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Faculty of Chemical Technology

Preparation of scaffold systems for cell cultivation using cryogenic grinding of thin layers of polymeric fibers

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ZADÁNÍ DIPLOMOVÉ PRÁCE

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Jméno a příjmení:	**Barbora Křížková**
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Název tématu:	**Příprava nosičových systémů pro růst buněk pomocí kryogenního mletí tenkých vrstev polymerních vláken**
Zadávající katedra:	**Katedra biologických a biochemických věd**

Zásady pro vypracování:

1. Vypracujte literární rešerši zaměřenou na problematiku možného využití polymerních mikrosfér s porézní strukturou jako nosičových systémů pro růst buněk. Popište základní parametry ovlivňující růst jednotlivých typů buněk na polymerních nosičích. Popište způsoby přípravy jednotlivých typů polymerních nosičových systémů. Zhodnoťte možnosti využití kryogenního mletí jako techniky přípravy porézních mikrosfér z tenkých vrstev polymerních vláken.
2. Navrhněte takový způsob kryogenního mletí tenkých vrstev polymerních vláken z PCL, který umožní získat mikrosféry s co nejužší distribucí velikostí částic a zachovanou porézní strukturou. Popište vliv jednotlivých parametrů kryogenního mletí (počet a délka mlecích cyklů, doba mezi mlecími cykly, doba mražení vzorku před započetím mletí, navážka materiálu, použitá mlecí kapalina, doba a způsob skladování mletého vzorku) na kvalitu výsledného produktu. Použijte navržený proces pro přípravu mikrosfér z kompozitních vláken PCL/PVA a popište rozdíly v chování obou materiálů v průběhu kryogenního mletí.
3. S využitím vhodných buněčných kultur proveďte kultivační in-vitro studii k ověření možnosti využití získaných materiálů pro potřeby tkáňového inženýrství. Diskutujte vliv získaných nosičových systémů na životnost a proliferaci použitých buněčných kultur

Rozsah grafických prací:

Rozsah pracovní zprávy:

Forma zpracování diplomové práce: **tištěná**

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V Pardubicích dne 23. února 2011

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Annotation

The work was focused on cryogenic grinding of electrospun poly-ε-caprolactone nano/micro-mesh submerged in liquid media to prepare a microsystem applicable as a scaffold for cell growth. Optimization of the grinding process involved examination of various parameters influencing the quality of the ground product. Characterisation and biocompatibility evaluation provided evidence of the applicability of the system in cell scaffolding.

Key words

Cryogenic grinding; poly-ε-caprolactone; nano/micro-mesh; microsystem; characterization

Název

Příprava nosičových systémů pro růst buněk pomocí kryogenního mletí tenkých vrstev polymerních vláken

Anotace

Tato práce byla zaměřena na kryogenní mletí tenkých vláknitých vrstev poly-ε-kaprolaktonu ponořených do kapalného média. Cílem práce bylo vytvoření mikrosystému využitelného jako nosič pro růst buněk. Optimalizace procesu kryogenního mletí zahrnovala prostudování různých parametrů, které mohou ovlivnit kvalitu výsledného produktu. Vytvořený systém je možné využít jako nosiče pro růst buněk, což bylo ověřeno charakterizací a posouzením biokompatibility daného produktu.

Klíčová slova

Kryogenní mletí, poly-ε-kaprolakton, tenké vrstvy polymerních vláken, mikrosystém, charakterizace

Acknowledgements

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List of abbreviations

RGD	arginine-glycine-aspartic acid
PCL	poly-ε-caprolactone
PLGA	poly(lactic-co-glycolic acid)
PLA	poly- lactic acid
PGA	polyglycolide
PDLA	poly D, L-lactide
PVA	poly(vinyl alcohol)
MSCs	mesenchymal stem cells
LN2	liquid nitrogen
SEM	scanning electron microscopy
AFM	atomic force microscopy
HOPG	highly ordered pyrolytic graphite
FBS	fetal bovine serum
DiOC6	3, 3'-Diethyloxacarbocyanine iodide
PBS	phosphate buffered saline
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
TRIS	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid

Summary

This study aimed to demonstrate a novel approach to pulverization of thin layers of polymeric fibers which employed cryogenic grinding of a poly-ε-caprolactone (PCL) submerged in a liquid medium. The goal was to produce a microsystem exploitable as a scaffold for cell growth with potential applications in tissue engineering. The electrospun PCL nano/micro-mesh submerged in a liquid was pulverized at the temperature of liquid nitrogen. The products of the grinding process were subsequently characterized mainly by laser diffraction and scanning electron microscopy (SEM). The examination revealed the presence of six particle types differing in morphology and size (fibrous particles, fibrous fragments, aggregates with and without an internal fibrous structure, lamellae and nanoparticles). The influence of the parameters of cryogenic grinding (type of the grinding solvent, sample storage conditions, weight of sample and different types of grinding) on the produced suspension was investigated with the goal of maximizing yield of the desired particle types, namely fibrous particles, fibrous fragments and agglomerates with an internal fibrous structure.

The applicability of the system as a cell growth support was evaluated by a cell cultivation study using mouse 3T3 fibroblasts. Cell growth and proliferation on the PCL microparticles established their potential as a cell scaffolding material.

This multidisciplinary approach opens a new potential research path in tissue engineering. The experiments presented in this work provide preliminary results. And additional studies have been outlined to optimize the properties of this scaffolding system.

Souhrn

Tato práce je zaměřena na prezentaci nového přístupu k rozmělňování tenkých vrstev polymerních vláken, jenž spočívá v kryogenním mletí vláknitých vrstev poly-ε-kaprolaktonu ponořených do kapalného média. Cílem bylo vytvoření mikrosystému, kterého by se dalo využít jako nosiče pro růst buněk s možnou aplikací v tkáňovém inženýrství. Tenké vrstvy poly-ε-kaprolaktonu, vytvořené elektrostatickým zvlákňováním, se ponořily do kapalného média a namlely při teplotě kapalného dusíku. Produkty mlecího procesu byly charakterizovány především za využití laserové difrakce a skenovací elektronové mikroskopie (SEM). Analýza odhalila přítomnost šesti typů částic, které se lišily morfologií a velikostí, jednalo se o vláknité částice, vláknité fragmenty, aglomeráty s vnitřní vláknitou strukturou a bez ní, lamely a nanočástice. Abychom maximalizovali výtěžek požadovaných částic, konkrétně vláknitých částic, vláknitých fragmentů a aglomerátů s vnitřní strukturou, musel být prostudován vliv jednotlivých parametrů kryogenního mletí na výsledný produkt. Mezi prostudované parametry patřily typ mlecí kapaliny, uchovávání vzorku, navážka vzorku a různé typy mletí.

Pro ověření, zda je možné využít získaný materiál jako nosičový systém pro růst buněk, byla provedena kultivační *in vitro* studie na myších fibroblastech 3T3. Proliferace buněk a jejich růst na mikročásticích PCL potvrdily, že se materiál jeví jako vhodný pro zamýšlené použití.

Multidisciplinární přístup prezentovaný v této diplomové práci otevřel novou cestu výzkumu v oblasti tkáňového inženýrství. Na základě uvedených výsledků byly navrženy další postupy, aby bylo možné vlastnosti potenciálního nosičového systému dále optimalizovat.

1. Introduction

1.1. Scaffolds in tissue engineering

Recent development of scaffolding systems for tissue engineering offers an attractive strategy to cure various defects. Scaffolding allows efficient localization of cells within the site of injury and controls the delivery of bioactive substances promoting the healing process. Tissue engineering is an interdisciplinary field that combines the use of cells, engineered support materials (scaffolds) and bioactive molecules. The major objective is the repair or replacement of tissues or organs [1]. An ideal scaffold should support the tissue regeneration by guiding cell growth and by controlling local microenvironment to favor desired cell proliferation [2].

The biological potential of a particular microsystem depends on multiple physical and chemical parameters. Microsystem morphology is important for cell attachment and survival. Highly porous structures with interconnected pores facilitate exchange of nutrients and waste products and promote higher cell density within the scaffold [3]. Mechanical properties of the scaffold should match those of the target tissue [1]. In addition to providing physical support to the target tissue, scaffolds may be loaded with growth factors that enhance tissue regeneration. Growth factors are involved in cell-cell signaling as well as in signal transduction between cells and extracellular matrix. They activate pathways of proliferation control, as well as the differentiation and migration of cells. Since growth factors are very potent signaling molecules, controlled release is important to maintain therapeutic concentration over the time period depending on the target tissue, and at the same time preventing side effects that may result from excessive dosage [4, 5].

1.1.1. Microcarrier based scaffolds

Different types of 3D structures including hydrogels, fibers, meshes, films, and sponges have been shown to support tissue regeneration. Recently microparticle based scaffold formulations have been widely studied [6]. Microsystem scaffolds have been widely utilized in the treatment of defective tissues, including bone [7], cartilage [8] and muscles [9]. Microspheres were utilized to deliver bioactive molecules (e.g. growth factors [10, 11], poly-L-lysine [12], DNA [13] and drugs [14]) and were combined with various scaffolding systems including hydrogels [8, 15], ceramics [16], titanium implants [17] and polymeric foams [18].

Microparticles have been extensively studied due to their potential in sustained delivery of growth factors as well as other drugs. Another advantage of microcarrier systems is that they are injectable thus minimizing the invasive routes of administration [19]. However, the control of release kinetics of growth factors from microspheres is a major challenge. While the control of release of drugs adsorbed or incorporated on the carrier surface is limited, embedding growth factors into the carrier seems to be more promising. The main benefit of this approach is the fact that the release of the active substance can be controlled through degradation rate of the scaffold itself [20].

Apart from drug delivery, microspheres have been explored in cell cultivation prior to implantation. Since cells adhere to and grow on the microparticle surface, trypsinization, which may cause damage to the cells, can be avoided, and the expansion of the culture can be achieved simply by adding fresh microspheres. Cell-attached microcarriers can then be used as injectable cell delivery systems [21]. The cells growing on the microcarrier surface are more likely to be washed off by shear stress compared to cells within the protective porous structure of microspheres [22].

Cell adhesion and proliferation can be enhanced by chemical surface modifications with bioactive molecules such as hydroxyapatite which promotes bone tissue formation [3]. Another frequently studied surface modification is the immobilization of arginine-glycine-aspartic acid (RGD) peptides onto the surface of microparticles which enhances cell adhesion via integrin – fibronectin interaction [22].

Of special note, composite scaffolds from microspheres and hydrogels can be effectively applied both as injectable drug delivery systems and also for the delivery of living cells [23]. Hydrogels are fibrous compact structures that swell upon exposure to aqueous medium. After swelling, the pore network permits transport and release of the entrapped drug. Such formulations are mainly designed for drug delivery activated by temperature, pH, glucose concentration, electric signal, pressure, light, ionic strength, antigen presence, etc. [24]. Hydrogels have also been studied as cell delivery vehicles. The properties of hydrogels allow homogenous cell dispersion within the matrix that mimics the environment of the target tissue. However, cell adhesion and growth support is not achieved within such systems. To overcome this limitation, hydrogels were combined with microcarriers to produce a novel system providing the advantages of both. Microcarriers provide the support for cell adhesion and growth, and the hydrogel ensures the immobilization of the cells within the target site [15].

1.1.2. Materials used in scaffold production

Several criteria need to be considered when selecting a suitable material for tissue engineering applications. Among the most important are biocompatibility, biodegradability, and bioresorbability. Biocompatibility is assessed by studying host responses, such as immunogenicity, carcinogenicity or thrombogenicity. Biodegradation means that the material is metabolized and the by-products are removed from the original site, but not necessarily cleared from the body. In contrast, bioresorbable polymers are eliminated completely, including all degradation by-products, with no residual side effects. Scaffolds need to be degradable so that they can be replaced by the newly formed tissue. Synchronization of the scaffold degradation with tissue growth is a major challenge in this field [1]. A great number of materials have been studied as scaffolds for tissue engineering. There has been a growing interest in natural polymers such as alginate [21, 25], chitosan [26] and collagen [10] because of their low toxicity, low manufacturing and disposal costs, and their potential biological function in cell signaling, adhesion, etc. [27]. Unfortunately, the natural polymers usually do not possess the physical and chemical properties, and the stability desired for scaffold formulations. Synthetic polymers for example poly-ε-caprolactone (PCL) [28, 29] or poly(lactic-co-glycolic acid) (PLGA) [30, 31], provide suitable physical and chemical properties, predictable and reproducible degradation, and can be modified for specific applications [27]. Drawbacks include potential toxicity, immunogenicity, and often an inability to promote desired biological responses. Composite materials can be designed to optimize the properties of the scaffold by combining advantages of different materials [27].

1.1.3. Poly-ε-caprolactone (PCL)

Poly-ε-caprolactone is a widely studied synthetic polymer that has shown a great potential development of drug delivery systems due to its hydrolytic degradability in biological conditions, high permeability to many drugs, and biocompatibility in physiological environments [32-34]. Other advantages of PCL over other commonly used polymers such as PLA (poly lactic acid) include its stability under ambient conditions, its widespread availability and lower price [2]. Thanks to its slow degradation, compared to other polymers, PCL is a suitable building block for long-term drug release systems [34]. The degradation rate of PCL is significantly slower than other polymers such as polyglycolide (PGA) or poly D, L-lactide (PDLA). This property makes PCL suitable for formulations that are required to remain active for time periods longer than 1 year. The degradation of PCL may extend over 2 -4 years. The can be altered by copolymerization with faster degrading polymers. PCL is degraded by a two-step process: initially, the ester groups are non-enzymatically hydrolyzed, followed by intracellular degradation of the polymer (as shown in Figure 1 [1]). Another important property of PCL is its bioresorbability. While biodegradability means that the polymer is degraded and disperses, bioresorbable polymers and their degradation products are eliminated completely from the body with no residual side effects [1].

The wide range of therapeutic substances that have been encapsulated on PCL drug delivery systems includes anticancer agents, diabetes drugs, growth factors, contraceptives, antifungal and antimicrobial agents [24, 33]. PCL has also been used in medical applications apart from drug delivery systems including suture materials, wound dressings, contraceptive devices, filling materials in dentistry. PCL has also played an important role in tissue engineering research [1].

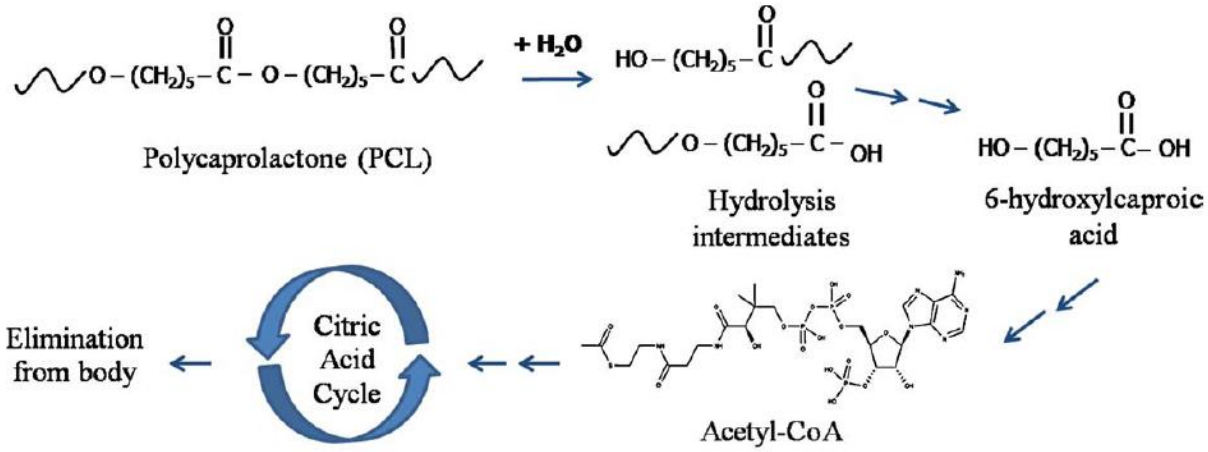

Figure 1. *Schematic diagram of hydrolytic degradation of PCL and its subsequent elimination from the body through citric the acid cycle.*

1.1.4. Poly(vinyl alcohol) (PVA)

PVA is a polymer known for its biologically friendly properties. It is biodegradable (degradation routes are shown in Figure 2 [35]), nontoxic, non-carcinogenic, and is compatible with tissue and blood. Another advantageous property of PVA is its thermal stability [36]. Mechanical properties of PVA such as flexibility and swelling capacity have proved beneficial in development of wound dressings. The use of this polymer is however limited by its instability in aqueous systems. This can be overcome by copolymerizing with another polymer or cross-linking [37]. The cross-linking of PVA can be achieved by physical or chemical methods. However, chemical procedures may alter the chemical structure resulting in loss of the desired properties, and physical cross-linking, e.g. by heat treatment, does not offer long-lasting products. Protection of PVA from the aqueous environment can also be provided by coating PVA fibers with another polymer, e.g. chitosan [38]. Linh et al. produced PVA/gelatin nanofiber composites with potential utility in bone repair and drug delivery systems [36].

The hydrophilic character of PVA allows the preparation of delivery systems from aqueous solutions and therefore promises opportunities for the delivery of sensitive hydrophilic substances [39]. On the other hand, this hydophobicity results in lower cell adhesion to PVA scaffolds, which is why this polymer has not been widely studied in tissue engineering research. The hydrophilic properties can be compensated by production of composite materials of PVA including other, more hydrophobic, polymers such as PCL [40].

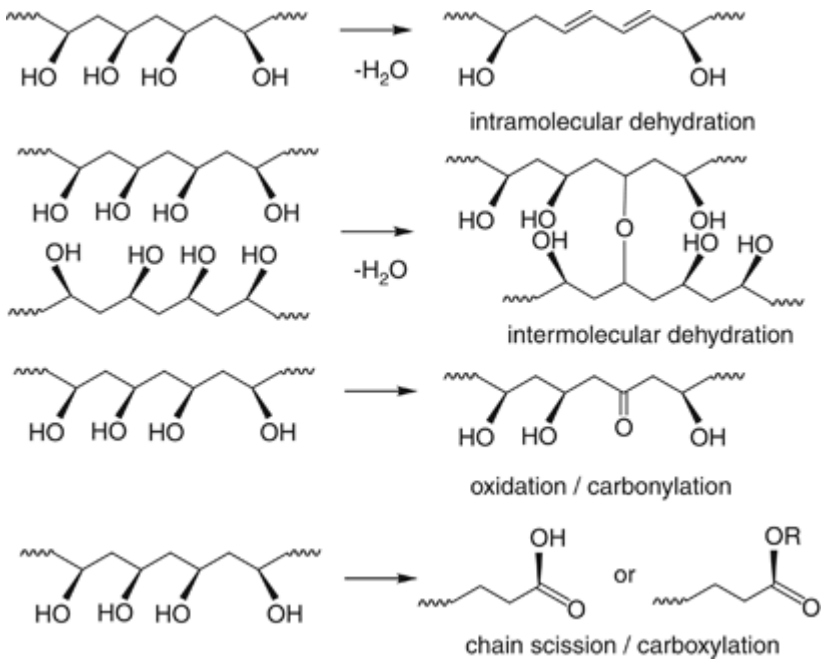

Figure 2. *Degradation routes of PVA proposed by Akhter et. al. [35]*

1.2.Electrospinning

1.2.1. Basic principles

For the preparation of nano/micro-mesh, electrospinning is one of the most commonly utilized techniques [41, 42]. This method is highly versatile since a wide range of polymers, from synthetic to natural, can be spun [42, 43]. In electrospinning, electrostatic forces are utilized to generate fibers from polymer solutions or melts [41, 44]. The fiber diameter ranges between several micrometers to tens of nanometers. In a typical electrospinning setup, liquid is forced through a capillary or needle connected to a high voltage supply. Increase in the electric field strength causes the Taylor cone formation, and upon further increase the self-organization of fiber jets from the cone apex. As the jets travel towards the grounded collector, they undergo bending instability, which lengthens the path to the collector (as shown in Fig. 3 [41]). This results in solvent evaporation and fiber thinning [44]. The phenomenon of self-organization of liquid jets explains why fibers thinner than the capillaries can be produced. Electrospinning can also be applied to either polymer melts or polymer solutions. The latter can also be set up as ‘co-axial’ or ‘core-shell’ spinning utilizing two different polymer solutions to produce composite fibers [41].

Several techniques have been employed to load bioactive agents into electrospun scaffolds. The simplest way is to immerse the electrospun fibers in the solution of desired substance. Although this method does not cause any interference with the active substance, no control of the release profile is available. A more complex technique involves covalent bonding of the bioactive agent to fabricated fibers and provides the possibility of release control. It is, however, a technically challenging method which requires optimization before it can be routinely used. Blend electrospinning, where a solution of the polymer is mixed with the bioactive agent and subsequently spun to

form hybrid fibers, also promises sustained release of biomolecules but the activity of sensitive proteins might be disturbed during the process. In co-axial electrospinning, the polymer solution is spun simultaneously with the biomolecule solution to produce fibers with core-shell structure. Thus active molecules are protected by the polymer shell and their release can be sustained for longer periods of time. A drawback of the technique is its complexity. Some interference with the activity of sensitive molecules has been observed, probably due to high voltage exposure [45].

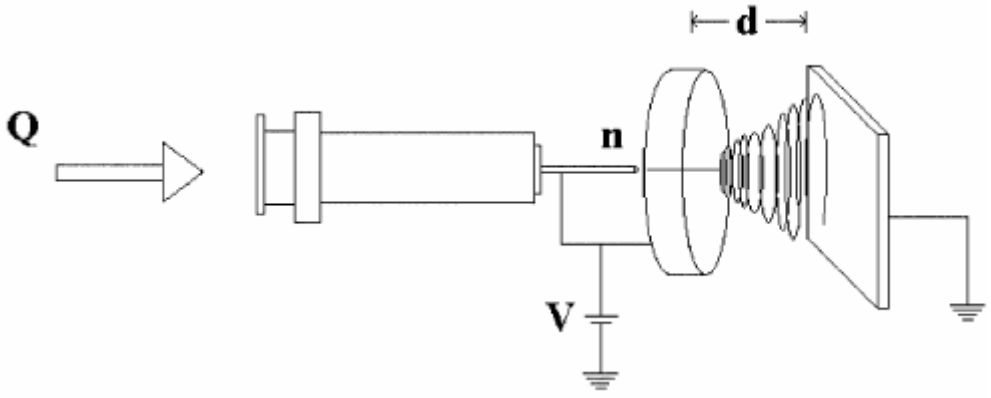

Figure 3. *Schematic representation of an electrospinning setup showing the solution supply with the flow rate (Q), distance between the needle/capillary tip and the collector (d), applied voltage (V) and the needle gauge (n).*

1.2.2. Parameters influencing the fiber formation

Since electrospinning is a complex process a number of variables can significantly affect the quality of produced fibers [40-42, 44]. The parameters influencing the process of electrospinning can generally be divided in three groups: (1) environmental factors, (2) properties of the polymer solution and (3) parameters of the electrospinning device. Environmental factors include ambient conditions such as humidity, temperature, air flow, etc. There are a number of properties of the polymer solution that affect fiber formation. Among these are viscosity of the solution, polymer concentration, conductivity, solvent volatility, surface tension, and additives. Parameters and settings of the electrospinning device also have significant effects on fiber production. These include types of electrodes and the collector, electric field strength, flow rate and the distance between the capillary and the collector. All of the above mentioned factors need to be balanced in order to prepare high quality fibers. Examples of commonly encountered problems are bead defect formation, fiber diameter variation, failure to form fibers at all, and separation into two distinct fiber in the case of co-axial electrospinning [41, 42, 44] .

1.2.3. Applications of electrospinning

Electrospun nano/micro-fiber layers are characterized by enormous surface to volume ratio, high porosity and an extracellular matrix resembling the structure determining their use in broad range of applications [43, 44]. Production of highly porous 3D structures resembling extracellular matrix promises the great potential in the field of tissue engineering [43, 46]. Gupta *et al*. [46] applied co-axial electrospinning in production of bicomponent core-sheath nanofibers. Two sets of samples were prepared with a natural polymer, gelatine or collagen, forming the sheath, and PCL, a synthetic polymer, as a core. The collagen-PCL nanofibers were selected for *in vitro* degradation studies which showed that the collagen sheath degraded within the observed period while the PCL core remained stable to provide further support for cell growth. The results indicated that the use of bicomponent fibers would offer advantages over monocomponent scaffolds. Yoshimoto *et al*. [47] prepared electrospun nanofibrous PCL scaffolds which were subsequently seeded with rat mesenchymal stem cells (MSCs). Copious extracellular matrix, mineralization and formation of type I collagen were observed after four weeks of culture suggesting that PCL scaffolds have potential for bone tissue engineering. Eriksen *et al.* [48] fabricated scaffolds of electrospun non-woven meshes of PCL incorporated with tricalcium phosphate nanoparticles. Cell culture tests with mouse preosteoblast cells (MC3T3-E1) revealed that changes similar to those occurring in a bone-cartilage interface could be observed within 4 weeks of culture.

Due to the vast selection of polymers that can be spun, the method has also been widely utilized in preparation of drug delivery systems with a variety of properties and release profiles [43, 44]. The versatility of the technique and ease of modification

of product properties predispose electrospinning to success in other biomedical applications such as wound dressing, fabrication of non-woven membranes [42-44].

1.3. Gryogenic grinding

Although the bottom-up approach by chemical synthesis predominates in nano/microparticle system preparation, top-down processes, such as grinding of larger particles, are also possible. The latter concept might offer easier control of preparation parameters [49]. However, the size reduction of particles produced by mechanical grinding techniques is limited. As the particle size decreases, the increasing surface results in agglomeration of particles caused by van der Waals forces and electrostatic interactions which prevents further particle size reduction. [50]. Another problem encountered during sample pulverization is an increase in temperature which can lead to alteration of properties or even degradation of the ground material. This issue becomes more serious with decreasing particle size since temperature of smaller particles increases faster [51]. This is of special concern when working with volatile compounds and biological samples as their components, such as proteins or DNA, are often heat-sensitive and can be irreversibly denatured by high temperatures.

The problem of size reduction limit of dry grinding was overcome by milling aqueous suspensions. Wet grinding techniques have been demonstrated to produce nanoscale suspensions [49]. In some cases however, wet grinding leads to problems in handling and drying of the sample, and it does not eliminate the heat-issue.

To overcome the problems of conventional grinding techniques, pulverization at low temperatures has been widely researched. The term cryogenic grinding describes pulverization of materials at temperatures below 0°C. However much lower temperatures are usually achieved since liquid nitrogen (LN2, T = -196°C) or the solid form of CO_2 (also known as dry ice, T = -78°C) are used as cooling media [52]. Cryogenic grinding is a well established method for bio-analytical, bio-technological

and pharmaceutical purposes. For example, it was used for pulverization of biological materials and tissues prior to extraction of DNA, RNA, proteins and tissue biomarkers [53-55]. The method was also used for preparation of scaffolds for bone tissue engineering [56, 57]. Cryogenic grinding can also substantially enhance bio-availability of low-water soluble and thermally sensitive drugs. By co-grinding with pharmaceutical excipients, it is possible to obtain stable amorphous drugs with enhanced solubility originating from a low-water soluble crystalline form [58-60].

Grinding at low temperature not only overcomes the problems of the heat generation and limited particle size reduction, it is often the only option for comminution of elastic materials such as polymers. Such materials are characterized by the ability of extensive reversible stretching under ambient temperatures. This property results in the loss of impact energy due to elastic deformation. The flexibility of the molecules is suppressed at low temperatures. As the sample temperature drops below the glass transition, the material becomes brittle and the impact energy required for fracture is decreased [51].

Niwa *et al.* proposed a novel approach of cryogenic grinding in a liquid medium as they used LN2 as the grinding liquid [50]. The use of LN2 offers a number of advantages including its easy availability, relatively low technical requirements, easy handling and no adverse environmental effects [51, 61]. Moreover, LN2 is an inert medium which ensures no dissolution of sample components in the liquid and fast evaporation allows for preparation of dry powders. [51].

1.3.1. Applications of cryogenic grinding

The range of fields where cryogenic grinding has been utilized is extensive and diverse. The following section will discuss some examples of applications of cryogenic grinding. In biological sciences cryogenic grinding has been exploited in homogenization of tissue samples intended for extraction of DNA, RNA or drug residues. The extraction of nucleic acids requires the temperature below -80°C in order to prevent thermal degradation. Cryogenic grinding provides an optimal method for homogenization of plant and animal tissue as part of sample preparation for DNA or RNA extraction because during the whole process the sample is cooled well below the critical temperature. A great advantage of utilization of cryogenic grinding in tissue sample preparation is the fact that even hard samples, such as bones and teeth, or flexible tissues, such as cartilage, can be pulverized due to the brittleness induced by cooling to low temperatures [62, 63]. The use of cryogenic grinding in tissue homogenization has also been exploited in analysis of biomarkers in cancer research as well as in ecotoxicological studies [54, 62, 64]. Lolo *et al*. demonstrated the benefits of sample homogenization by cryogenic grinding in extraction of antibiotic residues from muscle tissue. The researchers showed that pulverization at low temperatures resulted in complete rupture of the cells leading the exposure of all cellular contents to the extraction solvent [65].

Grinding at cryogenic temperatures has also found a widespread application in food industry. In milling of spices cryogenic grinding prevents the loss and degradation of volatile aromatic compounds and allows for production of finer powders compared to conventional grinding [55, 66]. Fine powder production by cryogenic milling has also been exploited in flour production and sample homogenization of food samples prior to further analysis [62, 67, 68].

The use of cryogenic grinding for pulverization of polymers is of major interest in this study. As mentioned above, fine powder production from viscoelastic polymers is virtually impossible at ambient temperature therefore cryogenic grinding is the technique of choice. The polymers undergo glass-transition upon cooling and thus become brittle [57].

Návesník in his thesis hypothesized that addition of liquid, in this case water, to the sample will improve the result of the grinding. He proposed that the frozen liquid will have abrasive effects on the sample. As the sample is immersed in LN2 the expansion of frozen liquid will cause tearing of the material. Moreover, during grinding the pieces of frozen liquid will act as grinding crystals resulting in further particle size reduction. The effects of water addition were studied especially on fibrous samples such as cotton, wool, cotton twill and cellulose. The results showed that the presence of the liquid improved grinding efficiency in all cases [52].

Figure 4. *Cryogenic mill Spex Freezer/Mill model 6750*

1.3.2. Parameters affecting the process of cryogenic grinding

A number of parameters may affect the results of cryogenic grinding. The settings of the mill usually allow for programming of several parameters. Total grinding time can be split into several cycles. It is essential to optimize the grinding time within each cycle since heat is generated during milling and the temperature of the sample may rise above the glass transition point leading to unsatisfactory results. Samples should be cooled between individual cycles to maintain the brittleness. Before actual pulverization, samples need to be cooled to become brittle. When selecting the pre-cooling time, the amount of the sample and the properties of the given material need to be taken into account [52, 62]. Not only the settings of the mill, but also other parameters of the sample, affect the results of the grinding process [52, 53, 62]. It has been shown that decrease in sample load for grinding may improve the results. Less material in the vial, however, might lead to extensive wear for the equipment also resulting in increased sample contamination [52, 69]. Another factor influencing the results of grinding is the concentration of the suspension [53].

All the above mentioned parameters are relevant for SPEX Freezer/Mill 6750, however, different conditions might need to be optimized when using other instruments. For instance, Ehmer [70] used a jet mill modified for cryogenic grinding for micronization of proteins. He found that the parameters that affect the grinding process were pressure, number of milling cycles and the milling gas temperature. However all these variables differ with the properties of the compound to be ground. Therefore no general conclusions could be drawn and the process needs to be optimized for each sample individually.

1.4. Aims and objectives

The major aim of this study was to implement a multidisciplinary approach to prepare porous microspherical scaffolds for cell growth by cryogenic grinding of electrospun fibrous polymer meshes. Firstly, the process of cryogenic grinding needed to be optimized to achieve the most favorable particle size and morphology of the ground material. This involved a study of effects of various parameters such as the type of the grinding medium, sample load, different types of grinding, and sample storage conditions on the resulting suspension. Secondly, the research aimed to characterize the results of cryogenic grinding in terms of particle size and morphology. Thirdly, the biocompatibility of the particle suspension obtained by cryogenic grinding, and their applicability in tissue engineering needed to be evaluated by cell cultivation studies with mouse 3T3 fibroblasts.

2. Materials and methods

2.1.Production of polymer microparticles

2.1.1. Fabrication of poly-ε-caprolactone (PCL) nano/micro-mesh

PCL nano/micro-mesh was prepared by a needleless electrospinning method from PCL with molecular weight 40,000 (Sigma Aldrich). Electrospinning of a 14 % solution (w/v) of PCL was carried out on a Nanospider^{TM} (Elmarco, CZ) device as previously described in detail [71]. Mean diameter of fibers was calculated from SEM images using image analysis software LUCIA (Laboratory Imaging, Czech Republic).

2.1.2. Optimization of cryogenic grinding

The cryogenic impact grinder with a self-contained liquid nitrogen bath (4-5L) model 6750 Freezer Mill (Spex, Certiprep, USA) was used. The samples were placed in a polycarbonate vial (model 6751C20 - volume 25 mL) together with a metal impactor. The vial was closed with a steel stopper and inserted in the mill. Number of grinding cycles, grinding time per cycle, pre-cooling time, cooling time between grinding periods and grinding frequency were set and the sample was subsequently pulverized by the magnetically driven impactor. The grinding program was suggested on the base of previous experience with grinding of a wide range of different types of matter and was expected to offer maximum disintegration effect. Pre-cooling time was set to 5 minutes and the applied impact bar frequency was 12 Hz in all experiments. The total grinding time of three minutes was split into three one-minute cycles with one minute of re-cooling between individual cycles. After grinding the samples were allowed to thaw and were transferred into a plastic test tube and stored either at room temperature or at - 20 °C for further work.

In a preliminary experiment dry cryogenic grinding of PCL nano/micro-mesh was tested (results not shown). The PCL layer was cut into 5×5 mm pieces, placed into a vial, and cryogenically ground. The properties and small amount of the sample in the grinding vial were possible reasons for quite unsatisfactory results of these initial conditions. The fibrous mesh was compressed rather than ground.

To improve the grinding efficiency, the technique of cryogenic grinding of the fibrous mesh submerged in a liquid medium was employed. 3 ml of the liquid were added to the polymer sample in the vial which was subsequently immersed in an LN2 filled bath for approx. four minutes to achieve sufficient cooling of the sample. Then a metal impactor was placed in the vial. The objective of this cooling step was to prevent trapping of the impactor by freezing. Addition of the solvent significantly increased the sample volume. Typically 0.2 g of PCL nano/micro-mesh was put into a vial with 3 ml of the liquid.

Since PCL fibers are partially hydrophilic thanks to the presence of carbonyl groups, water was selected as the grinding medium in the first attempts. However the ground particles showed a strong tendency to rapidly aggregate and formed a compact film on the water surface. To overcome the problem, three different liquid systems with different properties were tested, mannitol (10 w/v % water solution), Pluronic F-68 (10 w/v % water solution), and 2-octanol.

Influence of ground sample storage conditions on the quality of the ground product was examined. Cryogenically ground material was stored either at room temperature for 2 hours or at -20 °C. Samples were subsequently characterized in terms of particle size and morphology as described in the following section. A set of samples was also prepared immediately after the grinding for microscopic analysis so that the morphology and particle size could be examined in a freshly prepared product.

Effects of some variables directly associated with the grinding process were also tested. Firstly the influence of weight of PCL nano/mico-mesh loaded in the grinding vial was assessed. In this experiment 0.2 g or 0.1 g of the mesh was loaded into the vial together with 3 ml of mannitol (10 w/v % water solution). In another experiment, the ground PCL nano/micro-mesh underwent further grinding, this time in microvials (model 6753V – capacity 0.1 – 0.3 ml). Three stainless steel microvials were placed in a polycarbonate holder, 300 µl of the ground sample was added to each microvial and the whole set was immersed in LN2 for approx. four minutes. A stainless steel impactor was placed in each vial, the holder was plugged, and the set was inserted in the mill. The settings of the mill were as described above.

2.2. Characterization of cryogenically ground microparticles

The techniques utilized in the evaluation of particle size, shape and morphology included scanning electron microscopy (SEM), laser diffraction and atomic force microscopy (AFM).

The analysis of the size of particles involved the measurements of their hydrodynamic diameter using Mastersizer 2000 MU (Malvern Ins.) operating at wavelengths of 488 and 633 nm with water (for samples ground in mannitol and Pluronic) or ethanol (for samples ground in 2-octanol) suspension sonicated before analysis for 10 minutes. The retrieved signal was evaluated by Fraunhofer approximation in the range of 0.02 – 2000 μm, and the results were represented as the particle size distribution by volume.

Numerical distribution of particle types in terms of size and morphology was analyzed using scanning electron microscopy (SEM). The Scanning Electron Microscope Jeol JSM 5500 LV with accelerating voltage 10 kV was employed in visualization of typical representative samples. A few drops of samples (diluted with redistilled water 1:1 and sonicated for 2 hours) for SEM analysis were spin-coated on microscopic soda-lime glass etched with HCl (max. 1000 rpm). After the spin-coating procedure, the films were dried in vacuum at RT for at least 2 hours to completely evaporate the water.

Nanoscale components of samples were visualized by Atomic Force Microscopy (AFM). Samples for AFM were filtered with 0.8 μm membrane filters (Millex, type mixed cellulose esters) and samples were spin-coated on highly ordered pyrolytic graphite (HOPG) with similar procedure as described above. The topology of the surface was analyzed using Atomic Force Microscopy (AFM) Solver Pro-M (NT-MDT,

Russia) in semicontact mode with the high-resolution ”Golden“ silicon cantilevers NSG-03 (Au coating, cone angle less than 22° and force constant 1.5 Nm^{-1}).

2.3. Study of biocompatibility and applicability of the system

2.3.1. Cultivation of 3T3 fibroblasts

Mouse 3T3 fibroblasts were selected for the cell cultivation study as these cells are relatively inexpensive and easily cultured for preliminary experiments. Mouse 3T3 fibroblasts were cultivated in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin (100 I.U./ml and 100 μg/ml), 40 mM L-glutamine, 25 mmol HEPES, 40 μg/ml, sodium pyruvate, and 4.5 g/l glucose at 37 °C in a humidified atmosphere with 5% CO_2. The culture was passaged using the trypsin–EDTA method until confluence was reached. Fibroblasts were seeded on the microsphere scaffolds with a cell density of 2 000 cells per well. Prior to seeding cells, dried microsphere scaffolds (30 mg) were sterilized in 70% ethanol (v/v) for 30 minutes and then resuspended in the culture medium. Subsequently a 100-μl aliquot containing approx. 4 μg of microparticles was added to each well of a 96-well microplate. To prevent cells from adhering to the well surface, wells were modified with 1% Pluronic-127. A 50 μl aliquot of the cell suspension was added to each well and incubated for 2 h at 37 °C. Then 150 μl of cell culture medium was added to the samples and cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2.

2.3.2. Confocal microscopy

Adhesion of cells to the scaffolds was examined using confocal microscopy. Cells were visualized by propidium iodide and DiOC6 (3, 3'-Diethyloxacarbocyanine iodide) staining. Samples were fixed with cold methylalcohol (–20˚C) for 10 minutes, rinsed with PBS followed by DiOC6 (0.1-1 μg/ml in PBS; pH 7.4). After a 45-minute incubation at room temperature, the samples were rinsed with PBS (pH 7.4). Propidium iodide (5 μg/ml in PBS; pH 7.4) was subsequently added for 10 minutes, samples were rinsed with PBS (pH 7.4) again and visualized using a confocal fluorescence microscope (Zeiss LSM 5 DUO) (λ_{exc} = 484 nm and λ_{em} = 482–497 nm).

2.3.3. MTS assay

The viability and proliferation of the cells on the microparticulate scaffolds, was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) dye reduction according to the manufacturer's instructions. The test was performed on days 1, 7, 14 and 21 of cultivation. Unground electrospun PCL fibers were used as a control. Samples of either PCL microspheres or fibers were transferred to a new 96-well plate containing 100 μL of fresh culture medium per well, and 20 μL of CellTiter 96® Aqueous One Solution Reagent was added (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). The formazan absorbance in 100 μL of the solution was measured (λ_{sample} = 490 nm, $\lambda_{reference}$ = 690 nm) after a 2-hour incubation at 37°C and 5% CO_2, with a Biotek EL800 plate reader. The absorbance of the samples without cells was subtracted from the cell-seeded samples.

2.3.4. Cell proliferation analysis by PicoGreen

Numbers of cells growing on the microcarrier scaffolds were evaluated by the PicoGreen assay which quantifies DNA. The PicoGreen assay was carried out using the Invitrogen PicoGreen assay kit (Invitrogen Ltd., Paisley, UK). The proliferation of 3T3 fibroblasts on scaffolds was tested on days 1, 7 and 14. To process the material for the analysis of DNA content, 250 μL of cell lysis solution (0.2% v/v Triton X-100, 10 mM Tris (pH 7.0), 1 mM EDTA) was added to each well containing a cell seeded scaffold sample. To prepare the cell lysate, the samples were subjected to three freeze/thaw cycles. Scaffold samples were first frozen at -70 °C and thawed at room temperature. Between each freeze/thaw cycle scaffolds were vigorously vortexed. Lysed samples were stored at -70°C until analysis. The DNA content was determined by mixing 100 μL PicoGreen reagent with 100 μL of DNA sample. Samples were loaded in triplicates and florescence intensity was measured on a fluorescence platereader (Synergy HT, λ_{ex} = 480–500 nm, λ_{em} = 520 – 540 nm).

3. Results and discussion

3.1. Preparation of the PCL microsystem

Microspheres used in this study were prepared from PCL nano/micro-mesh composed of electrospun fibers. Fibers were prepared by needleless electrospinning and the morphology of PCL mesh was determined using SEM microscopy. Results showed that both nanofibers and microfibers were present in the fibrous mesh (see Fig. 5). The average diameter of microfibers was 1.9 μm ± 0.8 μm. The average diameter of sub-micro-fibers was found to be 0.4 μm ± 0.2 μm. Values represent mean ± standard deviation of 200 measurements.

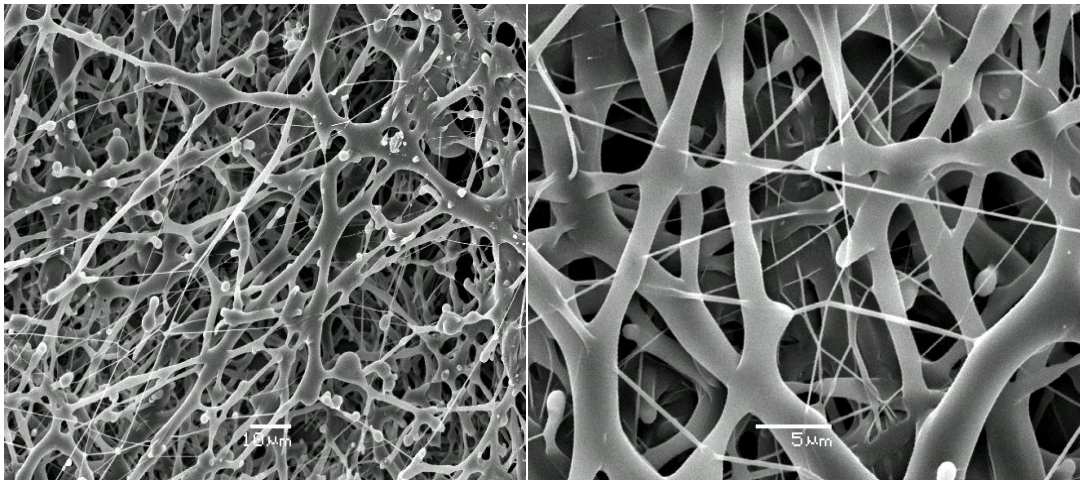

Figure 5. *Scanning electron micrographs of the electrospun PCL nano/micro-mesh*

PCL fibers have high elongation potential, high tensile stress resistance [72] and temperature dependent elastomeric properties [73]. These properties prevent the use of mechanical methods for fragmentation of PCL nano/micro-mesh into microspheres at room temperature. The glass-transition temperature of PCL was determined to be approximately -71°C [74]. Cryogenic grinding at the temperature of liquid

nitrogen (-196°C) was therefore proposed for disintegration of the thin polymer layers to produce nano- and microparticles.

The preliminary experiments with dry cryogenic grinding of PCL layers did not produce satisfactory results. The fibrous mesh was compressed and no desired disintegration and microparticle formation was observed. This was probably caused due to the physical properties of the ground material as well as the small amount in the grinding vial. To overcome the problem, an original procedure based on cryogenic grinding of polymeric nano/micro-layers in liquid was adopted based on Niwa *et. al* [75]. Three ml of a liquid were added to each sample prior to pulverization. Sample volume was significantly increased with the addition of the liquid and micro-crystals of frozen liquid could potentially facilitate sample fragmentation. Material storage, analysis, and separation of particles with desired parameters would also be facilitated by sample suspension.

Due to the presence of hydrophilic groups in the PCL structure, water was initially tested as the grinding medium. However, the ground particles showed a strong tendency to rapidly aggregate and formed a compact film on the water surface. This behavior may be explained by the high surface tension of water [76], higher density of water compared to PCL and a higher affinity of PCL particles for each other due to non-covalent interaction between particles during melting in the grinding vial.

To improve the suspension, a selection of a novel grinding solvent was necessary. Three liquids with different properties were tested. Mannitol (10 w/v % water solution) was chosen as an anti-aggregation additive since it is a widely used pharmaceutical dispersion stabilizing agent [77]. The surface tension of mannitol solutions is similar to water, but the sugar enables stabilization of particle suspensions [78]. Pluronic F-68 (10 w/v % water solution), a hydrophilic surfactant that lowers

surface tension [79], was chosen for its ability to increase colloidal stability of various particles [80]. Due to the only slightly hydrophilic nature of PCL microparticles, a hydrophobic solvent was tested too. 2-octanol was selected as a representative of hydrophobic solvents because of its optimal melting temperature, lower surface tension [81] and insolubility of PCL fibers in this solvent.

3.2. Characterization of the cryogenically ground PCL nano/micro-mesh

3.2.1. Size and morphology analysis of the particles ground in mannitol

To characterize particles produced by cryogenic grinding of the electrospun PCL nano/micro-mesh in mannitol/water solution the suspension was analyzed by laser diffraction, SEM and AFM microscopies. The particle size distribution by volume was analyzed by laser diffraction in suspension. The suspension produced by cryogenic grinding of the PCL in mannitol was polydisperse. The most prevalent particle size was 22 μm and the broad distribution of particles with the diameter ranging between 5 and 75 μm contained 80 % of all particles (see Fig. 6, solid line). At least two other sets of particles were distinguishable: 1) smaller particles (size < 2 μm) and 2) large particles comprised of residual starting material, fragments or aggregates (size > 75 μm). The populations were minor (10 %) in both cases. The method of particle size analysis employing laser diffraction assumes all particles to be spherical and their equivalent spherical volume diameter is calculated. This technique enabled rapid characterization of the particle size distribution in terms of volume.

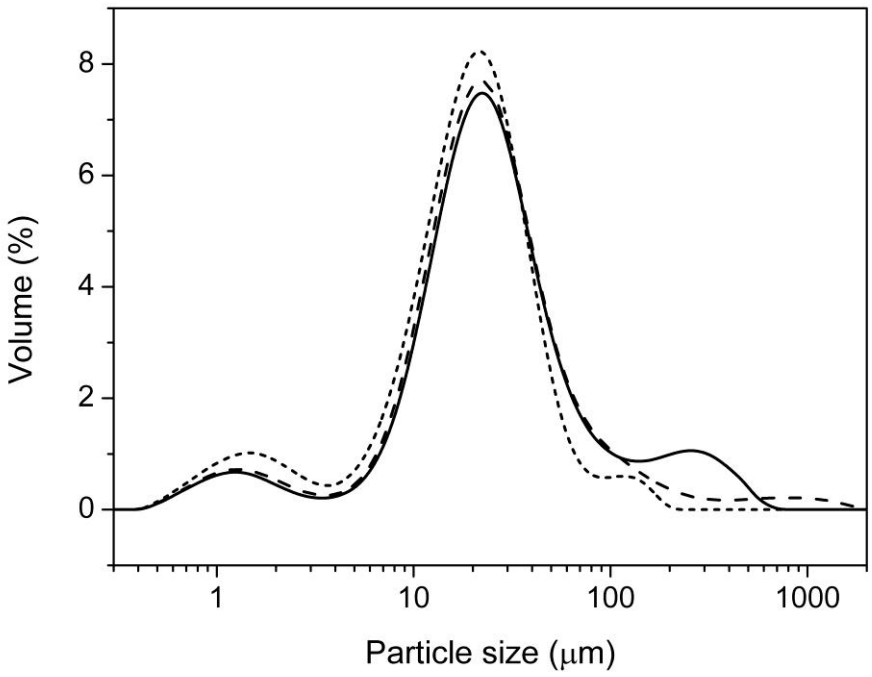

Figure 6. *Particle size distribution determined by laser diffraction analysis for samples ground in mannitol (solid line), Pluronic F-68 (dashed line) and 2-octanol (dotted line).*

Due to the fibrous structure of the starting polymer with high aspect ratio (length/diameter), another approach was necessary for complementary characterization of samples in terms of particle size and morphology. The information about particle size and morphology is necessary for evaluation of the system applicability. The visual inspection of SEM images was performed for retrieving the data of the inner structure and shape of particles. By SEM, 6 types of particles were detected in all studied samples (see Figs. 7, 8):

a. **Fibrous particles** with length from 300 μm to 1 000 μm, and width between 5 and 50 μm. These particles are characterized by low width to length ratio and are formed by disintegration of the original nano/micro-mesh.

b. **Fibrous fragments** with varying size and shape. The typical length was approximately 50 μm and maximum length was 300 μm. The width to length ratio is higher than in fibrous particle fraction. These particles are produced by disintegration of the fibrous mesh or by further fracture of fibrous particles.

c. **Fibrous aggregates** are globular particles with fibrous internal morphology and size varying between 10-100 μm. They are probably gradually formed from individual fibers, fiber fragments and lamellae. The morphology of individual fibers in the internal structure is variable since some aggregates are formed after the grinding process and some are formed during grinding which is apparently due to flattening of the aggregates in one direction. Due to their porous morphology, the fibrous aggregates have a potential for use as a scaffolding microsystem.

d. **Aggregates without an internal structure** are globular structures with size varying between 50 – 300 μm. Two possible mechanisms for formation are: 1) when the aggregates are present early in the grinding process, they are compressed and annealed together, destroying their fibrous structure leaving undefined agglomerates without an internal structure are formed; 2) aggregates without an internal structure could originate from non-fibrous defects produced during the electrospinning process. Such polymeric structures cannot be fragmented even by cryogenic grinding.

e. **Lamellae** are two-dimensional particles with flat morphology. They are residues from the impact of the grinding impactor on a fiber sample. The size of lamellae is relatively uniform with length approximately 30-40 μm and thickness of < 1 μm.

f. **Nanoparticles** were detected by AFM in the filtrate after filtration through a 0.8 μm membrane. These nano-particles displayed fibrous structure with a round cross-section 100 - 150 nm in diameter with lengths up to 40 μm. For visualization purposes, the nanofiber edges were highlighted on the images with Prewitt filter [82].

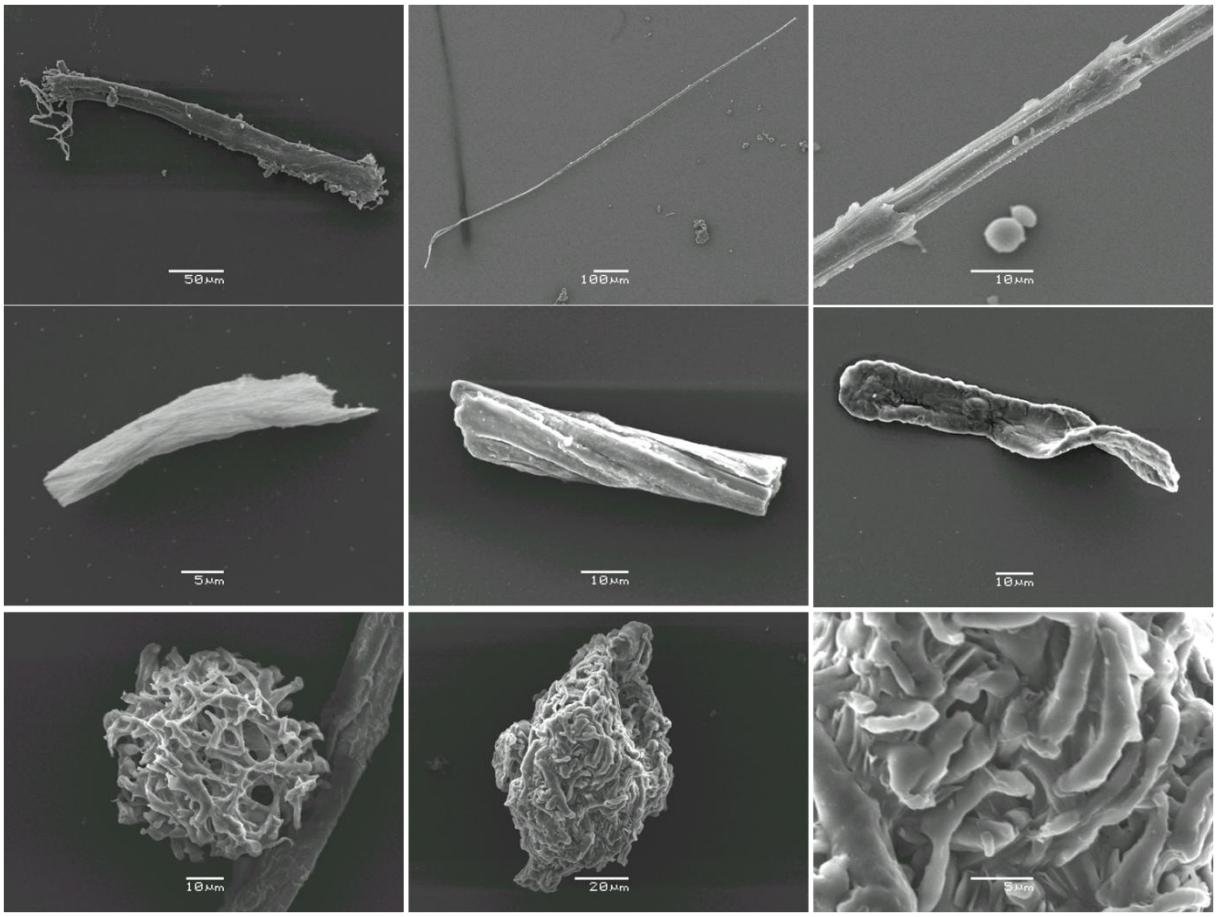

Figure 7. *Scanning electron micrographs of typical particles obtained by cryogenic grinding of PCL nano/micro-mesh in water solution of mannitol: upper row – fibrous particles; central row – fibrous fragments; lower row – fibrous aggregates.*

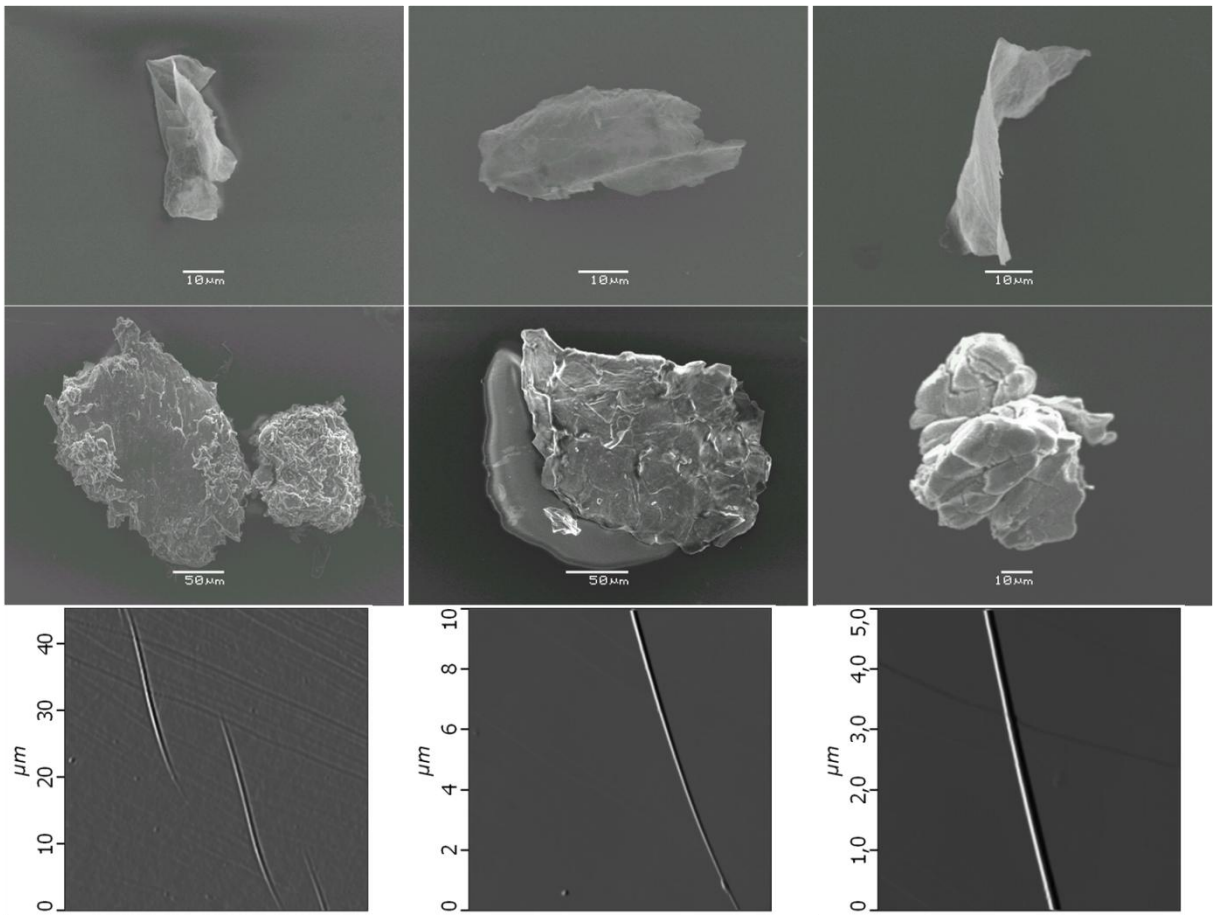

Figure 8. *Scanning electron micrographs of typical particles obtained by cryogenic grinding of PCL nano/micro-mesh in water solution of mannitol: upper row – lamellae; central row – aggregates without an internal structure; lower row – AFM images of nanoparticles, for reader's convenience the Prewitt filters were used for edge highlighting.*

Cryogenic grinding produced a polydisperse suspension of micro- and nanoparticles. Particles of different sizes and morphology can be exploited in different applications. Fibers and fragments can potentially be utilized as injectable micro-systems for controlled delivery of bioactive substances. Due to their size they would be a suitable drug delivery system for administration of bioactive substances into the colon and lungs [83, 84]. The smaller nanoparticles could be exploited for intravenous administration of bioactive substances.

Importantly for our purposes, microspheres formed from aggregates with fibrous structure can be utilized for cell scaffolding and potentially for *in vitro* cultivation of cells. The major advantage of these particles is their porous character. Cell attachment and growth is a complex process affected by a variety of physical and chemical parameters, thus many parameters should be considered in designing microspheres as an effective carrier of cells. The most important properties are surface topography, roughness and surface chemistry.

Microspheres could better perform their role as a cell carrier when macroporous. Larger pore size enables migration of cells into the microsphere interior and supports higher cell densities [28]. Chung *et al*. observed higher chondrocyte proliferation on macroporous microsphere scaffolds prepared by a gas-foaming method when compared to non-porous microspheres [30]. Those specific macroporous scaffolds have only limited drug-delivery potential for susceptible bioactive agents, such as growth factors. Our novel system combines the drug delivery potential of electrospun nanofibers with the advantages of porous microparticles. Porous particles could also be integrated into composite scaffolds with hydrogels and polymeric foams. Of special importance, the microspherical form can be effectively applied as an injectable system for minimally invasive surgery opening new potential for electrospun nanofibers. Lamellae and aggregates without fibrous structure were also produced by the grinding process. These particles are waste products of the process and should be eliminated.

3.2.2. Selection of appropriate grinding medium

To investigate the influence of the grinding medium on the distribution of individual types of particles in the resulting product, different liquid systems were tested. PCL nano/micro-mesh samples were ground in a water solution of mannitol (10 %) (part 3, Figs. 7, 8), in a water solution of Pluronic F-68 (10 %) and in 2-octanol (Fig. 9). Pluronic F-68 is a hydrophilic surfactant lowering surface tension [79] and increasing colloidal stability of various particles [80]. As PCL is only slightly hydrophilic, a hydrophobic solvent was tested too. 2-octanol was selected as an appropriate hydrophobic grinding medium because of its optimal melting temperature, lower surface tension [81] and insolubility of PCL nanofibers in this solvent. The SEM micrographs and laser diffraction analysis (Fig. 6) revealed production of similar particle types for all three liquids, however, the morphology of the particles showed variability. When Pluronic F-68 was used as the grinding solvent, the fibers were markedly flattened and more lamellae types of fibers were formed (see Fig. 9, left part). The fibers were also more branched than those ground in mannitol. Interestingly, samples ground in 2-octanol showed more fibrous morphology and presence of smaller aggregates with fibrous structure (Fig. 9, right part). This fibrous character of the material ground in 2-octanol resulted in the highest population of the smallest particles (12 % of population) and the lowest population (3 %) of original fibrous material with particle size > 75 μm, as shown in Fig. 6. The population of the particles > 75 μm were medial in the case of the Pluronic system (8 %).

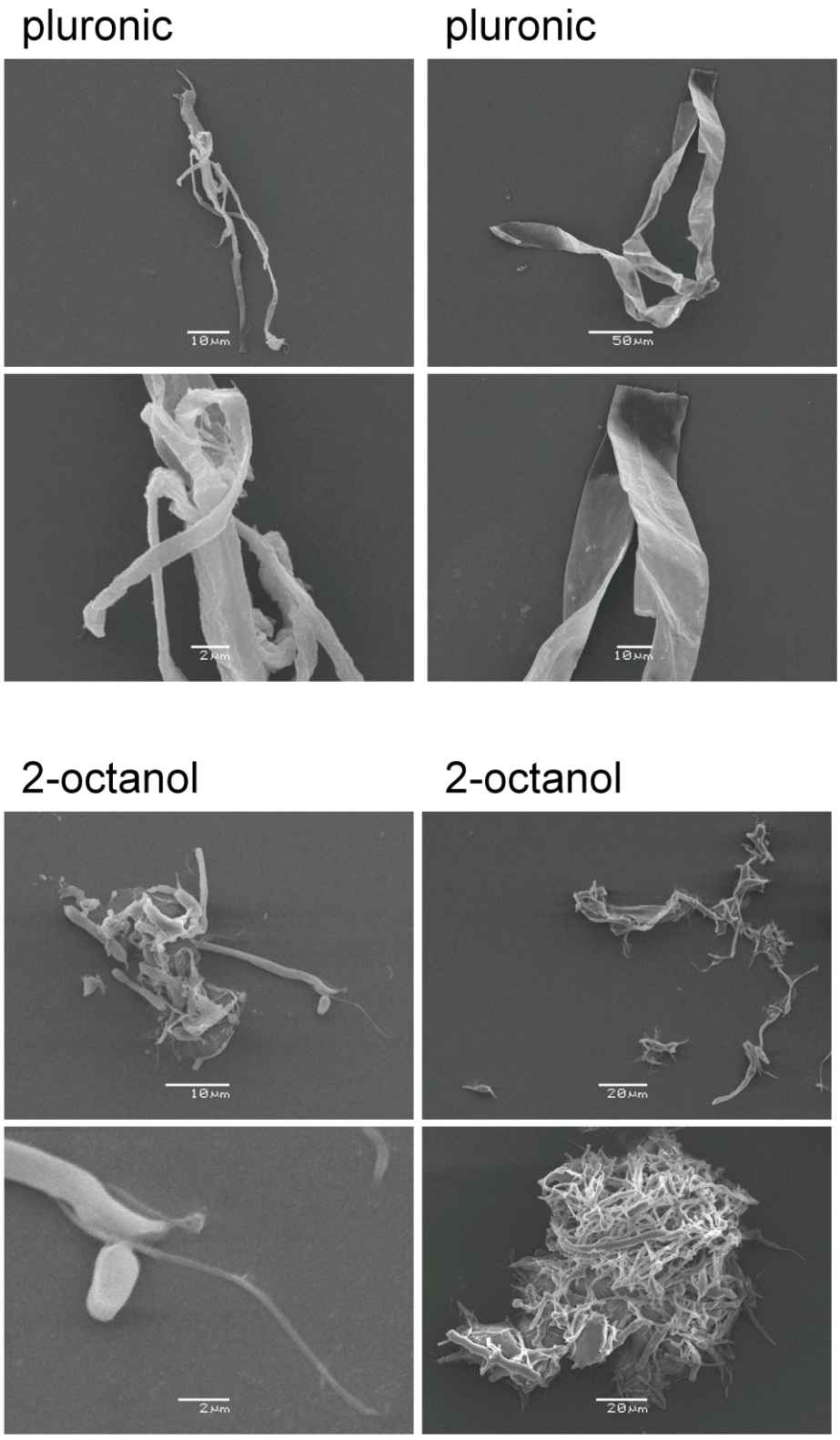

Figure 9. *Scanning electron micrographs of typical particles obtained by cryogenic grinding of PCL nano/micro-mesh in the water solution of Pluronic (upper two rows) and in 2-octanol (lower two rows).*

The numerical particle type distribution was evaluated using SEM images and represented by a pie chart (Fig. 10). The accuracy of the analysis of 5 particle types with detection of more than 100 individual particles was estimated to be 2 – 5 %. The results showed variability in particle type distribution among the three solvents. Samples ground in mannitol revealed high incidence of aggregates either with internal fibrous structure (32 %) or without internal structure (47 %). This high proportion of aggregates without internal structure indicates aggregation of particles during the grinding process when the fibrous agglomerates are flattened upon contact with the impactor and the internal structure collapses. On the other hand, the sample exhibited the highest presence of fragments (13 %) and the lowest presence of lamellae (2 %). A high proportion of aggregates without fibrous internal structure (37 %) was also observed in samples ground in Pluronic F-68, however the fraction was lower than in mannitol. Samples ground in Pluronic F-68 also revealed high proportion of fibrous aggregates (39 %) and similar amounts of fragments (8 %) and fibers (10 %). In the case of 2-octanol, the proportion of fibers and fragments noticeably increased. The proportion of fibrous aggregates (52 %) was notably higher than the proportion of aggregates without fibrous structure (15 %). These observations could result from a different grinding process or lower tendency to agglomerate during and after grinding attributed to surface tension, lower density and higher hydrophobicity of the solvent.

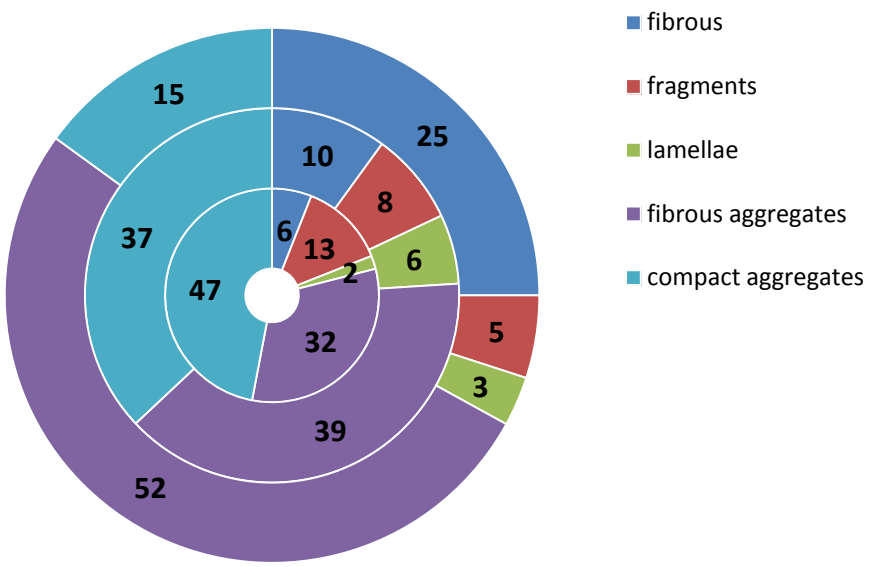

Figure 10. *Pie charts representing the numerical distribution of individual particle types in the product of cryogenic grinding in different grinding liquids: in mannitol (10% w/v in water) - inner chart; in pluronic (10% w/v in water) - middle chart; in 2-octanol – outer chart.*

The differences in distribution of particular particle types in the final product ground in different media could be caused by the character of interaction between the processed material and the liquid. 2-octanol, as a typical hydrophobic solvent, penetrates deep into the structure of the fibrous mesh and effectively fills the space between individual fibers. This leads to improved thermal conductivity during the freezing process since the direct contact between insufficiently frozen fibers and formation of air bubbles is prevented. The properly solvated sample is consequently cooled to a lower temperature in shorter time period and becomes highly fragile.

When aqueous solutions are utilized as grinding media, supposedly poorer penetration of the liquid into the pores is achieved due to increased polarity of the medium compared to 2-octanol. This results in formation of air bubbles within

the mesh, and poorer heat transfer during freezing, leading to the collapse of the structure on the direct impactor action on the insufficiently frozen material. Additionally, separation of the fibers from the solvent due to differences in density may occur. That would increase the possibility of interaction between polymeric particles resulting in their aggregation. Utilization of surface active solutions, in this case Pluronic F-68, was expected to enhance the results of cryogenic grinding by improving penetration of the solvent into the pores. The assumption was confirmed by the subtle increase in the fibrous aggregates portion at the expense of compact aggregates compared to the samples ground in mannitol. However this process needs to be further optimized.

The best results in terms of production of microspheres with desired porous structure were achieved with 2-octanol as the grinding medium. Unfortunately, toxicological characteristics of this substance seem to be prohibitive in terms of potential medical or pharmaceutical applications. As mentioned above, 2-octanol was selected for this study for its favorable properties such as hydrophobicity, surface tension, melting temperature, and insolubility of PCL fibers in this solvent. Another reason for selecting this liquid was the fact that it is relatively inexpensive and readily available. Our results indicate that another solvent with similar properties as 2-octanol but without the toxicological issues should be identified in further studies.

The portions of the desired porous particles in products from mannitol and Pluronic were almost comparable. Substantially lower environmental and health risks make, mannitol more desireable for technological use. Subsequent experiments were performed only with mannitol (10% w/v in water).

3.2.3. Influence of weight of loaded micro/nano-mesh sample

Not only the settings of the mill, but also other parameters of the sample, affect the results of the grinding process [52, 53, 62]. Decreased sample load for grinding may improve the results. Less material in the vial, however, might lead to extensive wear for the equipment also resulting in increased sample contamination [52, 69]. In the present study, the influence of the weight of sample loaded in the grinding vial was investigated by characterisation of particle suspensions produced by grinding of 0.1 g and 0.2 g of PCL nano/micro-mesh in 3 ml of solvent.

Numerical particle distribution was determined by SEM. Results showed that with increased weight of the sample the distribution profile did not change dramatically (Fig. 11). However, with the increased weight of loaded PCL mesh the proportion of non-fibrous aggregates was slightly increased (from 45% to 47%) and the presence of lamellae was slightly decreased (from 5% to 2%). This observation supports the hypothesis about formation of these particles. Lamellae are probably produced by the direct contact of the grinding impactor on the fibrous particle. This phenomenon is more probable when the smaller amount of the sample and thus lower density of particles and larger aggregates are present in the grinding chamber. Since the efficiency of the process, in terms of consumption of energy, liquid nitrogen and time, is higher when a larger amount of sample is loaded in one grinding lot, 0.2 g of PCL was loaded in all subsequent experiments.

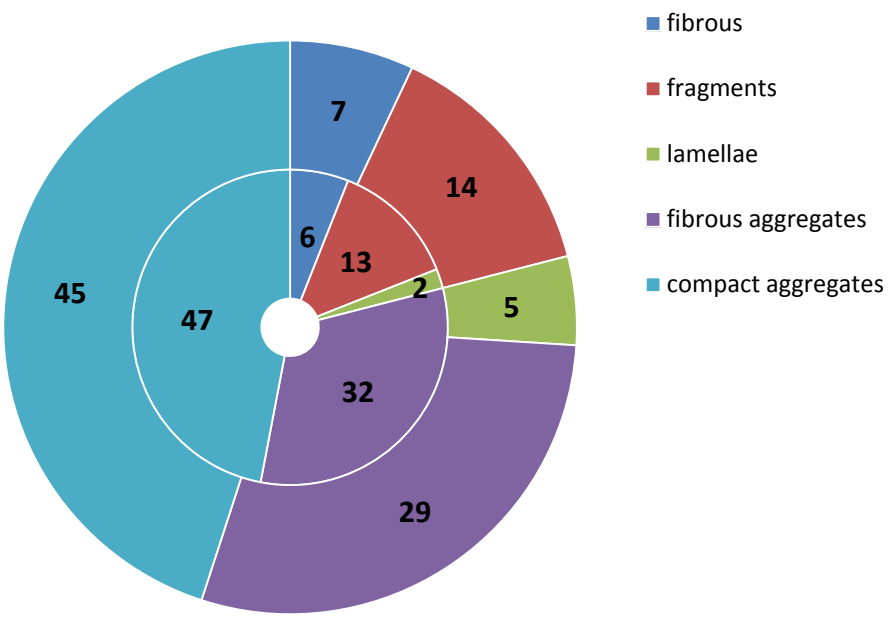

Figure 11. *Pie charts representing the numerical distribution of individual particle types depending on weight of PCL mesh loaded determined by SEM: 0.2 g - inner chart; 0.1 g – outer chart.*

Another experiment assessed the use of microvials for further disintegration of the ground material. The goal was to improve the results of the grinding process through further pulverization of a significantly smaller amount of the sample in a microvial. For simplicity, the results produced by grinding in a larger polycarbonate vial and followed by re-grinding in a microvial will be designated as results of "micro-grinding". As shown in Fig. 12 a decrease in the number of compact aggregates without the internal morphology from 36% to 28% was observed in the micro-ground material. Also the proportion of fibrous agglomerates was slightly lower (22% as opposed to 25%). In contrast, the proportion of smaller particles, namely fibrous particles (16%) and lamellae (13%), was found to be higher.

The increase in numbers of smaller particles at the expense of large aggregates supports the theory that the use of microvials improves the disintegration of the material into smaller particles due to increased probability of the action of the impactor on individual particles. However the increase in the total number of potentially exploitable particle types, namely fibrous particles, fibrous fragments and fibrous agglomerates, was only subtle (from 55% to 59%). These results together with the drawbacks of grinding in microvials, including higher time and energy consumption, and complicated handling, lead to the conclusion that micro-grinding does not increase the efficiency of the grinding process and thus was not used in further experiments.

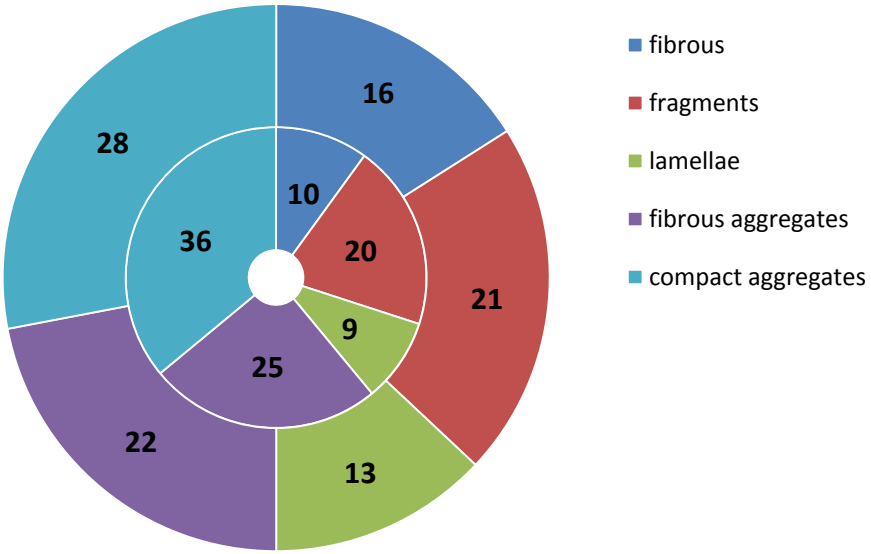

Figure 12. *Pie charts representing the numerical distribution of individual particle types in the ground PCL mesh (inner chart) and in the material reground in microvials (outer chart) determined by SEM.*

3.2.4. Influence of sample storage conditions on the particle distribution

Storage conditions were observed to be a crucial parameter determining the distribution of all types of micro and nano-particles. Influence of storage either at room temperature or at -20°C was investigated. The samples were prepared by cryogenic grinding in the mannitol water solution and an aliquot was spin-coated either immediately after grinding (Fresh), after 2 hours at room temperature (Room) or after freezing at -20°C (Freeze). The particle type distribution was determined by SEM (Fig. 13).

The incidence of fibers, fragments and lamellae was substantially higher in the samples prepared immediately after grinding, in the Fresh sample (the total of 46 %). In the sample stored at room temperature for 2 hours (Room), the proportion of the above mentioned particle types decreased, assumably due to their aggregation and formation of fibrous aggregates. An increase in the distribution of fibrous aggregates from 9 % to 29 % was observed.

The results showed no significant difference in particle type distribution between the samples prepared after two hours at RT and the samples stored frozen at - 20°C. This observation could be explained by the tendency of small ground particles to aggregate. Aggregation was visible to the naked eye in the ground samples shortly after the grinding process thus the aggregates could have formed before the samples were transferred to the freezer and during freezing.

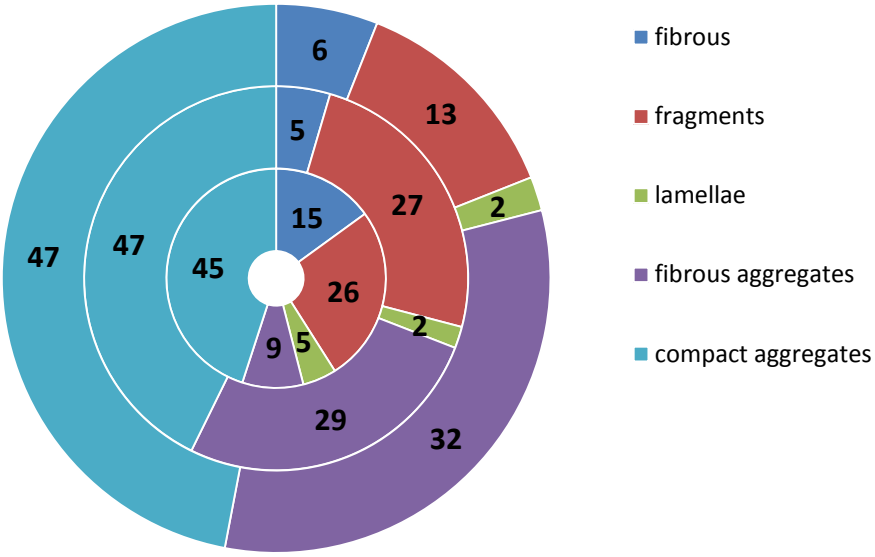

Figure 13. *Pie charts representing the numerical distribution of individual types of particles depending on storage conditions: samples spin-coated right after grinding (denoted in text as Fresh) – inner chart; after 2 hour at room temperature (Room) – middle chart; after freezing at -20°C (Freeze) –outer chart.*

3.3. Study of biocompatibility and applicability of the system

3.3.1. Cell adhesion on microparticles ground in different liquid systems

To evaluate the potential of prepared porous microparticle systems for tissue engineering, cultivation study with mouse 3T3 fibroblasts was performed. Cells were seeded on the microparticle systems prepared by grinding and cell adhesion was analyzed after one day of cultivation by confocal microscopy. Cell membranes were stained by $DiOC_6$ and propidium iodide was used as a counterstain for cell nuclei. Results of confocal microscopy showed adhesion of cells to all three samples. Particles ground in mannitol solution (Fig. 14A) and 2-octanol (Fig. 14B) showed good cell adhesion. Biocompatibility of particles ground in 2-octanol is quite surprising and could be explained by 2-octanol washing out during the sterilization of particles in ethanol. Interestingly, the sample ground in Pluronic F-68 (Fig. 14C) exhibited the lowest cell adhesion. Despite the fact that the particles ground in mannitol and 2-octanol showed comparable cell adhesion, mannitol was selected for further studies due to potential toxicological issues associated with the use of 2-octanol.

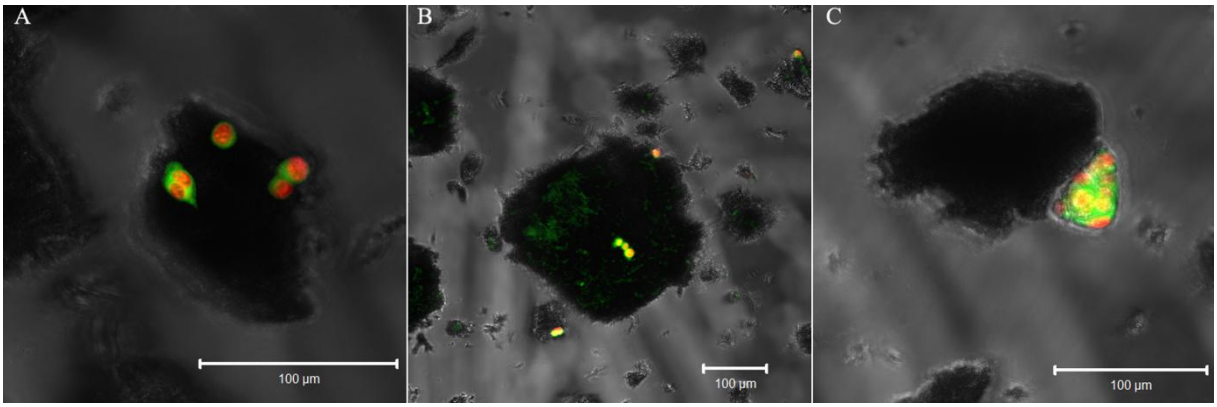

Figure 14. *Adhesion of 3T3 fibroblasts on microparticles: (A) microparticles ground in 10% manitol, (B) micropaticles ground in 2-octanol, (C) microparticles ground in 10% Pluronic F-68. Cells were stained for membranes (DiOC6, green) and nuclei (propidium iodide, red).*

3.3.2. Cell viability and proliferation on particles ground in mannitol

In order to examine cell proliferation and metabolic activity of 3T3 fibroblasts on PCL microparticles, a 21 day cultivation study was performed. Cells were directly seeded on PCL microspheres ground in mannitol using the intact PCL mesh as a control. The DNA concentration in the samples was quantified on days 1, 7, 14 and 21 by the Picogreen assay (Fig. 15A). Values represented the mean ±SD of four independent measurements. Similar cell growth was observed on day 1 on microspheres and fibers. Interestingly, cell proliferation by days 7 and 14 was higher on microspheres. This result could be caused by higher free surface of microparticles. On day 21 the number of cells on microspheres decreased due to metabolic deprivation. On the other hand, the number of cells on the intact mesh continued to increase even on day 21.

Metabolic activity of cells was measured by MTS reduction (Fig. 15B). Values represented the mean ±SD of four independent measurements. Cells on the microspheres showed the highest metabolic activity on day 7 which is in agreement

with the results of the Picogreen assay. Interestingly, the metabolic activity of the cells on microspheres was lower compared to the intact fibers on day 14 and 21. This result could be explained by higher shear stress exposure of the cells on microspheres, and depletion of nutrients, which would result in lower proliferation rate and reduced survival of the cells.

The results of the study showed that microparticles produced by cryogenic grinding could be utilized as scaffolding systems for cultivation of cells in suspensions. Based on the size and morphology of the system, the main potential application would be in the field of microinjectable composite systems. The structure and morphology of a compact fibrous mesh would make a microinvasive operation inevitable. Thus the potential of polymeric fibrous meshes for treatment of musculoskeletal defects (e.g. cartilage, bone and intervertebral disc) is decreased. The grinding approach presented in this study could enable efficient implantation of electrospun-mesh-derived microspheres in combination with polymeric gels or foams. Microspheres could also serve as a delivery system for molecules promoting tissue regeneration. Additionally, we have shown that cells can be directly seeded on the microparticles and the surface of microparticles provides favorable conditions for their proliferation. Thus the proposed particles could serve simultaneously as a drug delivery system and porous cell scaffolding material; however, further optimization of particle properties is necessary for promoting successful regeneration of desired tissues.

In general, porous microspheres produced by cryogenic grinding proved to be suitable for cultivation of cells. They could serve as a scaffolding system and adherent cells could be cultured in microsphere suspensions. The proliferation rate and metabolic activity were higher on first 7 cultivation days peaking on day 14. Interestingly, the

standard deviation of microsphere samples was higher than that of the fibers due to the size heterogeneity of the system.

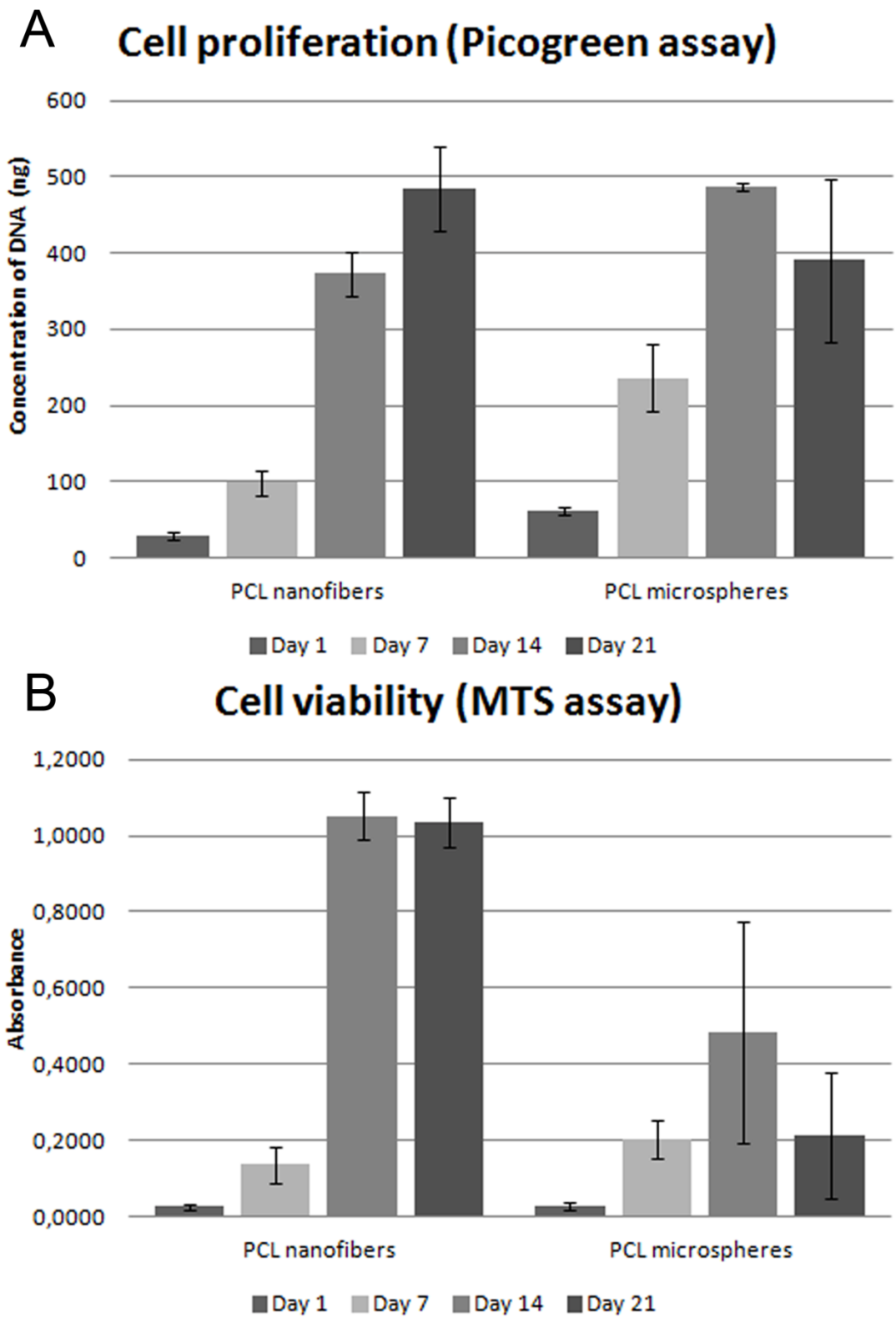

Figure 15: *Proliferation and metabolic activity of 3T3 fibroblasts on either PCL nanofibers or microspheres. (A) Proliferation of 3T3 nanofibers measured by Picogreen assay. (B) Metabolic activity of 3T3 nanofibers measured by MTS reduction.*

3.4.Future prospectives

The multidisciplinary approach presented in this work has provided a starting platform for further investigations that could potentially lead to the preparation of a scaffold for tissue regeneration. The technique of cryogenic grinding of polymeric fibers in frozen liquids has proved its potential in fragmentation of large polymeric fibers. However the results indicate that further testing of grinding liquid is warranted to maximize the yield of desired particle types. Based on the results, the ideal solvent needs to be non-toxic, and should have physical and chemical properties similar to 2-octanol, in order to achieve sufficient wetting and subsequently improved freezing of the PCL mesh. A separation of the particle products by size will be required to obtain fractions of defined sizes for further evaluation of biocompatibility. To move the cell culture studies to the next stage, additional cell lines will be necessary. Mesenchymal stem cells are widely explored in tissue engineering for their ability to differentiate in bone, cartilage and adipose tissue [85].

Tissue engineering combines the support of cell growth with the delivery of bioactive substances promoting the restorative processes. Therefore the ideal scaffold should serve simultaneously as a guidance material for cell growth and as a drug/growth factor delivery system. Hence, loading of the PCL fibrous mesh with bioactive agents such as growth factors should be studied. Encapsulation of some hydrophobic substances in PCL fibers, however, may be difficult. The problem might be approached by combining PCL with a more hydrophilic polymer such as PVA to produce a composite system. Fabrication of core-shell fibers with PVA as a core and PCL as a shell would be advantageous because the hydrophilic substance would be encapsulated in hydrophilic PVA which would be protected from the aqueous environment by the PCL shell.

4. Conclusion

An original technique of cryogenic grinding in liquids for pulverization of the PCL nano/micro-mesh prepared by electrospinning has been presented. The particle system combines the advantages of electrospun nanofibers and microspheres, and has shown a high potential for utilization as injectable gels, microinjectible systems and scaffolds for cell cultivation. Micro and nanoparticles with different morphologies were identified in the resulting product, and suggested for potential applications. The influence of the grinding medium, sample weight, different types of grinding, and sample storage on particle type distribution was studied. The most valuable particles obtained by this procedure were micrometric fibrous particles, fibrous fragments and fibrous aggregates. Lamellae and compact aggregates are waste products and their proportion of the product should be minimized in further work. The cell cultivation study revealed that the PCL microparticles support 3T3 mouse fibroblast adhesion, proliferation, and are promosing as a scaffolding system for cellular biomedical applications. Further experiments should be focused on maximization of the yield of the desired particle types as well as on the separation of distinct particle fractions, their stabilization during long-term storage and a detailed study of their biocompatibility.

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