters needed for configuration (i.e. distance between plate and syringe, voltage, flow rate, polymer concentration) were established.

The general parameters for monoaxial ES were the following:

- Voltage: 15.9 kV;
- Flow rate: 0.5 – 0.8 mL/h;
- Distance: 12 – 14 cm;
- Amount of solution per ES session: 0.05 mL = 50 μ L;

As coaxial ES is setup-wise relatively similar, the parameters were adopted from the monoaxial approach. It should be highlighted that for the coaxial ES the sheath/outer phase was set up (for configuring the flow rate) on the additional syringe pump. The concentric nozzle is shown in **Figure 9** where the core/inner phase is connected to the ESR200RD device and runs from left to right and the sheath phase (connected via PVC hose) runs down to up.

The general parameters for coaxial ES were the following:

- Voltage: 15.9 kV;
- Flow rate (core solution): 0.5 – 0.8 mL/h;
- Flow rate (sheath solution): 0.6 mL/h;
- Distance: 12 – 14 cm;
- Amount of solution per ES session: 0.05 mL = 50 μ L;

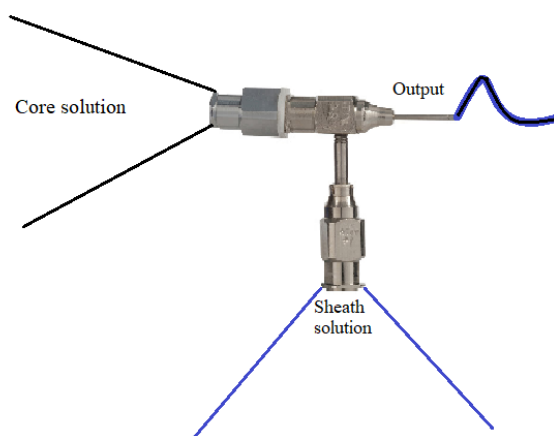


Figure 9. Coaxial ES concentric nozzle for two different phases (modified image from www.electrospintech.com)

The fiber mats were collected onto aluminium foil. The samples were stored in airtight plastic zip-lock bags 0% RH in a desiccator. The analysis of the electrospun samples took place on the same day (bacterial viability) or within 24h after the experiments (other analyses such as confocal fluorescence microscopy).

6.2.3 Analysis of electrospun fibers

For initial observation of ES fibers, a polarizing light microscope (*CETI MagtexT, United Kingdom*) was used (40x magnifier). The diameters, porosity and general morphology were examined by using scanning electron microscopy (SEM) (*Zeiss EVO MA, Germany*). The samples originated from the electrospun fiber mats collected on the foil. A small segment of the mat was allocated to a carbon tape which was intact to an aluminium base. Before the analysis, the sample was either sputtered with gold or platinum (~5nm thick layer). The SEM measurements were performed at the Institute of Ecology and Earth Sciences at the University of Tartu by supervisor G.M.Lanno. The implemented magnification varied: 2K, 5K, 10K, 20K. The diameters (100 measurements from one sample) of the fiber mats were analyzed with *ImageJ* software (version: 1.53e). The porosity of the fibers was evaluated by implementing a Python program (Python version: 3.8.5, Geany IDE version 1.36) created by the author. The data were ultimately visualized using R (Rstudio version 1.4.1106). The porosity of the fibers was established by processing the images using OpenCV library (see workflow in **Figure 10**) – the grayscale SEM micrograph was converted into a black-white image using a fixed threshold value. All the fibers became white and the room between fibers

(pores) became black. The ratio between the number of black pixels and all pixels of one image shows the prevalence of pores (porosity) in a micrograph.

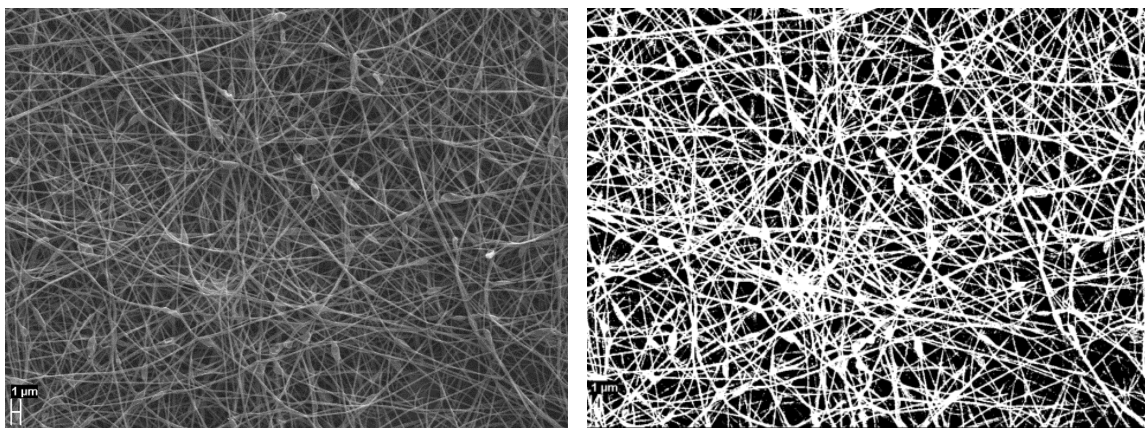


Figure 10. Workflow for obtaining porosity. The simple OpenCV function `cv2.threshold*` was used to convert the grayscale SEM micrograph (left) to a black-white image (right). The second argument of the function is a threshold value that classifies the pixel color value (black or white) to the pixel. The style of thresholding here was `cv2.THRESH_BINARY`. Thereafter, the number of black pixels was divided by all of the pixels and for percentage format, multiplied by 100.

* The `cv2.adaptiveThreshold` was also a function to consider if the lighting conditions would have been more varying (this was not the case for SEM micrographs).

6.2.4 Analysis of electrospun bacteria

The viability of bacteria electrospun (ES) monoaxially into the fiber mats was determined with the drop-plate method. The hydrophilic polymer (PEO) mat easily dissolved in PBS, which released the bacteria, through which growth was determined. The ES fiber mat was cut into two equal parts. Half of the fiber was suspended in 1mL of PBS. This dispersion was put onto ice until the next step. The solution consisting of dissolved bacteria-loaded ES mat was serially diluted onto the microtiter plate (refer back to **Table 2**). The serial dilution was performed so that all of the wells contained 90 µL of PBS initially, the 10 µL of suspension (e.g. dissolved ES mat in PBS) was added to the first well and serially diluted thereafter (diluted 6-12 times). Then 5 µL from every well was spotted onto the plates up to 6 times. The plates were incubated for 24hr at bacteria-specific temperature (*E.coli* – 37 °C,

L.lactis – 30 °C). The colonies were counted per drop and finally expressed as the concentration of bacteria in the suspension. Additionally, different controls (i.e. bacteria, bacteria + polymer and bacteria+polymer in a syringe after ES) suspensions were included as references and used for final calculations.

The viability and activity of coaxially ES bacteria were analyzed using confocal fluorescence microscopy. As hydrophobic polymer was used for the shell layer of the core-shell fibers, the drop-plate method could not be used. The viability and metabolic activity were evaluated using reporter protein Timer based on the green and red fluorescence.

6.2.5 Data analysis

All of the data are presented as an arithmetic mean and standard deviation (SD). The bacterial viabilities were computed by comparing the concentration of an electrospun bacteria to the same bacteria before ES. The effect of PEO was evaluated by comparing the concentration of bacterial suspension (pellet to medium) -to the concentration of bacterial suspension mixed with PEO.

The significance of data was analyzed using the f-test and t-test. The data were interpreted using both LibreOffice Calc (LibreOffice version 7.1) and Microsoft Excel (MS Office 2016) programs. The green and red fluorescence intensity was measured using ImageJ MicrobeJ plugin from confocal images of the samples. The green and red intensity ratio was calculated with Microsoft Excel which corresponds to the cellular activity - constitutively expressed protein has branched maturation pathway with rapid emergence of green fluorophores and delayed formation of red fluorophores. (Claudi *et al.*, 2014)

6.3 RESULTS AND DISCUSSION

6.3.1 Monoaxial ES

In monoaxial ES, 3% PEO solution was used and it was attempted to encapsulate two different bacteria – ECN and *L.Lactis*. As the *E.coli* bacteria are more widely known and grows quickly, it was decided to start trying to encapsulate this first. It can be said that ES of *E.coli* depended on bacterial load, as solutions with higher bacterial concentrations increased the viscosity, making it difficult to spin (unstable ES, fiber mat smaller than usual). Hence, it was not possible to go much higher with the initial bacterial load than OD of 2.

However, slightly different initial bacterial concentrations were tested for ES. **Figure 11** shows two SEM micrographs side-by-side: one of bacteria-loaded ES fiber mat and the other with mats with just ES polymeric fibers. It is visibly clear that the fiber sizes with the encapsulated bacteria are bigger than just pristine PEO fiber sizes. As can be seen from the figure, the fibers with bacteria seem to be containing more bead-like formations which could refer to the presence of bacteria inside the fiber. Similar formations were found in other bacteria encapsulation works as well. (Zupani *et al.*, 2019)

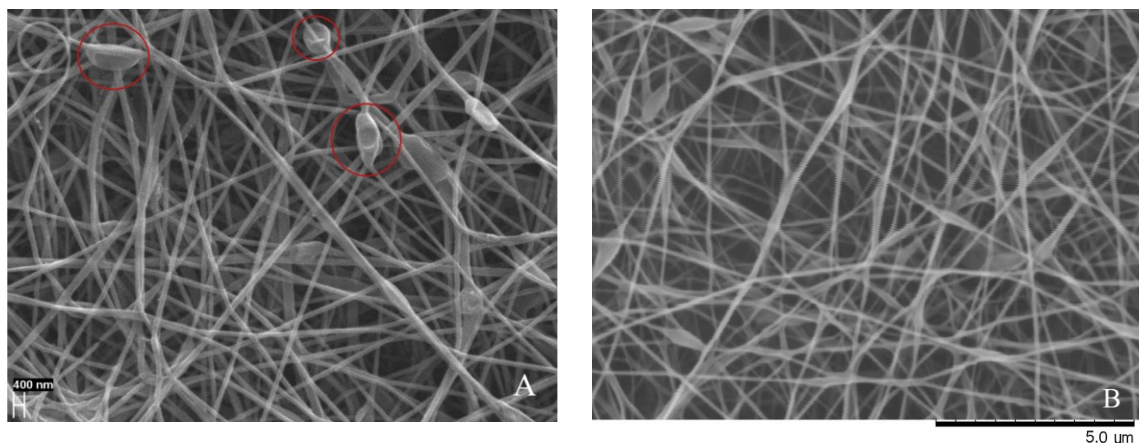


Figure 11. SEM micrographs of PEO ES fibers with (A) and without ECN (B). Red circles refer to the bead-like formations which could indicate where encapsulated ECN are located (magnification 10000X)

After several ESs of ECN, testing of *L.lactis* was initiated. Despite following the general protocol (in M17 growth medium, incubation in 30 °C) for growing *L.lactis*, it took several attempts to obtain considerable yield after growing for 24h. It is very likely that the difficulty was related to the M17 growth medium or the quality of the glycerol stock the *L.lactis* was stored in. Based on the OD (for OD₆₀₀ of 1.0 = 10⁹ cells/mL), the average and SD of ECN and *L.lactis* initial bacterial loads before spinning were $2.32 \pm 0.82 \cdot 10^9$ cells/mL and $0.612 \pm 0.17 \cdot 10^9$ cells/mL, respectively. In the work of Zupani *et al.* the *L.lactis* load was 8.19 ± 0.33 log CFU/mg. The reason for a marginally bigger bacterial load seen in Zupani *et al.* publication was probably obtained with growth medium M17. In their case, the M17 was supplemented with 0.5% glucose. Also, the overnight cultures were diluted in fresh medium and grown until a stationary growth phase was reached. (in our case the bacterial growth was in its exponential phase once taken from the overnight culture) (Zupani *et al.*, 2019) It was not discussed in their paper whether such high bacterial load caused any difficulties in ES.

Except for the difficulties growing *L.lactis*, there were no major differences in the ES of *L.lactis* and *E.coli*.

In order to understand the effect of bacterial concentration and additives on the fiber diameter, morphology and bacterial viability, SEM micrographs were investigated further.

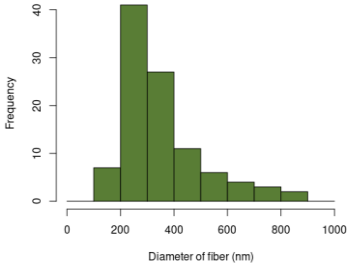
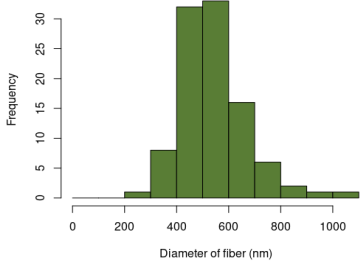
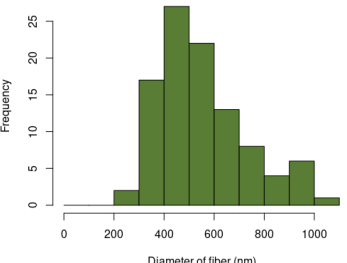
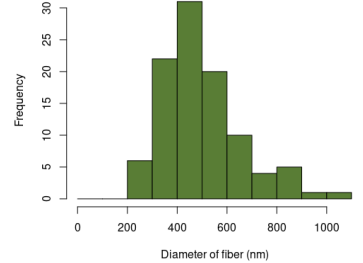
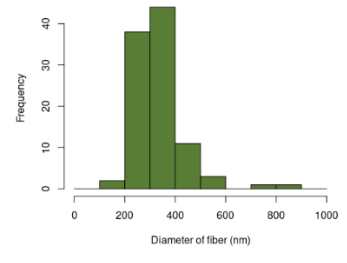
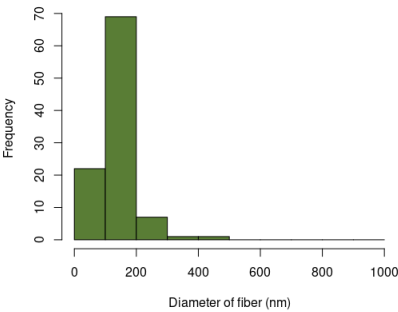
It is a well-known fact that sugar and amino acids improve bacterial growth. (Farrell and Finkel, 2003; Louis *et al.*, 1994) As sometimes bacteria are grown in specific pregrowth conditions (e.g. sugar or NaCl added to medium plate), it was interesting to obtain an understanding of how bacterial growth was affected with supplements on the plate or how the addition of supplements into the bacterial suspension prior to ES modified the viability of bacteria during ES. Hence, after encapsulation of just bacteria, the effect of sugar (trehalose dihydrate) to both bacterial mixtures (pellet+1mL medium) or combined amino acids (cAA) to ECN mixtures were tested on bacterial viability.

6.3.1.1 Fiber analysis

After successful ES of bacteria, the fiber mat was analyzed based on the porosity and fiber diameters which were compared to the fiber mats made using pristine polymer. It was confirmed that the bacteria-loaded fibers had larger fiber diameters compared to the PEO fibers (**Table 3**). Hence, the size of the fiber diameters can indicate whether a bacterial cell has been encapsulated into the fiber. Additionally, based on the SEM micrograph observations (**Figure 11**), the presence of „beads“ on the fiber could also be related to encapsulated bacteria.

Table 3 also allows to compare the fiber diameters with or without additives (trehalose dihydrate and cAA). Interestingly, it can be stated that fiber diameter of encapsulated *L.lactis* fiber mat is marginally bigger than encapsulated ECN. This could be reasoned with the fact that the morphology of *L.lactis* enables to form chains of bacteria which could result in the expansion of the fiber diameter. (Zupani *et al.*, 2019). The t-test showed that there were statistically significant ($p \leq 0.05$) differences in all the fiber diameters compared to PEO fiber diameter. The t-test also presented that the effect of cAA on ECN-loaded fiber diameters was statistically insignificant.

Table 3. The effect of different conditions on fiber diameters between encapsulated ECN and *L.lactis* (fiber diameters collected from SEM micrographs, 100 measurements were taken from one mat). Histograms illustrate the average fiber diameters in different conditions

Bacteria	<i>Escherichia coli</i> Nissle	<i>Lactococcus lactis</i>
ES bacteria	 Average: 348 ± 144 nm	 Average: 541 ± 129 nm
ES bacteria with added trehalose dihydrate	 Average: 553 ± 181 nm	 Average: 500 ± 159 nm
ES bacteria with added cAA	 Average: 331 ± 103 nm	*NA
ES PEO (control)	 Average: 137 ± 58 nm	

Key: cAA: combined amino acids, ES: electrospun, PEO: polyethylene oxide.

*NA – not applicable due to the difficulties of growing *L.lactis*, the addition of cAA could not be tested with this bacterium.

As may be noted from **Table 3**, the addition of lyoprotectant, trehalose hydrate, increased the fiber diameters of ECN-loaded fiber mats but not of *L.lactis*-loaded fiber mats. The work of Škrlec *et al.* showed that the inclusion of the same lyoprotectant to PEO + *Lactobacillus plantarum* solution slightly decreased the fiber diameters (Škrlec *et al.*, 2019). The addition of trehalose dihydrate also marked a small decrease in fiber diameters in the work of Dai *et al.* where PVP fibers encapsulated bacteriophage T7 (Dai *et al.*, 2014).

The likely reason for the increase in fiber diameters of ES ECN may be related to the initial bacterial load. Research has shown that it is not the relative humidity (RH), nor voltage of the ES conditions, but the bacterial concentration in the PEO solution that affects not only the fiber diameters and morphology, but also bacterial viability. (Škrlec *et al.*, 2019) This said the reason for increase in fiber diameter remains unknown, as the bacterial load (OD) on the experiment's day, when the addition of lyoprotectant to ECN was tested, was lower than the average OD (2.32) of ECN.

It can be said that the addition of cAA to the bacterial suspension showed no change on ES fiber diameters, as the average fiber diameter of the solely encapsulated ECN was in the same range.

The ES of bacteria experiments had day-to-day fluctuations which resulted in the varying standard deviations (SD) in fiber diameters. These changes can be explained with two points:

- inaccuracies in measuring fiber diameters;
- as experiments took place on different days, the humidity and temperatures in the ES system were varying (see **Figure 12**). The RH values varied from 7.6% to 34.8%, while temperature ranged from 21.9 °C to 27.4 °C. There were more changes in the RH than in the temperature during ES. Research has shown that higher RH improves the fiber quality/uniformity, but may as well induce pore formation on the fibers. (Ramos *et al.*, 2020)

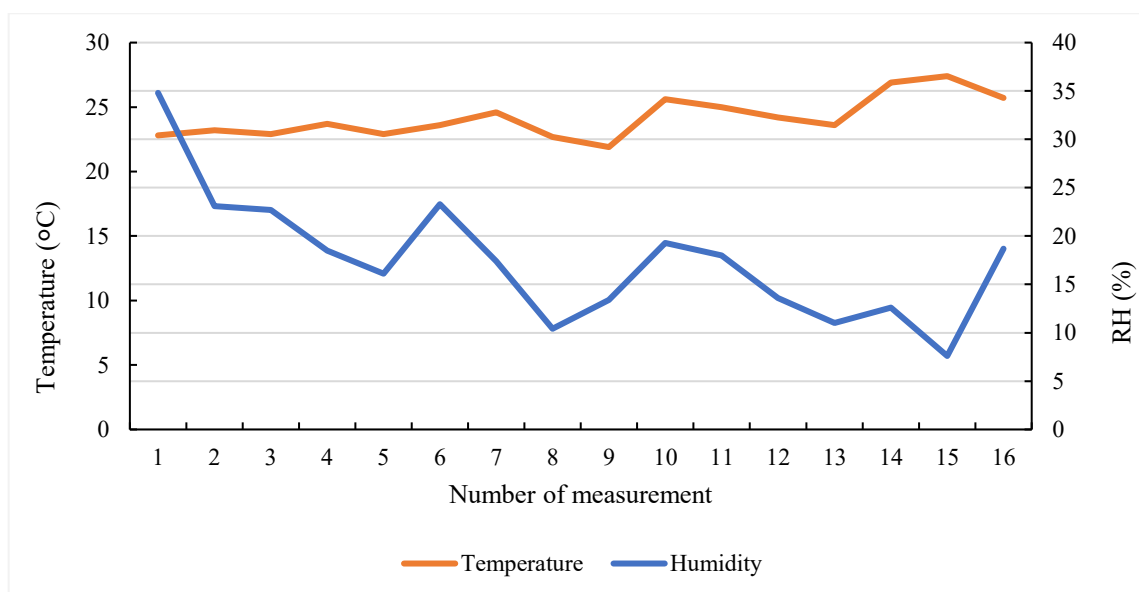


Figure 12. RH and temperature measurements taken throughout the ES experiments

As introduced previously, the two-dimensional porosity of the fibers was established with a Python program created by the author. **Table 4** shows the averages and SDs of porosity in percentages measured based on the SEM micrographs of different bacteria and conditions. From the t-test it can be said that all of the porosities of *L.lactis*-loaded mats were significantly different from pure PEO porosity ($p \leq 0.05$). However, the t-test unraveled that the porosities of mats with ECN-loaded mats together with cAA did not differ from the porosity of ECN-loaded and/or pristine PEO mats. On the contrary, the porosities of ECN-loaded mats together with trehalose dihydrate did significantly differ from the porosity of ECN-loaded and/or PEO mats. Whereas the porosities of *L.lactis*-loaded together with trehalose dihydrate mats showed no significant differences compared to the porosity *L.lactis*-loaded mats.

Table 4. Porosities of electrospun (ES) fiber mats in different conditions (N=6 mats per condition). Values represent the average porosity (area between fibers) of fibers $\pm$ SD.

Bacteria	<i>Escherichia coli</i> Nissle	<i>Lactococcus lactis</i>
ES bacteria	38.3 $\pm$ 12.7 %	49.8 $\pm$ 10.3 %
ES bacteria with added trehalose dihydrate	58.2 $\pm$ 13.9%	47.9 $\pm$ 11.0 %
ES bacteria with added cAA	32.2 $\pm$ 9.1 %	*NA
ES PEO (control)	31.3 $\pm$ 1.6 %	

Key: cAA: combined amino acids, ES: electrospun, PEO: polyethylene oxide.

*NA – not applicable due to the difficulties of growing *L.lactis*, the addition of cAA could not be tested with this bacterium.

The results from **Table 4** show that although the trehalose dihydrate increased the diameters of ECN fibers, it did not increment the coverage of the mat, which means that this sugar may have had an adverse effect or no effect on the amount of fibers per ECN or *L.lactis* mat, respectively. The addition of cAA to ECN-loaded fiber mat improved the coverage of fibers per mat, but reduction of fiber diameter was observed. From these tests, the increase in fiber diameter was connected to the rise of porosity values. However, research by Liu *et al.* showed the opposite – the porosity increased with the decrease of diameters. In this work three-dimensional porosity was obtained through real-life measurements: compression was applied to the mat with a known diameter and thickness and the pore volume was calculated by the difference of the total (known) and compressed volume. (Liu *et al.*, 2017)

6.3.1.2 Bacterial analysis

The viability of monoaxially ES bacteria were analyzed via the drop-plate method. The concentrations were diluted by a fold of 10 and this correlation was also expected to reflect in the number of colonies per concentration. However, during data analysis, there were huge variations, and dilutions did not always follow 10-fold. This can be justified by numerous factors – fluctuations in bacterial load (OD) and,- the bacterial uptake with 1 mL PBS from the mat was not always reproducible. Although 1 mL PBS was constantly used to suspend the ES bacteria in the fiber mat, the amount of the picked up PBS and mat solution was never exactly 1 mL. (some PBS left on the mat due to formation of bubbles during mixing PBS with the ES mat)

Figure 13 shows how ECN bacterial viability changes over the ES process (initial load of ECN $2.32 \pm 0.82 \cdot 10^9$ cells/mL) It can be seen that there were differences with replicate samples, and the variations were approximately 10-15%.

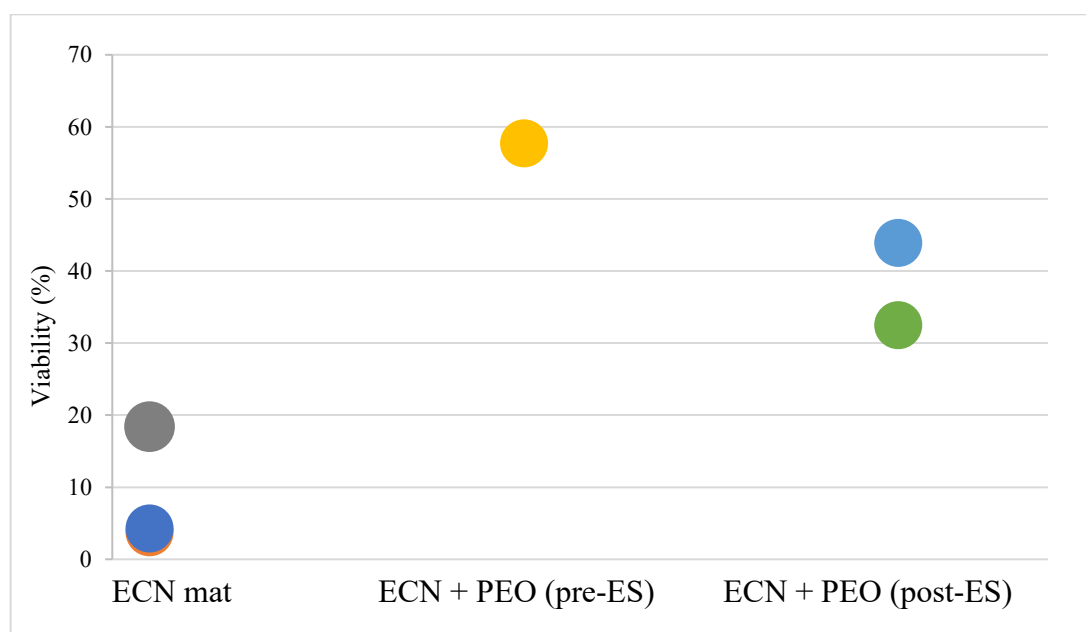


Figure 13. The average bacterial viability (N=3 replicates of every ES experiment) at different stages of ES. The dot values represent the relative viability (viability after spinning/viability before spinning). Key: ECN mat shows the viability of ECN in an ES mat. ECN+PEO (pre-ES) indicates the effect of PEO on ECN viability before ES and ECN+PEO (post-ES) (2 different measurements) marks the effect of PEO on ECN viability after ES. The difference in the number of measurements is due to the fact that some samples had a

very high bacterial load and it was not possible to count colonies from more than one/two serial concentration(s).

The graph reveals that after ES on average 9% of bacteria survived. Additionally, the PEO effect on bacterial viability was tested, as bacteria become in contact with the polymer prior and during ES. The results show that approximately 40% of the bacteria have died due to the presence of polymer. The bacterial viability in a polymer solution post-ES was reduced by 20% more. There are several likely reasons - the electric field during ES, bacteria are kept in an unfriendly environment for a long time or due to the fact that over time the bacteria and the polymer stratify and the viability obtained is covering a partial amount of the actual bacterial load.

Figure 14 asserts the fact that ES reduces bacterial viability, in this case the *L.lactis* viability decreased to 8%. The effect of PEO on *L.lactis* seems to reduce the bacterial viability by around 85%.

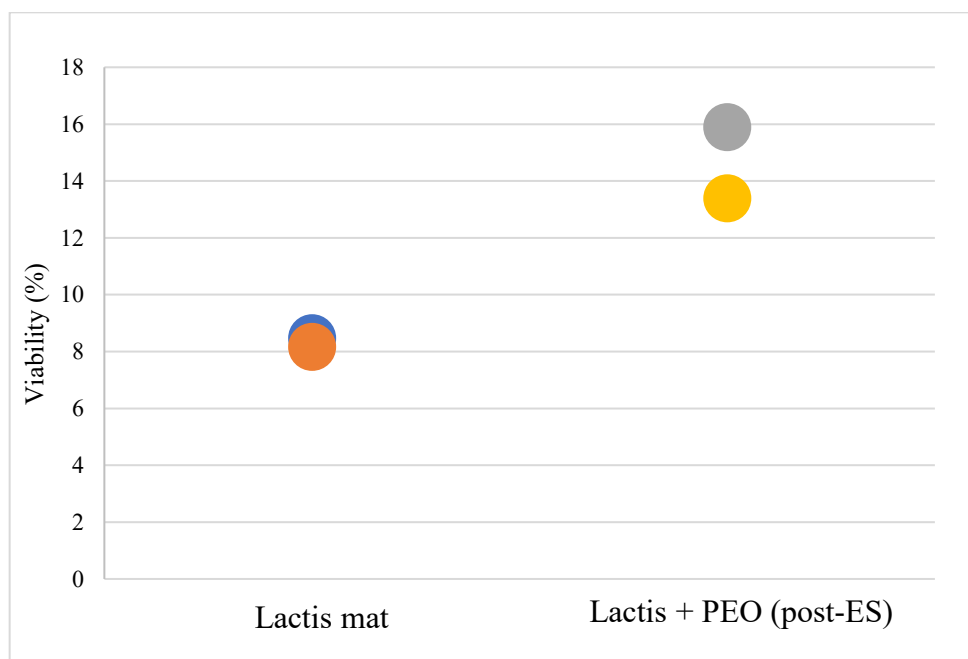


Figure 14. Average *L.lactis* viability (N=2 replicates from every ES experiment) during the ES process. Key: The *L.lactis* mat shows the bacterial viability after ES the bacteria into the fiber mat. Lactis+PEO marks the ultimate effect that PEO has on *L.lactis* viability after the ES process.

It seems that PEO has a more detrimental effect on the viability of *L.lactis* than ECN. However, it should be pointed out that the average bacterial viability of both *L.lactis* and

ECN show a similar result post-ES. Fewer variations were detected in the viability of *L.lactis* samples compared to ECN viabilities. But more experiments need to be conducted to confirm this statement.

The work by Škrlec *et al.* indicated, that the addition of lyoprotectants (sucrose, trehalose dihydrate) showed small improvements in the viability of *Lactobacillus plantarum* and showed no big differences in the diameters of the PEO nanofiber after ES (Škrlec *et al.*, 2019). However, the effect of trehalose dihydrate on ECN or *L.lactis* is seldom investigated.

We tested those effect on ECN and *L.lactis*. First, the effect of sugar was investigated on ES of ECN in pregrowth conditions. This means that 15% sucrose was added to the LB growth medium on the plate where the ECN was grown and used for ES with PEO. As can be seen from **Figure 15**, although other additives were tested out as well (NaCl, PEG), sucrose yielded the highest bacterial viability after ES.

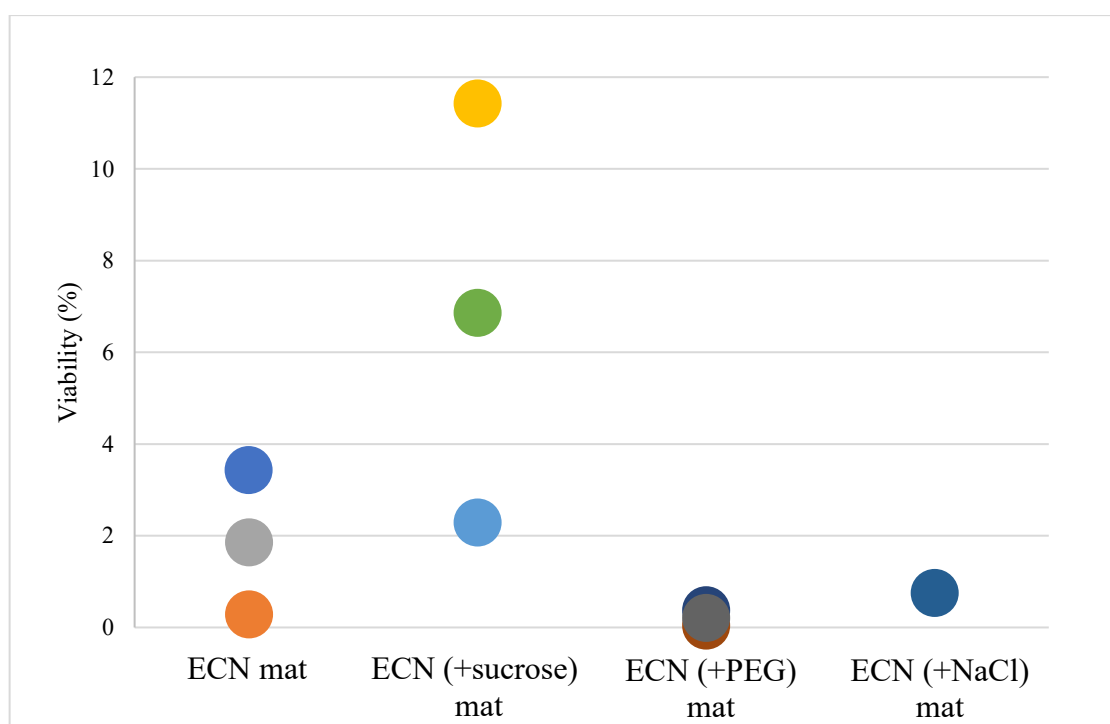


Figure 15. Effect of additives (added in pregrowth conditions) on ES bacterial viability (N=3 replicates from every ES experiment). Key: The term „mat“ refers to the after ES fiber mat. ECN mat is set as a reference point (bacteria grown on LB plate) to compare with the other modified pregrowth conditions. ECN (+sucrose) mat shows the post-ES bacterial viability of bacteria pre-grown on a 15% sucrose-LB medium solution. ECN (+PEG) mat shows the post-ES bacterial viability of bacteria pre-grown on a 5% PEG-LB medium solution. ECN

(+NaCl) mat shows the post-ES bacterial viability of bacteria pre-grown on a 100mM NaCl-LB medium solution.

It is worth mentioning that the bacterial viability in the ECN mat on **Figure 16** was very low during the day of testing (general average value of 8.82%) since the initial bacterial load was particularly small before ES on that day. Hence, variations between the different samples were observed.

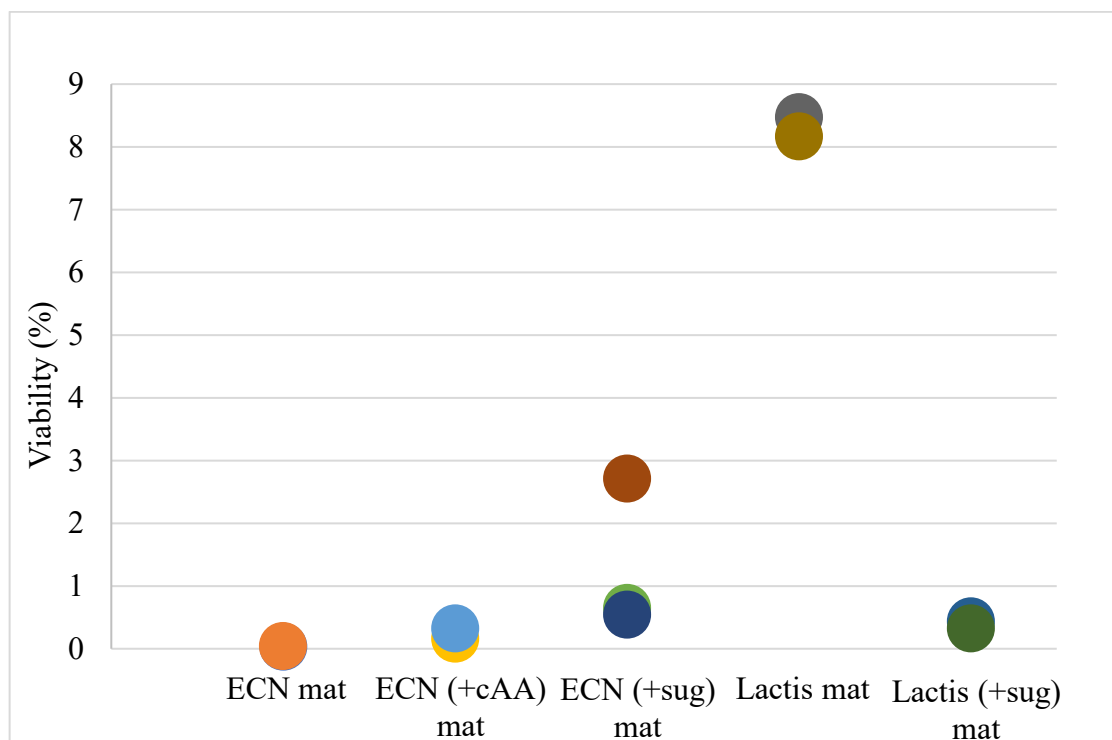


Figure 16. Effect of additives on average bacterial viability (N=2-3 replicates from every ES experiment). All of the additives [cAA, sug (trehalose dihydrate)] were added to the bacteria-polymer solution prior ES. Key: „mat“ refers to the ES fiber mat. The ECN mat shows the viability of ECN post-ES on that day, set as a reference point to compare the effect of additives. The ECN (+cAA) mat, ECN (+sug) mat indicates the effect of Casamino acids, trehalose dihydrate, respectively, on the bacterial viability after ES. Lactis mat illustrates the viability of *L.lactis* after ES. Lactis (+sug) mat notes the effect of trehalose dihydrate on the bacteria-polymer solution after ES. The difference in the number of measurements is due to the fact that some samples had a very high bacterial load it was not possible to count colonies from more than one/two serial concentration(s).

Nonetheless, the addition of cAA to the ECN bacteria suspension showed approximately 5x improvement in the viability of ECN.

The addition of trehalose hydrate had a better effect on the viability of ECN than *L.lactis*. The probable reason for the adverse effect of the lyoprotectant on the *L.lactis* is that perhaps the concentration of 4% was too high for the *L.lactis* strain due to the initially low bacterial load.

When comparing the bacterial viabilities of the modified pregrowth conditions to the additive effect on post-ES, it can be said that the addition of sucrose to pregrowth conditions showed a much higher result (6.86%) than the inclusion of trehalose dihydrate (1.30%) just before ES. This could raise a question about the effectiveness of the methodology in terms of adding the supplements right before ES, making the bacteria experience several, possibly inconvenient changes in the environment. (bacteria added to PEO, high voltage is being applied in ES etc.)

Figure 17 shows that the initial *L.lactis* bacterial load is in correlation with the bacterial viability within the ES mat. That is, the increase of OD generally results in increased viability. However, due to only a few technical replicates of the ES experiments with different *L.lactis* concentrations, it cannot be stated as a fact that the correlation exists and requires more tests at a wider range of ODs.

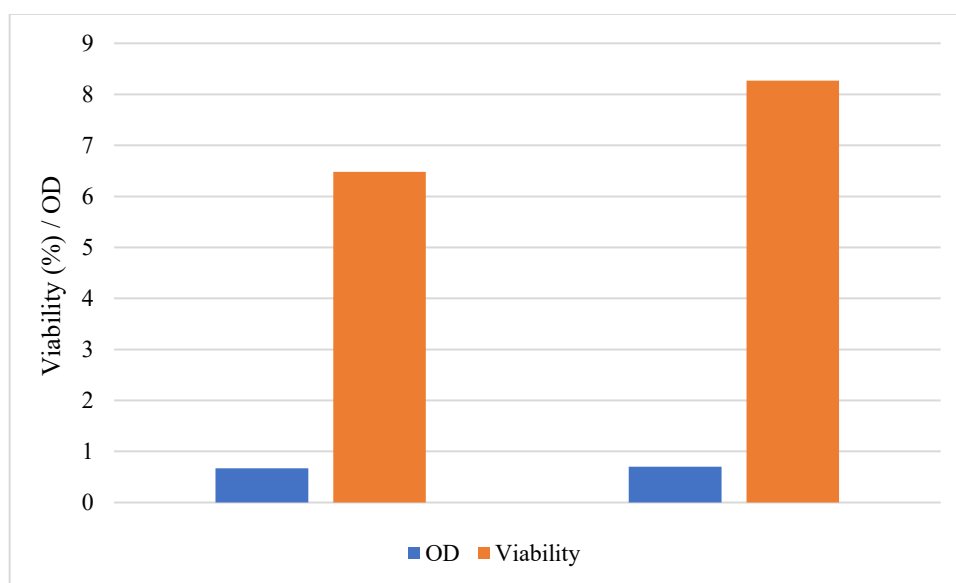


Figure 17. The effect of initial bacterial concentration (OD) on *L.lactis* bacterial average viability (N=3 replicates from every ES experiment). The OD was measured from the overnight culture before starting with the preparatory steps for the ES.

Additionally, the correlation between ECN bacterial load and ES ECN viability was analyzed (ODs ranged from 1.47 to 3.2) and it showed no connection between the initial ECN load and the ES ECN viability. (see **Appendix Figure A1**)

6.3.1.3 Polymer testing and selection for coaxial ES

Based on previous works in monoaxial ES, the hydrophilic PEO polymer had already shown reliable results for encapsulating bacteria. (Zupani *et al.*, 2019) However, as hydrophobic PDLA has features of a potentially benevolent biopolymer and research by Tsuji *et al.* has proved its electrospinnability as a stereocomplex (PLLA/PDLA) (Tsuji *et al.*, 2006), it was decided to test coaxial ES of living bacterial using PDLA with the proposed solvent by the supervisors.

Firstly, the most optimal flow rate for 15% w/w PDLA in organic solvent X was set by visually observing the effect of flow rate on the morphology of the fibers (the results are given in **Table 5**).

Table 5. The effect of flow rate on the morphology of the monoaxially ES fiber

Rate (mL/hr)	Consequence
0.4	Bursts of fibers, non-continuous flow
0.6	Continuous flow, one fiber
0.8	Fiber has many separate endings (brittle)
1	Fiber has even more separate endings (very brittle)

Based on the results, 0.6 mL/hr was the most optimal flow rate, the syringe with a PDLA solution was fixed at a distance of 16 cm from the collector plate, as it visually resulted in the best outcome. Next, the variation of voltage was tested.

As can be seen from **Table 6**, the most optimal voltage was ratified based on how brittle the fiber was, how long it took for the Taylor cone to form (the distance from the syringe when the fiber starts to spin). The presence of fibers on glass microscope slides was observed with a light microscope. Since 15% PDLA concentration did not show very uniform and numerous fibers, a 10% w/w PDLA in organic solvent X was created for comparison.

Table 6. The effect of voltage on the morphology of the fiber

PDLA, 15%, 16 cm distance, rate 0.6 ml/hr (visually solution is too thick)		PDLA, 10%, 16 cm distance, rate 0.6 ml/hr	
Voltage (kV)	Consequence	Voltage (kV)	Consequence
7-8	one output, sprays all over the place, thick layer	7-8	one output, spinning 40%-50% of the distance, formation of circle
9-12	Spinning from small distance to 60% of the fixed distance, many brittles	10	60% of the distance spinning
13	spinning from the very beginning of the needle (100% of the distance)	11	spinning 75% - 80% of the distance
14 - 17	Spinning from shorter distance to no spinning	12 - 17	No spinning

The following conclusions can be drawn from PDLA testing:

- ES 15% PDLA is more unstable than ES 10% PDLA solution;
- voltage of 13 kV was the most optimal for ES of 15% PDLA in organic solvent X, whereas, 11kV provides the best result for 10% PDLA.

These obtained parameters were adapted for coaxial ES of PDLA.

Due to the fact that the one other hydrophobic polymer X has been successfully coaxially ES in previous research at the Institute of Pharmacy at the University of Tartu, both hydrophobic polymers PDLA and polymer X were further tested for coaxial ES.

6.3.2 Coaxial ES

Core-sheath fibers were produced by coaxial ES to encapsulate EC101 (*E.coli* with the fluorescent protein expression plasmid). This specific bacterium was selected because the core fiber was hydrophobic and it was not possible to measure the bacterial viability as before (with serial dilution and plating). It is important to acknowledge that due to the hydrophobicity, polymer X, as well as PDLA, are suitable to serve as an outer layer and

protect the core (bacterial dispersion together with a hydrophilic polymer X). For wound dressing application it is more suitable to have a hydrophobic polymer matrix.

After the preparation of EC101 suspension, mixing with 0.625% hydrophilic polymer X solution and 15% polymer X solution, the two phases were set up to their separate microinject pumps. Coaxial ES by Xie *et al.* was achieved with the ratio of 1/6 between the flow rates of the shell and core solution (Xie *et al.*, 2016). Taken this standard into account, the optimal flow rates of polymer X and EC101 were 0.7 mL/hr and 0.1 mL/hr, respectively. Nonetheless, coaxial ES did not work properly, as the output of fiber was multi-streamed and the solution did not result in one core-shell fiber. Although the concentration of the sheath (copolymer poly(ethylene glycol)-polylactide) solution in the aforementioned paper was also 15%, the voltage applied was 20 kV, whereas the voltage applied in our case was 13kV. However, the increased voltage did not improve the given situation, so it was decided to decrease the concentration of the polymer solution to 12.5%. Alas, after multiple adjustments of the flow rates, visibly the process did not produce any core-sheath fibers. This assumption was also proved by the SEM analysis (example of micrograph shown in **Figure 18**) where abridged and non-uniform fibers were seen.

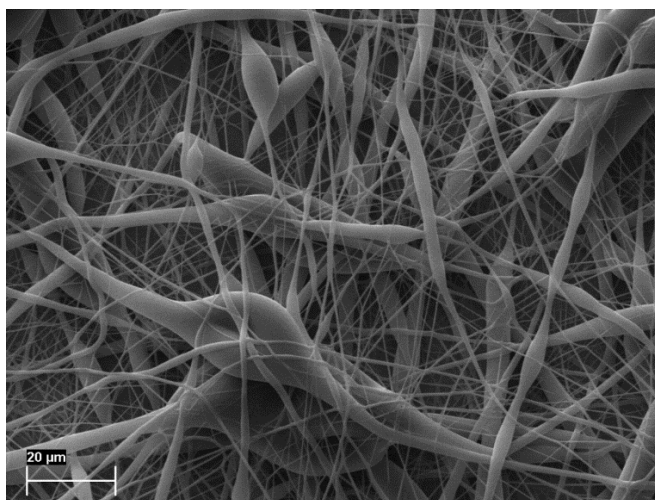


Figure 18. SEM micrograph of an unsuccessful Polymer X+EC101 core-sheath fiber mat (Magnification 2000X)

In the tested conditions, the ES was unsuccessful probably due to the dry conditions on that day (RH was 10.4%), however, more optimization and formulation should be done. (Change environmental conditions, test out different polymer X concentrations in monoaxial ES)

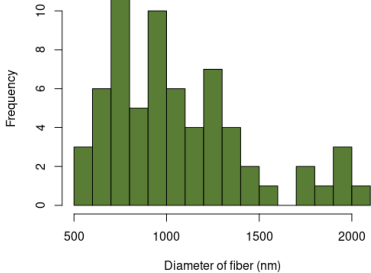
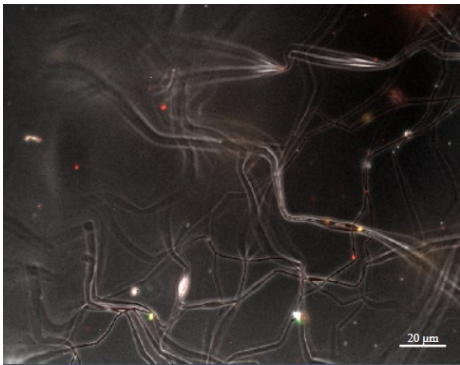
The parameters for coaxial ES of PDLA were adjusted based on the monoaxial polymer testing executed previously. This said a 10% PDLA was successfully coaxially ES with a

flow rate of 0.6 mL/hr (flow rate for bacterial suspension together with hydrophilic polymer was 0.1 mL/hr), with an input voltage of 13 kV and at a distance of 16 cm horizontally from the grounded collector. Therefore, living bacteria were encapsulated into core-shell fibers via coaxial ES. The consecutive fiber and bacterial analysis were based on the obtained PDLA-EC101 core-sheath fibers.

6.3.2.1 Fiber analysis

The fiber analysis for the coaxially ES PDLA and EC101 fibers is shown conclusively in **Table 7**. The marginal increase in diameters compared to monoaxial ES (see **Table 3**), refers to the double fibrous structure. Most of the fibers are in the 1000 nm region.

Table 7. Coaxial ES fiber analysis

Fiber diameters	Microcopy (confocal fluorescence microscopy image)
 Average: 1074 ± 390 nm	

6.3.2.2 Bacterial analysis

The bacterial viability was analyzed slightly differently from the monoaxial ES fiber mats as the viability was based on the $TIMER_{bac}$ expression of EC101. From the work of Claudi *et al*, the non-dividing and dormant cells have both fluorophores (red and green) and dividing cells have more green fluorophores (Claudi *et al.*, 2014). **Figure 19** expresses the green and red fluorophores in EC101 and ES EC101. It can be asserted that EC 101 has more green fluorophores with higher intensity, whereas, ES EC101 has more red fluorophores of the same intensity value. Based on **Figure 19**, it can be said that the amount of cells after ES with green fluorophores (with intensity over 2000) has decreased from 50 cells to 5 cells.

Meanwhile, the intensity of red fluorophores for the ES EC101 has also shown bigger values than EC101. Given these observations, it can be approximated that around 5 cells (showing bacterial viability of around 10%) are alive. However, the majority of the green fluorescent ES EC101 cells are indicating intensity lower than 1000 and show no notable increase in red fluorescence, it can be concluded that these cells have gotten damaged or are dead.

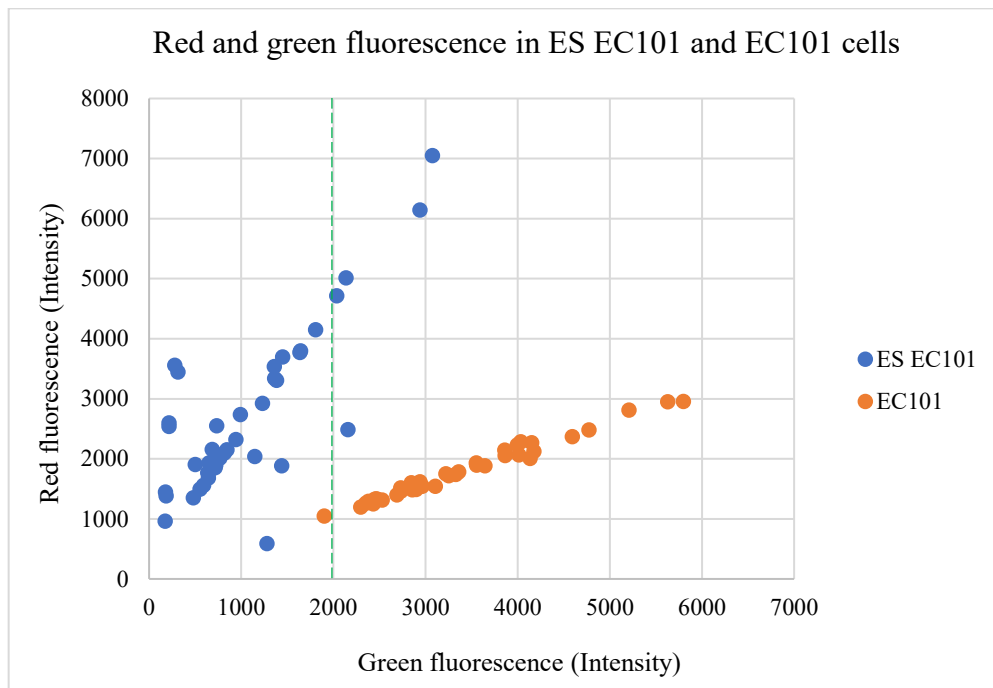


Figure 19. Green/red fluorescence intensity in ES EC101 and free EC101 cells. Data (N=50) are collected from confocal fluorescence microscopy images. The green dashed line shows the threshold intensity for considering bacterial viability.

6.4 Conclusions

This work has provided methods to design and develop functional biomaterials that contain living bacterial cells. The specific conclusions include:

- For monoaxial ES, encapsulation of both ECN and *L.lactis* was termed successful. This was achieved using PEO polymer (3% w/w) with H₂O as solvent. The ES conditions were the following:

- Voltage: 15.9 kV;
- Flow rate: 0.6 mL/hr;
- Distance between collector and pump: 13 cm.

The average environmental conditions were established based on data collected from experiment days and temperature was on average 23.1°C, whereas RH 23.2%.

- Regarding coaxial ES, the optimal shell polymer was 10% PDLA with organic solvent X. The core polymer was a combination of a hydrophilic polymer (0.625%) and *E.coli* with a Timer plasmid. The ES conditions were the following:

- Voltage: 13 kV;
- Flow rate 0.6 mL/hr (shell polymer) + 0.1 mL/hr (core polymer);
- Distance between collector and syringe: 16 cm.

The average environmental conditions were established based on data collected from experiment days and the temperature was on average 25.1°C, whereas RH was 13.5%.

- Throughout the experiments, higher bacterial concentration made the solution more viscous complicating the ES process. It can be said that the encapsulation of bacteria increased the ES fiber diameters. *E.coli* and *L.lactis* had similar viability within ES fiber mats. The *L.lactis* bacterial concentration showed only weak correlation between the viability of bacteria during ES but needs further research with significantly different bacterial loads.
- The effect of the tested additives (cAA, trehalose dihydrate) had no drawbacks regarding the ES process. The effect of trehalose dihydrate had a bacteria-dependent effect on the fiber diameter, as the fiber diameters of ES ECN increased, unlike the ES *L.lactis* fibers. Both additives only slightly increased the viability of ECN, yet trehalose dihydrate showed no rise in ES *L.lactis* viability. Conclusively, the effect of additives on ES bacteria requires further research.

SUMMARY

This thesis has provided methods to design and develop functional biomaterials that contain living bacterial cells. In this work, three types of bacteria were utilized in specific cases – *Escherichia coli* Nissle 1917 (ECN), *Lactococcus Lactis* IL1403 (*L.lactis*) and *Escherichia coli* with an electroporated plasmid pSC101-Timer-TetR-PtetA-BFP-noDeg (EC101). The bacteria were encapsulated using one of the two methods – monoaxial or coaxial electrospinning (ES). The polymers tested for ES were polyethyleneoxide (PEO), poly-D-lactide (PDLA), hydrophobic polymer X. The overall quality of an electrospun fiber mat was based on the physical properties of fibers and the viability of bacterial cells in the fibers. The quality assays included morphology evaluation with scanning electron microscopy (SEM), fiber diameter analysis with ImageJ, image processing using OpenCV to obtain information about the porosity of fiber mats, drop-plate method, and fluorescent protein expression for the bacterial viability testing.

Firstly, the monoaxial ES was implemented to test two types of bacteria – ECN and *L.Lactis* with a 3% PEO solution. The inclusion of bacteria increased the fiber diameters of ES mats. In order to improve bacterial viability and alleviate the drying of bacteria, different additives were included to the bacterial suspensions pre-ES. It can be concluded that the additives – trehalose hydrate and cAA – slightly increased bacterial viability compared to simply ES of bacteria with the carrier polymer on the day of these experiments.

Coaxial ES did not work with hydrophobic polymer X and EC101. Nonetheless, after PDLA polymer testing, the optimal parameters were exploited for coaxial ES, and encapsulation of EC101 was deemed to be successful. The bacterial viability proved to be higher than implementing monoaxial ES. Despite that, adjusting the system to produce a proper core-sheath fiber requires a considerable amount of effort. The author suggests several prospects in the light of continuing this research:

1. effect of additives on ES bacteria should be more tested;
2. the correlation between initial bacterial load and ES bacteria should also be more experimented with (varying ODs, checking viability);
3. coaxial ES should be more inspected as a method of encapsulating bacteria (determining more suitable polymers and/or solvents for bacteria).

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Appendix – The effect of initial bacterial concentration (OD) on ECN bacterial average viability

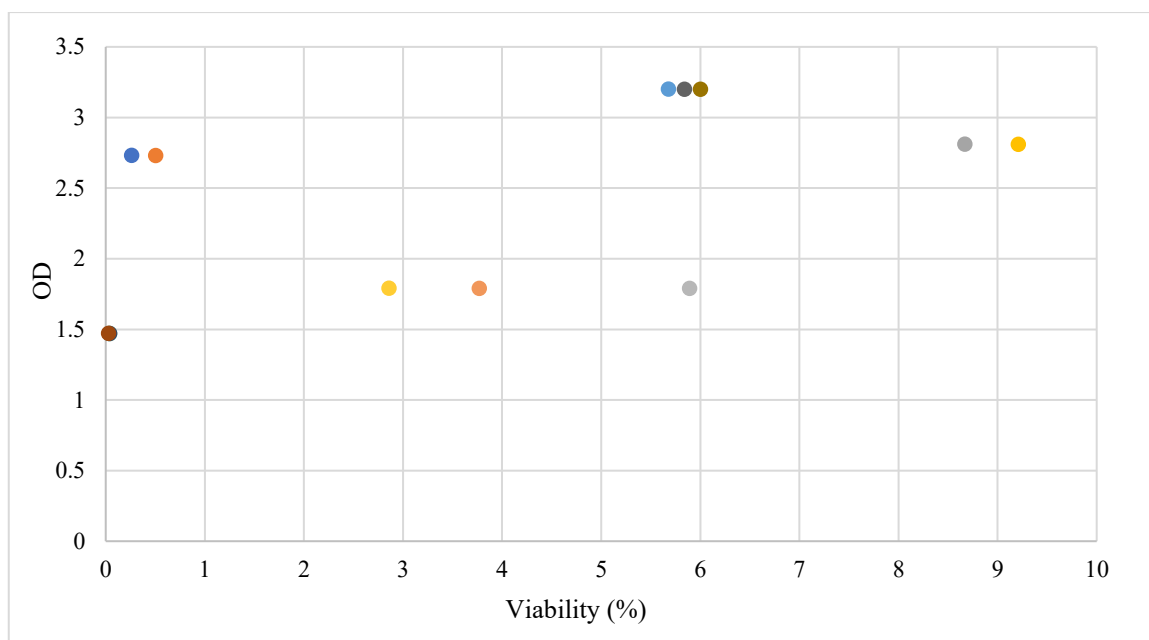


Figure A1. The effect of initial bacterial concentration (OD) on ECN bacterial viability (N=3 replicates from every ES experiment). The OD was measured from the overnight culture before starting with the preparatory steps for the ES. No correlation can be seen between the OD and viability.

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