Synthesis, Preparation, and Characterization of Living Biocomposites

DISSERTATION

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1. List of Publications

1. Poly(vinyl alcohol)-Hydrogel microparticles with Soft Barrier Shell for the Encapsulation of *Micrococcus Luteus*

*Mahsa Mafi,[1)] Ariel Kushmaro,[2)] Charles Greenblatt,[3)] Seema Agarwal[1)] and Andreas Greiner[1)]**

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2. Bioremediation of copper-ions by polymer encapsulated and immobilized *Micrococcus Luteus*

*Mahsa Mafi, Andreas Greiner**

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3. *Bacillus Subtilis* in PVA Microparticles for Treating Open Wounds

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2. Summary

The present thesis incorporates the manufacturing and development of polymer composites for the encapsulation of bacterial cells. The bacteria/polymer composites (based on the characteristic feature of bacteria) are applied in various industrial fields. In more detail, bacteria can be utilized in a wide range of applications, including bioremediation (e.g., wastewater treatment) and biomedical applications. Herein, two aerobic and gram-positive bacteria (*Micrococcus luteus* (*M. luteus*) and *Bacillus subtilis* (*B. subtilis*)) with a bacterial reference strain from an established culture collection were used for further studies. *M. luteus* has excellent potential for sequestration of heavy metals (e.g., copper in wastewater). Contrarily, *B. subtilis* is recently used for antimicrobial treatment.

The bacterial cell was protected via encapsulation in a polymeric matrix. A polymer matrix was produced by electrospinning or spray drying methods. Principally, a dispersion of poly(vinyl alcohol) (PVA) and bacteria was processed further with the mentioned methods. As a result, encapsulated bacteria in PVA particles (bacteria/PVA particles) were obtained. Based on this simple matrix, complex polymer composite structures, such as core/shell polymer particles and/or porous composite membrane of particles embedded in nanofibers nonwovens were designed.

In the first step, a bacteria/core/shell structure was synthesized and investigated. Comprehensively, *M. lutes*/PVA particles obtained from the spray drying process were chemically cross-linked with glutaraldehyde. The cross-linked particles are swelling in aqueous media, whereby the elasticity of the particles permits an easier proliferation of encapsulated *M. luteus*. However, in aqueous media, the release of *M. luteus* was observed. To protect the PVA particles and retard the release of *M. luteus*, surface polymerization of DEAEMA (*N*, *N*-diethylamino-2-ethylmethacrylate) monomer was performed using atom transfer radical polymerization (ATRP). PDEAEMA as a pH-responsive and antibacterial polymer was grafted

from *M. luteus*/PVA particles result in formation of core/shell particles (*M. luteus*/PVA/PDEAEMA). In acidic pH and by protonation of charged side groups in PDEAEMA chains, the steric repulsion occurs. Shell pores expand, which smooths the mass transport. Thereupon, elevated nutrient flow toward the PVA core benefits the growth and biological activity of encapsulated *M. luteus*. Diversely, the release of *M. luteus* from the *M. luteus*/PVA/PDEAEMA core/shell particles is conceivable regarding the antibacterial effect of protonated PDEAEMA shell. This method allows the long-lasting viability of bacteria in polymer core/shell particles.

Another approach is accomplished by a tea-bag concept, in which the bacteria/particles were surrounded by a porous nonwoven. In this method, the *M. luteus*/PVA cross-linked particles were supported against mechanical stresses, by using a nonwoven carrier. In particular, *M. luteus*/PVA cross-linked particles were embedded in a nonwoven of poly (lactic acid) (PLA) electrospun nanofibers. The nonwoven was achieved by a unique approach of polymer nonwoven manufacturing called the wet-laid method. The manufacture of such composite nonwovens requires a dispersion of shortcut fibers. Shortcut fiber dispersions are obtained by cutting electrospun PLA fibers in a blender. PLA short fibers serve as a carrier for *M. luteus*/ PVA cross-linked particles. The manufactured porous composite membrane of PLA nonwoven and *M. luteus*/PVA cross-linked particles have been successfully used for copper ion (Cu^{2+}) biosorption. The Cu^{2+} uptake by porous membrane composite was quantified by inductively coupled plasma-optical emission spectroscopy (ICP-OES), and by energy-dispersive X-ray spectroscopy (EDX) measurement of the accumulated Cu^{2+} in PVA cross-linked particles. The continuous biosorption capability of *M. luteus* for Cu^{2+} uptake was investigated by the specific time intervals.

Finally, the antibacterial treatment of open wounds colonized by pathogenic bacteria (e.g., methicillin-resistant Staphylococcus aureus (MRSA)) was investigated by encapsulated

Bacillus subtilis (*B. subtilis*) in polymer particles. In this method of treating open wounds, encapsulated *B. subtilis* in PVA particles (proceeded similar to the encapsulation of *M. luteus*) was used. *B. subtilis* is capable of producing antibacterial agents (e.g., Surfactin and Fengycin), that demonstrates activity against pathogenic bacteria. PVA particles provide adhesion to the skin, which consequently facilitates the healing of the wound. The implant of polymer matrixes with *B. subtilis* was investigated further for open wound treatment, in order to identify the antibacterial effect. The encapsulated *B. subtilis* produced Surfactin as an antibacterial agent after 4h up to 350 $\mu g.ml^{-1}$ in 12 h (the minimum amount to act as an antibacterial agent was determined to 200 $\mu g.ml^{-1}$). The antibacterial effect of *B. subtilis* was studied in vitro as well as in vivo by directly delivering the polymer/bacteria composites onto the wound.

3. Zusammenfassung

Die vorliegende Arbeit befasst sich mit der Herstellung und Entwicklung von Polymerkompositen zur Verkapselung von Bakterienzellen. Die Bakterien/Polymer-Verbundwerkstoffe können, basierend auf der charakteristischen Eigenschaft von Bakterien, in verschiedenen industriellen Bereichen eingesetzt werden. Genauer gesagt, werden Bakterien in einer Vielzahl von Anwendungen eingesetzt, einschließlich der Bioremediation von Abwässern und biomedizinischen Anwendungen. Hierfür wurden zwei aerobe und gram-positive Bakterien (*Micrococcus luteus* (*M. luteus*) und *Bacillus subtilis* (*B. subtilis*)) mit einem bakteriellen Referenzstamm aus einer etablierten Kultursammlung für weitere Untersuchungen ausgewählt. *M. luteus* hat ein hervorragendes Potenzial zur Abscheidung von Schwermetallen wie Kupfer aus dem Abwasser. Im Gegensatz dazu wird *B. subtilis* neuerdings zur antimykotischen Behandlung eingesetzt.

Die Bakterienzelle wurde durch Verkapselung in einer polymeren Matrix geschützt. Die Polymermatrix wurde durch Elektrospinn- oder Sprühtrocknungsverfahren hergestellt. Grundsätzlich wurde eine Mischlösung aus Poly(vinylalkohol) (PVA) und Bakterien weiterverarbeitet. Als Ergebnis wurden verkapselte Bakterien in PVA-Partikel (Bakterien/PVA-Partikel) erzielt. Basierend auf diesen einfachen Matrizen wurden komplexe Polymerkompositstrukturen, wie Kern/Schale-Polymerpartikel und/oder poröse Kompositmembranen aus Partikeln und Nanofaservliesen, erhalten.

Im ersten Schritt wurde Bakterien/Kern/Schale-Struktur synthetisiert und untersucht. *M. lutes*/PVA-Partikel wurden mit Glutaraldehyd chemisch vernetzt. Die vernetzten Partikel sind in wässrigen Medien quellfähig, wobei die Elastizität der Partikel eine leichtere Vermehrung des eingekapselten *M. luteus* ermöglicht. In wässrigen Medien wurde jedoch die Freisetzung von *M. luteus* beobachtet. Um die PVA-Partikel zu schützen und die Freisetzung von *M. luteus* zu verzögern, wurde eine Oberflächenpolymerisation von Poly(*N*, *N*-diethylamino-2-

ethylmethacrylat) (PDEAEMA) mittels Atomtransferradikalpolymerisation (ATRP) durchgeführt. PDEAEMA als pH-empfindliches und antibakterielles Polymer wurde auf *M. luteus*/PVA-Partikel aufgepfropft, wodurch Kern/Schale-Partikel (*M. luteus*/PVA/PDEAEMA) entstanden. Bei saurem pH-Wert und durch Protonierung der geladenen Seitengruppen in den PDEAEMA-Ketten tritt die sterische Abstoßung auf. Die Poren der Schale vergrößern sich, wodurch der Stofftransport erleichtert wird. Daraufhin begünstigt ein drastischer Nährstofffluss in Richtung des PVA-Kerns das Wachstum und die biologische Aktivität des verkapselten *M. luteus*. Hinsichtlich der antibakteriellen Wirkung der protonierten PDEAEMA-Schale ist eine Freisetzung von *M. luteus* aus den *M. luteus*/PVA/PDEAEMA-Kern/Schalenpartikeln denkbar. Diese Methode ermöglicht eine langanhaltende Lebensfähigkeit der Bakterien in den polymeren Kern/Schale-Partikeln.

Ein weiterer Ansatz wird durch ein Teebeutelkonzept erreicht, bei dem die Bakterien/Partikel von einem porösen Vlies umgeben wurden. Bei dieser Methode wurden die *M. luteus*/PVA-vernetzten Partikel gegen mechanische Belastungen und durch die Verwendung eines Vlieslträgers unterstützt. Im Detail wurden *M. luteus*/PVA-vernetzte Partikel in ein Vlies aus Poly(milchsäure)-(PLA)-elektrogesponnenen Nanofasern eingebettet. Das Nanovlies wurde durch einen einzigartigen Ansatz zur Herstellung von Polymervliesen, der so genannten "wet-laid"-Methode, hergestellt. Die Herstellung solcher Verbundvliese erfordert eine Dispersion von Kurzschnittfasern. Kurzschnitt-Faserdispersionen werden durch Schneiden von elektrogesponnenen PLA-Fasern in einem Mischer gewonnen. PLA-Faser-Kurzschnittfasern dienen als Träger für *M. luteus*/PVA-vernetzte Partikel. Die hergestellte poröse Kompositmembran aus PLA-Vlies und *M. luteus*/PVA vernetzten Partikeln kann erfolgreich für die Biosorption von Kupferionen eingesetzt werden. Die Kupferaufnahme durch den porösen Membranverbund wurde mittels ICP-OES quantifiziert, und das akkumulierte Kupfer in den PVA-vernetzten Partikeln wurde mittels energiedispersiver Röntgenspektroskopie (EDX) nachgewiesen. Die kontinuierliche Biosorptionsfähigkeit von *M. luteus* zur

Kupferaufnahme wurde durch Zugabe von Kupfer nach dem ersten Zeitintervall und Wiederholung der ICP-OES-Messung der zweiten Runde der Kupferaufnahme untersucht.

Schließlich wurde die antibakterielle Behandlung von offenen Wunden, die von pathogenen Bakterien (z. B. Methicillin-resistenter Staphylococcus aureus (MRSA)) befallen sind, mit Hilfe von verkapseltem Bacillus subtilis (*B. subtilis*) in Polymerpartikeln untersucht. *B. subtilis* ist in der Lage, antibakterielle Wirkstoffe (z. B. Surfactin und Fengycin) zu produzieren, welche Aktivität gegen pathogene Bakterien zeigen. Hierbei wurde zur Behandlung offener Wunden verkapselte *B. subtilis* in PVA-Partikeln verwendet (Verfahren ähnlich der Verkapselung von *M. luteus*). Die PVA-Partikel gewährleisten eine Adhäsion an die Haut, die die Heilung der Wunde erleichtert. Für die Behandlung offener Wunden wurde die Implantation von Polymermatrizen mit *B. subtilis* weiter untersucht, um die antibakterielle Wirkung zu ermitteln. Dies wurde sowohl in vitro als auch in vivo untersucht, indem die Polymer/Bakterien-Komposite direkt auf die Wunde aufgebracht wurden. Die verkapselte *B. subtilis* produzierte Surfactin als antibakteriellen Wirkstoff nach 4h bis zu 350 $\mu g.ml^{-1}$ in 12h. Die minimale Menge, um als antibakterieller Wirkstoff zu wirken, wurde zu 200 $\mu g.ml^{-1}$ bestimmt.

4. List of Symbols and abbreviations

M. luteus	*Micrococcus luteus*
Cu	copper
Th	thorium
Sr	strontium
U	uranium
mg	milligram
G	gram
Ppm	part per milion
B. Subtilis	*Bascillus subtilis*
E. Coli	*Escherichia Coli*
RNA	ribonucleic acid
CO_2	carbon dioxide
O_2	oxygen
PVC	poly(vinyl chloride)
PS	poly styrene
PVA	poly(vinyl alcohol)
°	degree
°C	degree centigrade
AFM	atomic force microscopy
PVP	poly(vinyl pyrrolidone)
HPC	hydroxypropyl cellulose
kV	kilovolt
Cm	centimeter
PEO	poly(ethylene oxide)
SEM	scanning electron microscopy
PAN	polyacrylonitrile
PMMA	poly(methyl methacrylate)
PLA	poly(lactic acid)
PVB	poly(vinyl butyral)
PCL	poly(caprolactone)
DMF	*N*, *N*-dimethylformamide
CVD	chemical vapor deposition

PPX	poly(p-xylylen)
ATRP	atom transfer radical polymerization
RAFT	reversible addition fragmentation transfer
NAMP	nitroxide mediated polymerization
LCST	lower critical solution temperature
PNIPAM	poly(*N*-isopropylacrylamide)
UCST	upper critical solution temperature
PNAGA	poly(*N*-isoproppylacrylamide)
PAA	poly(acrylic acid)
PDEAEMA	poly(N, N-diethylamino-2-ethylmethacrylate)
PMA	paramethoxyamphetamine
PTA	poly*(2*-tertbuthylamino) ethyl methacrylate
PEGMA	poly(ethylene glycol) methacrylate
Au	Gold
PG	Peptidoglycan
TA	teichoic acid
TVA	teichuronic acid
LPS	lipopolysaccharides
ICP-OES	inductively coupled plasma-optical emission spectroscopy
S. aureus	staphylococcus aureus
IMS	imaging mass spectroscopy
HPLC	high performance liquid chromatography
C. albicans	*Candida albican*

5. Introduction

Microorganisms, such as algae, fungi, yeasts, bacteria, and viruses are of considerable interest for a large variety of applications, including the food industry, agriculture, medicine, and bioremediation. In the food industry and by a dramatic rise in demands, industrial production of enzymes increased. Using microorganisms as the source of enzymes goes back to 1874. The use of microorganisms in food products can also provide the health for the host. As an example for this group of microorganisms, probiotics can be mentioned, which exist in most dairy products.[1-7] In agriculture, the use of chemical fertilizer can be exchanged by using microbial sources that increase biosafety and innovation in this field.[3] Microorganisms are also utilized for human health over the last 20 years in many diseases, such as inflammatory bowel, allergy, infection, and cancer.[8, 9] Another widespread application of microorganisms is in the decontamination of the ecosystem. Microorganisms have the most potential for the removal of a wide range of pollutants including heavy metals.[10-14]

Within the group of microorganisms, bacteria are known as the most abundant species. Using bacteria has several advantages compared to other microorganisms, such as enzymes, due to the low-cost production process and no requirement for purification.[15, 16] To retain the biological activity of bacteria and achieve a longer storage time, immobilization of bacterial cells is demanded. The immobilization of bacteria has several advantages over the free form of bacterial cells. The protection against unwanted environmental stress and controlled release and delivery are some of the most important advantages.

The current work was inspired by different fields of material science and biology that provide insights for the design of living biocomposites of polymer and bacteria. Within this framework, the production of polymer matrices with encapsulated bacteria is the major task. The polymer

matrix must ensure sufficient transport of oxygen and nutrient. Simultaneously, it should allow the removal of metabolically produced by bacteria.

The present dissertation focuses on the preparation and engineering of polymer matrices for the encapsulation of two different types of bacteria. The matrices were manufactured in the form of polymer nanofibers and/or polymer particles by using electrospinning and spray drying methods, respectively. In both methods, encapsulation of bacteria in terms of containing the biological activity is guaranteed. After that, the second essential factor is to maintain the mechanical and chemical stability of the matrices, especially in an aqueous medium (not to face the unwanted leakage of the encapsulated bacteria). Accordingly, the surface of the polymer matrices was modified with the help of polymer chemistry, and by polymerization of a hydrophobic shell. Furthermore, engineering of the polymer membranes requires polymer processing techniques like the production of the nonwoven membrane with the wet-laid method. As stated in the "tea-bag" principle, the living matrix of bacteria/particles was embedded in another polymeric carrier.

Finally, the encapsulated bacteria in polymer composites were applied in decontamination of wastewater, as well as biomedical fields.

5.1 Bacteria

Bacteria are known as the first organisms to evolve on the earth from ancient times. Although bacteria involve in many diseases, they are playing a role in a wide range of important contributions to the environment. In more detail, the viability of bacteria in almost every habitat makes life possible on the earth via processing enormous functions in the ecosystem. Decomposition of organic materials, capturing nitrogen, and photosynthesis are some examples of essential functions of bacteria.

The classification of bacteria based on their shape is into three basic structures: bacillus in straight and rod shape, coccus for spherical shape, and spirillus for those showing along and helical shape. However, in all three groups, the bacterial cell possesses a simple structure consisting of a cytoplasmic membrane and cell wall. The lack of nuclei is the noticeable difference between bacterial cells and all other organisms´ cells. The most important part of the bacterial cells is the cell wall, which contains peptidoglycan (poly (*N*-acetylglucosamine and *N*-acetylmuramic acid)). The cell wall of the bacteria plays an important role in structural integrity and the rigidity of the cells. Moreover, one of the crucial factors to differentiate bacteria is based on the composition of the cell wall, as the cell wall structure is not identical in all varieties of bacteria species. Based on that, two category types of bacteria are known: gram-positive and gram-negative bacteria.[10, 17]

5.1.1 *Micrococcus Luteus*

Micrococcus luteus (M. luteus) is from a group of cocci bacteria and displays a spherical shape. It is aerobic and gram-positive bacteria, that have been repeatedly found in harsh environmental conditions. The production of yellow water-insoluble pigments is a characteristic of this group of bacteria. Its easy handling feature is acknowledged since it is biologically active at low water activities and rapid change in osmotic pressure. *M. luteus* is capable to withstand a high concentration of copper (Cu) and sequestrating other heavy metals, such as thorium (Th), strontium (Sr), and uranium (U), and so forth. The highest metal biosorption of metal by *M. luteus* is approximately at pH= 4.[18-23] The uptake of different metal ions (mg) per g of *M. luteus* and pH values are summarized in Table 1.

Table 1. Biosorption of metal ions (initial concentration 50 ppm) by *M. luteus*.

Metal	pH	Biosorption $mg \cdot g^{-1}$	Reference
U	3.5	38.8	Nakajima and Tsuruta[24]
Th	3.5	77	Nakajima and Tsuruta[24]
Cu	5	33.5	Nakajima et al.[22]
Sr	4	25	Faison et al.[23]

5.1.2 *Bacillus subtilis*

Bacillus subtilis (*B. subtilis*) is gram-positive bacteria, which is naturally found on human skin. *B. subtilis* is used for pharmaceutical lipopeptides that functions as antifungal and antibacterial agents. Furthermore, for several decades, *B. subtilis* has been employed as a source of food-processing and industrial enzymes.[1, 25]

5.2 Encapsulation of bacteria in polymer matrices

The application of free bacterial cells is limited concerning different disadvantages explained as follows. The release of a massive number of bacteria in nature might alter the balance of natural bacteria in the environment. Therefore, separating the bacterial cells in the solution is a challenge for large scale recovery process. Additionally, the biological activity of bacteria might sink, due to the unfavorable environmental impacts. For these reasons and to improve the rate, efficiency, and time of the biological activity, immobilization of bacteria is recommended. Immobilization is defined by two terms: Limiting and preventing. In more detail, immobilization limits the mobility, free migration, and enzymatic activity of bacteria, likewise preventing the harsh impact of the environment, such as pH, temperature, and organic solvents.

The use of immobilized bacteria in various applications results in many advantages, a few examples are: decreased expenses, easier removal, efficiency, possible recovery and continuous use of bacteria, higher resistance of bacteria, right delivery to a particular receptor, and increased storage time through prolonged biological activities.[26-31]

The metabolic response of the bacterial cells to immobilization is difficult to define. This is not only due to the different genetic of bacteria and culture method but also to the material of the carrier and type of immobilization. The immobilized cell might respond to the immobilization in different ways. For instance, *Echeria coli* (*E. coli*) in different types of immobilizations shows various physiological responses, like slower degradation of ribonucleic acid (RNA), higher activity of the enzyme, and more oxidize glucose metabolites. The increase or decrease growth rate of bacteria, higher producibility, and increased resistance is the possible response to immobilization. The higher producibility and resistance are caused by the matrix, which is used for encapsulation. The increase in growth rate after immobilization has several reasons, the most important one is the protein, cellulose, and other nutrient provided by the matrices. For this reason, the chosen matrix is an important factor. Immobilization might also decrease the growth, which is a result of difficulties of mass transfer. In more detail, the oxygen and nutrient transfer, as well as the transfer of the produced CO_2 by metabolisms might be hindered by the matrix, which is like a membrane barrier between bacteria and the surrounding atmosphere. Furthermore, the accumulation of bacteria in the edge of the matrix because of the better attachment or higher access to the nutrient may cause the escape and leaching of the bacteria. Consequently, the growth rate slows down.[32]

The immobilization of bacteria also changes the concentration of surrounding nutrient media, which considerably results in a different environment for biological activity. Therefore, the osmotic pressure of the cell is reduced.[33]

Immobilization methods are divided into 7 cases: adsorption to the surface, covalent bond to the surface, cross-linking of the cells, flocculation, nano-coating, entrapment, and encapsulation.[32, 34] From all of the mentioned methods, encapsulation is attributed to the efficient protection of bacteria with a membrane (usually semi-permeable) from surrounding media. In this method, bacteria are approximately placed in the center of the matrix, or preferably have no direct interaction with the surrounding environment. In more detail, a barrier between the bacteria and the environment is made. Although the nutrient and produced CO_2 transfer, and also the proliferation of bacteria might cause problems in this method, it is still the most protectable compared to other immobilization methods.[34] As it is mentioned, growing the bacteria might generate mechanical damages to the supporting matrix. Therefore, the materials used for encapsulation have an essential effect. Some important features of the matrix are explained as follows: Firstly, the matrix needs to provide a bacterial-friendly environment for encapsulation, in which no antibacterial effect applies to bacterial cells. Secondly, the diffusivity of bacteria in the matrix should be possible. It is also necessary for a matrix not to be harmful or toxic to the surrounding media. Lastly, the water solubility of the matrix also plays an important role, as for the water purification applications, the matrix needs to be stable. In general, matrices are classified into two groups of inorganic and organic.[35] Inorganic matrices used for encapsulation like silicone show problems such as low binding groups for attraction of biological cells. The organic matrices are divided into two categories of natural (e.g., plant fibers, chitosan, alginate) and synthetic (e.g., Poly(vinyl chloride) (PVC), polystyrene (PS), poly(vinyl alcohol), (PVA)).[27] The advantages of synthetic matrices compared to natural organic ones is due to their mechanical strength and low cost. Moreover, they provide the possibility of controlling the molecular weight, porosity, and polarity of the molecular structure. There are several applications for encapsulated bacteria in artificial polymers, such as in microbial fuel cells, catalysis, biotechnical synthesis, textile, or bioremediation fields.[7, 18, 36-38]

PVA is one of the most used polymer matrices for the encapsulation of bacteria.[15] It is a nontoxic and water-soluble polymer with excellent mechanical durability.[39] In aerobic and anaerobic conditions, PVA has a biodegradable feature. Moreover, PVA is broadly used in biomedical applications, due to its biocompatibility and elasticity.[40] Furthermore, it is utilized in the food industry, cosmetic industry, lacquers, and resins. The water solubility of PVA hinders the use of this polymer in industrial applications. However, this polyhydroxy polymer provides many possibilities for low-cost physical and chemical cross-linking. In many literature studies, physical cross-linking of PVA with freeze-thawing cycles was shown, which is regarding the crystallinity of the polymer. Nevertheless, the aging, opaqueness, and thermal reversibility of physically cross-linked PVA is a hindrance in many applications. Therefore, glutaraldehyde or hexamethylene diisocyanate is used to provide chemical cross-linking. Although cross-linked PVA matrices are not soluble in water, there is a tendency to swell. This swelling tendency is proportional to the cross-linking density. The swelling feature is important in the context of the release pattern of bacteria from the PVA matrix.[15, 39-46]

The processing of the PVA matrices is achievable in different types of encapsulations, like encapsulation in fibers, or particles.[10, 34]

5.2.1 Encapsulation in PVA particles and nanofibers

5.2.1.1 Polymer particles and spray drying

Polymer particle engineering was attracted certain attention, in order to reduce the weight of the polymeric materials for a wide range of applications, such as the food industry, pharmaceutical, cosmetics. There are several technologies for particle formations. For instance, evaporation, emulsion polymerization, liquid jet, coacervation, and granulation can be mentioned here. [40, 47, 48] Most of these techniques are solvent-based, which require extra steps for removing solvent. On the contrary, spray drying has a beneficial processing in water. Thus,

this method is suitable for processing biopolymers, in consideration of the poor solubility in organic solvents. This is in contrast to many conventional methods of particle producing, in which only organic solvents are utilized. The history of spray drying dates back to the 1870s through the early 1900s, following by commercial application in milk drying production by the second decades of the twentieth century.[49] Furthermore, this method was used in the pharmaceutical industry, and for encapsulation of antibiotics, vitamins, and so forth. In the food industry, encapsulation of proteins against degradation was achieved. This technique also has the potential for encapsulation of corrosive and abrasive, as well as active materials like biological species. The process of spray drying can be divided into three steps of atomization, drying, and recovery. Spray drying is a cheap, one-scale, and rapid procedure technique, in which the formation of particles is performed by transforming from solution to solid powder. The process is starting by feeding polymer liquid in the form of a solution, suspension, or emulsion. In the atomization step, the polymer liquid is sprayed by a nozzle under the presence of atomization gas (air or nitrogen) into droplets. The cycle type in the spray drying process can be considered as an open or a closed-loop cycle. In an open cycle, atmosphere air is drawn to the loop. On the other hand, when the product is toxic or in an explosive atmosphere, inert gas like nitrogen is used. As a result, the formed droplets are exposed to a hot gas provided by an aspirator. The liquid phase is evaporated resulting in the formation of particles as dry powders. The high surface-to-volume ratio of droplets and high-performance evaporation is known as a drawback of this method since the hot flow might not uniformly touch all particles in the chamber. The recovery is done by exhaust gas using a filter bag or a cyclone. The whole three steps take place in a few seconds with minimal contact of the hot flow to the material (shown in Figure 1). Several parameters (e.g., viscosity, feed rate, and temperature) can control the particle size and morphology. The importance of these parameters is due to their influence on morphology, which governs adhesion, hygroscopicity, crystallinity, and many other important factors.[49-58]

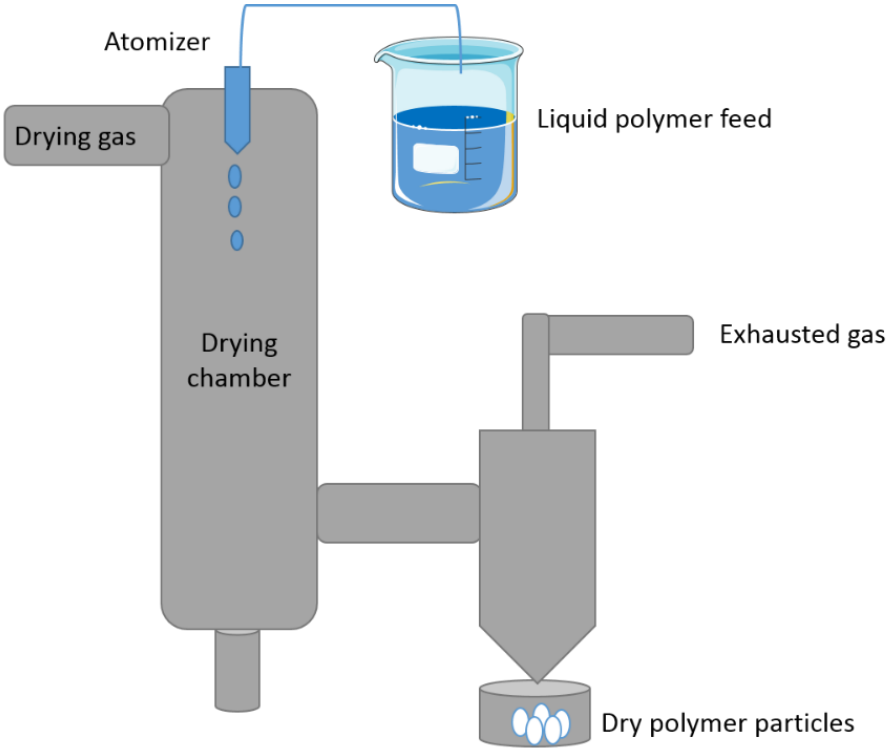


Figure 1. Scheme of the spray drying process, production of polymer particles.

5.2.1.2 Encapsulation of bacterial cell via spray drying

The encapsulation of bacteria in polymer particles is followed by the question of resistance of bacteria to high temperature (over 100°C). The work of Reich et al. revealed the fact of cell survival via this method, showing the encapsulated bacterial cells in particles (bacteria/particle) with Raman-AFM spectroscopy.[50] The state of the biologically active bacteria was tested with agar plate and formation of colonies. *M. luteus* and *E. coli* were successfully encapsulated in four different hydrophilic spray-dried polymer particles: PVA, poly(vinyl pyrrolidone) (PVP), hydroxypropyl cellulose (HPC), and gelatin. It was concluded that the proper temperature of the spray drying process concerning the survival of bacteria was 120°C for *E. coli*. In contrast, *M. luteus* can survive with a processing temperature of 150°C. The number of encapsulated bacteria in one particle and the number of the particles with encapsulated bacteria have remained without answer.

If the bacteria are orienting in the center of the particles, the leaching pattern might decrease. However, the nutrient transfer is possibly more difficult in contrast with when the bacteria are locating more in the outer part of the particles (see Figure 2). The encapsulated bacteria are surviving in particles for the minimum time of 14 months.[36]

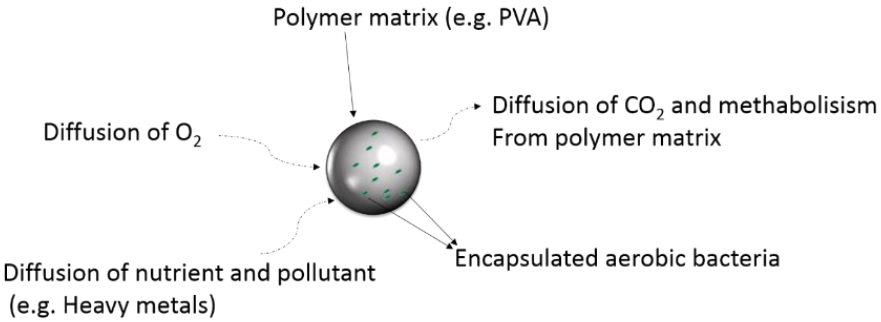


Figure 2. Encapsulated bacteria in polymer particles by spray drying. The nutrient and O_2 transport into the particles and exit of CO_2 gas.

5.2.1.3 Polymer nanofibers and electrospinning

Electrospinning has attracted numerous research interests, both from the fundamental science point of view and for perspective applications. In this method, the production of fibers in the range of nanometers up to a few micrometers is accomplished.[59-62] The process starts by feeding the polymer solution or melt from a spinneret connected to an electrode. The feed is led to the corresponding counter electrode with some distance to the spinneret by applying a strong electric field up to several $kV \cdot cm^{-1}$. The droplets induced by spinneret is forming a jet by whipping and stretching in the electric field and due to the Maxwell electrical stress. In more detail, when the electrostatic charge of the liquid polymer is larger than the surface tension, elongation will occur.[48, 63-65] The rapid solvent evaporation or the melt solidification are resulting in the production of fibers. Figure 3 is showing the setup of the electrospinning device.

Several parameters govern this method, including electrical conductivity, mass distribution, the viscosity of the solution, glass transition temperature, molecular mass range, the strength of the electric field, feeding rate, atmosphere condition (e.g., humidity and temperature), and so forth.[18, 64] The produced fibers are showing high permeability and surface area, small pore size, and ability of surface functionalization.[64, 65] Additionally, the process provides surface functionalization (e.g., increasing the hydrophilicity, bio-recognition, and biocompatibility).[66] Electrospinning has a wide range of applications including in: filtration, clothing, tissue engineering, biosensors, optic, energy conversion, food industry, drug delivery, electronic devices, and composite materials.[48, 64, 67-70] Electrospun membrane nonwovens have been used in bioremediation applications since they are suitable for encapsulation of enzymes and bacteria. The ease and low cost of the process, make electrospinning a stoning method of polymer processing.[8, 13, 16, 65, 67] Production of composites by this method is simple and achievable by adding different components to the polymer solution before electrospinning process.[18, 71, 72]

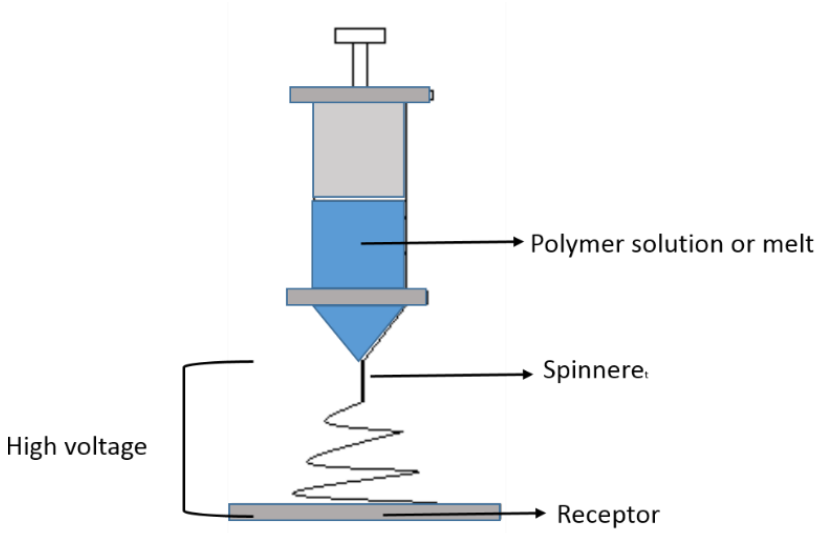


Figure 3. The basic setup of electrospinning process, production of polymer fibers.

5.2.1.4 Encapsulation of bacterial cell via electrospinning

The bacteria/polymer fiber composites have been studied in different researches. Salalha et al. reported the first work on encapsulation of biological materials like bacteria and viruses in electrospun PVA nanofibers in 2006.[71] In the work of Sarioglu et al. bacteria were trapped in nanofibers by natural adhesion force, which leads to the formation of biofilms. [65] The critical requirements of polymer-based material for encapsulation of bacteria via electrospinning are as follows: 1. Water-soluble polymers, such as PVA, PVP, PEO (poly(ethylene oxide)), etc. 2. continuous manufacturing ability, 3. Biocompatibility of polymers such as PVA, PEO, gelatin, and dextran. Therefore, a limited number of polymers are suitable for the encapsulation of bacteria in electrospun nanofibers. PVA and PEO are the most used polymers for this purpose that have all three parameters.[16, 73]

Bacteria pellet is priory mixed with the polymer solution and diffused in the fibers upon spinning.[74] The large surface-to-volume ratio of these structures allows an optimal contact of the substrate with bacteria while separating the microorganisms from the surrounding environment. The encapsulation of bacteria can be proved by scanning electron microscopy (SEM). The polymer matrix acts as a border for bacteria to the external environment (Figure 4). It is also important to know that a huge loss in bacterial activity might occur upon the electrospinning process. This is caused by a change in osmotic pressure upon evaporation of water and the production of solid nanofibers.

It is also feasible to double encapsulate the bacteria by electrospinning. In the research of Reich et al. and Gensheimer et al., the previously encapsulated *M. lutes*/PVA particles were re-encapsulated in nanofibers of polyacrylnitrile (PAN), poly(methylmethacrylate) (PMMA), polystyrene (PS), poly(lactic acid) (PLA), poly(vinylbutyral) (PVB), and polycaprolactone (PCL) forming meso-fiber nonwovens.[36, 50]

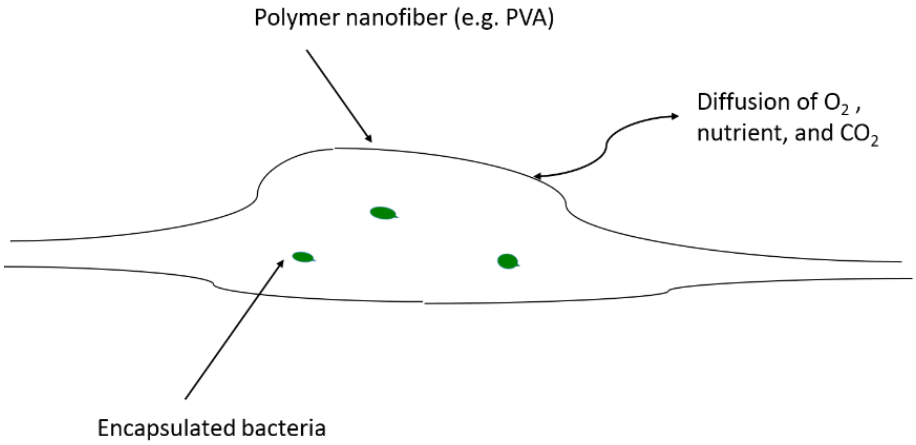


Figure 4. Encapsulated bacteria in polymer nanofiber made by electrospinning.

5.3 Modification of polymer matrices with encapsulated bacteria

The fundamental importance of protecting the polymer matrices with encapsulated bacteria is attributed to water solubility and chemically unstable features of the polymers. As the chosen polymers for encapsulation purposes are mostly water-soluble in order not to inhibit bacterial growth. Consequently, protection is required to assure the stability of the material in an aqueous environment. Additionally, mechanical damage to the matrices can be caused not only by an external force but also by the proliferation of the encapsulated cells. The stability of the matrix can be increased by post-treatment of the matrix including cross-linking or coating with hydrophobic polymer shells. Besides that, the post-treatment is controlling the release kinetics.[72, 75-78] The release profile is an important factor in encapsulation techniques, which has been influenced by several parameters. The release is firstly influenced by properties of the matrix such as solubility, cross-linking, polarity, and degradation. Secondly by thickness and conformity of the shell or coating. Finally, by external parameters, such as osmotic pressure and mechanical forces, and by shell responses to the stimulus.[79, 27, 79] Different studies were done on protecting the matrix with encapsulated bacteria. Therefore, various stabilization techniques were used by many projects like in Klein et al. using coaxial electrospinning of core-

shell fibers[27], and Knierim et al.[80] with the synthesis of hydrophobic poly(p-xylxlene) (PPX) by chemical vapor deposition (CVD).

Knierim and coworkers investigated core/shell particles with surface polymerization of PMMA on PVA particles with encapsulated *M. luteus*. Core/shell polymer particles have numerous applications in drug delivery, printing, catalysis, and biosource application. The presence of at least two materials in core/shell particles, in which the properties of the core and the shell separately can sum up and proceed to a special polymer composite.[81]

The post-treatment of bacteria-loaded matrices has no inhibition effect for bacterial growth. Although the free bacterial cells are not capable to survive in organic solvents, encapsulated bacteria can undergo treatment in organic solvents. The study of Gensheimer et al. in 2011 showed the survival of encapsulated *M. luteus* and *E. coli* after 340 h and by exposure to the different organic solvents.[36] Bacteria were encapsulated in PVA particles, following by double encapsulation in fibers by electrospinning of a suspension of particles in another polymer/ethanol solution. The encapsulated bacteria become resistant to toxicity, salinity, and pH. Non-water-soluble polymers used for electrospinning were dissolved in dimethylformamide (DMF). The exposure of the bacteria to the solvent was limited by PVA particles. The hydrophobic fibers also play an important role in retarding the bacteria leaching by preventing PVA particles from dissolving.[36, 65] Although the double encapsulation retards the release of bacteria, the mass transport for this immobilization model is retarded with the fibers, which cover the surfaces of the microparticles. Thus, a nonwoven is desirable, which makes no hindrance for nutrient passage through the PVA microparticles (like the tea-bag concept). Besides that, the preparation of high surface area non-woven composites with electrospun nanofibers is a time-consuming process, which can be optimized by preparation of similar nonwoven by use of short nanofibers' dispersion by "wet-laid method".[82] The procedure

offers the additional advantage of mixing other additives, in the short fiber dispersion just by mixing and forming the additive immobilized nonwoven.[82-84]

5.3.1 Surface modification of bacteria/particles via surface polymerization

Surface adjustment by applying a polymer coating becomes a powerful approach to tailor the surface properties, such as wettability, corrosion resistance, biocompatibility, and so forth. The polymer film can be sprayed to the surface and form a physical binding, or contrarily polymer chains can be covalently graft to the surface. Covalently grafted polymer chains are called polymer brushes. Precisely, polymer brushes are polymer films attached to a surface by individual chain end. The concept of polymer brushes has given a rise to surface modification techniques. There are two approaches for synthesis of polymer brushes. The first approach is by attachment of the preformed polymer to a surface with physisorption or chemisorption. Diversely, "grafting from" the approach is defined as the formation of polymer chains from the active site on the surface (Figure 5). The drawback of "grafting to" method, regarding the steric repulsion of polymer chains, hinders the arrangement of thick brushes. Therefore, surface modification with grafting from method is more versatile and robust. As it was declared, in "grafting from" method, the surface needs to be functionalized to initiate the polymerization.[85-88] Building up the polymer brushes with various methods of polymerization, including anionic, cationic, ring-opening, and controlled radical polymerization are acknowledged. Among all, controlled/living free radical polymerization has gained attention for widespread use. The reason is due to the satisfying control on the architecture and grafting density, as well as the possibility for performance in aqueous and organic media. Tailored polymers with controlled physical and chemical properties are attractive for a variety of applications. Therefore, the demand for the synthesis of polymers with specific architecture, composition, and functionality increases.

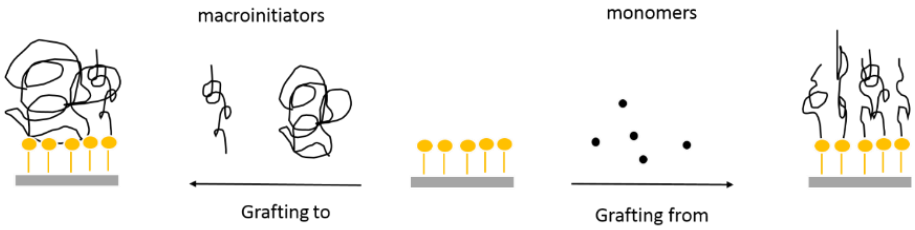
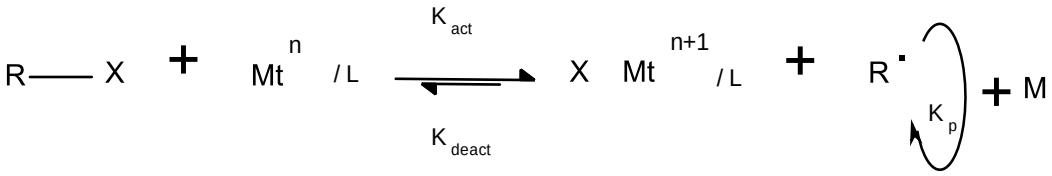


Figure 5. "Grafting from" and "grating to" method for synthesis of polymer brushes.

One of the subclasses of controlled/living radical polymerization is atom transfer radical polymerization (ATRP). ATRP has several advantages over other methods of controlled polymerization (e.g., reversible addition/fragmentation transfer (RAFT) or nitroxide mediated polymerization (NMP)) through its resistance to impurities. ATRP provides a mild condition in terms of pH and temperature, which is an essential factor in the polymerization process in the presence of sensitive materials, like biologically active components.[89-94] There are many studies regarding ATRP and grafting from a functionalized surface.[85, 86]

In this method, carbon-carbon bond formation is initiated with a dormant alkyl halide-terminated polymer chain end in the presence of a redox-active metal complex as a catalyst. The halogen atom (x) of the dormant initiator (R) is transferring to the metal complex (Mt/L), and oxidation of the transition metal complex occurs by the transfer of an electron from the metal complex to the halogen. As a result of the redox mechanism by the halogen atom, a radical is generated at the polymer chain end, which propagates through the addition of monomer (M) (see Formula 1).[93, 95-97] The equilibrium can be altered by adding the deactivating metal complex in an oxidation state directly to the polymerization solution. For instance, by using both Cu(I)/Cu(II) in the reaction, the controlled activation and deactivation of the mechanisms are achievable. This method was established by Wang and Matyjaszewky.[96]

Formula 1.



To manipulate the structure of attached polymer brushes and to achieve a smart surface, responsive polymers, such as solvent responsive, thermo- and pH-responsive, and ion-sensitive polymers can be used. Subsequently, the chemical composition and architecture are altering upon responding to external stimuli.[78, 88, 98, 99]

Thermoresponsive polymers are categorized by either a lower critical solution temperature (LCST) like poly(*N*-isopropylacrylamide) (PNIPAm), or upper critical solution temperature (UCST), such as poly(*N*-acryloylglycinamide) (PNAGA).[88, 92, 100]

In contrast to thermoresponsive surfaces, pH-responsive polymers respond to changes in the pH of the surrounding environment.[99, 101] A pH-responsive surface is achievable by surface polymerization of polyelectrolytes, such as poly(acrylic acid) (PAA) and poly(N, N-diethylamino-2-ethylmethacrylate) (PDEAEMA). In the study done by Brittian et al., polyacidic brushes were investigated at different pH.[102] The repulsion of the charged chains alters the brush thickness from 16 mm (pH = 2) to 26 mm (pH= 8).

PDEAEMA is another example of pH-responsive polymers of a cationic polyelectrolyte. The polymer chains transform from collapsed to expanded form by going from basic to acidic pH. At pH<6.5, the protonation of the amine group of PDEAEMA results in the expansion of the chains. In contrast, in neutral and/or alkaline medium the chains are coagulating and become hydrophobic.[39, 103-105]

Other than responsive surfaces, functional surfaces are achievable by using polymers with antibacterial polymers. Polymer brushes with antibacterial properties have an influential role in

biomedical applications to prevent the formation of biofilm. Antibacterial polymers alternatively can be used as antibiotics and biocides.[106] In general antibacterial polymers can be categorized into three groups (Figure 6): polymeric biocides, biocidal polymers, and biocide-releasing polymers. The first subdivision shows a biocidal effect (the repeating units act as biocide), which can kill the bacteria. In more detail, biocidal groups are attached to the polymer chains. The biocidal activity of PDEAEMA was studied by Russell et al.[107] upon surface polymerization of PDEAEMA on different substrates, such as glass slides and filter papers. Modification of the quaternary ammonium results in biocidal activity. Therefore, the impact of surface charge density is an important factor, which is expected to be higher than $1\text{-}5 \times 10^{15}$ accessible quaternary ammonium per square cm. The second group is dedicated to polymers having non-biofouling properties that reduce bacterial adhesion. As an example, polymer brushes of PMAA, para methoxy amphetamin (PMA), and poly(2-tert-butylamino) ethyl methacrylate) (PTA) can be mentioned. The third and last group is containing both biocidal and non-biofouling. The polymer chains carry biocides to attack microbes. This is shown by Yao and coworkers.[33] In this work, block copolymerization of poly(ethylene glycol) methacrylate (PEGMA) and PDEAEMA were used.

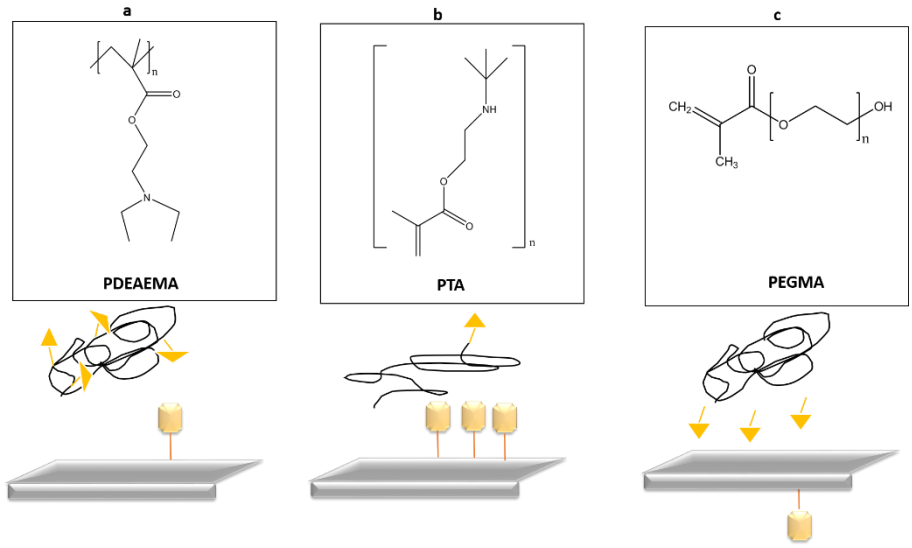


Figure 6. Antibacterial polymers principles: a) polymer biocides, b) biocidal polymer brushes, c) biocide releasing polymers.

5.3.2 Wet-laid method

The production of polymer fibers in terms of mass and surface area is time-consuming. Fiber entanglements are also known as other hindrances of the nonwoven procedures of electrospun fibers. For this reason, wet-laid nonwoven of electrospun polymer nanofibers is highly demanding in polymer composite preparation.[82] A suspension of short polymer nanofibers made by electrospinning in almost any kinds of solvent is going through the wet-laid process for huge scale nonwoven production. The process (Figure 7) starts with the cutting of the electrospun fibers into short length (depending on the application the time of the cutting and as a result, the length can differ), following by precipitation of the suspension and filtering through a membrane. The manufactured nonwoven can be formed in 2D as membrane nonwoven nanocomposite, or in 3D as polymer nonwoven sponges. There are various applications of wet-laid polymer nonwoven, such as filtration industry, or in microbial fuel cells. In this method except for the benefits of large-scale production, the weight area can be controlled. Moreover, comparing to the electrospinning of different polymers to make a nonwoven, which suffer from

many hindrances, like the use of different solvents, in this method mixing of nanofibers of different polymers is easily possible. For this purpose, a wide range of tunable properties is achievable.[82-84] The procedure permits the mixing of various components in short fiber dispersion. In more detail, the other components can be mixed and embedded in the nonwoven of short nanofibers by filtering through a membrane. Gao et al. and Reich et al. studied the combination of silver nanowires in polymer short nanofibers, which lead to flexible electronic nonwovens.[83, 84]

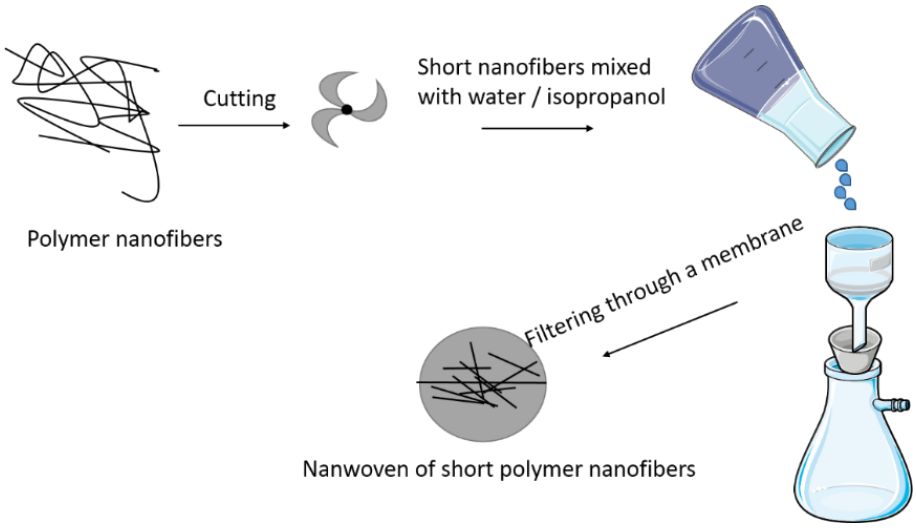


Figure 7. Scheme of the wet-laid process for short fiber nonwoven preparation.

5.4 Application of bacteria

As it was mentioned, in this work, the application of bacteria/polymer composites in bioremediation and/or biomedical field were studied.

5.4.1 Bioremediation of metals:

Metals are playing an important role in the ecosystem and are known as essential materials for human life. Due to the ultimate metal extraction and discharge of the metal waste, the standard

balance in the environment is raised. This increased level has an adverse impact and harm to the environment and human health. Three various categories of metals are named as follows: toxic metals (such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co, Sn, etc.), precious metals (such as Pd, Pt, Ag, Au, Ru, etc.) and radionuclides (such as U, Th, Ra, Am, etc.).[19, 108] Removal and recycling of the metal in a contaminated environment and waste stream is known as a challenge nowadays.[10]

There are various conventional approaches to remove metals from the environment such as precipitation (conversion of metal ions to insoluble form), ion exchange, electrochemical methods, reverse Osmosis, and solvent extraction. Most of the mentioned methods are suffering from the low-performance efficiency at a low concentration of metals. In addition, some membrane-based approaches increase the costs. For extraction methods, an organic phase is required. The pollutant in the aqueous phase is extracted and dissolved in the organic medium, following by the re-extraction of metals from this phase. This re-extraction is difficult in most cases, and the rate of toxicity is also high.[10, 19]

Aside from the so-called physical and chemical techniques of metal removal, for several decades, the concept of bio-removal of metals is carried out to make it a commercially viable technique. This technique has been attracting attention for a long time, which can be referenced to the work of Sheoran et al. in 1992.[109, 110]

Bioremediation of xenobiotic form environment is a cheap and harmless method, which is useful also to restore natural resources as metal contaminates.[11, 34, 111, 112]

Bioremediation is defined as using biomaterial for sequestrating and immobilization of organic and/or inorganic material. Several terms depict bioremediation, such as biosorption, bioaccumulation, bioconcentration, and so on. It is essential to distinguish the difference between the two terms of biosorption and bioaccumulation. The difference is due to the tendency of the dead and living cells for the sorption of heavy metals. In more detail,

biosorption is the ability of dead and live cells for metal uptake. The presence of some binding sites in dead cells facilitates the process and attracts metal ions. On the other hand, bioaccumulation is the ability of living and biological active cells.[11-13, 19, 28, 111, 113, 114] In the process of bioremediation two phrases are involved: biomass or biosorbent and sorbate. The sorbate is referred to as metals, which is existing in aqueous media, as well as in soil; and the biomass is microorganisms, plants, and exopolymers.

Cell walls of microorganisms have the capacity to sorb, bind or entrap many soluble and insoluble metal species.[111] Although, like breaking down the organic materials by microorganisms and using them as a source of carbon and energy, in the case of heavy metals, biological degradation is not feasible. For this purpose, microorganisms are changing the physical and chemical state of metals via metabolic activities. The high surface-to-volume ratio of microorganisms allows better interaction between metal ions and biosorbent. In the degradation process, metals transform from oxidation state to reduction. Consequently, the water solubility (hydrolysis) and toxicity will change.[34] The affinity of microorganisms to different types of metals is revealing the fact that they behave selectively for biosorption. The uptake of heavy metals by biosorbents, although they consider as toxic materials, are usually much higher than non-toxic alkaline earth metals (Ca, Na, K, Mg). Since one of the mechanisms of biosorption is ion exchange, the biosorbent tends to sorb cations (e.g., Cd, Cu, Ni, Pb, Cr^{3+}, etc.) or anions (e.g., As, Se, V, Cr^{6+}, etc.). There is one exception regarding gold, which is bound to biosorbent only in a specific cationic form of Au^{3+}.[14]

5.4.1.1 Biosorption process and parameters

Biosorption is known as a general feature of the biologically active, as well as non-active cells to absorb metals or other components into biomass. To reach the optimal level of metal removal by biosorption, a deep understanding of biosorption mechanisms and influencing parameters is required. The mechanism of biosorption is dependent on the different physicochemical

processes, from which ion exchange, chelating, adsorption, and diffusion through the cell wall can be mentioned here.[19, 115] The biosorption process can be dependent or independent of cell metabolisms. When the biosorption is not related to the metabolisms of the cells, uptake is by physicochemical interaction between the metal and the functional groups present on the microbial cell surface, such as physical adsorption, ion exchange, and chemical sorption.[116] The processes are dramatically depending on interactions of biosorbent and sorbate, which results in various binding types, such as covalent, electrostatic, and Van der Waals forces. The type of species is also the second influencing factor.

Heavy metal biosorption is affected by various factors. Different strains of a species can also vary the amount of metal uptake and the type of metal bound. Other important parameters such as external environmental and physiological factors, chemistry of the metal, and performance of the biosorbent are widely discussed in the following:

1. pH: Ion exchange is the most important process of biosorption. In different pH values, the diverse oxidation state of metals is observed. In more detail, metal charges and adsorbability at solid-liquid interfaces are changing by a change in pH. Therefore, pH strongly alters the bioremediation.

2. Temperature: Temperature can highly change the metabolisms of living cells. Cells are sensitive and might damage in high temperatures. On the other hand, adsorption and ion exchange are known as exothermic processes. Desirable metal uptake can proceed in a temperature range of 4-40°C.

3. Metal ion concentration in initial state: The higher concentration of ions in comparison to the lower concentration with respect to the available sites for biosorption has a different role for this process. In more details, at higher concentration, the available sites are limited, which increase the independence of the absorption on the initial

concentration of metal ions in the solution. Diversely, at low concentration, the available sites are better accessible. As a result, the biosorption is dependent on the concentration of ions.

4. Ionic strength: Ions would compete with the charges on the surface of biomass and electrostatic interaction in the aqueous phase. As a result, the biosorption will decrease by increasing the strength of ions.

5. Biosorbent concentration: Greater amount of biosorbent follows by an increase in the available binding sites to attract the metal ions. As the process of biosorption is complicated and highly depends on the available surface area of binding sites, the higher dosage of biosorbent might deactivate the sites adjacent to each other. This results in lower uptake with biosorbents.[12, 19, 114]

5.4.1.2 Biosorption of metals with bacteria

Bacterial growth and reproduction need energy and nutrient, which are provided from carbon sources. In general, based on the consumed carbon source, bacteria are divided into two groups: autotrophs and heterotrophs. Those which gain their energy from carbon in inorganic sources are called autotrophs unless otherwise, heterotrophs need organic sources.[17] Autotrophs are doing metabolisms depending on the inorganic sources, like metal binding. Metal uptake by bacteria relies on the microprecipitation method, which may occur by electrostatic attraction or complexation. For instance, in the case of complexation, N and O ligands in the cell wall appear, and in electrostatic attractions, charged groups in the cell wall play a role in the interaction. It is noteworthy to mention the difference between gram-positive and gram-negative bacteria. As it is shown in Figure 8, the cell wall in gram-positive bacteria is thicker compared to gram-negative cell walls. The presence of peptidoglycan (PG), which is a linear polymer consisting

of glucosamine and muramic acid with peptide side chains offers functional groups like the carboxyl group. Besides that, in the PG layer, some teichoic acids (TA) and teichuronic acids (TUA) are embedded that consisting of phosphoryl groups. TUA is responsible for the reduction mechanisms of metals in live cells, which is not observed when a cell is not alive anymore.[117] The phosphoryl and carboxyl groups are negatively charged and interact with positively charged metals. The PG layer of gram-negative bacteria bears less negatively charged carboxyl groups. For this purpose, biosorption of gram-negative bacteria is less than gram-positive ones. Whereas, in gram-negative bacteria, the presence of lipopolysaccharides (LPS) and phospholipids providing negatively charged phosphonate groups, possibly the primary metal-binding site.[10, 19]

As it was discussed so far, bacteria can sequestrate metals.[12, 19, 80, 108, 114] Due to their high surface to volume ratio and chemically active biosorption sites in the cell walls, metal uptake is rather efficient. Within different species of bacteria, Bacillus, Pseudomonas, Streptomyces, Micrococcus, and Escherichia coli are highly potential for metal biosorption.[114]

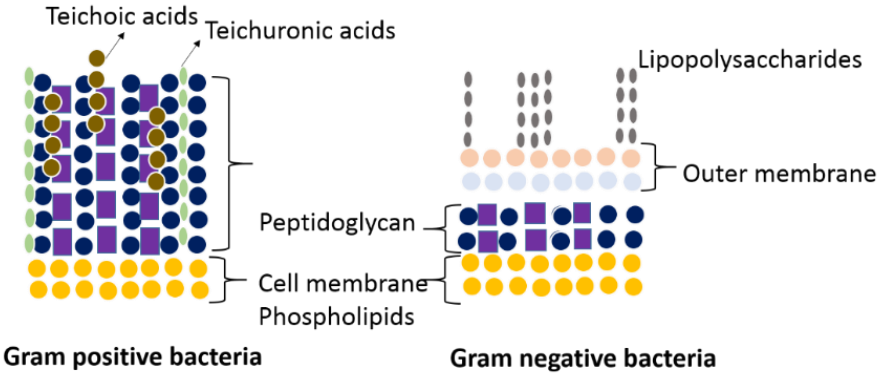


Figure 8. The cell walls of gram-positive and gram-negative bacteria.

5.4.1.3 Biosorption of metals with encapsulated *M. luteus*

Bioremediation of metal ions with immobilized *Micrococcus luteus* (*M. luteus*) in polymer matrices has been broadly studied for applications including wastewater treatment. Initially, it is important to acknowledge the maximum metal concentration on growth inhibition of *M. luteus*. The biosorption of Cu^{2+} by free cells of *M. luteus* was studied by Letnik et al.[20] It was shown that only 10% of cells with the initial concentration of 10^8 CFU can survive Cu^{2+} by the concentration of 370 ppm (6 mM), and a Cu^{2+} solution of 0.05 mM to 0.1 mM has no inhibition effect on bacterial growth. The maximum biosorption of Cu^{2+} by *M. luteus* cell was quantified as 59mg (Cu^{2+}/g of bacterial cells) in the solution concentration of 1 mM (80% uptake), which decrease to 14% for Cu^{2+}solution of 10 mM.[20]

In 2019, Reich studied the ability of encapsulated *M. luteus* to uptake Au (III) and catalytic conversion to gold nanoparticles (Au (0)).[117] The gold uptake was measured by Inductively coupled plasma-optical emission spectroscopy (ICP-OES) and quantified for a nonwoven of living *M. luteus* as 2500 ng Au per mg of fibers (for initial Au concentration of 1000 $\mu mol \cdot L^{-1}$). This amount was decreased to 1000 ng Au per mg of fibers for a nonwoven of dead cells of *M. luteus.*

4.4.2 Biomedical application of bacteria for treating wound infections

Non-pathogenic bacteria have the potential for biomedical applications and human disease therapy. The mechanism of live bacteria therapy for various diseases is complicated and might be resulted from the penetration of bacteria into the infected organ and the production of some cytotoxic molecules. Fungal and bacterial infection of the skin is known as one of the widespread diseases in human beings.[9] Administration of live bacteria provides various benefits and no side effects. This is in contrast to medical treatment with antifungal drugs that cause harm to kidney and other organs. Besides that, in antibiotic therapy, there is a risk of antibiotic resistance.[118]

Skin and epidermis as a protective barrier for the body consist of a diverse variety of microorganisms. Fungi, viruses, and bacteria are the existing microorganisms on the skin, which mostly are beneficial for the health of the host.[8, 118-122] Grice and Segre studied the molecular characterization of diverse microbial species on the skin and whether they provide some benefit to the host.[122, 123]

B. subtilis is a bacterium that naturally exists on the skin. The employment of *B. subtilis* for antimicrobial applications began with an understanding of the variety of antimicrobial molecules produced by *B. subtilis*. The secreted molecules can highly control the growth of surrounding microorganisms. Hence, *B. subtilis* is commercially used as a skincare product, which can act as antibiotics. Gonzalez et al. invest investigated the interaction between *B. subtilis and Staphylococcus aureus* (*S. aureus*) using imaging mass spectroscopy (IMS).[124] *S. aureus* is a human pathogen and antibiotic resistance that involves serious infections. The secretion of an array of antimicrobial molecules from *B. subtilis* is inhibiting the growth of *S. aureus*.[124]

Another study on the antifungal effect of *B. subtilis* was done in the work of Lufton et al.[25] *B. subtilis* was encapsulated in poly(ethylene oxide)-poly(propylene oxide)- poly(ethylene oxide) (Pluronic F-127). Pluronic-127 is an LCST polymer with critical temperature around body temperature. *B. subtilis* was incubated in a culture medium with the presence of Pluronic F-127. The bacteria/hydrogel mixture was applied on the skin at room temperature and solidifies on the skin with body temperature. The encapsulation of *B. subtilis* in polymeric matrices needs to provide good adhesion to the skin without any cell damage.

The antibacterial activity of *B. subtilis* against *Candida albicans* (*C. albicans*) as one of the reasons for surface infections was widely studied. Different molecules were secreted from *B. subtilis*, such as Surfactin and Fengycin lipopeptide.[125] The presence of both biomolecules can be ensured by high-performance liquid chromatography (HPLC). Surfactin is the most powerful

biosurfactant that has a dramatic antibacterial effect. Surfactin antibacterial effect is by adsorption to the cytoplasmatic membrane of the microbial cells.

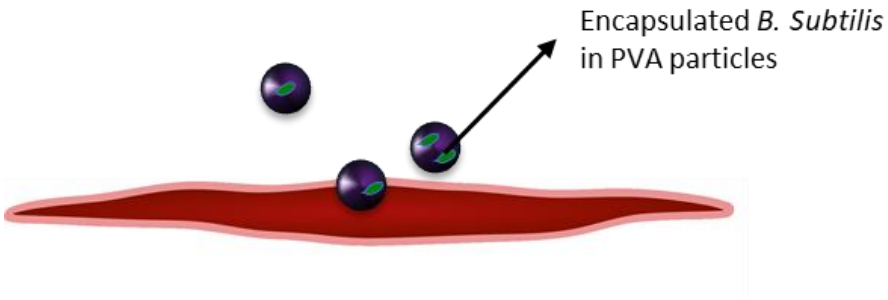


Figure 9. Microparticles with encapsulated *B. subtilis* were administered directly onto open ulcers or wounds.

5.5 Aim of the thesis

The interest in using encapsulated bacteria may be motivated by a broad spectrum of reasons. encapsulated bacteria are contributed for various industrial fields including decontamination of ecosystem or biomedical applications (Figure 10). The actual work aimed to accomplish novel designs of living biocomposites of bacteria/polymer by engaging different fields of science. In more detail, a combination of polymer science and biology leads to the encapsulation of bacteria in polymer composites with the lowest biological loss. The biocomposites preferably protect bacteria from unpleasant environmental impacts, such as organic solvents, harsh pH and temperature changes, toxicity, and so forth. Although the barrier between bacteria and the surrounding environment is acting as a protective membrane, it is expected that the polymer materials allow the nutrient, oxygen, and metabolic transfer for bacterial growth. As a result, the engineering and material of polymer composites play an important role in the long-term preservation of biological activity. Electrospinning and spray drying are two polymer processing methods, which are providing a suitable condition for encapsulation of bacteria in polymer nanofibers and particles, respectively. As was mentioned, the chosen polymer material is also an essential factor for the encapsulation of bacteria. Consequently, water-soluble polymer like PVA, which is also processable in both methods is a proper choice for a matrix. The stability of polymer matrixes is another critical parameter.

In practice, *M. luteus* and *B. subtilis* were encapsulated in PVA polymer particle for different applications.

For PVA particles made by spray drying, the water resistance of the matrixes is a problem, since PVA is swelling or is soluble in water, as a result, the release of bacteria in an aqueous medium is possible. The surface of the PVA particles with encapsulated bacteria was coated with a PDEAEMA shell. The surface polymerization of the shell was applied by polymerization of the

DEAEMA and using control radical polymerization. The production of core/shell polymer particles with encapsulated bacteria that ensure long-term reactivity remains a challenge.

PVA particles with encapsulated bacteria can also be embedded in a nonwoven carrier, made by PLA fibers. PLA nanofibers are electrospun, and cut into a short length, following by mixing with PVA particles and filtration through a membrane. As a result, the PLA nonwoven layer acts as a carrier and also retards the release of bacteria. The second section of the dissertation deals with the encapsulation of *B. subtilis* in PVA particles for topical treatment of open wounds.

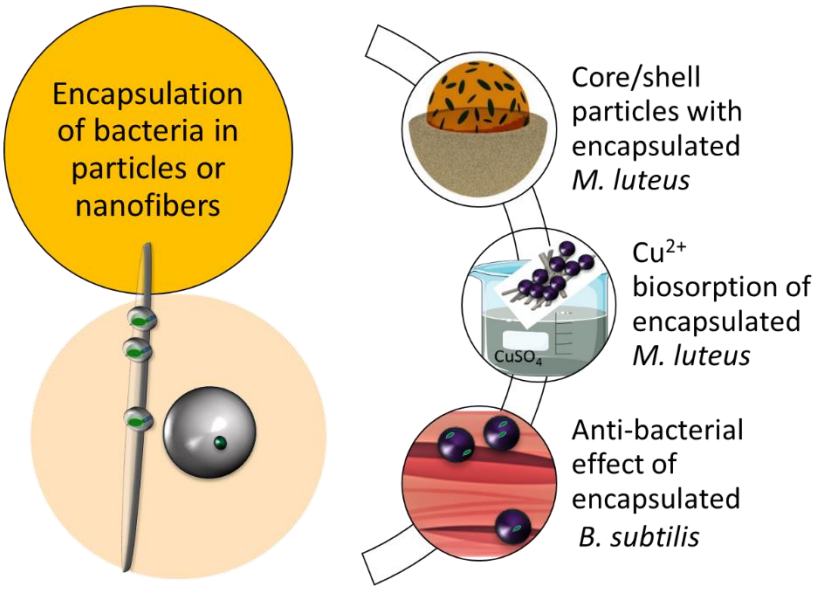


Figure 10: Encapsulation of bacteria in polymer particles or nanofibers: the bacteria/polymer composites were used in different applications.

6. Literature

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7. Synopsis

The focus of this thesis is on the preparation of biocomposites containing polymer and bacteria. The polymeric part plays a role as a matrix for bacteria and is used for immobilization. The importance of chosen polymer for a matrix is to keep the bacteria biologically active and block the escape of the bacteria from it. Although the polymer matrix is an essential factor, the immobilization techniques are also important. Different immobilization methods were broadly studied to emphasize their importance on the biological activity of bacteria, storage time, and application efficiency.

In the first two publications (parts 7.1 and 7.2), the application of biocomposites was aimed in the long term for wastewater treatment, and preciously for bioremediation of heavy metals such as Cu^{2+}. *M. luteus* was used for these two works, which has a high potential for sequestration of heavy metals.

In part 7.1, a biocomposite of bacteria/hydrogel-core/shell particle was synthesized. As encapsulation in hydrogel microparticles shows problems such as bacterial escape within an aqueous environment, we reported a new approach to encapsulation. In this method, the release behavior was retarded up to 5 weeks. Besides that, by the use of a polymer shell with special features, the exchange of metabolism products from hydrogel microparticles was accelerated. As a result, the produced core/shell microparticles guarantee a longer encapsulation time with high biological efficiency.

Although the core/shell microparticles presented in part 7.1 are showing an efficient mass transfer for the biological activity of *M. luteus*, the concept is not practical for bioremediation techniques on large scales. Removing the inserted microparticles in wastewater might not be easy after bioremediation. Therefore, using a carrier for the microparticles with encapsulated bacteria might be more efficient. In part 7.2, porous composite nonwovens with living *M. luteus* were prepared. The bacterial cells were encapsulated in PVA microparticles (*M. luteus*/PVA

microparticles) produced by the spray drying method and chemically cross-linked. The hydrogel microparticles with encapsulated *M. luteus* were embedded in a nonwoven of PLA electrospun short fibers provided by the wet-laid method. The encapsulated *M. luteus* in polymer composite nonwovens confirmed the continuous bioremediation of Cu^{2+} in aqueous media.

In part 7.3, the pharmacological activity of *B. subtilis*/PVA microparticles was successfully demonstrated in an open wound model. *In vitro,* the *B. subtilis*/PVA microparticles demonstrated a remarkable antibacterial activity against MRSA and *S. aureus* by producing antibacterial agents and inhibiting the growth of these pathogenic bacteria. This is in contrast to the *in vivo* with *B. subtilis*/PVA that is only in the first days effective. However, i*n vivo* study with empty PVA microparticles (without *B. subtilis*) healed the wound after 12 days, compared to 15 days for the non-treated wounds.

7.1 Poly(vinyl alcohol)-Hydrogel microparticles with Soft Barrier Shell for the Encapsulation of *Micrococcus Luteus*

This work was published by **Mahsa Mafi**, Ariel Kushmaro, Charles Greenblatt, Seema Agarwal, Andreas Greiner, *Macromol Biosci* **2021**, 2000419, 1-8

In this work, we showed encapsulation of *M. luteus* in PVA microparticles with the spray drying method, followed by a coating of a polymer shell with special features. The PVA microparticles with encapsulated *M. luteus* were prepared by spray drying method and chemically cross-linked with glutaraldehyde. The hydrogel PVA core required a polymer shell, which was grafted from the PVA microparticles and with surface polymerization. To synthesis the shell, the OH groups of PVA chains on the surface of the microparticles undergo functionalization with 2-bromo-isobuthyril bromide. The OH groups were replaced with halogen atoms (Br atoms), which were

more reactive and capable of providing free radicals. Afterward, the polymerization starts by adding DEAEMA monomer and initiation by active sites (Figure I). The average diameter after polymerization increased from 2.64 ± 0.96 μm for bare *M. luteus*/PVA microparticles to 3.25 ± 1.22 μm for *M. luteus*/PVA/PDEAEMA (core/shell microparticles with encapsulated *M. luteus*).

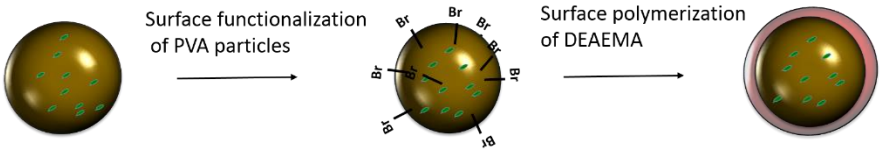

Figure I. Surface functionalization and polymerization of PVA microparticles. Firstly, the surface of the microparticles was functionalized with Br groups, and secondly, DEAEMA monomer was polymerized and graft from the PVA microparticles.

This polymer shell was highly susceptive for mass transport in and out of the microparticle core. These mass exchanges are based on the pH-responsivity of PDEAEMA shell. The pH-responsive PDEAEMA is affected by environmental parameters and forms an open porous structure (Figure II A), that accelerates nutrient transfer into the PVA core containing living *M. luteus*.

The diameter of the *M. luteus*/PVA/PDEAEMA microparticles increased from 3.25 ± 1.22 μm at pH= 7 to 7.51 ± 3.99 μm in an aqueous medium at pH= 1 (Figure II B). Besides that, the increased size of the microparticles in acidic pH indicated that the elasticity and ability of such core/shell structures also allowed bacterial growth.

Although the mass transport through the PVA particles was allowed, the release of *M. luteus* was not wanted. The antibacterial effect of PDEAEMA retards the escape of *M. luteus*. This effect of PDEAEMA was examined by suspending the microparticles in a nutrient medium and an agar plate test. The PDEAEMA shell was retarding the release of *M. luteus* for up to 5

weeks. In conclusion, the mass transfer experiment and release behavior show that the core/shell microparticles with encapsulated *M. luteus* provide a promising living composite since higher metabolism efficiency with retarded leakage of bacteria was achieved.

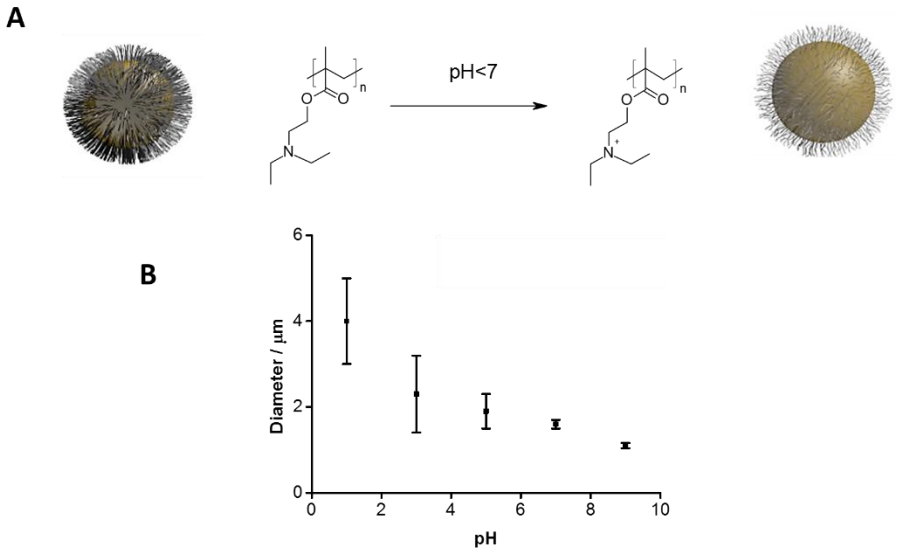

Figure II. A) The pH responsivity of DEAEMA on acidic comparing to neutral pH. B) SLS measurement on diameter size of the *M. luteus*/PVA/PDEAEMA (core/shell microparticles with *M. luteus*) in different pH.

7.1.1 Individual Contribution to Joint Publications

The synthesis, processing, and characterization of the polymer core and shell were carried out by me. SLS, as well as biological tests were also performed by me. The manuscript was written and revised by me. Prof. Dr. Andreas Greiner and Prof. Dr. Seema Agarwal, Prof. Dr. Charles Greenblatt, and Prof. Dr. Ariel Kushmaro (corresponding author) were responsible for supervising, participating in the discussion, designing concept, and correcting the manuscript.

7.2 Bioremediation of copper-ions by polymer encapsulated and immobilized *Micrococcus Luteus*

This work was published by **Mahsa Mafi**, Andreas Greiner, *Macromol Biosci* **2021**, 2100086, 1-8

In this work (see Figure III), immobilized *M. luteus* has been used for bioremediation of copper (Cu^{2+}). Encapsulated bacterial cells in PVA microparticles (*M. luteus*/PVA microparticles) were produced by spray drying method and chemically cross-linked (Figure III A). The diameter size of the PVA microparticles with encapsulated *M. luteus* is in the range of 2.58 ± 0.89 µm. Porous composite nonwovens with living *M. luteus* were prepared by embedding the hydrogel microparticles with encapsulated *M. luteus* in a nonwoven of PLA electrospun short fibers (Figure III B and III C). Electrospun PLA fibers with average diameters of 240 ± 115 nm undergone a fiber cutting process to get short fibers of an average length of 0.45 ± 0.29 mm.

Two different methods of composite nonwovens, in which the place position of the hydrogel PVA microparticles with encapsulated *M. luteus* were prepared. In Method 1. Both *M. luteus*/PVA microparticles and short PLA fibers were re-dispersed together in water and filtered through a mesh. In this method, the position of *M. luteus*/PVA microparticles and short PLA fibers were randomly distributed (Sample R).

In Method 2. Firstly, PLA short fiber dispersion was filtered through a PET mesh, on top of which *M. luteus*/PVA microparticles were filtered. Afterward, they were covered by filtration of another batch of PLA short fibers. Consequently, *M. luteus*/PVA particles were sandwiched between two layers of PLA porous nonwovens (Sample S). To study the effect of encapsulated *M. luteus*, the amount of microparticles was varied, while the amount of short PLA fibers was kept the same for each method (Sample S2 and R2 have 2 times more microparticles than S1 and R1). The thickness of the nonwoven formed by short-fiber and microparticles dispersion

filtration in both methods is in the range of 1.68±0.12 mm. The pore size measurement of the PLA nonwoven showed the size range of the pores roughly about 119.95±67.55 µm.

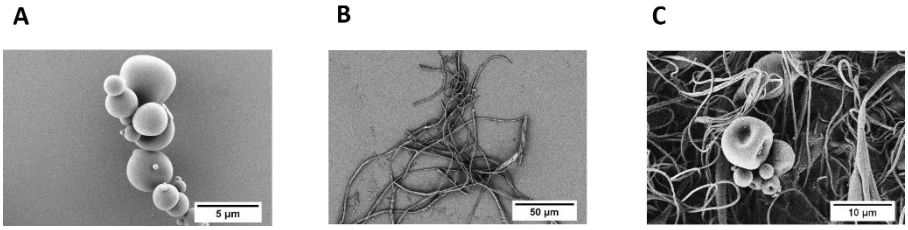

Figure III. SEM micrograph of samples: A) *M. luteus*/PVA microparticles after spray drying. B) electrospun PLA short nanofibers (after cutting). C) the cross-section of the nonwoven, Sample S.

As it was aimed, the composite nonwovens were used for the remediation of Cu^{2+} by the encapsulated *M. luteus*. Furthermore, the continuous bioremediation of *M. luteus* by the addition of Cu^{2+} and the self-renewing bioremediation ability was investigated. The Cu^{2+} biosorption with *M. luteus* was quantified by ICP-OES (Figure IV A and IV B). For this purpose, composite samples (made with methods 1 and 2) with the size of 1cm×1cm were placed in 10 mL Cu^{2+} solutions with different concentrations (5, 10, and 15 mM) at 37 °C for 48 h. Control samples of encapsulated dead cells of *M. luteus* in polymer composites with methods 1 and 2 were used (the result of bioremediation with control samples of different models were approximately in the same range). The Cu^{2+} uptake with the composite nonwovens with living *M. luteus* was 3 to 4 times more than the uptake with the dead cells (for all Cu^{2+} concentrations) (Figure IV A). A low concentration of Cu^{2+} solution (5 mM) resulted in approximately 90% uptake by the composite nonwovens of all types. The uptake for 10 mM Cu^{2+} solution was different for different composites, and a higher amount of Cu^{2+} solution (15 mM) led to a decrease in uptake.

For the continuous bioremediation, the composite nonwoven samples were kept in 5, 10, and 15 mM Cu^{2+} solutions (10 mL) at 37 °C for 48h. Afterward, the process continued by the addition of 5, 10, and 15 mM Cu^{2+} solutions for another 48h. The result (Figure IVB) is revealing the ability of encapsulated *M. luteus* to grow and continue the biosorption. After 96h and by addition of Cu^{2+} salt to the composite samples with living *M. luteus*, bioremediation of roughly 60-70 % and 25-45 % for Samples made by different methods were observed.

Figure IV A and IV B show, in Cu^{2+} concentrations of 10 mM and 15 mM, samples made by method 2 (S1 and S2) perform the bioremediation better than the randomly made composites. The significantly reduced amount of uptake for Sample R1 and R2 is due to the release of the encapsulated *M. luteus* from the surface of the samples. However, for sample S1 and S2 (method 2), the PLA short fibers covering the top and bottom of the composites and reducing the exposure of *M. luteus*/PVA microparticles with nutrient medium or Cu^{2+} solution. This PLA layer was playing role in preventing the release of *M. luteus*.

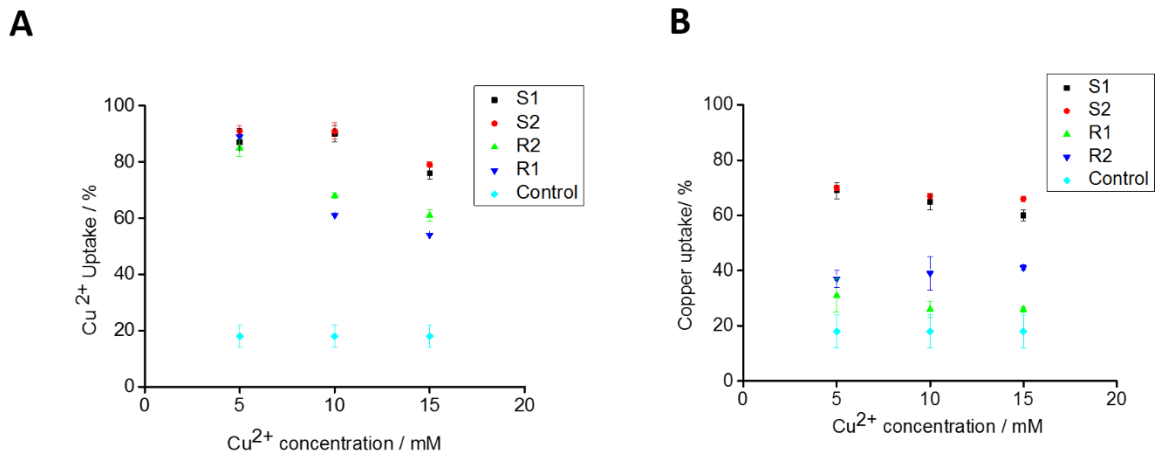

Figure IV. A) ICP-OES measurement of Cu^{2+} uptake by composite Nonwovens: after 48h at 37°C, Comparison of the Cu^{2+} uptake of different samples (the control sample (Sample 10, Table 2) consists of the dead cell of *M. luteus*) measured by ICP-OES at different Cu^{2+} concentrations (5, 10, and 15 mM). B) after 96h the effect of additional Cu^{2+} to the ability of

different composite samples with living *M. luteus* and dead *M. luteus* (for control samples) for second time bioremediation, continuous bioremediation was operated at 37°C and pH= 5.

7.2.1 Individual Contribution to Joint Publications

The synthesis, the biological characterizations of the porous composites were done by me. SEM measurement and ICP-OES analysis were done by me and Carmen Kunert, respectively. The manuscript was written and revised by me. Prof. Dr. Andreas Greiner (corresponding author) were responsible for supervising, participating in the discussion, designing concept, and correcting the manuscript.

8.3 *Bacillus Subtilis* in PVA Microparticles for Treating Open Wounds

This work was published by Noa Ben David, **Mahsa Mafi**, Abraham Nyska, Adi Gross, Andreas Greiner, Boaz Mizrahi, *ACS Omega* **2021**, 6, 13647-13653.

In this work, the biomedical application of biocomposites with another strain of bacteria (*B. subtilis*) was studied. *B. subtilis* shows antibacterial activity (by the production of antibacterial molecules) against *MRSA* and *S. aureus* (pathogenic bacteria), and is useful for treating infected open wounds. Therefore, in this work, *B. subtilis*/ PVA microparticles were embedded directly to an open wound (like a dressing for the wound). The manufactured dressing provides a moist environment while protecting the wound from bacterial contamination.

The effect of the spray drying process on *B. subtilis* was evaluated dynamically by spectrophotometer and by the study on the growth rate of the cells (Figure VA). The growth of an equivalent number of free cells non-encapsulated *B. subtilis* (as a control sample) was also

measured. Both encapsulated and non-encapsulated bacteria cells reached a plateau about 8 h from the beginning of growth. The delay in growth kinetics of encapsulated *B. subtilis* can be explained by the "wake-up" period and several factors which may have inhibited bacterial growth.

In vitro, the antimicrobial activity of *B. subtilis* was measured by LC/MS. The release of antimicrobial agents (Surfactin and Fengycin) from *B. subtilis* is shown in Figure VB. The bacteria's ability to produce and secret Surfactin started after 4 h, reaching a maximum of 350 µg/ml in 12h. The Fengycin production was after about 6 h and its concentration reaching a maximal concentration of about 80 µg/ml in 12h. The minimal inhibitory concentration (MIC) is 200 µg/ml for Surfactin against *S. aureus*. As a result, 250 µg/ml fully inhibited its growth.

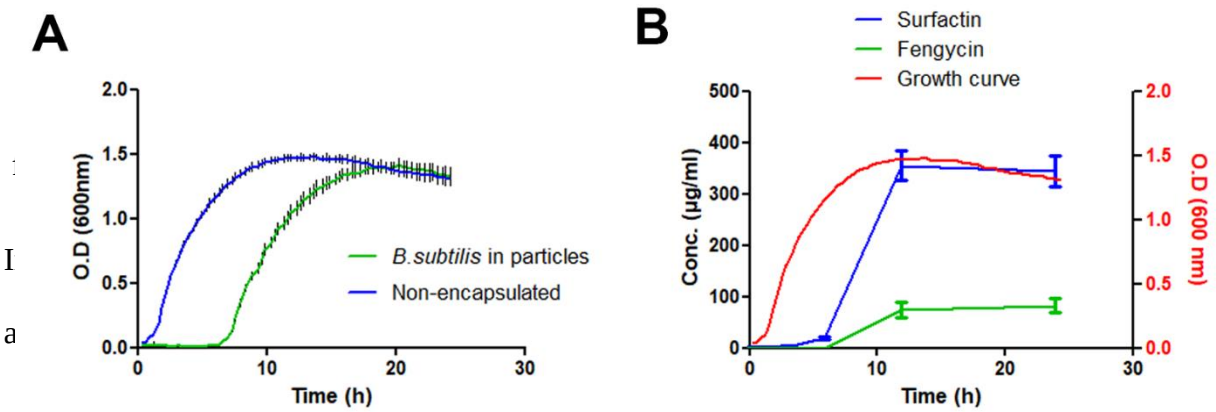

Figure V. A) Growth diagram of encapsulated as well as non-encapsulated *B. subtilis.* B) Surfactin and Fengycin produced by *B. subtilis* and measured by LC/MS.

For *in vivo*, an open wound model in C57BL mice was studied. A single open wound was created by removal of the skin (1x1 cm^2) in the dorsal region of mice, and was treated every 24h with empty or with *B. subtilis* PVA particles. A control sample of a wound was prepared

by removal of the skin (1x1 cm^2), and left untreated. The wound area was measured daily. Mice were sacrificed after 5 or 15 days and tissues from the injured area were harvested for histological analysis. Healing was evaluated by daily measuring of wounds size (Figure VI A and VI B) and by the toxic effect of the treatment as was evaluated by histology (Figure VI C).

PVA particles (empty and *B. subtilis*) were immediately attached themselves to the surface of the wound upon administration, remaining there until fully dissolved. Three days after injury, significant differences in wound closure were noticed between the PVA treated wound and the untreated wound. These observations were also confirmed by histology where five days post injury, a healthy healing process was evidenced by the formation of crust and by the appearance of granulation tissue (marked by green arrows). After 12 days, both PVA treated wound showed a complete reepithelization and hair growth, compared to 15 days required for the untreated group to heal.

In comparison to empty PVA particles, *B. subtilis*/PVA particles, which demonstrated excellent *in vitro* performance, was relatively limited in the healing process *in vivo*. The bacterial effect was only noticed in the first nine days of the experiment, then they had no apparent influence on healing process. This discrepancy may be attributed to crust formation observed above the wound area 5 days after the beginning of the experiment (Figure VI C**,** marked by blue arrows).

The histology data is indicating the lack of any toxic effect related to PVA particles or to the *B. subtilis* incorporated in them. Comparison of the data between the 3 tested groups indicates the presence of comparable changes, in nature and severity. Moreover, the presence of the *B. subtilis* did not cause any delay in the healing of the induced wound, when comparing to the data evaluated at the 5- and 15-days' time-points. It can be concluded that under the present experimental conditions, the use of the *B. subtilis* incorporated in PVA particles in the wound healing model in mice is not associated with any adverse effects, and should be safe.

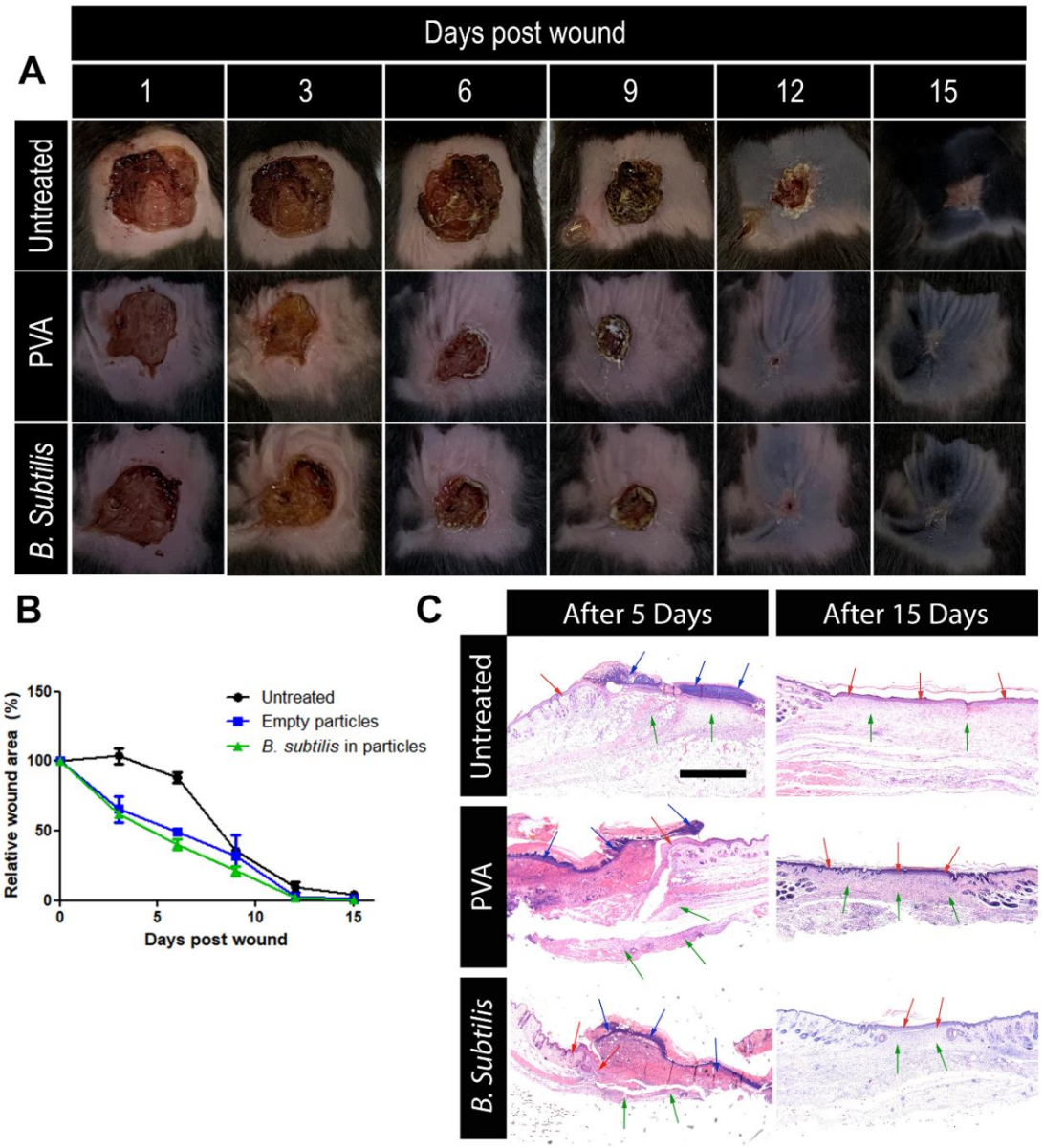

Figure VI: Wound closure in C57BL mice: **A**. Representative photos showing the time course of wound healing. **B.** Wound closure, normalized to the original size of the wound, expressed as mean ± SD. **C.** Representative images of *in vivo* response of skin to particles with and without *B. subtilis* after 5 and 15 days and untreated group (scale bar= 1 mm). Red arrow - epidermis; blue arrows - crust; green arrows - granulation tissue.

7.2.1 Individual Contribution to Joint Publications

The encapsulation and spray drying of the bacteria/PVA microparticles were carried out by me. SEM measurements were also performed by me. The in vivo and in vitro experiments were performed by Noa Ben-David. The manuscript was written and revised by Noa Ben-David and

me. Prof. Dr. Boaz Mizrahi and Prof. Dr. Andreas Greiner (corresponding author) were responsible for supervising, participating in the discussion, designing concept, and correcting the manuscript.

8. Reprints of publications

The manuscripts are reprinted in the form of journal articles with the permission of the respective publishers.

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8.1 Poly(vinyl alcohol)-Hydrogel microparticles with Soft Barrier Shell for the Encapsulation of *Micrococcus Luteus*

This work was published by **Mahsa Mafi**, Ariel Kushmaro, Charles Greenblatt, Seema Agarwal, Andreas Greiner, *Macromol Biosci* **2021**, 2000419, 1-8

RESEARCH ARTICLE

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Poly(Vinyl Alcohol)-Hydrogel Microparticles with Soft Barrier Shell for the Encapsulation of *Micrococcus luteus*

Mahsa Mafi, Ariel Kushmaro, Charles Greenblatt, Seema Agarwal, and Andreas Greiner**

The encapsulation of bacteria in polymers results in hybrid materials that are essential for the long-term biological activity of bacteria and formulations in practical applications. Here, the problem of bacterial escape and the exchange of metabolism products from hydrogel microparticles within an aqueous environment are addressed. Bacteria are encapsulated in chemically cross-linked poly(vinyl alcohol) (PVA) hydrogel-microparticles followed by their encapsulation in a pH-responsive and soft antibacterial shell of poly(*N*,*N*-diethylamino ethyl methacrylate) (PDEAEMA). This polymer shell acts selectively with regards to the mass transport in and out of the microparticle core and is affected by environmental parameters, such as pH and antibacterial effect. The pH-responsive PDEAEMA shell forms an open porous structure that accelerates nutrient transfer into the PVA core containing living *Micrococcus luteus* (*M. luteus*). Results show that the antibacterial effect of PDEAEMA retards the escape of bacteria up to 35 days when the shell is open. Additionally, the permeation of a small molecule into the gel, for example, methylene blue dye through the core/open-shell structure, certifies a flexible barrier for mass transport, which is required in the long term for the biological activity of encapsulated *M. luteus*.

1. Introduction

Bioremediation using bacteria is becoming an emerging field for reclaiming wastewater and decontamination of the ecosystem.[1–5] The use of bacteria for bioremediation affords various advantages over conventional methods, as it is environmentally friendly, non-toxic, inexpensive, and useful for the elimination of a wide variety of pollutants, among a broad spectrum of bacteria groups, for example, *Bacillus*, *Pseudomonas*, *Streptomyces*, *Micrococcus*, and *Escherichia coli* are potentially the most well-established for heavy metals' bioremediation.[5] Micrococcus luteus (*M. luteus*) is an anaerobic, non-pathogenic, Gram-positive bacterium that has the capability of sequestrating heavy metals, such as copper (Cu), gold (Au), and strontium (Sr).[6–9]

Different environmental factors can negatively affect the physiological state of the bacteria. Such unfavorable impacts may be prevented by encapsulation in polymer matrices enabling their use in bioremediation.[1,2,6,10–12] Encapsulation of these bacteria provide not only protection against harsh environmental conditions but also provides other advantages, such as prolonged storage time, the stability of physiological activity, and ease of handling.[6,11,13] In particular, encapsulation in polymeric matrices endows protection to bacterial cells to retain their biological functionality. Natural and synthetic polymers are used for encapsulation purposes. Commonly used natural polymers are profitable and are characterized by biocompatibility, hydrophilicity, low cost, and some of them are biodegradable.[1] Chitosan and alginates are typical examples of such polymers. Despite their benefits, the low mechanical and chemical resistance of natural carriers limits their functionality for encapsulation purposes. Synthetic polymers show a range of properties, differing in porosity, polarity, and wetting behavior. Poly(vinyl chloride), poly(vinyl alcohol) (PVA), polystyrene, and polypropylene belong to such a group of synthetic polymers.[6]

Polymer particles are broadly used for the encapsulation of sensitive materials in a wide variety of applications. Polymer particles are prepared by several methods, including emulsion and suspension polymerization, evaporation, and supercritical fluid technology.[14,15] Most of these methods

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are organic solvent-based. Therefore, water-soluble polymers like biopolymers are not the desired choice for the aforementioned methods due to the low solubility in organic solvents. Contrarily, spray drying has no limitation for the production of polymer particles from water-soluble polymers. Spray drying is a rapid one-step technique for manufacturing dry particles and encapsulation since the early 1940s.[16] In this method, a polymer solution (emulsion or suspension) is atomized with an atomization nuzzle, followed by exposure to a hot gas stream. Evaporation of the solvent results in the formation of dried polymer particles. As the evaporation process takes place in a few seconds, a minimal loss of encapsulated material is achieved, resulting in their ability to encapsulate heat-sensitive materials such as catalysts, enzymes, bacteria, pharmaceuticals, antibiotics, and vitamins.[15,17,18] Reich et al. reported the encapsulation of two different strains of bacteria in polymer particles prepared by spray drying.[19] Despite this, the uncontrolled release of bacteria from the particles is still a challenge, as is the uptake of nutrients for the bacteria in the particles. Indeed, bacteria were released from pure PVA particles immediately after contact with aqueous nutrient media. Suppression of the release of bacteria was achieved previously by the encapsulation of the bacteria in hydrophobic shells of poly(vinylidene fluoride)[7] and by poly(p-xylylene).[20] The bacterial release was retarded significantly by wrapping the particles in a shell of poly(methyl methacrylate) by polymerization. It is possible that this hydrophobic shell blocks the diffusion of nutrients into the bacteria-containing particles.[6]

The limitations of hydrophobic shells for the encapsulation of hydrogel particles containing bacteria could be overcome by the use of shells made of responsive polymers. The release of the encapsulated material, therefore, is dependent on the composition of the outer shell. A dense layer of a responsive polymer permeable to water and nutrients or other small molecules, for example, metal ions or dye molecules, can be manipulated to release compounds by changing the pH or temperature, or medium. Chen et al. successfully established a method for drug release with pH-responsive polymeric micelles in tumors since the tumorous pH is different from normal physiological pH.[21,22] Poly(*N*,*N*-diethylamino ethyl methacrylate) (PDEAEMA) is a well-known pH-responsive polyelectrolyte with pK_b 6.9.[23] In a basic pH range, PDEAEMA coagulates and forms a closed pore membrane. A pH below 6.5 results in open pores in the shell due to the protonation of the amine group and steric repulsion of the chains in the membrane (**Scheme 1**A).

Herein, we report on a concept for the encapsulation of *M. luteus* in microparticles covered by an additional shell to prevent the uncontrolled release of *M. luteus* and provide the potential for the release and uptake of small molecules, including nutrients. Following Scheme 1B, *M. luteus* was encapsulated in PVA microparticles (*M. luteus*/PVA microparticles) using spray drying. *M. luteus*/PVA microparticles were chemically cross-linked by glutaraldehyde. Subsequently, the surface of the hydrogel microparticles was functionalized with an initiator for atom transfer radical polymerization (ATRP). This macroinitiator initiated the polymerization of *N*,*N*-diethylamino ethyl methacrylate (DEAEMA). The final result of this process was PVA hydrogel microparticles with a shell of the pH-responsive PDEAEMA. We explored the effect of this PDEAEMA cover on the release of *M. luteus* from the hydrogel PVA microparticles. In particular, at low pH, where the shell is quasi-open (the PDEAEMA is in its quaternized stage), it is antimicrobial for *M. luteus*.[24–27] Further, we analyzed the permeation through the shell at low pH using the dye methylene blue as a model for small molecules.

2. Result and Discussion

The PVA microparticles without *M. luteus* were prepared by spray drying a 2.5% PVA solution in phosphate-buffered saline (PBS) (sample 1). These spherical microparticles ranged from a few hundred nanometers to ≈2.8 µm in size (**Figure 1**A and Figure S1A, Supporting Information). Following this experiment (see also Scheme 1B), we encapsulated colloidal *M. luteus*, which has a spherical shape in a size range of 0.5–1 µm (Figure 1B). The encapsulation by spray drying yielded microparticles ranging from 2.64 ± 0.96 µm in size (sample 2, Figure 1C, and Figure S1B, Supporting Information). The successful encapsulation of live *M. luteus* in the PVA microparticles was proven using agar plate tests and observation of characteristic yellow colonies. In our previous work, we showed the encapsulation of bacteria in PVA microparticles by advanced characterization method, such as Raman-AFM.[19] Since some of these PVA microparticles also contained dead *M. luteus*, we analyzed the survival of *M. luteus* in additional steps using the live/dead analysis test and by differentiating the live and dead cells (microparticles with dead *M. luteus* cells [sample 3] were used as control samples for live/dead test).

Next, we studied the morphology of non-cross-linked and cross-linked PVA microparticles as well as the effect of cross-linking on *M. luteus* in the microparticles with live (sample 4) and dead bacteria (sample 5). Cross-linking of the microparticles was done by glutaraldehyde. The amount of glutaraldehyde was below its minimum bactericidal concentration/minimum inhibitory concentration (MBC/MIC) for *M. luteus* (MBC = 0.7 mg L^{-1} and MIC = 0.001 mg L^{-1}). The amount of glutaraldehyde varied in the range of 0.03–0.12 ([glutaraldehyde]/[*M. luteus*/PVA microparticles] g g^{-1}) to study the swelling behavior of the PVA microparticles to achieve low swelling of PVA microparticles in the water at the lowest possible amount of glutaraldehyde. As seen from Figure S2A, Supporting Information, the concentration ratio of 0.06 glutaraldehyde to PVA microparticles was used to ascertain the toxicity of glutaraldehyde toward *M. luteus* while attaining minimum swelling of the PVA microparticles. According to scanning electron microscopy (SEM) and environmental scanning electron microscopy (ESEM) analysis (Figure 1D,E) of the swelling of the *M. luteus*/cross-linked PVA microparticles (sample 4), the average size of the microparticles increased from 2.64 ± 0.96 to 5.28 ± 1.91 µm after 24 h at room temperature at a concentration of 0.1 wt% in water. The swelling ratio of cross-linked microparticles decreased with a larger amount of glutaraldehyde (Figure S2A, Supporting Information), while the soluble residue of non-cross-linked PVA (Figure S2B, Supporting Information) increased with decreasing amount of glutaraldehyde. The shape of the microparticles did not change significantly using

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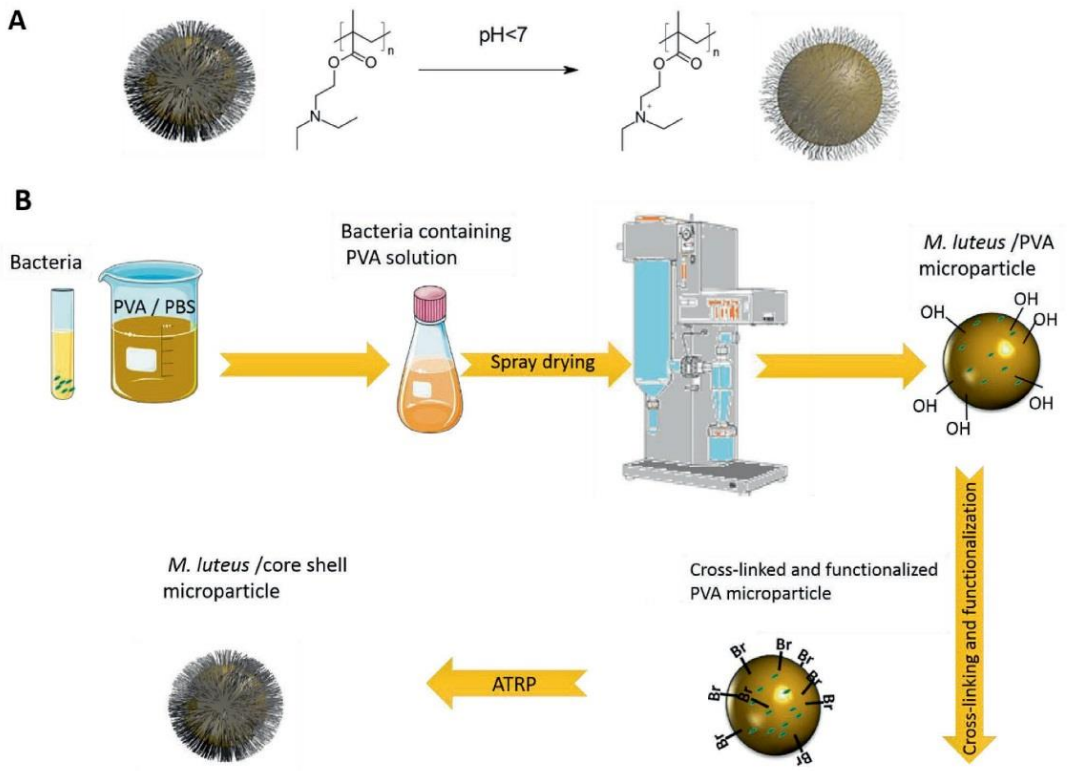

Scheme 1. A) Core/shell microparticle with a pH-responsive PDEAEMA shell; the effect of pH on the shell. B) Preparation of PVA microparticles containing *M. luteus* followed by cross-linking and surface functionalization. The polymerization of the PDEAEMA shell was performed on cross-linked and functionalized PVA microparticles.

the ratio of 0.06 glutaraldehyde to PVA microparticles (Figure S2C,D, Supporting Information).

Next, we studied the grafting of the cross-linked PVA microparticles loaded with live *M. luteus* and dead *M. luteus* by DEAEMA, as well as the grafting of microparticles containing methylene blue (Scheme 1B, samples 6–9). The encapsulated *M. luteus* in PVA hydrogel microparticles survive harsh conditions, such as treatment with organic solvents in comparison to the unprotected *M. luteus* as shown in our previous work.[12] The surface grafting of DEAEMA on the microparticles was accomplished by ATRP at room temperature. The results of these grafting reactions were cross-linked microparticles with a shell of PDEAEMA. The grafting of PDEAMA was confirmed by FT-IR (Figure S3, Supporting Information). The IR-spectra showed a band at 3500 cm^{-1}, which corresponds to the amine group of PDEAEMA. The average diameter of the microparticles (100 particles were counted) with encapsulated *M. luteus* increased from 2.64 ± 0.96 µm for bare *M. luteus*/PVA microparticles (sample 2) to 3.25 ± 1.22 µm for sample 6 (see Figure S4, Supporting Information, for size distribution). The shell of PDEAEMA did not increase the size of the microparticles significantly according to SEM analysis (Figure 1F). It is obvious that SEM is not an appropriate method to conclude about the influence of the macromolecular layer on the microparticle diameter, notably due to the very broad distribution of the particle diameter and a very thin layer of the shell. Therefore, the PDEAEMA shell thickness of sample 6 was determined by transmission electron microscope (TEM) measurement. The PDEAEMA shell was stained using uranyl acetate, followed by embedding in epoxy resin and microtome sectioning, resulting in a shell thickness of ≈500 nm (**Figure 2**A).

To test the fitness of the encapsulated *M. luteus* over the whole process of spray drying, synthesis, and storage, we applied a live/dead analysis test. In this method, live *M. luteus* were indicated by green and dead by red color in a confocal laser microscope (Figure 2B). Sample 7 was also probed with staining of dead *M. luteus* to ensure the red and green dots are not an artifact of confocal microscopy (Figure S5A, Supporting Information). The survival of *M. luteus* was also confirmed using the agar plate test and SEM imaging of the microparticles after disassembly of the core/shell morphology (Figure S5B,C, Supporting Information).

The escape of *M. luteus* from non-cross-linked and cross-linked microparticles was investigated with and without PDEAMA shell. The escape of live *M. luteus* cells was analyzed by keeping the microparticle samples in a nutrient culture

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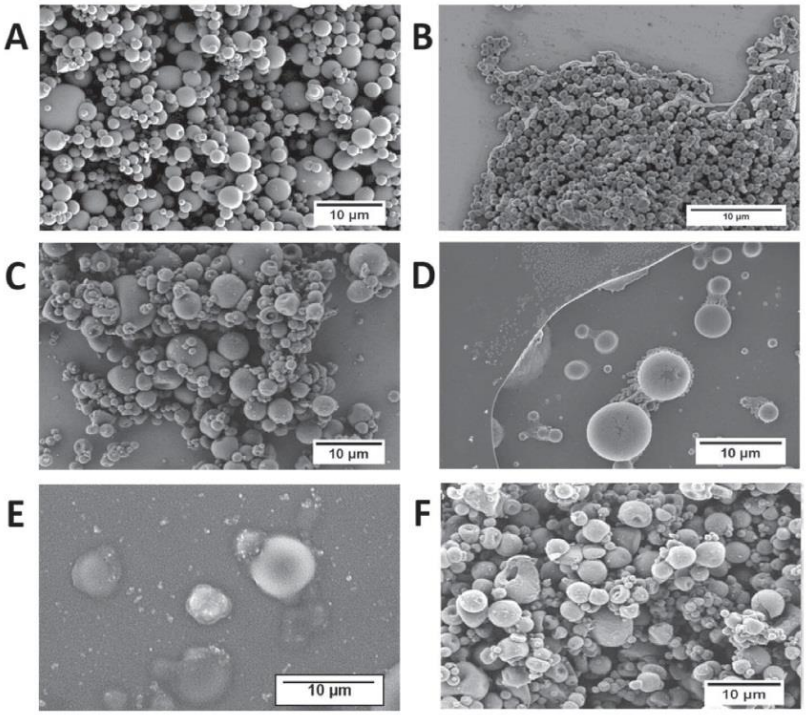

Figure 1. SEM image of A) PVA microparticles obtained by spray drying (sample 1). B) ESEM of *M. luteus* in PBS solution. C) SEM image of PVA microparticles with encapsulated *M. luteus* (sample 2). D) SEM micrographs of *M. luteus*/cross-linked PVA microparticles using the ratio of 0.06 glutaraldehyde to PVA microparticles (sample 4) after 24 h in water. E) ESEM image of *M. luteus*/cross-linked PVA microparticles (sample 4) in water. F) SEM image of core/shell microparticles (sample 6).

medium, followed by an agar plate test and detection of colonies. As expected, the escape of *M. luteus* from the non-cross-linked microparticles was observed within a few hours.[6] This occurred due to the swelling of the microparticles in water. The same result was observed for the cross-linked microparticles (Table S3, Supporting Information). In contrast to this, the escape of *M. luteus* was significantly retarded for the cross-linked microparticles with the PDEAMA shell. Therefore, we studied the microparticles with the PDEAMA shell in more detail. Sample 6 was suspended in a buffer solution of different pH values, and the diameter of the swollen microparticles was determined by SLS measurement (Figure 2C). Although the SLS measurement showed a significant increase in size of the microparticles by decreasing pH to acidic region, due to the broad size distribution, confocal laser microscopy was further used to confirm this effect. The confocal laser microscope image of sample 6 in aqueous media with pH = 1 is shown in Figure S6, Supporting Information. The diameters of 100 microparticles were counted and averaged (7.51 ± 3.99 μm). The average diameter of sample 6 particles in the dry state was 3.25 ± 1.22 μm as determined from SEM (Figure 1F).

The PDEAEMA shell significantly reduced the escape of *M. luteus* out of core/shell microparticles even when the shell of PDEAEMA is in its open state at pH = 5 (**Table 1**). To quantify this, the release of *M. luteus* was examined for the sample using grafted PDEAEMA shell from the surface of *M. luteus*/PVA microparticles (sample 6) and compared to the sample without a PDEAEMA shell (sample 4, Table S3, Supporting Information). The same study for the non-cross-linked PVA microparticles was shown in the work of Knierim et al.[6] Samples were kept separately in a nutrient medium (pH = 5), and the leaching of *M. luteus* was quantified by taking samples from the nutrient medium at weekly intervals. The release of bacteria was proven by the presence of yellow colonies on agar plates (Table 1). As yellow colonies appeared only after five weeks, it is likely that the release of *M. luteus* was significantly delayed by the presence of the PDEAEMA shell. In contrast, *M. luteus* from the microparticles without the shell (sample 2) was evident after 72 h in pH = 5.

The microparticles with the open shell of PDEAMA should allow the mass transfer of small molecules easily. To probe the mass transfer, we have used methylene blue as a model for small molecules. As the mass transport via the PVA hydrogel microparticles (cross-linked microparticles without PDEAEMA shell) occures immediately (due to the swelling behavior), the dye transfers (in and out) were studied for the sample with the shell. This experiment was carried out by loading the core/shell microparticles (sample 8) with methylene blue at pH = 5 (open pore structure was formed by protonated PDEAEMA shell) (Figure S7, Supporting Information). The extra amount

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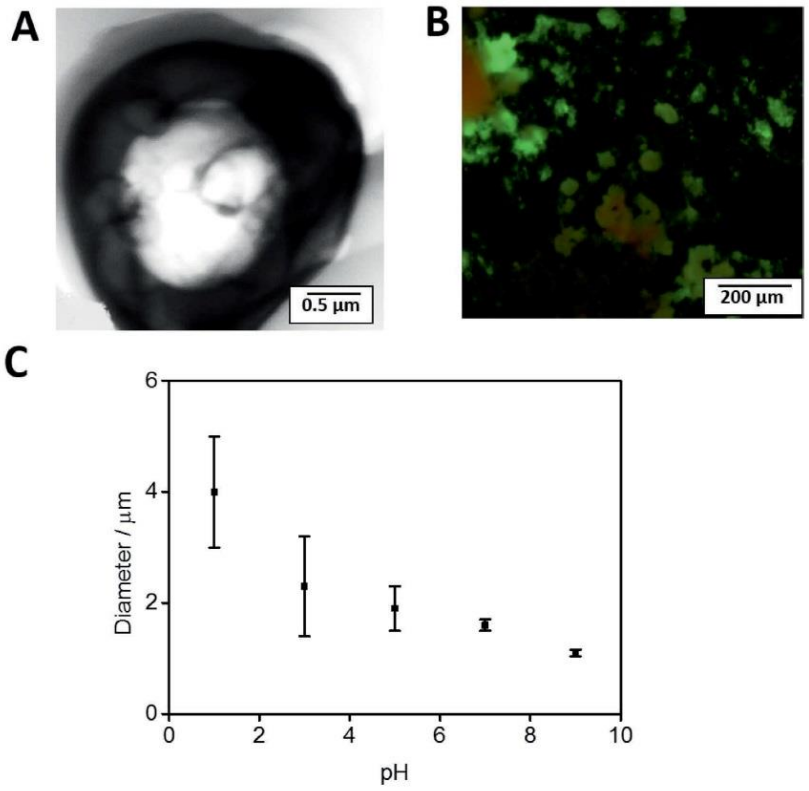

Figure 2. A) Microtome section embedded in epoxy resin of sample 6. B) Live/dead analysis of *M. luteus* using confocal laser microscopy, red: dead *M. luteus*, green: living *M. luteus*. C) Diameter change of core/shell microparticle (sample 6 in water) at different pH (pH = 1, 3, 5, 7, and 9), measured by SLS.

of methylene blue on the surface of the microparticles was washed with water, and samples were kept in pH = 9 (closed-shell) for 48 h to keep the methylene blue inside the microparticles. The release of the dye was quantified in acidic pH = 5, by absorption measurement with UV/vis. These measurements reveal that 10 wt% of the methylene blue leached within the first 24 h. The experiment proves sufficient nutrient transfer toward the suspension of core/shell microparticles with encapsulated *M. luteus* and vice versa.

3. Conclusion

The synthesis of the core/shell microparticle of PVA/DEAEMA was shown as a proof of concept for a system for the encapsulation of *M. luteus* in core/shell microparticles with a reduced bacterial escape but the potential for the mass transfer of small molecules. The pH-responsive design of the shell of these microparticles makes them of particular interest, for example, in wastewater treatment by bioremediation. The mild polymerization conditions for the grafting of the PDEAMA shell by ATRP was very important for the fitness of *M. luteus* after the grafting reaction. The diameter of the *M. luteus*/PVA/PDEAEMA microparticles increased from 3.25 ± 1.22 µm at pH = 7 to 7.51 ± 3.99 µm in an aqueous medium at pH = 1, which indicated that the elasticity and ability of such core/shell structures also allowed bacterial growth, which is another important characteristic of a functional microbial system.

The efficiency of the core/shell microparticle morphology in terms of the physiological activity of encapsulated *M. luteus*

Table 1. Release of *M. luteus* from *M. luteus*/PVA/PDEAEMA microparticles (sample 6) at 37 °C and pH = 5, the effect of PDEAEMA shell on release pattern.

Time [weeks] Samples	1	2	3	4	5
M. luteus/PVA/PDEAEMA microparticles (sample 6)	no colony	no colony	no colony	no colony	colonies observed

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was demonstrated by performing a live/dead analysis test. The antibacterial effect of PDEAEMA was examined by suspending the microparticles in a nutrient medium and an agar plate test, retarding the release of *M. luteus* up to 5 weeks. We conclude from the mass transfer experiment with methylene blue that mass transfer of nutrient and metabolism products will be possible from microparticles. As a result, the core/shell microparticles with encapsulated *M. luteus* provide a promising living composite for a wide variety of applications since higher metabolism efficiency with retarded leakage of bacteria was observed.

4. Experimental Section

Materials: PVA (M_w = 13000–23000 g mol^{-1}, 99% hydrolysis), glutaraldehyde (70 wt% in H2O), 1,1,4,7,10,10-hexamethyl triethylenetetramine (HMTETA) (97%), α-bromoisobutyryl bromide (98%), butyl acrylate (>99%); purchased from Sigma Aldrich; and ethyl-2-bromoisobutyrate (98%, Acros) were used as received. Copper bromide (CuBr) (98%, Acros) was flushed with argon before use. DEAEMA (Sigma Aldrich, 99%) was distilled under vacuum and stored under argon. Dichloromethane (DCM), pyridine, tetrahydrofuran (THF), anisole, and acetone were distilled prior to use.

Analytical Methods: ESEM (QuantaTM FEG 250, EFI) was performed to investigate the morphology of microparticles in aqueous media (temperature = 2 °C; pressure = 220 Pa). The measurements were carried out on a Wet-STEM, enabling control of humidity and temperature at an acceleration voltage of 1 kV. A gaseous secondary electron detector and circular backscatter detector detectors were used.

The particle size was measured by SEM; a Zeiss LEO 1530 (Jena, Germany) with a Schottky field emission cathode was used. The samples were adhered to a sample holder with double-sided adhesive tape and subsequently coated with 2.0 nm of platinum by a high-resolution sputter coater (208 HR, Cressington). A secondary electron (SE2) detector was used for SE2 images at an acceleration voltage of 3 kV and a working distance of ≈4.6 mm.

Energy-dispersive X-ray spectroscopy measurements were performed using a Zeiss Ultra Plus (Jena, Germany) with a Schottky field-emission cathode with an acceleration voltage of 10 kV. The samples were vapor-coated with platinum using a Balzer Union MED 010 before measurement.

For the shell characterization, an elastic bright-field TEM (Zeiss 922 Omega EFTEM, Jena, Germany) at a voltage of 200 kV was used.

A confocal laser microscope was performed using a Leica TCS SP8 (Model DMI 6000, HyD Hybrid-Detector; PMT-Detector) with an argon-laser (488 nm).

SLS measurements were performed on an LS spectrometer of LS Instruments AG (Fribourg, Switzerland) using a HeNe laser (maximum 35 mW constant output at 632.8 nm) as a light source. Two APD detectors in pseudo-cross-correlation were used to detect the scattered light. The time average scattering intensities were measured at a scattering angle of 90°.

Encapsulation of Micrococcus luteus in Poly(Vinyl Alcohol) Microparticles Using Spray Drying: *Cultivation of M. luteus*: A mixture of meat extract (lysogeny broth [LB] culture medium) (Roth) in extra pure water provided by a Milli-Q Plus system (conductivity = 0.072 µS cm^{-1}, pH = 7) was used as a nutrient medium for culturing *M. luteus* (DSM-No. 20030, DSMZ Braunschweig). The mixture was sterilized with an autoclave at 121 °C. Utilizing encapsulation, *M. luteus* bacterial cells were harvested after 72 h of growth in LB culture medium at 37 °C. The cell pellet was achieved by centrifuging at 4000 rpm for 10 min. Subsequently, the bacterial cells were washed with PBS (pH = 7.4).

Agar plates were prepared by mixing 15 g LB culture medium and 12 g agar-agar (Roth) in 750 mL water, followed by sterilizing the mixture.

In all the steps of the encapsulation and synthesis, a survival test was done by incubating the *M. luteus* on agar plates for 72 h at 37 °C. The growth of yellow colonies revealed the fact that *M. luteus* is biologically active.

Encapsulation and spray drying: In general, a solution of 2.5 wt% PVA in PBS, pH = 7.4, was prepared and sterilized in an autoclave (121 °C). The bacterial pellet was washed and resuspended in the PVA solution at room temperature (Scheme 1B). Dry PVA microparticles with encapsulated *M. luteus* were prepared by spray drying, using a mini spray dryer b290 (Büchi, Switzerland). The atomization gas was set to 600 L h^{-1} (50 mm) with an inlet temperature of 110 °C. A mixture of PVA solution with *M. luteus* was delivered to the device with a feeding rate of 2.5 mL min^{-1}. The experiment was carried out under an ambient atmosphere, using an open-loop system, in which the atmospheric air was proceeding to the device and heated for evaporation of the solvent. Parameters used for spray drying are listed in Table S1, Supporting Information. Products (sample 2, **Table 2**) were collected and stored at 4 °C.

Two control samples were manufactured (samples 1 and 3, Table 2): sample 1 was prepared by spray drying of PVA microparticles without

Table 2. Spray-dried PVA microparticles with encapsulated *M. luteus* (non-cross-linked and cross-linked microparticles (using the ratio of 0.06 glutaraldehyde to PVA microparticles), and the synthesis of core/shell microparticles of PVA/PDEAEMA.

Samples	Cores	*M. luteus*/PVA microparticles [g g^{-1}]	Diameter [µm]
1	PVA particle	—	1.68 ± 1.12
2	Alive *M. luteus*/PVA microparticle	1/20	2.64 ± 0.96
3	Dead *M. luteus*/PVA microparticle	1/20	2.64 ± 0.96
4	Alive *M. luteus*/cross-linked PVA microparticle	1/20	2.64 ± 0.96
5	Dead *M. luteus*/cross-linked PVA microparticle	1/20	2.64 ± 0.96
	Core/shell microparticles	Core/shell ratio	
6	Alive *M. luteus*/cross-linked PVA/PDEAEMA microparticle	1/6	3.25 ± 1.22
7	Dead *M. luteus*/cross-linked PVA/PDEAEMA microparticle	1/6	3.25 ± 1.22
8	PVA/PDEAEMA microparticle	1/6	—
9	Methylene blue/PVA/PDEAEMA microparticle	1/6	—

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encapsulated *M. luteus*. For sample 3, dead *M. luteus* cells were encapsulated in PVA microparticles. To produce sample 3, *M. luteus* pellet was separated from the culture medium and washed with ethanol (for killing *M. luteus*) before encapsulation. Dead cells were mixed with 2.5% PVA solution before spray drying. The agar plate test resulted in formation of zero colonies, which reveal the encapsulation of dead cells in PVA microparticles.

Cross-linking and functionalization of M. luteus/PVA microparticles: PVA microparticles with encapsulated *M. luteus* (live and dead cells, samples 2 and 3) were chemically cross-linked with glutaraldehyde at the concentration ratio (g g^{-1}) of 0.06/1 ([glutaraldehyde]/[*M. luteus*/PVA microparticles]) (Scheme 1B). An argon-flushed flask was charged with glutaraldehyde (70 wt% in H_2O) (0.027 g) and THF (25 mL). PVA microparticles with encapsulated *M. luteus* (0.29 g) were added to the mixture and stirred for 4 h at RT. The cross-linked microparticles (samples 4 and 5) were filtered and washed with THF.

Synthesis of functionalized M. luteus/cross-linked PVA microparticles: *M. luteus*/cross-linked PVA microparticles were functionalized to be used as a macroinitiator (Scheme 1B). Cross-linked *M. luteus*/PVA microparticles (0.1 g) were dispersed in 25 mL DCM and 1.6 mL (0.02 mol) pyridine. α-bromoisobutyryl bromide (0.87 mL, 7 mmol) was added, and the mixture was kept stirring at RT while purging with argon for 24 h. The microparticles were filtered and washed with DCM and acetone.

Surface polymerization of PDEAEMA on M. luteus/PVA microparticles using ATRP: Core/shell microparticles (samples 6–9, Table 2) were synthesized using surface-initiated ATRP (Scheme 1B). The core/shell ratio was calculated by regarding the weight (g) of PVA microparticles and the feeding ratio of the DEAEMA monomer for surface polymerization. In the following, the surface polymerization of sample 7 was described, Table 2, as an example. The cross-linked and functionalized *M. luteus*/PVA microparticles (macroinitiator) (0.404 g) were dispersed in 20 mL anisole and hexamethylenetetramine (HMTETA) (0.55 g, 3.9 mmol). The polymerization was started by the addition of CuBr (0.2 g, 1.4 mmol). Mixtures were kept for 24 h while purging with argon at RT. The core/shell microparticles were filtered and washed thrice with acetone and DCM. In order to check the survival of bacteria, sample 6 was immersed in liquid nitrogen, followed by scratching between two glass slides. The encapsulated bacteria were depicted with SEM micrograph.

Encapsulation of methylene blue in core/shell polymer microparticles (methylene blue/PVA/PDEAEMA, sample 9, Table 2): A stock solution of 25 mg L^{-1} (w/v) methylene blue in water was prepared. Core/shell microparticles (sample 8, Table 2) (0.025 g) were added to the mixture (pH = 3) and kept for 72 h. Afterward, the microparticles (sample 9, Table 2) were centrifuged and washed with water at basic pH (pH = 9). The release of dye was examined under acidic pH (pH = 3).

Staining with ruthenium oxide: PVA microparticles (sample 1, Table 2) were added to a mixture of 0.01 g ruthenium oxide in 1 mL water. After 5 min, the microparticles were centrifuged and washed thrice with water.

Staining with uranyl acetate: *M. luteus*/PVA/PDEAEMA microparticles (core/shell, sample 7, Table 2) were stained with uranyl acetate before embedding in epoxy resins. Uranyl acetate (0.04 g) was dissolved in 4 mL water. This was followed by the addition of 0.1 g of sample 10, followed by shaking, precipitation, and washing the microparticles thrice with water.

Live/dead analysis: The viability of *M. luteus* was tested with a confocal laser microscope (LSM510, Zeiss, Oberkochen, Germany) and via staining (BacLight Bacterial Viability Kit, Life Technologies [Eugene, OR]) of *M. luteus* (live/dead stain). The green color of Syto9 (green, fluorescent nucleic acid stain) showed living bacteria, and the red color of propidium iodide represented the dead bacterial cells.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Keywords

antibacterial polymers, core/shell polymer microparticles, encapsulation, *Micrococcus luteus*

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8.2 Bioremediation of copper-ions by polymer encapsulated and immobilized *Micrococcus Luteus*

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RESEARCH ARTICLE

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Bioremediation of Copper-Ions by Polymer Encapsulated and Immobilized *Micrococcus Luteus*

*Mahsa Mafi and Andreas Greiner**

Bioremediation of copper (Cu^{2+}) with immobilized *Micrococcus luteus* in polymer matrices has been broadly studied for a wide range of applications including wastewater treatment. Herein, the bioremediation efficiency based on modified immobilization techniques and by the addition of Cu^{2+} is investigated. Porous composite nonwovens with living *M. luteus* (living polymer composites) are prepared by encapsulation of the bacterial cells in poly(vinyl alcohol) (PVA) microparticles (*M. luteus*/PVA microparticles) produced by spray drying method. The *M. luteus*/PVA microparticles are chemically cross-linked. The hydrogel microparticles with encapsulated *M. luteus* are embedded in a nonwoven of poly (lactic acid) (PLA) electrospun short fibers provided by wet-laid method. Two different models of composite nonwovens are reported, in which the place position of the hydrogel PVA microparticles with encapsulated *M. luteus* and PLA nonwoven can affect the bioremediation process.

1. Introduction

Copper (Cu), as a trace element like many other heavy metals, beyond a certain amount, is capable of providing nutrients for the metabolism of microorganisms and regulating plant functionality. The human activities have altered the environmental copper content, resulting in copper pollution in the aquatic system. The excess amount of copper and especially highly toxic copper (II) (Cu^{2+}), is affecting human bodies and is known as a harmful ion in wastewater.[1]

Removal of Cu^{2+} from wastewater is performed with various physicochemical methods, such as absorption, cementation, nonwoven filtration, and dialysis. Absorption of Cu^{2+} with modified natural carriers like biopolymers has attracted numerous attentions in wastewater treatment. Modified biopolymers are widespread viable and environmentally friendly. Polysaccharide polymers as a well-known group of biopolymers are industrially attractive for reducing the Cu^{2+} concentration in wastewater upon absorption to the functional groups.[2] Parkash et al. showed the absorption ability of chitosan to uptake Cu^{2+} with the uptake amount of 87 mg per g of chitosan.[3]

Another prevalent method of Cu^{2+} removal is bioremediation, which is a noncostly and least harmful method. Bioremediation with microorganisms is highly potential for the decontamination of wastewater. Microorganisms such as fungi, bacteria, algae, protozoa, or viruses are breaking down the chemical components and using them as a source of carbon, whereas in bioremediation of heavy metals, biological degradation is not possible. The mechanism with microorganisms is based on the change from oxidative state or complex to another state (valance transformation), volatilization, and extracellular chemical precipitation.[4–9] Bacteria are confessed as the most abundant microorganisms in the ecosystem with a high surface to volume ratio.[10] The bioremediation efficiency is depending not only on the ambient conditions, such as pH and temperature, but also to the type of bacteria.[11] The most attractive feature of bacteria and a key to achieve excellent bioremediation is the presence of chemisorption sites in the cell wall. The presence of negative chemisorption sites in gram-positive bacterial cell wall provides higher sequestration of metal cations.[1] *Micrococcus Luteus* (*M. luteus*) is an aerobic gram-positive bacterium, which is exclusively withstanding the environmental stress.[12] In the study of Letnik et al. it was shown that only 10% of *M. luteus* cells with the initial concentration of 10^8 CFU are able to survive Cu^{2+} by the concentration of 370 ppm (6 mM).[13] In contrary, a Cu^{2+} solution of 0.05–0.1 mM has no inhibition effect on bacterial growth. The maximum bioremediation of Cu^{2+} by *M. luteus* cell after 1 h was determined as 59mg (Cu^{2+}/g of bacterial cells) in the solution concentration of 1 mM (80% uptake), which decrease to 14% for Cu^{2+}solution of 10 mM.

Despite the merits of bacterial bioremediation, continuous bioremediation has not been studied so far. The bioremediation process can be achieved by biologically active as well as inactive bacterial cells. Besides that, bacteria ability to self-producing is remarkable. These features can provide infinite possibilities for self-renewing bioremediation.[14,15]

Another essential factor maintaining the biological activity of bacteria, for higher bioremediation achievement is to protect

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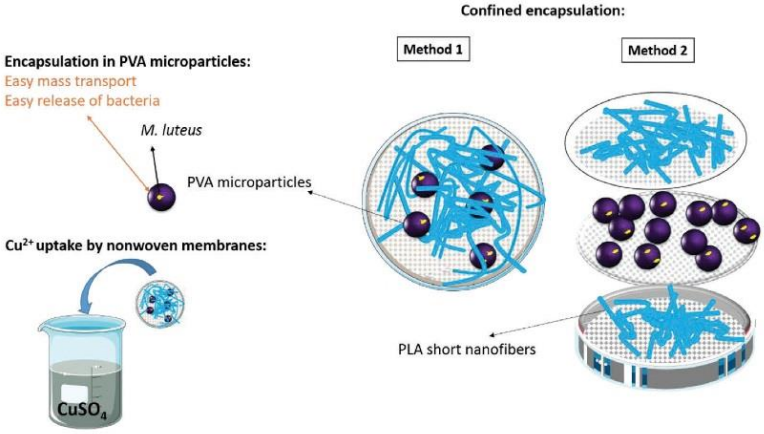

Scheme 1. Production of porous composite nonwovens containing *M. luteus*. Encapsulation of *M. luteus* in PVA microparticles followed by embedding in fiber nonwovens. Method 1: randomly mixed *M. luteus*/PVA microparticles and PLA short fibers. Method 2: *M. luteus*/PVA microparticles placed between 2 PLA nonwoven layers. Biosorption of Cu^{2+} with nonwoven membrane.

bacterial cells against unwanted environmental stresses. Immobilization of bacteria in polymer matrices offers advantages in terms of higher resistance to unfavorable environmental impacts, higher storage time, and reusability.[4,16,17] "Living composites" of bacteria/polymers promise to change the way of bioremediation methods with various advantages. Polymer particles and fibers are well known for encapsulation of active materials, such as catalysts, enzymes, microorganisms, nanoparticles, and pharmaceuticals, antibiotics, vitamins for a wide variety of applications. A wide variety of processing methods is well known for particle production, including emulsion polymerization, evaporation, and spray drying. Among all the mentioned methods, spray drying has attracted significant attention for its easy and fast manufacturing procedure. In this method, a polymer mixture in the form of a solution, emulsion, or suspension is atomized with atomization nuzzle, following by exposure to a heated gas stream. As a result, the formation of powder of solid particles by evaporation of the solvent is achievable.[18–21] Reich et al. introduced the encapsulation of bacteria in polymer microparticles by spray drying of gram-positive and negative bacteria in different polymer solutions.[22]

Another commonly used method for the preparation of polymer composites is electrospinning, in which fibers in the range of 10–100 nm in diameter is achievable.[23–26] Electrospun fibers have the unique feature highly porous structure and a wide variety of different fiber morphologies. As a result, the emerging use of fiber nonwovens in the production of nonwoven for traditional filtration, tissue engineering, biosensors, and composite materials is observed. Besides that, fibers are widely used for the immobilization of enzymes, bacteria, viruses, proteins, and other biologically active materials.[8,16,17,27–30] Salalha et al. reported the first work on encapsulation of biological materials like bacteria and viruses in electrospun PVA fibers in 2006.[30] In the work of Sarioglu et al. bacteria were trapped in fibers by natural adhesion force, which leads to the formation of biofilms.[31] Gensheimer et al. studied the double encapsulation of bacterial cells in polymer particles and fibers.[29] In more detail, *M. luteus* was firstly encapsulated in PVA particles, followed by electrospinning of a suspension of particles in another polymer/ethanol solution. Although the double encapsulation retards the release of bacteria, the essential nutrient transports through the particles were also reduced.

However, the preparation of high surface area nonwoven composites with electrospun fibers is a time-consuming process, which can be optimized by preparation of similar nonwoven by use of short fibers' dispersion by a wet-laid method.[32] The procedure offers additional advantage of mixing other additives, in short fiber dispersion just by mixing and forming the additive immobilized nonwoven.[32,33]

The objective of the current work is to emphasize on immobilization techniques of *M. luteus* cells for continuous sequestrating of Cu^{2+}. Encapsulation of *M. luteus* in PVA microparticles (*M. luteus*/PVA microparticles) was achieved by the spray-drying method. The microparticles were chemically cross-linked, to attain hydrogel structure that swells in exposure to aqueous media. This swelling behavior grants the elasticity to the microparticles upon bacterial growth. In this work, for retarding the bacterial leakage from PVA microparticles, a carrier of electrospun PLA fibers was used (Scheme 1). The effect of porous composite nonwoven preparation on mass transport for bioremediation was studied with two methodologies. Following **Scheme 1**, the immobilization of *M. luteus*/ PVA microparticles in PLA short fibers was made in two different forms (the *M. luteus*/ PVA microparticles were sandwiched or randomly mixed in PLA short fibers). Bioremediation of Cu^{2+} by alive encapsulated *M. luteus*, as well as encapsulated dead cells was studied, and identified by analytical methods.

2. Result and Discussion

Two different strategies were adopted in order to immobilize *M. luteus*/PVA microparticles in nonwovens with the aim to study

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the effect of the preparation method on the bioremediation efficiency. In the first approach (the work of Genscheimer et al.), *M. luteus*/PVA microparticles can be encapsulated in electrospun polymer fibers, which means double encapsulation of *M. luteus*. As a result, fibers act as a shell for encapsulated microparticles.[29] The mass transport for this immobilization model is retarded with the fibers, which cover the microparticles surfaces. Therefore, a nonwoven is desirable, which makes no hindrance for nutrient passage through the PVA microparticles.

In the second approach, *M. luteus*/PVA microparticles are dispersed in short length fibers by help of wet-laid method (Scheme 1). In this method, nonwoven matrix has less limiting effect on mass transport.

For both strategies, and to protect *M. luteus* from environmental stress, and increase the storage time with respect to the biological activity, *M. luteus* was successfully encapsulated in PVA microparticles using spray drying method (Sample 1, Table 2, **Figure 1**a,b). The proof of the efficiency of the encapsulation in the PVA microparticles was shown in the work of Reich et al. and by a Raman-atomic force microscopy (AFM) measurement.[22] In this study, the presence of *M. luteus* inside the PVA microparticles was shown. The diameter size of the PVA microparticles with encapsulated *M. luteus* is in the range of 2.58 ± 0.89 μm (Figure S1, Supporting Information). *M. luteus*/PVA microparticles were cross-linked (Sample 3, Table 2, Figure 1c), and survival of *M. luteus* was tested with bacterial culture in agar plate test. The observation of characteristic yellow colonies revealed the fact of the biologically active *M. luteus* in the cross-linked PVA microparticles. The cross-linked microparticles were kept in water for 48 h in order to study the cross-linking efficiency (Figure 1d). The cross-linked PVA microparticles were immobilized in PLA short fibers nonwoven. Electrospun PLA fibers with average diameters of 240 ± 115 nm (Sample 4, Table 2) (Figure 1e, Figure S2, Supporting Information) underwent a fiber cutting process to get short fibers of an average length of 0.45 ± 0.29 mm (Sample 5, Table 2) (Figure 1f). PLA short fibers nonwoven were prepared by wet laid procedure.[32–34] The method utilizes a dispersion of short fibers, and subsequent filtration leading to the formation of a self-standing nonwoven by the percolation of fibers. In the first method, the *M. luteus*/PVA microparticles were dispersed together with PLA short fibers and filtered through a filtration funnel to obtain PLA nonwoven immobilizing the microparticles (Samples 6 and 7, Table 2). Whereas, in second method the *M. luteus*/PVA microparticles were sandwiched between two PLA nonwovens by a three step procedure (Sample 8 and 9, Table 2): making PLA nonwoven by wet-laid process using a dispersion of short PLA fibers, filtration of *M. luteus*/PVA microparticles through PLA wet-laid nonwoven followed by covering the particles with another layer of PLA made by wet-laying short PLA fiber dispersion. The thickness of the nonwoven formed by short-fiber and microparticles dispersion filtration in both methods is in the range of 1.68 ± 0.12 mm. The pore size measurement of the PLA nonwoven showed the size range of the pores roughly about 119.95 ± 67.55 μm.

SEM micrographs of wet-laid nonwovens with PVA microparticles containing the encapsulated *M. luteus* are shown in Figure 1g–j. The cross-section of the wet-laid nonwoven in Figure 1h,j showed the distribution of *M. luteus*/PVA microparticles throughout the PLA short fibers nonwovens.

Bacterial leaching test was performed by agar plate tests for porous composite nonwovens of two different approaches (Method 1 and 2), and also with different amounts of *M. luteus*/PVA microparticles (**Figure 2**a). The release behavior was studied for the time period of 72 h, and the formation of yellow colonies confirmed the release of *M. luteus*. The release study showed less leaching of *M. luteus* as observed by the formation of less colonies for composite nonwovens of sandwich samples (Method 2, Samples 8 and 9) in comparison to the randomly mixed nonwovens (Method 1, Samples 6 and 7). As a result of the investigation in release behavior, and in order to prevent leakage of *M. luteus*, the bioremediation ability of encapsulated *M. luteus* was required to be studied in 48h. In this step, composite samples (made with different approaches and amount of components) with the size of 1 cm × 1 cm were placed in 10 mL Cu^{2+} solutions with different concentrations (5, 10, and 15 mM) at 37 °C for 48 h.

In order to analyze the functionality of the composite nonwoven, we have investigated the remediation of Cu^{2+} by the encapsulated *M. luteus*. The remediation of Cu^{2+} was quantified by ICP-OES. To compare the passive and active bioremediation (with live and dead cells, respectively), control samples of encapsulated dead cells of *M. luteus* in polymer composites with methods 1 and 2 were used (Sample 10, Table 2). The result of bioremediation with control samples made in different models (method 1 and 2) were approximately in the same range (narrow error bar in Figure 2b). The Cu^{2+} uptake with the composite nonwovens with living *M. luteus* was 3 to 4 times more than the uptake with the dead cells (for all Cu^{2+} concentrations). A low concentration of Cu^{2+} solution (5 mM) resulted in approximately 90% uptake by the composite nonwovens of all types. The uptake for 10 mM Cu^{2+} solution was different for different composites, and a higher amount of Cu^{2+} solution (15 mM) led to a decrease in uptake. In Cu^{2+} concentration of 10 mM and 15 mM, the significantly reduced amount of uptake for Samples 6 and 7 (Table 2) is due to the release of the encapsulated *M. luteus* from the surface of the samples, which was also observed in the study of the release behavior. The increased release of *M. luteus* for Samples 6 and 7 (Table 2) was studied by Agar plate test (Figure 2a), and observation of more yellow colonies compared to Samples 8 and 9. However, for Samples 8 and 9 (Table 2), the PLA short fibers covering the top and bottom of the composites made by model 2 were reducing the exposure of *M. luteus*/PVA microparticles with nutrient medium or Cu^{2+} solution, which was playing role in preventing the release of *M. luteus*. The bioabsorbed Cu^{2+} in the nonwoven composites with encapsulated *M. luteus* were identified by SEM using an energy dispersive X-ray detector SEM using a back scattered electron (BSE) detector (Figure 2c,d).

The study on continuous bioremediation of *M. luteus* by the addition of Cu^{2+} and the self-renewing bioremediation ability was carried out in this work. Composite nonwoven samples (the same size and same procedure as explained before) were kept in 5, 10, and 15 mM Cu^{2+} solutions (10 mL) at 37 °C for 48 h, followed by the addition of the second batch of Cu^{2+} solution with the same concentrations after 48 h. After the second addition of Cu^{2+}, the amount of Cu^{2+} bioabsorbed was quantified by ICP-OES measurement. The control sample was prepared as mentioned in the previous section. The result (**Figure 3**) is revealing the ability of encapsulated *M. luteus* to grow and continue the uptake process.

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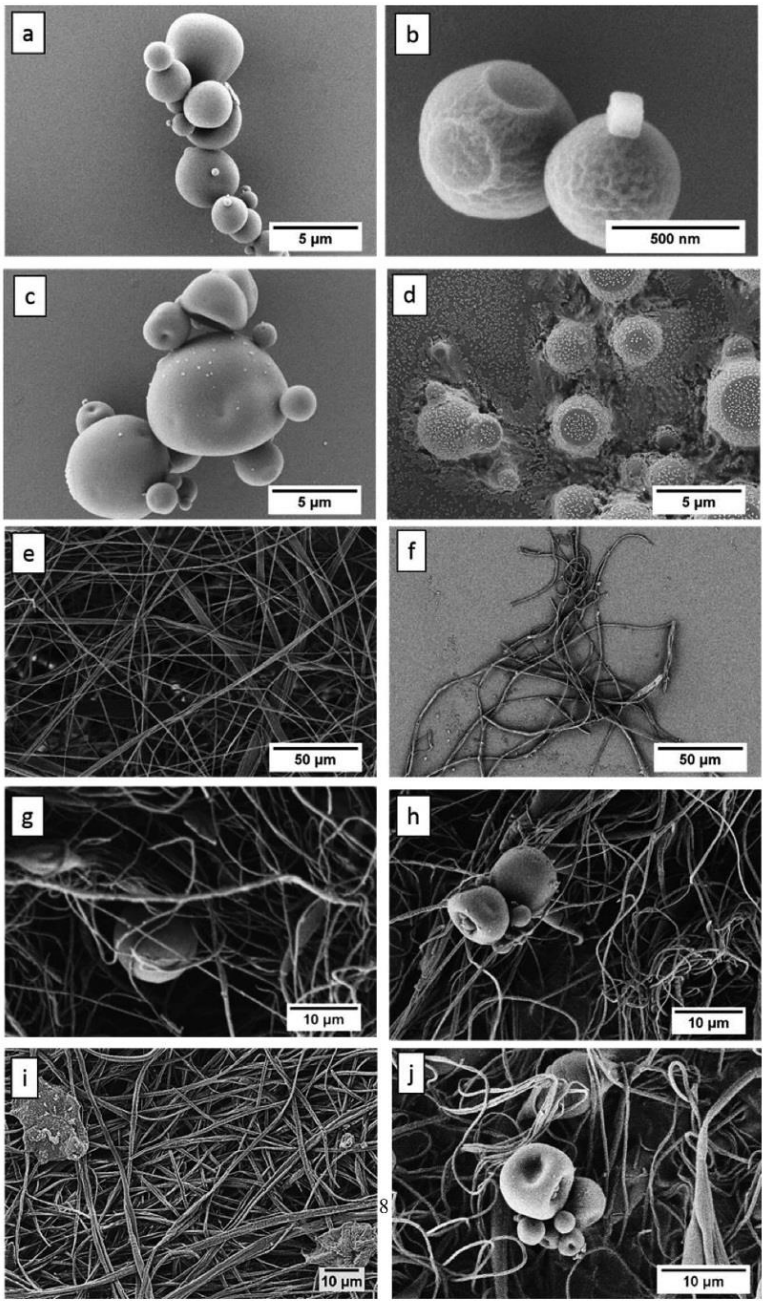

Figure 1. SEM micrograph of samples in Table 2. a) *M. luteus*/PVA microparticles after spray drying, Sample 1. b) Surface of the dry *M. luteus*/PVA microparticles (the salt crystal is due to the use of PBS for spray drying), Sample 1. c) *M. luteus*/ PVA microparticles after cross-linking with glutaraldehyde, Sample 3. d) *M. luteus*/ cross-linked PVA microparticles after 48 h immersion in water, Sample 3. e) Electrospun PLA fibers, Sample 4. f) PLA short fibers, sample 5. g) *M. luteus*/PVA microparticles in short fiber nonwoven, the surface of Sample 6 (the gel formed on top of the sample is due to the cross-linking of $CaCl_2$ and sodium alginate). h) The cross-section of the nonwoven, Sample 6. i) *M. luteus*/PVA microparticles in short fiber nonwoven, the surface of sample 8; j) the cross-section of the nonwoven, Sample 8.

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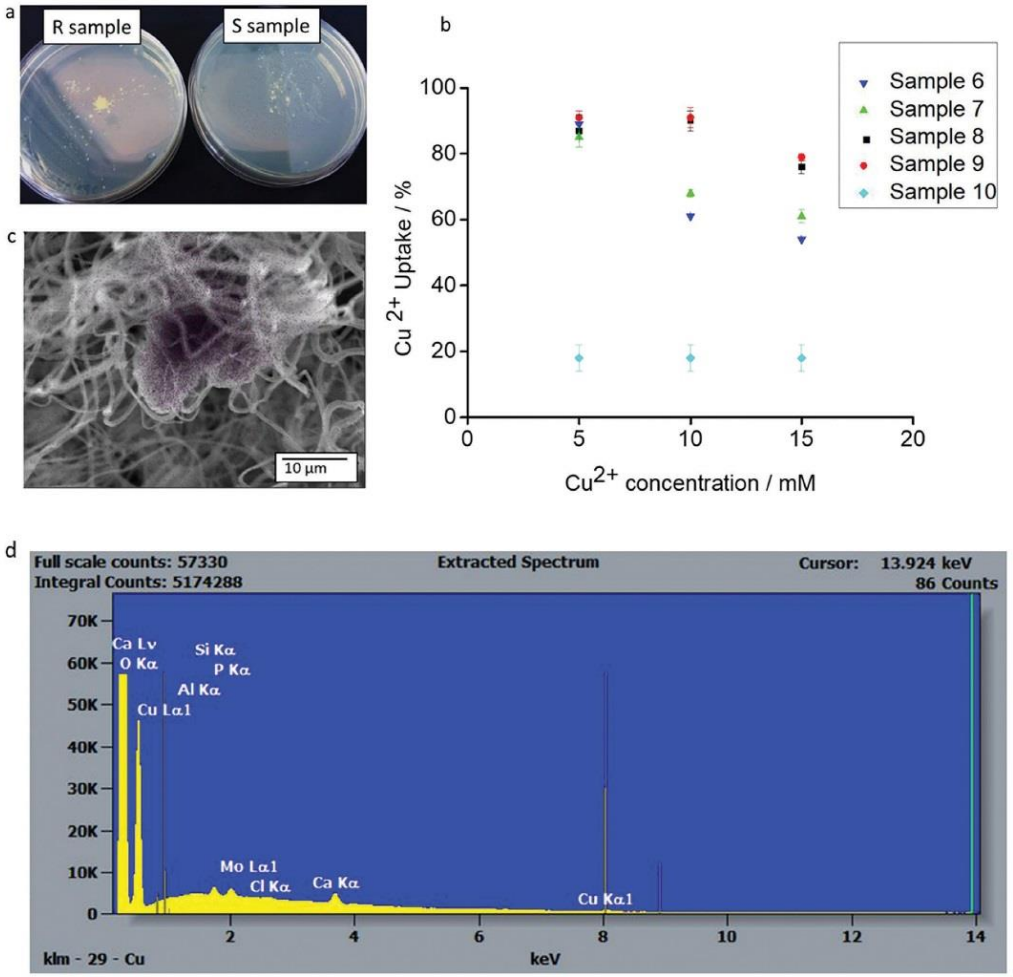

Figure 2. a) Release of *M. luteus* from porous composite nonwovens of different methods.) Characterization of Cu^{2+} uptake by composite nonwovens after 48 h at 37 °C, Comparison of the Cu^{2+} uptake of different samples (the control sample (Sample 10, Table 2) consists of the dead cell of *M. luteus*) measured by ICP-OES at different Cu^{2+} concentrations (5, 10, and 15 mM). c) SEM/BSE image of PVA/PLA nonwoven Sample 9 (Table 2). d) SEM/EDX spectra of *M. luteus*/PVA microparticles embedded in PLA short fibers, Sample 9.

Figure 2 shows that the sandwich composites (method 2, Samples 8 and 9) perform the bioremediation better than the randomly made composites (method 1, Samples 6 and 7), which is also observed in the current experiment. After 96 h and by the addition of Cu^{2+} salt to the composite samples with living *M. luteus*, bioremediation of roughly 60–70% for Samples 8 and 9 and 25–45% for Samples 6 and 7 were observed.

3. Conclusion

Two different porous composite nonwovens with encapsulated living *M. luteus* were prepared, and the bioremediation of Cu^{2+} as an existing heavy metal in wastewater by encapsulated *M. luteus* was quantified. The porous composite nonwovens were prepared by the encapsulation of *M. luteus* in PVA microparticles with a diameter of 2.58 ± 0.89 µm, followed by embedding the microparticles in PLA short fibers with average diameter of 240 ± 115 nm and length of 0.45 ± 0.29 mm. PLA nonwoven used as a carrier and made by wet-laid method. Two types of samples with alive and dead *M. luteus* cells were prepared. 1:By filtering the microparticles and short fibers together, and 2: a sandwich structure of two layers of short fibers with a middle layer of microparticles. The amount of microparticles with encapsulated *M. luteus* was doubled for the composite nonwovens of each model (model 1 and 2) to show its impact on bioremediation efficiency *M. luteus* remained active for 48 h without leaching from porous composite

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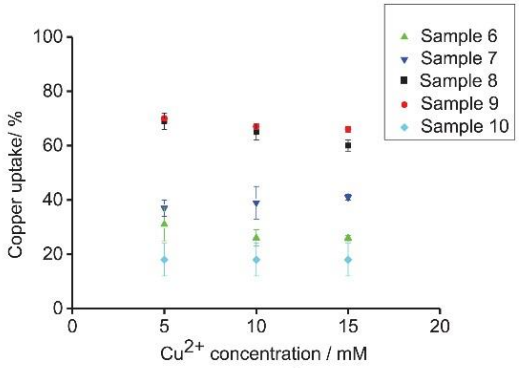

Figure 3. ICP-OES measurement of Cu^{2+} uptake after 96 h: the effect of additional Cu^{2+} to the ability of different composite samples with living *M. luteus* and dead *M. luteus* (for control samples) for second time bioremediation, continuous bioremediation was operated at 37 °C and pH = 5.

nonwovens of two different models, and samples were successfully applied for Cu^{2+} removal. The bioremediation of Cu^{2+} by encapsulated *M. luteus* was quantified by ICP-OES. The uptake for composite nonwovens of all types with initial Cu^{2+} concentration of 5 mM was approximately 90%, which reduced by increasing the initial Cu^{2+} concentration to 10–15 mM. The continuous uptake was quantified by prolonging the time of the experiment for the second 48 h and by the addition of Cu^{2+} salt to the porous composite samples with living *M. luteus*. The Cu^{2+} bioremediation of 60–70% for method 2 and 25–45% for method 1 were observed. Therefore, the composite nonwovens made by method 2 were more efficient for bioremediation applications, due to the higher protection of *M. luteus*/PVA microparticles with two layers of PLA nonwovens. The current work confirmed the ability of encapsulated *M. luteus* in polymer composite nonwovens for continuous bioremediation of Cu^{2+} in aqueous media. With the present concept, a new system is available which will provide optimum mass transport for bioremediation while the unwanted escape of the bacteria from their carrier is suppressed and thereby the environmental pollution by the microbes.

4. Experimental Section

Materials: PVA (M_w = 13 000–23 000 g·mol^{-1}, 98–99% hydrolysis, Sigma Aldrich) and PLA (IngeoTM 4043D, NatureWorks LLC) were obtained as indicated. Glutaraldehyde (70% in H_2O) (Sigma Aldrich), copper sulfate ($CuSO_4$) (Roth ≥98%), calcium chloride ($CaCl_2$) (Grüssig GmbH), alginic acid sodium salt ($NaC_6H_7O_6$) (Merck), and acetic acid (CH_3COOH, 100%, VWR) were used as received. Tetrahydrofuran (THF), chloroform ($CHCl_3$), dimethylformamide (DMF) were distilled before use.

M. luteus (DSM-No. 20030) from DSMZ Braunschweig was cultivated in a mixture of Lysogeny broth (LB) (Roth) in extra pure water provided by a Milli-Q Plus system (conductivity = 0.072 μS·cm^{-1}, pH = 7) at 37 °C for 72 h.

Analytical Methods: Scanning electron microscopy (SEM) (Zeiss LEO 1530, Jena, Germany) was used to analyze the morphology of microparticles and fibers with a secondary electron (SE2) detector, and an acceleration voltage of 3 kV and a working distance of ≈4.6 mm. The samples were stuck onto a sample holder with double-sided adhesive tape and subsequently coated with 2.0 nm of platinum by a high-resolution sputter coater (208 HR, Cressington).

Table 1. Parameters of spray dryer for particle producing.

Aspirator [%]	Inlet temperature [°C]	Inlet temperature [°C]	Pump [mL min^{-1}]	PVA solution [%]
100	110	67	2.5	2.5

Energy-dispersive X-ray spectroscopy (EDX) measurements were performed with a Zeiss Ultra Plus (Jena, Germany) with a Schottky field-emission cathode with an acceleration voltage of 10 kV. The samples were vapor coated with platinum using a Balzer Union MED 010 before measurement.

Inductively coupled plasma-optical emission spectrometry (ICP-OES) was performed on a VARIAN Vista-Pro (40 MHz, Argon Plasma) equipped with an ASX-510 autosampler, an Echelle polychromator, and an argon humidifier. Before measurement, samples were dissolved in 0.5 mL nitric acid and diluted with 9.5 mL of ultrapure water. A CCD semiconductor detector was used for the detection.

Encapsulation of M. Luteus in PVA Microparticles and Cross-Linking Procedure: Cultivated *M. luteus* pellet was centrifuged (10 min, 4000 rpm), washed, and re-suspended in the solution of 2.5 wt% PVA in phosphate buffered saline (PBS) (with 8.0 g NaCl, 0.2 g KCl, 1.42 g Na_2HPO_4, and 0.24 g KH_2PO_4 per liter of millipore water, pH = 7.3). The ratio between *M. luteus* and PVA polymer for one setup of spray drying was set as 1:20 g/g. The PVA solution was sterilized by autoclaving at 121 °C for 20 min.

A mini spray dryer b290 (Büchi, Switzerland) was utilized to encapsulate *M. luteus* in PVA microparticles. The bacteria/polymer solution was fed to the device with a feed rate of 2.5 mL·min^{-1} and the atomization gas of 600 L·h^{-1}. The inlet temperature based on the boiling point of water was set to 110 °C. The experiment was carried out under an ambient atmosphere. Further parameters used for spray drying are listed in **Table 1**. The encapsulated *M. luteus* in PVA microparticles (*M. luteus*/PVA microparticles) were collected and stored at 4 °C for further analysis (Sample 1, **Table 2**, Scheme 1A).

Besides the production of PVA microparticles with biologically active *M. luteus*, dead cell encapsulation was also performed. In this case, the centrifuged pellet of *M. luteus* obtained after cultivation was washed three times with ethanol. The encapsulation of dead cells in PVA microparticles was similar to the encapsulation of live *M. luteus* (Sample 2, Table 2).

The spray-dried PVA microparticles were chemically cross-linked with glutaraldehyde (Sample 3, Table 2). An argon-flushed flask was charged with glutaraldehyde (70% in H_2O) (0.035 g) and THF (25 mL). The amount of glutaraldehyde was decided based on testing its effect on *M. luteus* by minimum bactericidal concentration (MBC)/minimum inhibitory concentration (MIC) tests. Considering the result, the amount of glutaraldehyde below 0.14 mg L^{-1} has no inhibition effect on the growth of *M. luteus*. *M. luteus*/PVA microparticles (0.29 g) (Sample 1, Table 2) was added to the glutaraldehyde-THF solution under stirring for 4 h at RT.

Electrospinning of PLA: Electrospinning was carried out using a 7 wt% PLA solution in the mixture of $CHCl_3$/DMF/CH_3COOH (v/v/v, 70/15/15). The solution feeding rate was 1 mL h^{-1}. The applied voltage was 20 kV. The fibers (Sample 4, Table 2) were collected on a collector covered with a parchment paper (cellulose-based paper) with the distance of the collector to the nozzle of 12 cm.

Preparation of PLA Short Fibers Dispersion and M. Luteus/PVA Microparticles Immobilized in Porous Composite Nonwovens by a Wet-Laid Method: PLA fibers (8 g) (Sample 4, Table 2) were dispersed in water (1 L) and cooled down to −18 °C. The cutting process was operated by adding the dispersion in a blender for 1 h at 4000 rpm. The PLA short fibers (Sample 5, Table 2) were collected for the next step.

Porous composite nonwovens of PLA with immobilized *M. luteus*/PVA microparticles (Scheme 1) were prepared by two methods.

Method 1. Both *M. luteus*/PVA microparticles (Sample 2, Table 2) and short PLA fibers (Sample 5, Table 2) were re-dispersed together in water

Table 2. Details of components used for the preparation of *M. luteus*/PVA microparticles immobilized porous composite nonwovens.

Samples	Components
1	Live *M. luteus* /PVA microparticles
2	Dead *M. luteus* /PVA microparticles
3	Cross-linked PVA microparticles with encapsulated live *M. luteus*
4	PLA nanofibers
5	PLA short nanofibers nonwoven

Samples	Composite nonwovens and method	Amount of PLA short fibers [g]	Amount of microparticles [g]
6	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 1	0.5	0.5
7	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 1	0.5	1
8	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 2	0.5	0.5
9	Live *M. luteus*/PVA microparticles /PLA short fibers made by method 2	0.5	1
10	Dead cells of *M. luteus*/PVA microparticles/PLA short fibers made by method 1 and 2	0.5	0.5 or 1

and filtered through a mesh of poly (ethylene terephthalate) (PET) (Franz Eckert GmbH) with a pore size of 100 µm. In this method, the position of *M. luteus*/PVA microparticles and short PLA fibers were randomly distributed (Samples 6 and 7 were made by this method, Table 2).

Method 2. Firstly, PLA short fiber dispersion (Sample 5, Table 2) was filtered through a PET mesh (pore size 100 µm) to make a PLA porous nonwoven by the percolation of short fibers as shown in the previous works for different applications,[32,33] on top of which *M. luteus*/PVA microparticles (Sample 2, Table 2) were filtered. Afterward, they were covered by filtration of another batch of PLA short fibers. Consequently, *M. luteus*/PVA particles were sandwiched between two layers of PLA porous nonwovens (Samples 8 and 9, Table 2). To study the effect of encapsulated *M. luteus*, the amount of microparticles was varied, while the amount of short PLA fibers was kept the same for each method (Table 2). To prevent the unwanted release of the *M. luteus*/PVA microparticles from porous nonwovens, a solution of 1 and 2 wt% Alginic acid sodium salt and $CaCl_2$, respectively, were added in the last step and after the porous nonwoven composites of microparticles and short fibers were made (5 mL of each solution). The cross-linking of alginic acid by $CaCl_2$ is well-known in the literature.[35] Table 2 represents the preparation methods and the amount of the components used in sample preparation.

All the steps of cross-linking or preparation of porous nonwovens were carried out for Sample 2, Table 2 for comparison purposes.

Cu^{2+} Uptake by Encapsulated M. Luteus: To quantify the Cu^{2+} depletion by encapsulated *M. luteus* in porous composite nonwovens with ICP-OES, 10 mL solutions of 5, 10, and 15 mM Cu^{2+} in extra pure water (milli-Q system) were prepared. Porous composite nonwoven samples made as explained previously with the size of 1 × 1 cm and weight of 0.1g were kept in the solutions for 48 h (temperature 37 °C, pH = 5, and shaking speed 100rpm). After the first interval (48 h), the polymeric matrices (PLA and PVA matrix) were destroyed by keeping the samples in aqua regia for 24 h. This is part of the sample preparation for required for the destruction of measurement by ICP-OES.

To measure the continuous sequestration of Cu^{2+} by bacteria/polymer porous composite nonwovens, the first experiment was repeated with the same size and weight of the samples (37 °C and pH = 5). After 48 h, the samples were taken out of the solutions and were washed with water. The washed samples were placed again in newly made 10 mL Cu^{2+} solutions of 5, 10, and 15 mM. The second interval of 48 h was considered, followed by removing the polymeric matrices.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that supports the findings of this study are available in the supplementary material of this article.

Keywords

bioremediation, encapsulation, polymer microparticles, wet-laid nonwovens

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8.3 *Bacillus Subtilis* in PVA Microparticles for Treating Open Wounds

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Article

Bacillus subtilis in PVA Microparticles for Treating Open Wounds

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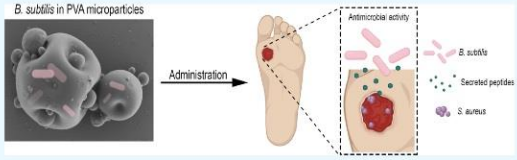
ABSTRACT: Open wound dressings should provide a moist environment, protect the wound from bacterial contamination, and shield it from further damage. These requirements, however, are hard to accomplish since such wounds are colonized by pathogenic bacteria, including resistant species such as methicillin-resistant *Staphylococcus aureus* (MRSA). A new approach for treating open wounds that is based on sticky and dissolvable polyvinyl alcohol (PVA) microparticles containing live *Bacillus subtilis* (*B. subtilis*) is described. Microparticles, fabricated by the spray-drying technique, were administered directly to an open wound while *B. subtilis* continuously produced and secreted antimicrobial molecules. *B. subtilis* in PVA microparticles demonstrated remarkable antibacterial activity against MRSA and *S. aureus*. In in vivo experiments, both *B. subtilis* and empty PVA microparticles were effective in decreasing healing time; however, *B. subtilis* microparticles were more effective during the first week. There was no evidence of skin irritation, infection, or other adverse effects during the 15 day postoperative observation period. This concept of combining live secreting bacteria within a supportive delivery system shows great promise as a therapeutic agent for open wounds and other infectious skin disorders.



1. INTRODUCTION

Skin healing is critical for maintaining the barrier between the body and the external environment. Hydration is considered to be the single most important factor for optimal wound healing by providing a protective barrier, reducing dermal necrosis, and accelerating wound re-epithelialization.[1] There is also an urgent need for effective infection control to promote rehabilitation and avoid systemic inflammation.[2] This requirement, however, is hard to accomplish since open wounds are invariably colonized by pathogenic bacteria including *Staphylococcus aureus* (*S. aureus*) and methicillin-resistant *Staphylococcus aureus* (MRSA), which express virulence factors.[3,4] Consequently, the preferred open wound dressing should not only provide a moist environment but also protect the wound from bacterial contamination, shield the wound from further damage, and promote granulation tissue formation.[5]

Currently available treatments for open wounds are often less than ideal as drug delivery systems. Antiseptics, for example, are toxic to human keratinocytes and fibroblasts and may increase the intensity and duration of skin inflammation.[2] The antibacterial activity of topical therapy lasts only up to 12 h, and dressing must be replaced routinely.[6] Topical formulations also suffer from inherent shortcomings, particularity those containing an oily phase such as ointment and emulsions. These eventually dry, and the dressing tends to stick to the surface of the wound, causing unnecessary pain, cell damage, and impaired wound healing.[7] Finally, oral and topical antibiotics have not always been shown to improve healing rates of chronic ulcers,[8] probably due to the rapid emergence of resistant bacteria.[9]

The objective of this study was to design and study a new class of topical formulae for treating open wounds. We hypothesized that a live bacterial formulation that continuously produces antibacterial molecules and delivers them directly to the open wound surface may be an alternative therapy. Our structure motif is based on incorporating *Bacillus subtilis* (*B. subtilis*), a Gram-positive bacterium that produces and secretes a variety of potent antibacterial agents,[10] into polyvinyl alcohol (PVA) microparticles. PVA was selected as the carrier due to its water solubility, hygroscopic properties, and excellent ability to adhere to the skin.[11,12] In addition, owing to its very low modulus of elasticity, PVA formulations cause minimal mechanical irritations, exhibit excellent biocompatibility with body tissues, and do not require removal or replacement.[13,14] From an industrial point of view, PVA is scalable and ideal for fabricating microparticles due to its semicrystalline structure and physical properties.[11,14] *B. subtilis* has been marketed as a biocontrol agent for various crop diseases thanks to its ability to protect against bacterial and fungal attack in plants.[15] *B. subtilis* has been designated Generally Regarded as Safe (GRAS) by the U.S. Food and Drug Administration.[16−19] In this study, we describe a particulate system that can be administered directly onto the open wound surface, seal it, and

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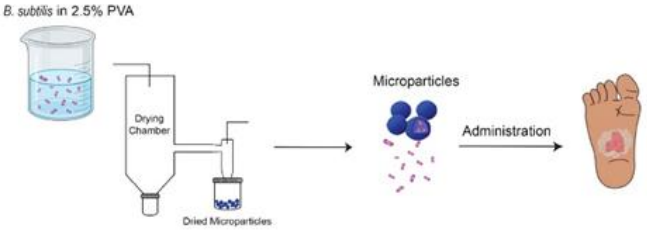

Figure 1. Preparation and administration of live bacterial microparticles. Microparticles, formed by spray drying, can be administered directly onto open ulcers and lesions.

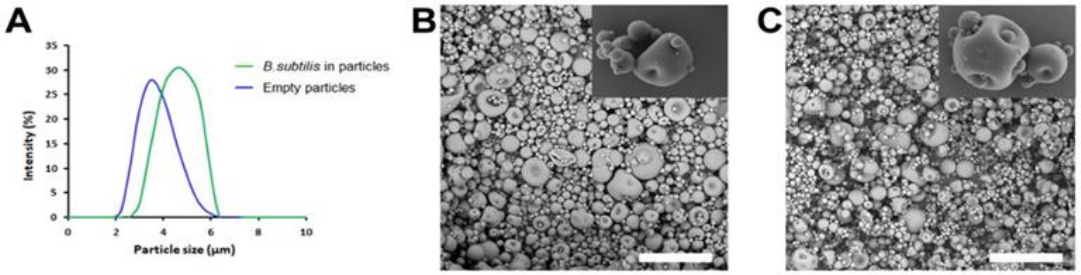

Figure 2. Size and morphology: (A) Microparticle size distribution of microparticles with *B. subtilis* compared with empty microparticles as determined by DLS. (B) PVA microparticles and (C) *B. subtilis* encapsulated in PVA microparticles. Scale bar: 20 μm.

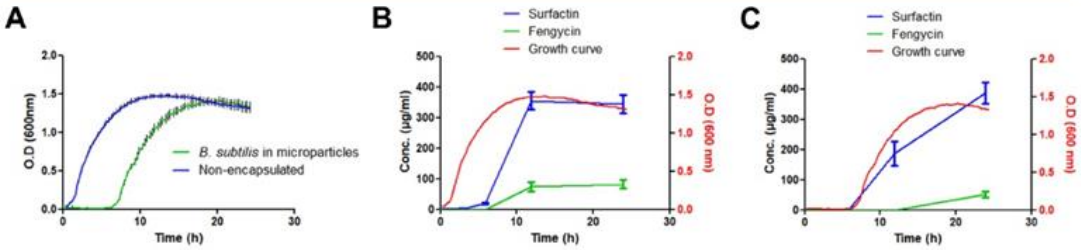

Figure 3. Characterization of *B. subtilis*. (A) Growth of *B. subtilis* in microparticles compared with fresh bacteria at 37 °C (n = 4). (B) LC/MS analysis of surfactin and fengycin produced and extracted from free bacteria. (C) LC/MS analysis of surfactin and fengycin produced and extracted from encapsulated bacteria.

maintain a humid atmosphere while continuously producing and secreting antimicrobial molecules (Figure 1).

2. RESULTS AND DISCUSSION

2.1. Microparticle Formation and Characterization. Microparticles containing *B. subtilis* were manufactured by spray-drying a bacterial suspension in PVA aqueous solution (2.5 wt %). Spray drying is a known method for the production of PVA microparticles in large quantities. The effect of *B. subtilis* on microparticle size and morphology was documented using dynamic light scattering (DLS) and scanning electron microscopy (SEM), respectively (Figure 2).

Empty microparticles showed an average diameter of 3.5 μm (Figure 2A), whereas *B. subtilis* microparticles exhibited a slightly larger diameter, around 4.5 μm. This increase in size can be attributed to the increase in viscosity of the PVA solution due to the presence of the bacteria.[20] Increasing the viscosity in the spray-drying process was found to increase droplet size distribution, which resulted in larger microparticles.[21] Regardless of the bacterial presence, microparticles larger than 1 μm in diameter tend to exhibit an anisotropic buckled morphology compared with the smaller microparticles, which were more spherical (Figure 2B,C). Studies on the formation of microparticles via spray drying have revealed that buckling is caused by the emergence of particle-dense regions at the surface of the droplet.[22] As the process of solvent evaporation progresses, the capillary forces exceed the electrostatic forces, causing deformation of the shell leading to nonspherical microparticles.[23] In other words, during the drying stage of the spray drying process, the interface of each droplet becomes solid-like and, as a result, the larger microparticles can only shrink further by crumbling of the outer shell.[24]

2.2. *B. subtilis* Analysis. To achieve possible effects of the spray-drying process on the fitness of bacteria, the growth rate of PVA-encapsulated bacillus was evaluated dynamically by a spectrophotometer (Figure 3A). By way of comparison, a similar experiment was carried out for equivalent amounts of both free, nonencapsulated bacteria and empty microparticles.

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Both groups reached a plateau about 8 h from the beginning of growth. However, while free bacteria started growing within 1 h, the encapsulated bacteria showed a lag of around 7 h. This "wake-up" period can be explained by several factors:[25] (1) extra time is required for rehydration and for the polymeric matrix to release the bacteria; (2) the environmental stress exposure during the spray-dry procedure may have decreased bacterial viability,[26] and (3) bacterial cells were exposed to both thermal and dehydration inactivation during spray drying, which may have inhibited bacterial growth. This delay in growth kinetics, previously documented for other bacteria strains,[27] could potentially be addressed by decreasing particle size or modifying process parameters. We note that *Bacillus* was identified before and after this assay with 99.92% accuracy (Crystal Gram-Positive ID Kit, BD, Maryland, USA). Thus, this in vitro assay supports our hypothesis that spray-dried *B. subtilis* may be used as a form of pharmaceutical dosage without compromising the viability, sterility, and shelf life of such formulations.

2.3. Surfactin and Fengycin Extraction. The antimicrobial activity of *B. subtilis* is based on the bacteria's ability to produce and secrete antimicrobial agents.[28] We therefore measured the release of surfactin and fengycin from free and from encapsulated *B. subtilis* (Figure 3B) by LC/MS (Figures S1−S3, Supporting Information). Surfactin production by *B. subtilis* started after 4 h, reaching a maximum of 350 μg/mL in 12 h. Fengycin production began after about 6 h, and its concentration reached a maximum of about 80 μg/mL in 12 h. Release of surfactin and fengycin from encapsulated *B. subtilis* showed comparable patterns with a 6 h delay, mirroring the delayed growth curve of the bacteria (Figure 3C). This manner in which surfactin is secreted before fengycin was attributed to the hemolytic and biofilm activities of the former.[29] In this regard, it is noteworthy that 200 μg/mL was found to be the minimum inhibitory concentration (MIC) for surfactin against *S. aureus*, while 250 μg/mL fully inhibited its growth.[30]

2.4. In Vitro Study. The antibacterial activity of *B. subtilis* microparticles against MRSA and *S. aureus* was examined using the disk-diffusion method. A lawn of each bacterium (100 μL of 10^8 CFU/mL) was spread on lysogeny broth (LB) agar Petri dishes using sterile beads followed by placement of microparticles (1 mg) at the center of each dish. After 48 h at 37 °C, the radius of inhibition was measured and compared with those of empty PVA microparticles (Figure 4A and Figure S4). The bacillus microparticles demonstrated antibacterial activity against both MRSA and *S. aureus* (inhibition zone diameters were 11 and 12 mm, respectively, $p < 0.01$), while empty PVA microparticles did not exhibit any activity. The activity of *B. subtilis* microparticles can be attributed, at least partially, to the secretion of a wide range of antibacterial molecules including surfactin and fengycin.[31] Similar experiments using pure surfactin, with concentrations between 100 and 400 μg/mL, were successful in inhibiting *S. aureus* and *Escherichia coli* (*E. coli*).[30,32] Surfactin is known to reduce and inhibit the formation of biofilm by *S. aureus*, causing an interruption in surface adhesion.[33] *S. aureus*, a human skin pathogen that causes a diverse range of serious hospital infections worldwide, has developed significant resistance against a variety of antibiotics.[34] Thus, the concept of administrating beneficial bacteria to environmental wounds can be seen as an alternative to antibiotics, including for the elimination of pathogenic bacteria such as *S. aureus* and MRSA.

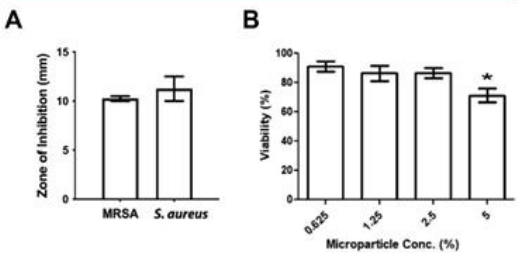

Figure 4. (A) Antibacterial activity of *B. subtilis* encapsulated in microparticles, against methicillin-resistant *S. aureus* and *S. aureus*. Empty particles showed no zone of inhibition. (B) NIH 3T3 fibroblast viability 48 h after exposure to increased PVA microparticle concentration. * = statistically significant difference from all groups. $P < 0.01$ ($n = 4$).

We next evaluated the cytotoxicity of empty PVA microparticles on skin NIH 3T3 fibroblast cells using the cell viability assay (MTS) method (Figure 4B). Cells were seeded in wells (100,000 cells/well) and exposed to empty PVA microparticles at concentrations ranging between 0.625 and 2.5%, which is way below the maximum concentration allowed for PVA in cosmetics and skincare products.[35] Results show that cell survival is very high, around 90% (compared with nonexposed cells). Nevertheless, at a PVA concentration of 5%, a 30% cell death is observed, which is in agreement with another report.[36] Our results demonstrate well the potential of loaded PVA microparticles for treating skin wounds.

2.5. In Vivo Study. Next, tissue reaction to *B. subtilis* microparticles was determined using an open wound model in C57BL mice.[37] A single open wound was created by removing the skin (1×1 cm^2) in the dorsal region of mice. Wounds were administered once daily with PVA microparticles, with or without *B. subtilis*. As a way of comparison, the dorsal region of mice of the same age and weight was also removed but left untreated. Lesion dimensions were measured, and wound area was documented daily by ImageJ. Mice were sacrificed after 5 or 15 days, and tissue from the injured area was harvested for histological analysis. Healing was evaluated by daily measuring wound size (Figure 5A,B) and by the toxic effect of the treatment as evaluated by histology (Figure 5C).

PVA microparticles (with and without *B. subtilis*) immediately attached themselves to the surface of the wound upon administration, remaining there until fully dissolved. Three days after injury, significant differences in wound closure were noticed between the two PVA-treated groups and the untreated group, with the former showing a reduction of 25% in wound size compared with the untreated group, which exhibited no difference or a slight increase in wound size. After 6 days, the wound area of the untreated group was around 95% of its initial size, compared with around 50% for the treated groups. Furthermore, the untreated wound was rougher and darker than the treated groups. These observations were also confirmed by histology, in which 5 days post-injury, a healthy healing process was evidenced by the formation of a crust and by the appearance of granulation tissue (marked by green arrows). This healthy process was verified when fresh epidermis tissue was created after 15 days.[38,39] After 12 days, both PVA groups showed complete re-epithelization and hair growth, compared with the untreated group, which required 15 days to heal.

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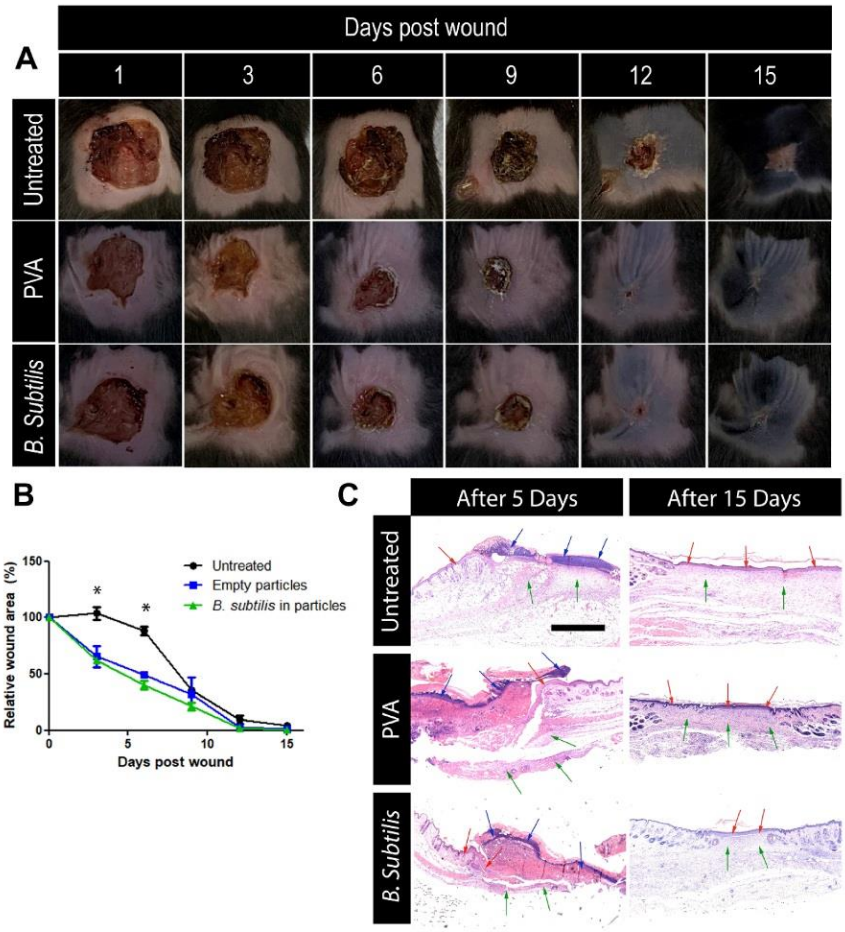

Figure 5. Wound closure in C57BL mice: (A) Representative photos showing the time course of wound healing. (B) Wound closure, normalized to the original size of the wound, expressed as mean ± SD. (C) Representative images of the in vivo response of skin to microparticles with and without *B. subtilis* after 5 and 15 days and the untreated group (scale bar = 1 mm). Red arrow - newly regenerated epidermal coverage; blue arrows - crust coverage of the wound site; green arrows - granulation tissue within the dermis and subcutis. *$P < 0.05$ versus empty and *B. subtilis* particles.

The incorporation of *B. subtilis* into PVA microparticles, which demonstrated excellent in vitro performance, was relatively limited in the case of the in vivo healing process. A nonsignificant bacterial effect was noticed only during the first 9 days of the experiment, after which there was no apparent influence on the healing process. This discrepancy may be attributed to crust formation observed over the wound area 5 days after the beginning of the experiment (Figure 5C, marked by blue arrows). It is likely that the crust prevented bacteria and bacterial peptides from reaching the wounded area, thus limiting their efficacy to the first days of treatment. Further experimental studies are needed, however, to ascertain whether crust formation is indeed involved. Nevertheless, regardless of the bacterial presence, both PVA microparticle groups exhibited a faster healing rate than the untreated group, which is a prerequisite for decreasing the risk for early contamination.[40]

The histology data indicates no toxic effect related to PVA microparticles or to the *B. subtilis* incorporated in them. Data comparison between the three test groups indicates the presence of comparable changes, in both nature and severity. Moreover, the presence of *B. subtilis* did not delay healing of the induced wound, compared with the data evaluated at the 5 and 15 day time points. It can therefore be concluded that under the present experimental conditions, the use of *B. subtilis* incorporated into PVA microparticles in the wound healing model in mice is not associated with any adverse effects[41] and should be considered as safe.

3. MATERIALS AND METHODS

3.1. Materials. PVA (M_w = 13,000−23,000 g/mol, 99% hydrolysis), glucose, surfactin, and fengycin were purchased from Sigma Aldrich (Sigma Chemicals, St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco-Invitrogen Corp.

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(Grand Island, NY). Also used were Bacto Tryptone, Bacto Yeast extract, Bacto Agar, Bacto peptone (Becton Dickenson, NJ, USA), NaCl (Bio-Lab Chemicals, Jerusalem), $MgSO_4$ (Spectrum Chemical, CA, USA), KH_2PO_4 (Riedel-de Haën, Munich), HCl 37% (Daejung Chemicals, Korea), and acetonitrile (J.T. Baker, NJ, USA). *B. subtilis* 3610 was generously provided by Prof. Ilana Kolodkin-Gal's lab,[42] Weizmann Institute of Science, Israel. Bacterial cultures were American Type Culture Collection (ATCC) strains: *S. aureus* 25923 and methicillin-resistant 43300.

3.2. Microparticle Formation and Characterization. PVA microparticles containing *B. subtilis* were prepared using a Mini Spray Dryer B290 Advanced (Ëchi, Switzerland). PVA/bacterial solutions were prepared by suspending harvested bacteria pellets (2×10^9 CFU/mL) in sterile 2.5% aqueous PVA solution (80 mL). *B. subtilis* was incubated overnight at 37 °C and 200 rpm. The bacterial suspension was connected to the designated spray drier's pump, which was adjusted to deliver 2.5 mL/min at an air flow of 600 L/h, with inlet and outlet temperatures set to 110 and 45 °C, respectively. Dried microparticles were collected from the cyclone and stored in a sterile Falcon tube at 4 °C. Control microparticles were also fabricated under similar conditions without bacteria. Microparticle morphology was characterized by SEM (SEM, Zeiss LEO 1530, Jena). Before imaging, samples were coated with 2.0 nm platinum using a sputter coater (208 HR Cressington, UK). The effect of the *B. subtilis* presence on microparticle size was measured using DLS (Malvern Panalytical, Cambridge, UK).

3.3. *B. subtilis* Analysis. Bacterial isolates from microparticles were plated on LB agar Petri dishes and incubated for 24 h at 37 °C. A single colony was then transferred to a tube of inoculum fluid supplied with the BBL Crystal kit (Gram-Positive ID Kit-BD, Maryland, USA), and identifications were carried out according to the manufacturer's instructions. To evaluate the concentration of *B. subtilis* in PVA microparticles, microparticles (2 mg) were suspended in 100 μL of LB medium. The suspension was plated on LB agar Petri dishes (n = 4) and incubated for 24 h, and colonies were counted using ImageJ software (NIH, Maryland, USA). As a way of comparison, control microparticles without bacteria were also evaluated. The possible effect of spray drying on the viability of *B. subtilis* was assessed by comparing the growth kinetics of encapsulated bacteria with those of a similar amount of free, nonencapsulated bacteria. Microparticles and free bacteria were cultured at 37 °C, and growth curves were dynamically monitored by a spectrophotometer (Synergy H1 Plate Reader Biotech Instruments Inc., Winooski, VT, USA) at λ = 600 nm over 24 h.

3.4. Surfactin and Fengycin Extraction. The antimicrobial properties of *B. subtilis* are based on the ability of the bacteria to produce and secrete antimicrobial agents. The release of antibacterial molecules by *B. subtilis* in microparticles was, therefore, monitored using encapsulated bacteria and was compared with that of free bacteria. Bacteria was diluted in 250 mL of an extract medium containing 20 g of glucose, 30 g of peptone, 7 g of yeast extract, 1.9 g of KH_2PO_4, and 0.45 g of $MgSO_4$ in 1 L of DDW. The bacterial medium was incubated at 37 °C at 200 rpm. The bacterial medium (50 mL) was centrifuged at 8000g, at 4 °C for 10 min. The supernatant was adjusted to pH 2 with 6 M HCl, stored at 4 °C overnight, and centrifuged at 11,000g, for 20 min at 4 °C. The pellet was redissolved in DDW and lyophilized, and samples were redissolved in acetonitrile/DDW (80:20) and analyzed by LC/MS. Analysis was performed on a Waters UPLC H-class system equipped with a Waters Acquity C18 column (50 × 2.1 mm, 2.6 μm particle; injection volume 7 μL). The mobile phase consisted of solvent A (DDW containing 0.1% trifluoroacetic acid) and solvent B (acetonitrile containing 0.1% trifluoroacetic acid). The following linear gradient elution was used: 50% A at 0 min, decreased to 3% A from 0 to 10 min, held at 3% A from 10 to 12 min, then increased to 50% A from 12 to 12.5 min, and further held at 50% A until 17 min. The flow rate was set at 0.4 mL/min, and effluent absorbance was monitored at 210 nm. Mass spectrometry was performed using a Waters Xevo G2 QTof system operating in electrospray ionization positive-ion mode. Cone and probe capillary voltages were 45 V and 3.0 kV, respectively. Source and desolvation temperatures were 120 and 400 °C (desolvation gas flow (N_2), 800 L/h, cone gas flow, 1 L/h). Results were compared with those obtained from commercial surfactin and fengycin.

3.5. In Vitro Study. The antibacterial activity of the PVA microparticles was determined against *S. aureus* and MRSA using the disk diffusion method.[43] In brief, a lawn of each bacterium (100 μL of 10^8 CFU/mL) was spread on LB agar Petri dishes using sterile beads. Microparticles (1 mg) were placed at the center of each dish using a sterile spatula. Petri dishes were incubated for 48 h at 37 °C, and the radius of inhibition was measured. As a way of comparison, the activity of empty microparticles was also measured using the same method.

Microparticle cytotoxicity was evaluated by exposing NIH 3T3 fibroblast cell lines to increased concentrations of PVA microparticles. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cultures were maintained at 37 °C in a 95% air/5% carbon dioxide atmosphere, at 95% relative humidity. Cytotoxicity was assessed after 24 h by the MTS assay using CellTiter 96 solution according to the manufacturer's instructions. Results are expressed relative to unexposed cells (n = 4).

3.6. In Vivo Study. Animals were cared for in compliance with protocols approved by the Council for Animal Experiments, Israel Ministry of Health, in conformity with the Animal Welfare law guidelines (published in 1994). A total of 21 eight-week-old C57BL mice (Envigo, Jerusalem, Israel) were anesthetized with 1% isoflurane (Piramal Critical Care, Inc. PA, USA) in oxygen. The dorsal region was removed, creating an open wound (1×1 cm^2). Mice were randomly assigned to one of the following three groups: empty PVA microparticles, PVA microparticles containing *B. subtilis*, and a control group that did not receive any treatment ("no treatment"). Each treatment was administered daily for the entire period of the experiment, and lesion dimensions were measured using ImageJ software (NIH, Maryland, USA). Mice were sacrificed after 5 or 15 days, and dorsal skin samples were harvested and kept in 10% formalin for histology analysis. Skin samples were stained with hematoxylin and eosin and Masson's trichrome for histology evaluation, which was performed by a board-certified toxicologic pathologist (AN).

3.7. Statistical Analysis. Results of surfactant extraction, inhibition rates, cytotoxicity MTS assays, and in vivo studies are presented as mean values ± SD. Statistical comparisons were performed using Prism 5, GraphPad (San Diego, CA). The t test was used to analyze the significance of the

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differences between the treated groups; p values <0.01 were considered to reflect statistical significance (n = 4).

4. CONCLUSIONS

The pharmacological activity of PVA microparticles was successfully demonstrated in an open wound model. Microencapsulation techniques for live bacteria include extrusion, emulsification, freeze-drying, and spray drying.[44] The delivery system described here, which is based on spray drying, was chosen based on its advantages over the other techniques: microspheres formed by extrusion tend to be less stable and do not lend themselves to large-scale production.[45] Emulsification involves organic solvents, often toxic to the bacteria; although freeze drying is considered to be very efficient, it is, however, limited by very high production costs.[46] Thus, spray drying is the most widely used technique for microencapsulation of live bacteria, being relatively simple and cost effective, and amenable to large-scale production. Microparticles were produced by one simple step that is amenable to large-scale production. Wounds treated with PVA microparticles were closed and healed after 12 days, compared with 15 days for the nontreated groups. There was no evidence of skin irritation, infection, or other adverse effects during the 15 day postoperative observation period. In vitro, the bacillus microparticles demonstrated remarkable antibacterial activity against MRSA and *S. aureus*, but in this study, the bacterial effect was limited to the first days of the in vivo study, probably due to crust formation on the wound surface. Nevertheless, it was shown that the use of live bacterial formulations has the potential to continuously produce antimicrobial agents and inhibit the growth of pathogenic bacteria. Since *B. subtilis* peptides were proven to be therapeutic agents for open wounds and skin disorders,[47,48] a live bacterial delivery approach that continuously secrete antimicrobial peptides could open the door to a new class of delivery systems for biological molecules, with potential in many acute and chronic skin diseases. PVA microparticles may be administered as compact (pressed) powder using a fluffy sponge or a brush, dispersed in liquid oils (e.g., paraffin or vegetable oil), or applied using a powder blower or microneedles.[49]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c00790.

LC/MS data and standard curves of surfactin and fengycin and images of inhibition zones of *B. subtilis* particles against *S. aureus* and MRSA (PDF)

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Notes

The authors declare no competing financial interest.

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9. Outlook

As it is shown in this dissertation by use of a number of examples, encapsulated bacteria in polymer matrices are attracting a growing interest in research as well as in a wide range of applications. In recent years, considerable progress has been made in understanding the effect of polymeric supports on encapsulation. Nevertheless, several questions remain to which there is no answer and which require further and more precise studies.

The application of bacteria/polymer composites is highly considerable in wastewater treatment. Besides that, the bacteria/polymer composites open a broad field of possible applications in the field of biomedicine, as shown in this thesis. Such a system could be used for wound care and by controlled release of antibiotics into open skin lesions.

In this case, PVA as a water soluble polymer provides a bacterial-friendly environment. The cross-linked PVA particles allow the proliferation of the encapsulated bacteria by swelling in water and by the resulted elasticity. To protect particles containing encapsulated bacteria, two methods were carried out in this thesis: the first one is based on the surface modification of particles, and surface polymerization of a hydrophobic shell. The second approach is based on embedding the particles in another polymer matrix.

For the first approach, surface polymerization of DEAEMA monomers was carried out. The resulted PDEAEMA shell could play an important role in inhibiting the unwanted release of bacteria from PVA particles as they can show a significant antibacterial impact. Moreover, the change in pH alters the polymer morphology from open-shell structure to aggregates. The pH-responsivity of the substituents seems to be the main factor influencing the formation of open-shell structure, which allows mass transport through the PVA core.

To protect bacteria/PVA particles, a nonwoven carrier of PLA nanofibers was also used. The produced porous membrane provides a better manual use for large-scale applications, and

especially in an aqueous environment. The porous membrane is also environmentally friendly, due to the compatibility of both PVA and PLA polymers.

10. Conference's participation

2017 German-Israel-Palestine trilateral cooperation (GIP) meeting, Rhodes Greek (presentation)

2017 Bayreuther Polymer Symposium, Bayreuth

2018 Macromolecular Colloquium Freiburg

2019 Bayreuther Polymer Symposium

2019 SFB, Bayreuth (poster presentation)

2019 Leadership Seminar, Wirsberg

2020 Macromolecular Colloquium Freiburg

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12. (Eidesstattliche) Versicherung und Erklärungen

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Hiermit versichere ich eidesstattlich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe (vgl. Art. 64 Abs. 1 Satz 6BayHSchG).

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