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Pure mycelium materials: characteristics and applications

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Abstract

Mycelium is the vegetative part of Fungi. It is made up of an interconnected network of hyphae, which are elongated cells with a complex cell wall. Fungi are able to grow on a wide range of substrates thanks to the extended catalytic capabilities of the enzymes they secrete and they have a modular nature, thus they grow by apical extension and lateral branching. They are exploited for a large variety of applications, from bioremediation to drug production. Recently, mycelial biocomposites obtained from the growth of a strain on waste agricultural substrates have been used as materials, which have great thermal and hydrodynamic properties, a low cost of processing and they are easily biodegraded. Previous works from *Smart Materials* group showed that even pure mycelia have promising features to be employed as materials and in nanotechnological applications. These conclusions are further investigated in the Thesis.

The Introduction is an overview of the issues related to plastic use and production and contains a review of the main solutions and alternatives considered up to now. In the second part of the Introduction, mycelia and their applications are described. In Chapter 1, the possibility of finely tune morphological, chemical, hydrodynamic and mechanical properties of pure mycelial materials is investigated by analyzing growth of *Ganoderma lucidum* in liquid substrates enriched in different components. In Chapter 2, both the porous structure and the chemical and hydrodynamic features of mycelia from *Ganoderma lucidum* and *Pleurotus ostreatus* are exploited in the development of suitable bioscaffolds for attachment and growth of Human Adult Fibroblasts (HDFAs).

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Introduction

Plastics. How we arrived to the global pollution

The research project examined in this thesis arises in the first place from concern towards the problem of plastics. The term 'plastic', when used to define a group of polymeric materials and not simply something characterized by plasticity, does not correspond to a well-defined designation (Hartmann et al.). Usually it is used to include the most important thermoplastics (polyethylene, polypropylene, polyvinylchloride, polystyrene, polyester and polyurethane) and in general petroleum-based polymers, thus the issue of plastics mainly concerns the problems both of the unsustainability of the origin of most of the materials used nowadays and of the persistence of these materials in the environment. However, this issue is even broader, explaining why it is hard to have a unique definition of plastics. The following paragraph will examine the topic in detail.

The first material regarded as plastic is the celluloid invented in the second half of the XIX century by mixing nitrocellulose and castor oil. The idea of inventing a new material came from the necessity of overcoming the use of some scarce natural sources, like ivory (Freinkel). This seems a paradox since scientists are now facing the same issue with regards to all the petroleum-based materials, used in daily life. The very first, fully synthetic, material was Bakelite, a thermoset phenol formaldehyde resin developed by Leo Baekeland in 1907. Its development occurred from the need to find a substitute for shellac, a resin secreted by some bugs, and it constitutes the real onset of a new way of thinking, that it was possible to stop imitating nature and start rearranging it in new and imaginative ways. Cellulose acetate, polystyrene, nylon, polyethylene soon came out in

this new horizon, contributing to validate it. As the New York Times art critic Hilton Kramer said, these new polymers represented "an entire family of materials which can be made to assume virtually any size, shape, form or color the mind of man can conceive" (Kramer). The great success and fast development of plastics was related to their malleability, low cost and possibility of mass manufacture.

Plastics properties depend on a great variety of factors: the chemical composition; the chain length; the origin of the monomers, whether natural or synthetic; the manufacturing method (addition or condensation polymerization, co-polymerization); modifications obtained after synthesis by chemical reactions like crosslinking (Hiemenz and Lodge). The combination of all these variables allows using the same polymer in different applications and tuning its properties. One good example is polyethylene, which is mostly commercialized either as low-density (LDPE) or high-density polyethylene (HDPE). The two densities are obtained just by changing extrusion pressure and temperature. LDPE has lower barrier properties and it is used mostly in the form of films and for the coating of cartons. HDPE is injection-molded in containers (bottles, tanks etc) or used in the form of films which possess high fat resistance and better aroma barrier properties than LDPE. Copolymerization is an excellent way to tune a polymer's characteristics, as in the case of polystyrene (PS) which can form hard and transparent products, but it is brittle and sensitive to stress-cracking. Copolymerization of styrene with acrylonitrile and polybutadiene generates ABS (Acrylonitrile Butadiene Styrene) material, more resistant to stress and in fact used for the realization of automotive components. SAN (Styrene Acrylonitrile) is another copolymer of styrene, which has higher thermal resistance than PS alone, even if they have similar mechanical properties.

Plastics are furthermore classified in thermoplastics, when they are moldable above a certain temperature and solidify cooling under it, in a reversible way, and thermosets, if they form irreversible chemical bonds during the curing process. In both cases, polymers go across phases in which they can be easily shaped and molded. A wide number of industrial processes have been developed to exploit these features and allow production of goods of different shape, size, thickness, from both types of polymers. This is an important factor that contributed in the success of plastics because the same machine can be employed to process different polymers, by varying only temperature and pressure conditions.

Eventually, it should be evident that the limit of plastics is in their sustainability. Of the features praised at the beginning of the XX century when they started to be produced on a large scale, *i.e.* malleability, low cost and possibility of mass manufacture, only the fact that plastics are inexpensive is not true: if the cost is not directly evident from industries bills, the price is paid anyway in environmental terms. The environmental cost of plastics depend on different factors (Nielsen et al.).

First, most of the monomers used for the synthesis of plastics comes from the refining of petroleum, whose extraction and use unavoidably cause pollution and several environmental hazards. Petroleum in fact must be found by seismic techniques and with the need for drilling once a site is recovered, thus with very invasive methods (Kharaka and Dorsey). Extracted petroleum is then transported to refineries, where it is separated into a wide array of products, included naphta which is the main source for plastic monomers (*How Plastics Are Made*). Refining processes poison air, water and soil in the

proximity of the refinery. Transfer from the extraction site can cause hazards for the environment, too, for the risk of oil spills. Moreover petroleum is a non-renewable resource: oil reserves take millions of years to form, so the total amount available on Earth can be considered finite (“Petroleum - Status of the World Oil Supply”). The Hubbert curve is a dumb-bell curve that predicts the production rates for non-renewable resources. It is given by the ratio between reserves to production. Since new wells are still discovered, it is not easy to trace the curve on a long period of time, but it is clear oil reserves will be depleted (Aguilera et al.).

Moreover, usually plastics include additives, which work as plasticizers, antioxidants, heat stabilizers or as colorants, fillers and reinforcements. Some molecules are added also to ameliorate blending between polymers forming a copolymer (Hahladakis et al.). Eventually, unreacted monomers or oligomers can persist in the final plastic polymer. All these compounds are rarely chemically bound to the matrix and usually have a high octanol/water partition coefficient (K_{ow}), *i.e.* they are hydrophobic. These two parameters extremely increase their bioavailability, since they easily disperse in the environment and accumulate in the tissues of living organisms which absorb them through water, through their diet or directly by ingesting plastic fragments and microplastics (Hermabessiere et al.; Gallo et al.). Unfortunately, many of these molecules have also been recognized as potential toxic compounds, supposed to act as endocrine disruptors. The most famous case is Bisphenol A (BPA), used especially in polycarbonates to increase thermal and mechanical properties. Incorporation of BPA in baby bottles has been banned in Europe since 2011 and in 2019 its inclusion in general has been regarded by the General Court of the European Union of "high concern on account of its properties as a substance toxic

for reproduction" (Barboza et al.; Liguori et al.). Similar concerns can be true also for many other compounds used as additives. However, determination of minimum harmful dose or of the conditions in which migration from plastic to living tissues happens is not easy, due to the complexity of required analyses and to dependence of migration on various parameters, from time and temperature of contact phase to initial concentration of the migrant component.

Finally, plastics persist in the environment. Polymer chains present very stable and strong covalent bonds, which require a high energy to be broken down (Figure 0.1) (Moharir and Kumar; Danso et al.). Degradation can occur naturally via physical forces, such as mechanical forces, UV lighting and heat; chemical reactions, prompted by acid and alkali compounds; biological action by enzymes secreted by microorganisms or invertebrates (F. Zhang et al.). The efficacy of these factors in fully degrading dispersed plastics depend on several features of the polymeric object, such as density, thickness, weight and chemical composition. However, plastics are hardly degraded completely to carbonic anhydride and water in natural conditions and environment, but more probably form smaller pieces, which are called microplastics when they have dimensions inferior to 5 mm. The effects of microplastics on living organisms and the environment are still under study, but many toxic effects have already been observed (Wang et al.; De-la-Torre). Plastics and microplastics can be ingested by animals, interfere with invertebrates fundamental activity in the soil and negatively interact with plants. Humans can get in contact with microplastics by air, through the skin or via the food they eat, especially in the case of food coming from aquatic environment (Prata et al.). Plastics contribute in dispersing toxic contaminants not only by releasing the additives they may contain, but

also functioning as optimal adsorption platforms for many organic pollutants, which preferably adhere on hydrophobic surfaces (Zettler et al.). In addition, microrganisms use microplastics as "rafts" to be transported, as biofilms, in niches where they could not otherwise survive. The microbial community found on the surface of microplastics is different from the one identified in the surrounding habitat and has acquired the name of *Plastisphere*. This is of special concern when pathogen microrganisms exploit microplastics surfaces to be carried and live in different areas and for longer times (Krause et al.).

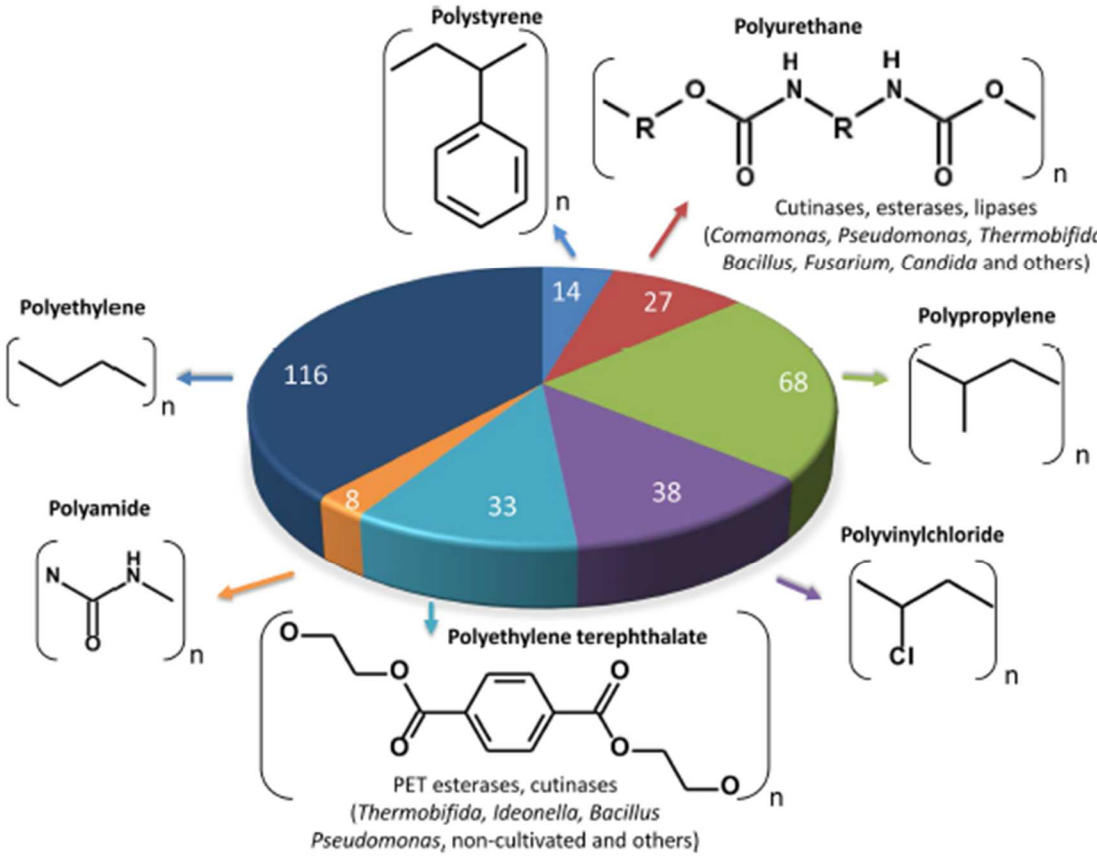

Figure 0.1. Main synthetic polymers produced in 2016 and relative chemical structure. Enzymes able to degrade some of these polymers are indicated. (Picture reproduced from (Danso et al.))

Solutions

Recycling

The most straightforward and known solution to the problem of plastics pollution is recycling. Recycling can determine a reduction both in the pollution from plastic waste and in the oil consumption for plastic production. ASTM D5033-00 distinguishes four types of recycling (Figure 0.2) (Hopewell et al.; Okan et al.):

- Primary recycling is the mechanical reprocessing of discarded, uncontaminated plastic waste for reuse in a product with equivalent properties. For this reason, it is also called closed-loop recycling.
- Secondary recycling is the mechanical reprocessing of materials into products with lower value (downcycling).
- Tertiary recycling, or chemical recycling, is the recovery of petrochemical constituents in form of gases, liquids or solids. These chemicals could serve either as polymer monomers or as combustibles.
- Quaternary recycling is the energy recovery through incineration.

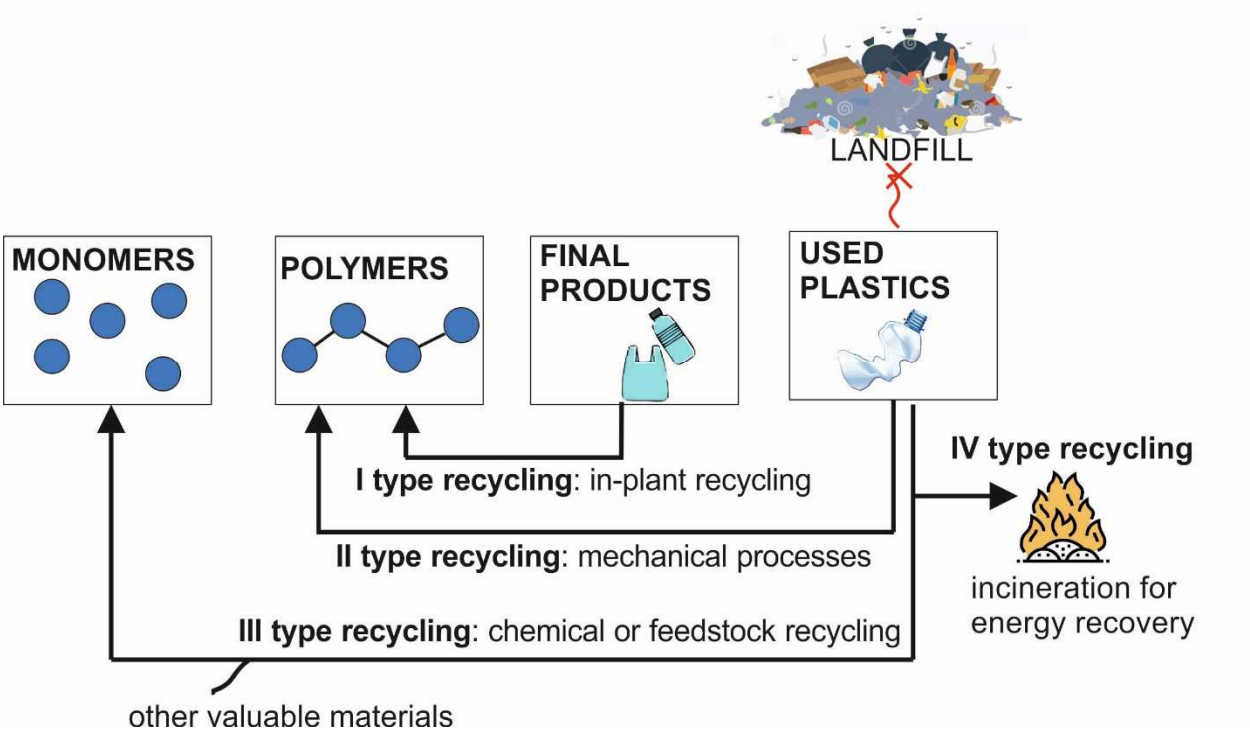

Figure 0.2. Recycling processes of plastics.

Primary and secondary recycling processes only involve physical treatment of collected materials and they are also known as mechanical recycling methods. They both start with the collection and separation of materials. So far, primary recycling has worked only with very few plastics, mainly PET and HDPE bottles, due to the presence of contaminations and to the limited cycles of reuse for any material. Primary and secondary recycling can involve only thermoplastic polymers, since thermosets cannot be melted and reshaped. Consequently, thermosets are recovered only in part to be used as fillers, after grinding. The additives and fillers they may contain can be recycled, too. Thermoplastics must be separated according to chemical composition, density, surface properties and color: mixing of polymers differing for these features would impair the melting process or lower even more quality of the resulting plastic. Tools based on FTIR, Raman and NIR have

been developed to support the separation process, together with dry or wet processes for density and surface properties-based separation. A good waste disposal and a starting manual separation step, however, are still necessary and improve recycling. The need for separation and cleaning of plastic waste from dyes, adhesives etc. is the main limitation for this recycling process and even among thermoplastics, blends, composites and polymers with high-melt viscosities cannot be recycled (Sheldon and Norton).

In tertiary recycling, valuable monomers or petrochemical resources are obtained from plastics via thermolytic, chemical or other degradation treatments (Lee and Liew). Thermolysis may consist in pyrolysis, hydrocracking or gasification. The first can produce gasoline and naphta, but it needs high temperatures and thus it has a high cost in terms of heat energy required. Hydrocracking proceeds by polymer cracking through addition of hydrogen: it is very expensive due to the cost of hydrogen and of the equipment necessary to maintain the high pressures demanded. Gasification produces a syngas composed mainly of methane and hydrogen which can serve as combustible gas, but with low calorific power. Moreover the process has a poor throughput and determines the production of toxic gases such as NO_x. The generation of toxic sub-compounds is also the main drawback of chemolysis, where polymers are transformed in reusable monomers via alcoholysis, hydrolysis, glycolysis or methanolysis, at relatively mild temperatures (80-280 °C) thanks to the use of catalysts. However, large-scale application of these techniques is limited by the different operating conditions required by the diverse plastic polymers.

Degradation pathways can involve both abiotic and biotic processes: the former would require a high amount of energy to completely degrade plastic without any other contribution, while the latter alone would be very slow. Moreover, chemical bounds in polymer chains are in many cases inaccessible to enzymes. Thus, the two strategies are usually coupled (F. Zhang et al.). Abiotic processes comprehend thermodegradation, photodegradation and mechanochemical degradation and aim at reducing length of polymer molecules and create reactive sites to favor biotic degradation. In thermodegradation, applied temperatures are lower than in pyrolysis and consequently the process takes longer to complete. Photodegradation exploits near-UV light to cause the production of reactive species in the polymers. Photosensitizer compounds can be introduced in polymers to enhance this process: the problem is to avoid premature onset of degradation. In mechanochemical degradation ultrasonic vibrations determine breakage of polymer chains, thus there are high energy costs and requirements. Identification of microorganisms and fungi able to degrade polymers is a hard task due to methodological and natural difficulties (Danso et al.; Moharir and Kumar). On the one hand, in fact, these studies are usually led in laboratory cultures and by observing the decrease in plastic weight in the time, in presence of defined microbial populations. The obstacle then is to relate any degradation activity observed to a specific enzyme and especially not to be biased by the easy degradation which can occur for additives and impurities present in the polymer, but which is not associated to degradation of the macromolecule itself: reduction of polymer weight is not a reliable variable to observe. On the other hand, as reported above in this chapter, fossil-based polymers present very stable and strong covalent bonds, which could hardly react with naturally occurring

enzymes. Up to now, only enzymes able to attack and degrade PET molecules have been fully characterized (Figure 0.1). Studies have occurred on biodegradation of PS, PE, polyamides, but enzymes responsible for the degradation process have not been identified, if any biodegradation has been observed. Recently, invertebrates have also been observed for the ability of degrading Styrofoam and PE. Apparently, these larvae are able to mechanically grind plastics, favoring biodegradation from their gut microbiome (Gu). The advantage of exploiting biodegradation pathways of plastics is the possibility of operating at mild pressure and temperature conditions, if excluding a preliminary abiotic treatment step. However, on a large scale other issues should be addressed such as the possibility of maintaining optimal conditions for long times and of minimizing microbial stress exposure. Further studies and developments should be performed to industrially scale up these discoveries (Amobonye et al.).

Finally, incineration has a very high economic value, since a large quantity of energy can be obtained from polymers. Problems are obvious: production of Greenhouse Gases (GHG), especially CO and NO_x, and of other toxic compounds as dioxins. Some solutions have been proposed, from the use of special filters active against these poisonous sub-products to incorporation of additives in the virgin plastics to reduce their production. However, incineration only allows the recovery of energy, but it does not affect the recycling of carbon, while increasing its emissions in the environment. This is not the optimal solution in a scenario of climate change as the actual.

New plastics

Alternatives to petroleum-based plastics may be conceived to solve the issues of the unsustainability of the origin from fossil fuel and/or the persistence in the environment, without releasing toxic compounds that can derive from the disposal of waste. Two different categories of such alternative plastics can be distinguished: bio-based plastics, which derive from natural sources such as plant biomass, and biodegradable plastics, which are fully mineralized to full mineralization into CO_2, H_2O, biomass and inorganic salts by microorganisms (Figure 0.3). The two categories can overlap for some plastics, but not all the bio-based plastics are biodegradable and viceversa (RameshKumar et al.; Narancic et al.).

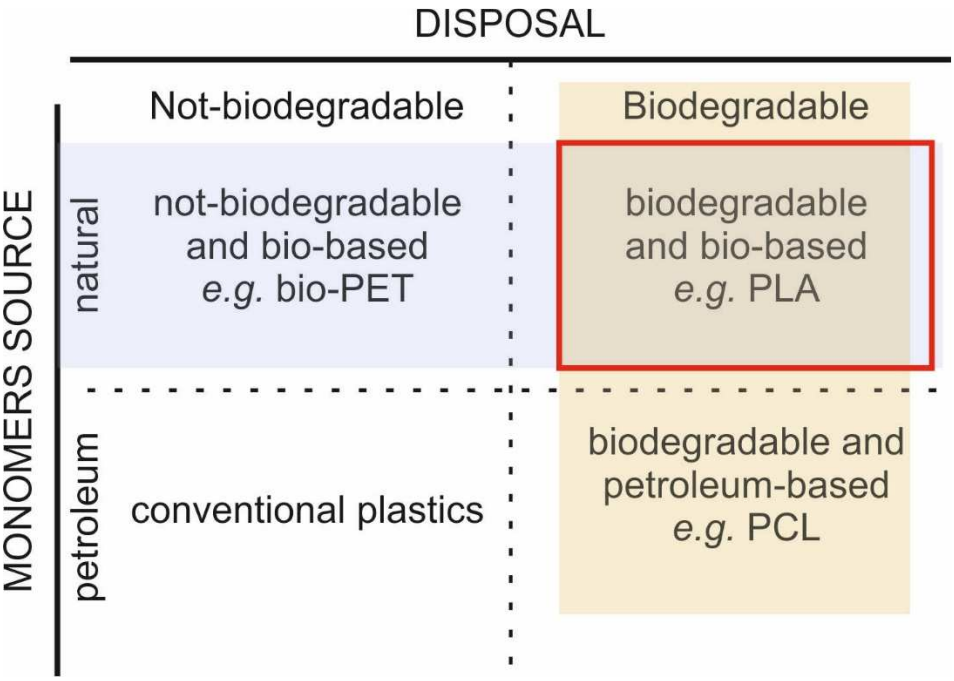

Figure 0.3. Plastic classification according to origin of the employed monomers and occurrence of biodegradation, which is intended as complete mineralization of the material. The ideal material is bio-based and biodegradable.

Bio-based plastics can either be *drop-ins*, which have the same chemical structure of petroleum-based plastic even if they derive, totally or in part, from renewable sources, as in the case of bio-PET; or they can be completely new polymers (Sheldon and Norton). In the latter case, it is possible to distinguish among plastics synthetically made from bio-based monomers, like polylactic acid (PLA); plastics produced directly as polymers in fermentation processes, as in the case of polyhydroxyalkanoates (PHA); eventually, plastics derived from modification of biopolymers, usually cellulose or starch. The advantage of *drop-ins* is that they can be processed by the same equipment of petroleum-based plastics and have well-known properties and usage. However, they keep also the same problems such as that of degradation. Bio-PET is synthesized from bio-ethanol and fossil p-xylene. A completely bio-based polymer, equivalent to PET, is polyethylene furandicarboxylate (PEF), in which the terepthalic acid of PET is substituted with a furanic ring. PEF is synthesized from 2,5-furandicarboxylic acid (FDCA) and monoethylene glycol (MEG) obtained from the fermentation of wheat- or corn-based sugars or of waste or bagasse. PEF is not biodegradable, neither.

Development of completely new plastics is not trivial since has to face several challenges (Filiciotto and Rothenberg; Gandini and Lacerda). First, these materials should own mechanical, thermal and other intrinsic properties corresponding to those retained by petroleum-based plastics, in order to have the chance to replace them in some fields. This is not immediate because in the production of bio-based materials many variables cannot be easily controlled, especially for plastics derived from biopolymers or totally synthetized in microorganisms, like PHA. In these cases many and complex pathways control the final output and changes never have a linear response. For this reason,

research has focused also on the design and generation of monomers to be bound synthetically (Hatti-Kaul et al.). The glass transition temperature (T_g) is a fundamental parameter which determines mechanical, thermal and other physical properties of amorphous materials. It has been observed that T_g can be regulated by introducing aromatic units: this is an example of how synthetic routes from bio-based monomers can be more easily engineered. Like T_g, crystallinity and melting temperature can be tuned by the introduction of specific chemical units or by specific design of the chemical chain.

The possibility of relying on the same industrial infrastructures used for petroleum-based plastics, from injection-molding to calendering, is another target of research on new bio-based materials. Actually, industrial processing has been successfully implemented mostly with PLA, PHA and starch-derived materials, even if with some limits. PLA mechanical properties could allow its production by different processes, like extrusion, injection and blow molding, but its viscosity and temperature of degradation still constitute big constraints, which can be overcome by the use of viscosity enhancer or through creation of blends. Starch, in the form of gelatinized starch called TPS (thermoplastic starch), can be processed by extrusion, even if its high hygroscopy causes the necessity of long drying procedures (Reichert et al.; Thomas).

Even the issue of biodegradability is not so straightforward (Mazhandu et al.). First, biodegradability is not always a praised feature and traditional plastics became popular also for their durability. Second, biodegradability can comprehend a variety of cases. As described in the previous paragraphs, any plastic experiences some kind of degradation in the environment. Biodegradation is defined though as the complete mineralization to

carbon dioxide, water, ammonium, nitrogen, hydrogen and biomass by microorganisms: no toxic compounds are produced in the biodegradability process. Having fixed this definition, biodegradation comprehends different processes, with various time-scales, and occurring in diverse environments (soil, fresh or marine water). Beyond environmental temperature and humidity, also chemical composition, structure and physical properties of the bioplastics influence the rate of biodegradation. This explains why there is a wide variety of analytical tests to perform to assess the biodegradability of a material, following diverse standard procedures (Walker and Rothman). Some plastics can thus be named "compostable" if through biodegradation they generate an organic-rich material: even in this case, polymer should be distinguished in industrially compostable and home-compostable, where in industrial composting higher temperatures and humidity are used. Moreover, in waste treatment plants, pre-treatment processes such as UV-irradiation, which enhance biodegradation, can be employed. Eventually, any of these standard procedures up to now is able to evaluate the production of microplastics, which is in fact a step in the biodegradation process that goes across three phases, *i.e.* biodeterioration, biofragmentation and finally assimilation. Table 0.1 lists biodegradation processes available for the most used biodegradable plastics. Recycling, especially chemical recycling which could recover starting monomers, can be employed for bioplastics, too, but it is still highly limited by the necessity to sort out precisely the different polymers, in order to avoid excessive downgrading of the recovered material (Folino et al.; W. Meereboer et al.).

Table 0.1. Sustainable end-of-life options for most common biodegradable plastics. Adapted from (RameshKumar et al.)

Biodegradable polymer	**Source/Feedstock**	**Sustainable end-of-life options**
TPS, cellulose, cellulose acetate, starch blends	Biomass, agro-residues, lignocellulosic derivatives	HC, IC, AD
PLA and PLA blends	Lactic acid from dairy whey, corn starch or organic residues	IC, MR, CR
PHA, PHB	Volatile fatty acids, glucose/glycerol from fermentation of municipal solid waste or any carbon feedstocks	HC, IC, AD, CR
PCL	Chiral hydroxy acids, lactones	HC, IC, CR

AD, Anaerobic Digestion; MR, Mechanical Recycling; CR, Chemical/Catalytic Recycling; ED, Enzymatic Depolymerisation; IC, Industrial Composting; HC, Home Composting.

Life cycle assessment (LCA) is a tool that allows to determine the environmental footprint of products or processes. It can be calculated either from cradle-to-gate, *i.e.* from the raw materials to the generation of the product, or from cradle-to-grave, *i.e.* from the raw materials to end of life (Blanco et al.). LCAs are not always more favorable for bioplastics in comparison to petroleum-based materials. This is due to several factors, first the absence of a unique standard for LCA calculation in terms of scope, data quality, assessment method or impact categories. Only in 2013 the European Union commission has published guides to Product Environmental Footprint (PEF) and an Organisation Environmental Footprint (OEF) assessments (*Single Market for Green Products - Environment - European Commission*). However most studies in literature are hardly comparable due to differences in approach and calculations. Moreover, bioplastics

research is running fast and evaluations on processes and related environmental cost must be updated very often. In general, from studies published so far, it turns out that the highest impact of bioplastics on environment is given by a larger aptitude to cause eutrophication and acidification, while they are more affordable in terms of GHG emissions than fossil-fuel derived counterpart (Walker and Rothman). With regards to the economical cost, that of bioplastics production is still higher than for the petroleum-based counterpart, but this is mostly related to the fact that price for oil extraction and distribution is kept low for economical and geopolitical reasons. This constraint could be overcome only by proper legislation by governments and international organisms. In general, principles of Extended Producer Responsibility should be introduced, by which the largest industrial plastics producers would be forced to take care of collection and recycling of produced materials, thus encouraging development of biodegradable materials and in general minimization of production (Sheldon and Norton).

The highest percentage of plastic waste (39.9%, (*Pubblicazioni :: PlasticsEurope*)), in fact, derives from packaging, which is conceived to protect its content just for a short period of time: decreasing the use of packaging could therefore determine a large reduction in plastic waste production (Pauer et al.). Food quality and shelf life depend on temperature, oxygen concentration, carbon dioxide concentration, humidity and temperature, thus packaging can help in preventing food losses and by assuring that these parameters stay in the best range (Kalpana et al.). However, food waste, which accounted for 149 billion euros in 2015 only in the European Union, can be ascribed to different factors, mainly oversupply and undervaluing. Indeed, food waste is by far less in low income countries ("Unwrapped"). Reduction of packaging demand and production

could be achieved only by changing the system of distribution and organization of the supply chain. On account of this, the European Union is approving a new strategy, called Farm-to-Fork, which promotes shorter supply chains and more direct form of retailing, thus avoiding the need for packaging which only can support long-distance transportation and large scale selling (BINNS). The realization of these goals is not so straight: an article interviewing representatives of 7 European companies shows how reduction of packaging usage or even substitution with more sustainable alternatives is impaired by worries on the possibility of harming the product or the inability to guarantee its safety and protection (Ma et al.). Moreover, companies do not have any control neither on logistics infrastructure nor on the recycling one: the effort should be aimed at unifying control and rationalization of all the steps, from food collection to retailing. The certainty that new materials can assure the same protection on packaging content as the traditional petroleum-based plastics can come only from research: states and organizations should invest on these fields, standing up for an holistic reorganization of the whole supply chain. The report Talking Trash, published by the Changing Market Foundation, describes how, notwithstanding all the largest plastic producing companies in the world are committing to reduce plastic pollution by taking care of recycling, none of them is in fact really doing it or is respecting the same targets they decided to assume (Changing Markets). The reason is that these goals are not economically convenient in a system like the predominant capitalistic one, nor the times required by a true reorganization of the supply chain and necessary to benefit of all the efforts implemented from now are compatible with those of the actual production. A revolution of the whole productive system is necessary to really face the environmental issue.

Mycelia

Eukarya domain is traditionally divided into four main groups, which are Protists, Fungi, Plants and Animals. Discovery of new living organisms and the use of phylogenomic techniques have complicated this scheme, but the principle that each eukaryote cell has a nucleus is still the main criterion, which stays valid. Fungi own several peculiarities with respect to the other kingdoms. First, they digest their substrates externally: fungi secrete enzymes in the environment and can only absorb digested products. This has led to the evolution of extracellular mucilages, membrane components and enzymes which improve their control on the surrounding environment prevailing on possible competitors to whom digestion products would be available, too (Webster and Weber). Secondly, they are modular organisms: they grow from the elongation of a unique cell, which can then divide or branch. Each fungal cell is just a segment of a tubular cell, named hypha, and is thus totipotent since it is able to form alone an entire new organism. The interconnected network of hyphae which arises from the growth is called mycelium (Moore). Oomycota and Zygomycota have aseptate hyphae with a unique cytoplasm where all the nuclei stay, so they are called *coenocytic* fungi. Asco- and Basydiomycota have septate hyphae and each segment can contain one, two or more nuclei, identical or not. Finally, some fungi do not grow as hyphae but as discrete cells: these are the yeasts.

Hyphae are long, tubular cells that grow only at their tips, thus fungal morphogenesis depends only on the placement of hyphal branches. This modular structure is an effective solution for the acquisition of nutrients by a non-motile organism: as soon as nutrient resources become depleted in an area, production of further modulus permits to find a

new one. It is said that plants can adopt a *guerrilla* strategy, when they grow for example in stolons, thus in loosely bound ways, but expanding in wide areas so that they can luckily survive in some; otherwise plants can pursue a *phalanx* strategy, which means that they grow in restricted areas but being very resistant to competitors attacks or physical damages. Mycelia can adopt both these strategies by affecting hyphal rate extension and branching frequency. Given that the order of a hyphal branch is the number of branches that separates it from the main hypha, it can be modeled that there is an inverse logarithmic relation between the number of branches of an order and the number of orders of branches. This allows the mycelium to colonize the maximum surface area by the minimum length of filaments, but it also let the mycelia to choose either to branch or to elongate according to nutrient availability (Gow and Gadd). The modular nature makes the mycelium more resistant to physical injuries or attacks by predators: if a modulus does not survive, the others cannot be affected. Moreover, in septate fungi, septal pores constitute another defense since they can be closed to avoid the passage of toxic substances or opened to bring support to a stressed branch.

Another peculiarity of Fungi is the presence of a cell wall. The cell wall is indispensable to support the cytoplasm, to adhere to external structures or receive communications, to permeate molecules outside selectively. The cell wall is mainly composed by polysaccharides and proteins, but the chemical composition differs among different fungal families, at different stage of development and depending on the environmental conditions, from pH to the available nutrients (Bueno and Silva). Polysaccharides mainly consist in glucans (both alfa and beta), mannans and heteropolysaccharides like galactomannans. Proteins constitute only from 3 to 20% of the chemical compounds of

the cell wall, but are fundamental to support the cell structure, to increase its hydrophobicity and to make it interact with the surrounding environment, since they can have enzymatic activity (Fricker et al.). The polysaccharide chitin also contributes to cell wall structure and hydrophobicity. Chitin is a polymer of N-acetylgluocsamine, which in Zygomycota can be deacetylated to generate chitosan. In Fungi, chitin is only in the alfa form, *i.e* with antiparallel chains that determine its high mechanical strength. Together with chitin, a group of cysteine-rich proteins, the hydrophobins, is responsible of hyphal hydrophocibity. These proteins in fact are amphipathic and form a rodlet layer. Eventually, pigments as melanin can be present in the outer layer of the cell wall, to protect against UV or enzymatic damage (Ruiz-Herrera).

The modular nature of fungal growth and the presence of a cell wall are advantageous, and make fungi perceive and respond to the environment at a micrometric scale, but at the same time, they can share resources and coordinate activity across all the space spanned by the mycelium (Peay et al.). There are of course also some limits. First, maintaining a long structure like the mycelium, which has a wide area/volume ratio thanks to its tubular shape, is very expensive in terms of generating all the macromolecules present in the membrane and in the cell wall. Especially nitrogen, a limited nutrient in the environment, is necessary for the generation of the proteins, which are fundamental to keep cell structure and resistance. Moreover, if the cell wall can be very strong against different attacks from predators and chemicals, the growing hyphal apex is vulnerable, even if indispensable for growth and exploration.

However, the features described above open a wide range of possibilities and reasons why study mycelia (Figure 0.4). Mycelial network can be modeled by mathematical equations (Vidal-Diez de Ulzurrun, Baetens, Van den Bulcke, and De Baets). Models differ according to the scale of focus, either microscopic (cell scale), mesoscopic (hyphae level) or macroscopic (biomass exchange between mycelium and environment). Microscopic analyses cannot be easily upgraded to larger perspectives, while macroscopic ones hardly take into account the processes that drive hyphal development. Mesoscopic level is thus the best to understand both the biomass exchange with the environment and the internal hyphal movements (Vidal-Diez de Ulzurrun, Baetens, Van den Bulcke, Lopez-Molina, et al.). Models can study mycelia in two dimensions (especially by using a lattice model) or in three dimensions, which is more realistic but more difficult to implement without risking to lose information (Bezzi and Ciliberto). These models are useful for field experiments where it is important to foresee mycelium development, for example in bioremediation applications where the location of toxic compounds is known and position of inoculum must be optimized. However, modeling of hyphal network can be fruitful also for computer and algorithm development. Slime mold *Physarum polycephalum* (a protist which grows in filamentous form) has been found able to solve mazes and optimization problems like the traveling salesman one (Nakagaki et al.). Experiments are designed as circuits with the mold at one side and nutrients located at interesting points. A fungal inspired algorithm has been developed and used to plan Chinese transport system (Liu et al.). The advantage in using hyphae as a model, in this case, is the possibility to study both the biomass transport occurring inside the hyphae and the biomass exchange with the outside (Daws et al.).

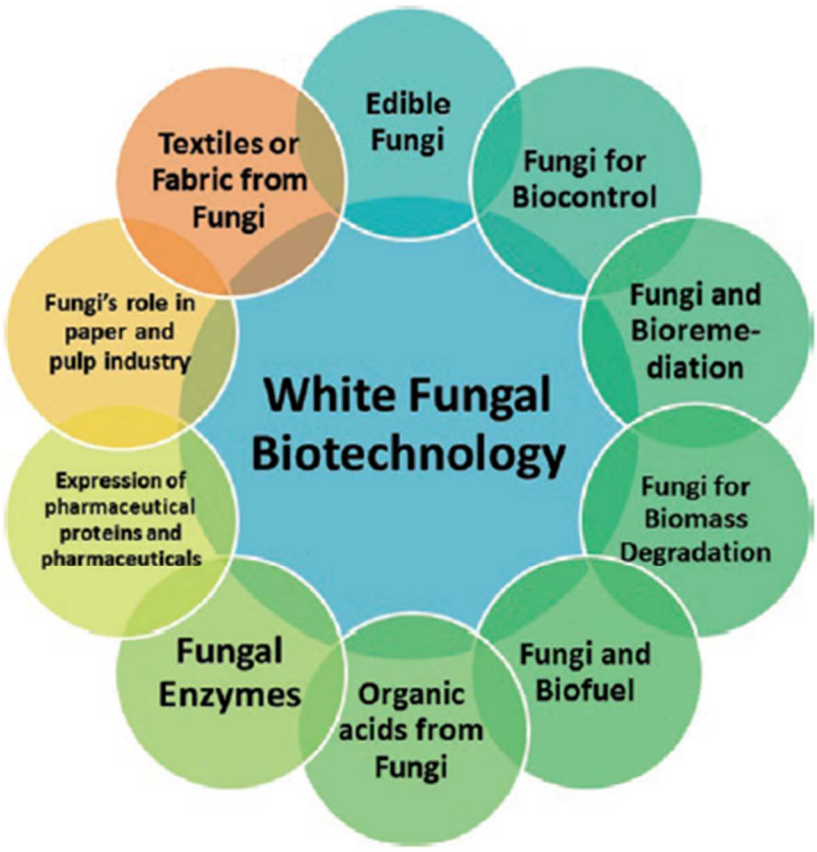

Figure 0.4. Fields of application for Fungi. Reproduced from (Yadav).

Other applications rise from the numerous trophic roles that fungi assume in the environment. On account of this, Fungi can be roughly divided into four categories: **saprophytic**, which are able to digest organic decayed biomass; **mycorrhizal**, when they establish mutualistic associations with plants contributing to their development; **endophytic**, when they live inside the plant itself, with beneficial effects in many cases; **parasitic** (Stamets). These roles come from fungal ability to digest a lot of substrates, recovering nutrients which can be shared with other species, and to the production of molecules able to communicate and to exert toxic or beneficial effects to other living organisms (Branco). Fungi have been thus employed as cell factories for the production

of organic acids, antibiotics, compounds with medical applications (Hyde et al.). Since the first decades of the XX century, *Aspergillus niger* has been used for the production of citric acid, widely employed in food and beverage industry as an acidulate, a flavoring agent, but also as antimicrobic agent (Show et al.). Antibiotics have been discovered from Fungi: Alexander Fleming first observed the toxic effect of penicillin, secreted by the mold *Penicillium rubens*, versus the bacterium *Staphylococcus aureus*. Since then, some other fungal compounds have been isolated with antibacterial properties, *e.g.* chephalosporins and beta glucans. For a matter of competition, Fungi can secrete also molecules with antimycotic activity, like the griseofulvin, discovered in 1939 and efficacious against dermatophytosis (Kück et al.). Being able to synthetize such a wide range of molecules, Fungi can be easily engineered to produce compounds of industrial interest. Yeasts in fact have been the first model organisms for genetic engineering in eukaryotes (Botstein et al.).

The capability of Fungi to grow on substrates recalcitrant for many other species, such as the decayed organic biomass rich in lignocellulosic material, demonstrate the large catalytic activities owned by their enzymes (Tortella et al.). This can be employed against toxic or persistent compounds of human origin. Different experiments of bioremediation by using Fungi have been attempted, both *in situ*, *i.e.* by directly growing or augmenting a population of mushrooms observed to be able of degrading a molecule of interest, and *ex situ*, *i.e.* by isolating enzymes with catalytic activity against any toxic substance to use them in specific reactors (Gadd and Gadd). Bioremediation has several limitations, from the difficulty of controlling all the numerous parameters of growth in the environment affecting the species employed for bioremediation, up to the laboriousness of extracting

one hazardous chemical from a location to convey it to the bioremediation reactor (Yadav). However, diverse oxidoreductases, laccases, catalases and peroxidases have been usefully employed from Fungi. Laccases are especially interesting because they can attack a wide variety of substrates and have been successfully used to remove dyes from wastewater streams (Rodríguez-Couto; Senthivelan et al.). Oxidoreductase are searched for their activity against chlorinated compounds. Moreover, some Fungi have shown their capability to absorb heavy metals thanks to metal-chelating groups exposed in their cell walls and membranes. Mycelia can thus act even as mycofilters, both *in situ* and *ex situ* (Stamets). Trophic role of Fungi can be exploited in agriculture and forestry, enhancing plants and trees wellness by the association of proper fungal species in the soil, and thanks to the numerous compounds Fungi can secrete having pesticide activity. Eventually, Fungi living in extreme environments (*i.e.* high temperatures or in presence of toxic compounds) can be studied to find enzymes active even at harsh conditions, usually found in industrial plants (Lange et al.).

In this thesis, Fungi were employed in the production of materials. Many of the features described above make mycelia interesting even in the materials science. First, mycelia can grow on a wide variety of substrates developing either a two- or three-dimensional network (Crowther et al.). Fibrous materials can thus be obtained without high energy investments, but just exploiting these microorganisms self-growth ability. Exact modeling of growth rate and branching of mycelia in a certain substrate is not easy because diverse variables affect the process (Swadel). However, it seems proved that branching occurs more frequently in nutrient-lacking media, while hyphae elongate faster in nutrient-rich ones. The final architecture of mycelium depends on several factors, starting from the

possibility for the mycelium to penetrate deep in the substrate or not. Final density of the mycelium is determined also by the strain, since some species can produce more than one type of hypha (Fricker et al.). In particular, hyphae can be generative, usually aerial, bringing reproductive cells at the extremes; skeletal, if they are narrower and with thick cell walls and they branch laterally to generative hyphae; ligative, when they are thick-walled and short, binding together more hyphae (Bueno and Silva). Strains are called monomitic, dimitic or trimitic depending on the number of hyphae they can produce (Jones et al.). Some species can also generate special types of hyphae, like stolons, long aerial hyphae that allow the mycelium to expand fast; appressoria, attachment elements employed to attack another living organism; rhizoids and haustoria, to absorb nutrients from the environment or from a parasitized organism. Nutrients distribution in the substrate is one of the main driving factor for mycelium growth. As described above, growth can occur only from the apical hypha, while branching can result both from the apex and, most commonly, laterally. Lateral branching seems to be more frequent in nutrient-poor media, where the mycelium needs to explore wider areas. However, strategies can differ among strains and according to the nutrient available (Heaton et al.). Even the traditional inverse relationship between growth and enzyme production has been questioned recently and related to the type of enzyme, the environmental conditions and the strain (Zheng et al.).

Diverse works in recent years have attempted to determine the best substrate for a specific strain to develop a material with certain features (table 0.2). This effort is complicated by the range of factors that influence fungal growth at the same time, as written above. However, mycelium-based materials open the possibility of exploiting

many waste and recalcitrant substrates and research should go in this direction. Agricultural waste has been used in particular for this kind of application. Saprophytic fungi in fact are able to digest lignocellulosic material, which is otherwise very hard to dispose of (Salvachúa et al.). The main limitations with the use of these substrates is the fact that they can be very poor in nutrients, thus determining a slow growth of mycelia or the development of a loose hyphal network. However, different strategies can overcome these limits, from the addition of specific chemical compounds, which could enhance growth, to the realization of mutualistic associations or symbioses, which could favor nutrient assimilation even in poor media. Moreover, development of mycelium-based materials could be coupled to one of the beneficial applications described above, from bioremediation to mycofiltration. Research is still at its infancy in this field.

Chitin is one of the most abundant biopolymers together with cellulose. It is present in the shell of various marine organisms, of insects and in the cell wall of fungi. It is made up of 2-(acetylamino)-2-deoxy-d-glucose units and confers rigidity and strength to the structures in which it is found (Muzzarelli et al.). These properties, together with the ability to reduce water absorption, have been exploiting since 1970s, with applications in a lot of fields, from textiles to packaging (Crini). First experiments were led with the aim of recovering materials from the huge amount of wasted shrimp shells. Eventually, optimal properties of chitin, especially in biomedical field, were discovered: in bioengineering, deacetylated derivative of chitin, chitosan, is widely employed, having more hydrophilic surface properties than chitin. The problem with this biopolymer is the unsustainability and cost of its extraction: currently, chitin can be recovered either by pulping or in ionic-liquids (Shamshina et al.). Enzymatic extraction is not applied at industrial scale, yet.

Pulping makes use of strong acids and bases at high temperatures and damages chitin molecules with its scission in smaller weight compounds. Ionic-liquids extraction allows to extract the biopolymer without damaging it, but it is still an expensive technique; moreover not all the solvents used are biodegradable. Development of mycelium-based materials could thus determine the possibility of using chitin-rich materials, without the need of extracting it. It has been reported that the highest chitin synthase activity is observed at the apex and at the branching points: this means that fast-growing mycelia should be richer in chitin (Feofilova). On the other hand, a proper amount of nitrogen should be present in the growing medium to assure the generation of this molecule.

As reported in table 0.2, most of the works concerning development of mycelium-based materials have focused on the production of composites where mycelia grow on agricultural feedstock to form composites with their growing substrates. In these materials, mechanical properties are enhanced by the contribution of the fibers digested by Fungi and the mycelial component response to soft strains, producing a multiscale response to mechanical stress (Elsacker et al.; Islam et al.). Mycelium moreover improves thermal resistance of the composite and is able to influence its hydrodynamic behavior: according to the fungal strain employed, composites can be more or less prone to absorb water, but they can also show high water contact angles, thus being highly hydrophobic. This is also affected by the extension of chitin and hydrophobin production by mycelia, which is related to strain, chemical composition of the substrate, environmental conditions of growth and manufacturing procedures (Appels, Dijksterhuis, et al.). Only few works exploit mycelia also for their fibrous and porous structure (Hao et al.; Jiang et al.). Usually polymers need to be processed by special techniques as electrospinning or 3D-printing

to form three-dimensional networks, while mycelia can grow these structures on their own.

Table 0.2: main publications regarding mycelium-based materials

Reference	Fungal strains employed	Product	Composite with agricultural waste
(Hao et al.)	Not known	3-D network activated carbon for supercapacitors	
(Jiang et al.)	Ecovative manufactured	Bioresin infused then cured mycelium-based sandwich-structure	Yes
(Soh et al.)	*Ganoderma lucidum* *Dendrocalamus asper*	Mycelium composite	Yes
(Appels, Camere, et al.)	*Ganoderma lucidum* *Trametes multicolor*	Mycelium composite	Yes, and PET-G molds
(Sun et al.)	Ecovative manufactured	Mycelium composite	Yes and cellulose nanofibrils
(Bruscato et al.)	*Pycnoporus sanguineus* *Pleurotus albidus* *Lentinus velutinus*	Mycelium composite	Yes
(Appels, Dijksterhuis, et al.)	*Schyzophyllum commune* (also genetically engineered)	Pure mycelium (sandwich of two)	No
(Pelletier et al.)	Basidiomycetes	Mycelium-based acoustic absorbers	Yes
(Attias, Danai, Ezov, et al.)	*Pleurotus pulmonarius, ostreatus and salmoneo* *Agrocybe aegerita*	Mycelium composite	Yes
(Attias, Danai, Tarazi, et al.)	*Colorius* spp. *Trametes* spp. *Ganoderma* spp	Mycelium composite	Yes
(Elsacker et al.)	*Trametes versicolor*	Mycelium composite	Yes
(Islam et al.)	Ecovative manufacture	Mycelium based material	No
(Tudryn et al.)	Not known	Mycelium composite	Yes
(Matos et al.)	*Lentinula edodes*	Mycelium composite	Yes

Objectives & novelty of the current research study

In this thesis, strains belonging to Basydiomycota phylum are studied not in composites, but as pure mycelium materials to employ both structural and chemical, thermal, hydrodynamic properties of fungi in the development of advanced materials. Basydiomycota is one of the most numerous phylum in Fungi, together with Ascomycota. It comprehends saprophytic fungi able to grow and digest decayed organic biomass rich in lignocellulosic content (Schmidt-Dannert; Sandargo et al.). In particular, member of this phylum have traditionally been distinguished in brown-rot fungi, when keen on oxidizing cellulose up to their complete degradation, but not able to depolymerize lignin, and white-rot fungi, provided with hydrolases that allow them to digest even this aromatic compound. Genomic and proteomic analyses are recently questioning this distinction and the contribute of aromatic catalyzing bacteria in the environment to the achievement of these processes is becoming more and more central (Riley et al.). However, it is true that white- and brown-rot fungi are furnished with a wide range of enzymes with high catalytic abilities. Mycelia belonging to this phylum have therefore been chosen to open the possibility to grow pure mycelium materials on a large variety of substrates, even waste or recalcitrant ones, or to provide to this application extended value by coupling the material production with bioremediation or enzymatic activity in the production reactor.

In particular, two strains are employed in most of the experiments. *Ganoderma lucidum* and *Pleurotus ostreatus* are basydiomycetes, white-rot fungi able to grow on lignocellulosic substrates. Both belong to biosafety level 1, thus they can be manipulated with no risk of hazard by humans. They are also edible and commonly consumed in Asian

countries. *G. lucidum* has been extensively studied for its ability to secrete molecules with biomedical effect, especially as anti-cancer, anti-inflammatory, anti-oxidant, immunomodulatory, anti-diabetic, anti-viral, anti-bacterial, anti-hypertensive compounds (Kk et al.; Martínez-Montemayor et al.). *P. ostreatus* has been considered especially for bioremediation from heavy-metals and phenolic compounds, thus being employable for dyes and coal processing, oil refinining, detoxification of wastewater streams, as well as bleaching and delignification of cellulose pulp (Bettin et al.; Feldman et al.). The two strains thus seem available for application in diverse fields, from bioengineering to bioremediation.

Previous work from *Smart Materials* group had evaluated growth of these two strains on standard fungal medium Potato Dextrose Broth (PDB) and on micro-cellulose derived films (Haneef et al.). *G. lucidum* and *P. ostreatus* can be cultivated in a variety of conditions and in the examined cases could develop homogenous mycelia, with preserved hydrodynamic and thermal properties and with the largest differences related to chemical composition of the cell wall and hyphal network structure. In this thesis, the possibility of tuning mycelial materials properties by changing substrate of growth is further investigated by growing *Ganoderma lucidum* on standard fungal medium enhanced in some components (D-glucose or lignin) (Antinori et al.). In this work, artificial media are preferred to complex substrates such as agriculture feedstock to better distinguish the main variables affecting mycelial network development. Moreover, notwithstanding the large attention given to mycelium-based materials in the last 7 years and the advancement in manufacturing procedures, their commercialization is still scarce. This can be due to different factors, but it is clear that the lack of industrial viability and

the length of manufacturing contribute a lot. On account of this, development of mycelial materials in laboratory conditions and for advanced nanotechnological applications seem an interesting field of investment. In this context, many of the fungal wonderful properties could be exploited without suffering from competition against petroleum-based plastics, still more advantageous in terms of cost and easy of processing. As described in the first part of this introduction, other materials like PLA and PHB could more readily be an alternative to commodity plastics. For this reason, an application of mycelia as tissue engineering bio-scaffolds is proposed in chapter 2 of the thesis.

Much of the work of the thesis has also been addressed to the development of methods suitable for the study of these materials: the compresence of a micro- (the hyphal level) and macro-scale (the final material) make the analysis of the material response to morphological, hydrodynamic, mechanical, chemical analyses quite complex. In many cases, it is necessary to complement information from diverse techniques: this is the case of mechanical characterization, where both tensile test and Dynamic Mechanical Analysis (DMA) were employed to describe quantitatively the mechanical behavior of developed materials. The same is true for porosity analysis, where Scanning Electron Microscopy (SEM) complemented Mercury Intrusion Porosimetry (MIP) results, and for chemical characterization, where both Attenuated Total Reflectance Fourier Transformed Infrared (ATR-FTIR) and elemental analysis were integrated. Post-harvesting processing of mycelial materials can affect material properties, too. In this work, only deactivation, either by mild-temperature treatment (Chapter 1) or by autoclave (Chapter 2), was considered. Further analysis could evaluate the possibility of employing other procedures, such as hot pressing, mild acid or basic treatments, blending.

Chapter 1

Fine-tuning of physicochemical properties and growth dynamics of *Ganoderma lucidum* mycelium

INTRODUCTION

Ganoderma lucidum is a member of Basydiomycota fungal phylum, belonging to white rot fungi mycelia, able to fully digest lignocellulosic material (Riley et al.). It can grow on wood and agricultural waste, but it has a cosmopolitan distribution thanks to the wide catalytic abilities displayed by its enzymes. Traditionally, it has been employed in food and medicine as a dietary supplement for its anti-oxidant, immunomodulatory activities (Kk et al.). These properties are related especially to secreted polysaccharides that are being studied recently for their antitumoral activities (Martínez-Montemayor et al.).

In this Chapter, its properties as free-standing, pure mycelium material are evaluated. This strain in fact has been employed in few works regarding mycelial composites (Appels, Camere, et al.; Attias, Danai, Ezov, et al.), but none of these works study mycelium *per se*, *i.e.* not embedded in a composite. Some authors studied pure mycelia to model their hyphal network (Bezzi and Ciliberto) or to understand fungal physiology, especially in fermentation systems (Veresoglou et al.; Krull et al.). Previous work from *Smart Materials* group has shown, however, that *Ganoderma lucidum* has very promising properties even for applications as a self-standing material. In particular, it was able to grow on hard substrates as microcellulose-derived films and owned excellent hydrodynamic and thermal properties, being hydrophobic and able to resist to

temperatures of 290°C (Haneef et al.). As described in the Introduction, mycelia could be optimally employed as materials since they develop porous structures at environmental temperatures (27°C), on a wide variety of substrates, and only need a simple inactivation step before use. Bacteria can also grow on various organic substrates and generate polyhydroxyalkanoates or bacterial cellulose, but with several drawbacks. The main limitation of polyhydroxyalkanoates is that they are produced intracellularly, thus their purification process is long and expensive (Kourmentza et al.). Bacterial cellulose sheets form extracellularly but are less versatile than mycelia, since bacteria able to secrete cellulose must be cultured in liquid media, while mycelium can also grow on solid substrates, widening the range of possible growth media.

Objectives

In this Chapter, *Ganoderma lucidum* is grown on liquid substrates slightly differing in chemical composition to check the possibility of finely tuning morphological, chemical, hydrodynamic and mechanical properties of the final material. In particular, *Ganoderma lucidum* is cultured in a standard fungal medium, potato dextrose broth (PDB), adding either D-glucose or alkali lignin. In the former case, a component already present in the original medium, D-glucose, is increased in concentration, thus a sharpening of properties already observed in PDB is expected. Moreover, polysaccharides could increase hydrophilicity of final materials. In the latter condition, a new molecule, alkali lignin, is introduced in the medium, thus radical changes in some properties can be predicted. Alkali lignin is a partially hydrolyzed version of the complex molecule present in the wood,

but it has been reported to be functionally equivalent to the original one, since it is metabolized with the same enzymes and at comparable times (Picart et al.). As a white rot mycelium, *Ganoderma lucidum* is able to digest it.

MATERIALS AND METHODS

Chemicals

Potato dextrose broth (P6685), D-glucose (G7021) and alkali lignin (471003) were purchased from Merck.

Strain, media and growth conditions

Ganoderma lucidum DSM9621 active culture was purchased from DSMZ, Germany. Culture was maintained in 100 mm Petri dish with Potato Dextrose Broth (PDB) and monthly transferred to fresh medium. A piece of 20 days grown mycelium was inoculated in 100 mm Petri dishes containing 30 mL of either PDB 24 g/L (P medium), or PDB 24 g/L with D-glucose 30 g/L (G medium), or PDB 24 g/L with alkali lignin 2 g/L (L medium; table 1.1). PDB medium was used in the standard concentration suggested by Merck. Different concentrations of D-glucose and alkali lignin in PDB were tested to find the optimal one for *Ganoderma lucidum.* Average growth observed in the tested media is reported in table 1.2. Tested concentrations of D-glucose ranged from 5 to 50 g/L, thus they were always inferior to glucose limit of solubility in water (909 g/L), but greatly superior to concentrations used in standard high glucose cell culture media (usually about 5 g/L). Growth was good at any tested concentration and an intermediate one was chosen. Solubility limit of alkali lignin in water is not reported, so tested concentrations

were randomly chosen in a range from 0.2 to 10.0 g/L. *G. lucidum* growth was highly impaired at concentrations higher than 5 g/L, so an intermediate concentration was chosen. Water was the solvent used for every medium and all media were autoclaved before use. Mycelia were incubated in a climatic chamber (Memmert, HPP 260) at 27°C and 78% water humidity, in the dark. Every 7 days, for a total of 28 days, five mycelia for every condition were collected and cleaned from the substrate by using a spatula. Mycelia were then dried in an oven at 50°C for 15 hours before further analyses.

Table 1.1 label and relative content (% wt.) of the main components of the substrates used in this work

Label	Substrate composition	PDB % wt.	D-glucose % wt.	Alkali lignin % wt.
P	Potato Dextrose Broth 24 g/L	100	0	0
G	D-Glucose 30 g/L + PDB 24 g/L	44.4	55.6	0
L	Alkali lignin 2 g/L + PDB 24 g/L	92.3	0	7.7

Table 1.2 average mycelium area after 28 days of growth in media with different composition in PDB, D-glucose and alkali lignin

Medium composition	Average mycelium area after 28 days growth (cm^2)
PDB 24 g/L (P)	51.9±4.6
PDB 24 g/L + D-glucose 30 g/L (G)	59.5±2.7
PDB 24 g/L + D-glucose 50 g/L	69.6±1.0
PDB 24 g/L + lignin 2 g/L (L)	56.6±4.0

PDB 24 g/L + lignin 5 g/L	53.2±3.3
PDB 24 g/L + lignin 10 g/L	29.7±8.0

Growth rate and morphological evaluation

Mycelium area was measured by using ImageJ software on pictures taken approximately every day by a camera (Nikon). Area values were normalized with respect to the initial surface of every inoculum. Weight of dry samples was measured with an analytical balance. Skeletal density was measured by Thermoscientific Pycnomatic Evo helium pycnometry furnished of a 4 cm^3 chamber. Measurements were performed at 20°C. The skeletal volume of the sample, *i.e.* the volume of the sample when pores are not accessible to the gas, was measured by detecting the change in pressure due to the volume of helium that is displaced by the sample within the sealed and pressure-equilibrated chamber. The skeletal density is obtained by dividing the sample mass by the skeletal volume. It is assumed that helium atoms are able to penetrate all open pores within the mycelium. Ten measurements of the same sample were averaged. Porosity was determined by mercury intrusion porosimetry (MIP) performed with Pascal 140 Evo and Pascal 240 Evo mercury porosimeters (Thermo Fisher Scientific). The pressure of mercury intrusion was set at 0.0136 MPa and continuously increased up to 200 MPa, with a rate of 6–14 MPa min^{-1}. The contact angle of mercury with the samples and the surface tension of pure mercury were assumed to be 140° and 0.48 N m^{-1}, respectively. Washburn equation was used to calculate the pore size from the applied pressure, assuming that the pores are of cylindrical shape. For the measurement, the sample was rolled up in a special support for membrane analysis and inserted in the dilatometer.

Morphology was observed by Scanning Electron Microscopy (SEM, JEOL JSM 6490LA) using a 10 kV accelerating voltage. For the observation, samples were covered with a 10 nm thick gold layer and mounted on aluminum stubs, with double-stick carbon tape.

Chemical analyses

Infrared spectra of samples were obtained with an attenuated total reflection (ATR) accessory (MIRacle ATR, PIKE Technologies) coupled to a Fourier transform infrared spectrophotometer FTIR spectrometer (Equinox 70 FT-IR, Bruker). All spectra were recorded in the range from 3800 to 400 cm^{-1}, with 4 cm^{-1} resolution, accumulating 64 scans. The sample was gently placed on a spot of ATR accessory and slowly pressed, with either the part grown in contact with the substrate (named “bottom”) or with air (named “top”) on the ATR crystal, to distinguish the chemical composition of both surfaces. To ensure the reproducibility of obtained spectra three samples of each type were measured. Spectra analysis was performed with Origin pro 2016 software. Elemental analysis was performed by using an Elemental Analyzer CNHS EA3000 (Eurovector).

Hydrodynamic surface characterization

A contact angle (CA) goniometer (DataPhysics OCAH 200) was used for static water contact angle measurements at room temperature. 5 µL droplets of water were deposited on the corresponding surfaces and side view images of the drops were captured after 10 s. CA were automatically calculated by fitting the captured drop shape. Up to 15 contact angle measurements were carried out on every sample at random locations and their average values and standard deviation were reported. For the water uptake, dry

samples were weighed on a sensitive electronic balance and then placed in different humidity chambers. Samples were dried by conditioning in a desiccator for 24 hours, weighed and then put in 100% humidity conditions for additional 24 hours, before being weighed again. The amount of adsorbed water was calculated based on the initial dry weight.

Mechanical characterization

Tensile tests were performed on a dual column universal testing machine (Instron 3365). 4 mm wide, 25 mm long strips were cut from samples with thickness ranging between 0.1 and 0.3 mm, and mounted on the machine clamps. The samples were deformed at a rate of 1 mm/min until failure. The Young's modulus E, ultimate tensile strength UTS and elongation at fracture were extracted from the stress-strain curves. Ten measurements were conducted on each sample in order to confirm the reproducibility of the tensile tests, and results were reported as average values and standard deviation. All tests were carried out at room temperature, 23 °C. Storage modulus *E'* and tanδ (*i.e.* the ratio between loss and storage moduli, representing the relative energy dissipation) were measured also with a Q800 DMA testing machine (TA Instruments). Samples were cut in 20 × 35 mm^2 rectangles and tested at room temperature in uniaxial tensile mode, applying a sinusoidal deformation with an amplitude of 20 μm, and frequencies of 7, 10 and 16 Hz.

RESULTS AND DISCUSSION

Morphology and growth parameters

Growth in L-medium (PDB 24 g/L and alkali lignin 2 g/L) proceeded in a significantly different way than in the other two conditions as shown both by morphology (Figure 1.1) and growth rate (Figure 1.3). Images taken with a photographic camera (Figure 1.1, circular insets) show that macroscopically, the sides grown in contact with the substrate (named “bottom” sides) are yellowish in all conditions, while “top” sides are white in P- (PDB 24 g/L) and G-samples (PDB 24 g/L and D-glucose 30 g/L) and with concentric darker rings in L-mycelia. Final aspect of these samples thus resembles growth pattern observed during expansion in L-medium (Figure 1.2).

SEM images show that bottom sides are similar among samples, too, and present numerous spores (pointed by arrows in Figure 1.1), while top sides are more filamentous. On this side, the samples grown on the substrate with lignin show a smoother and less entangled hyphal network with respect to the samples grown in the other two conditions. Using the same definitions used in the previous work from *Smart Materials* group (Haneef et al.), in the L-grown samples only elongated thread-like features can be found, while the P-grown samples demonstrate both short and highly entangled tube-like as well as elongated thread-like features. In the case of the G-grown samples, the tube-like features become predominant.

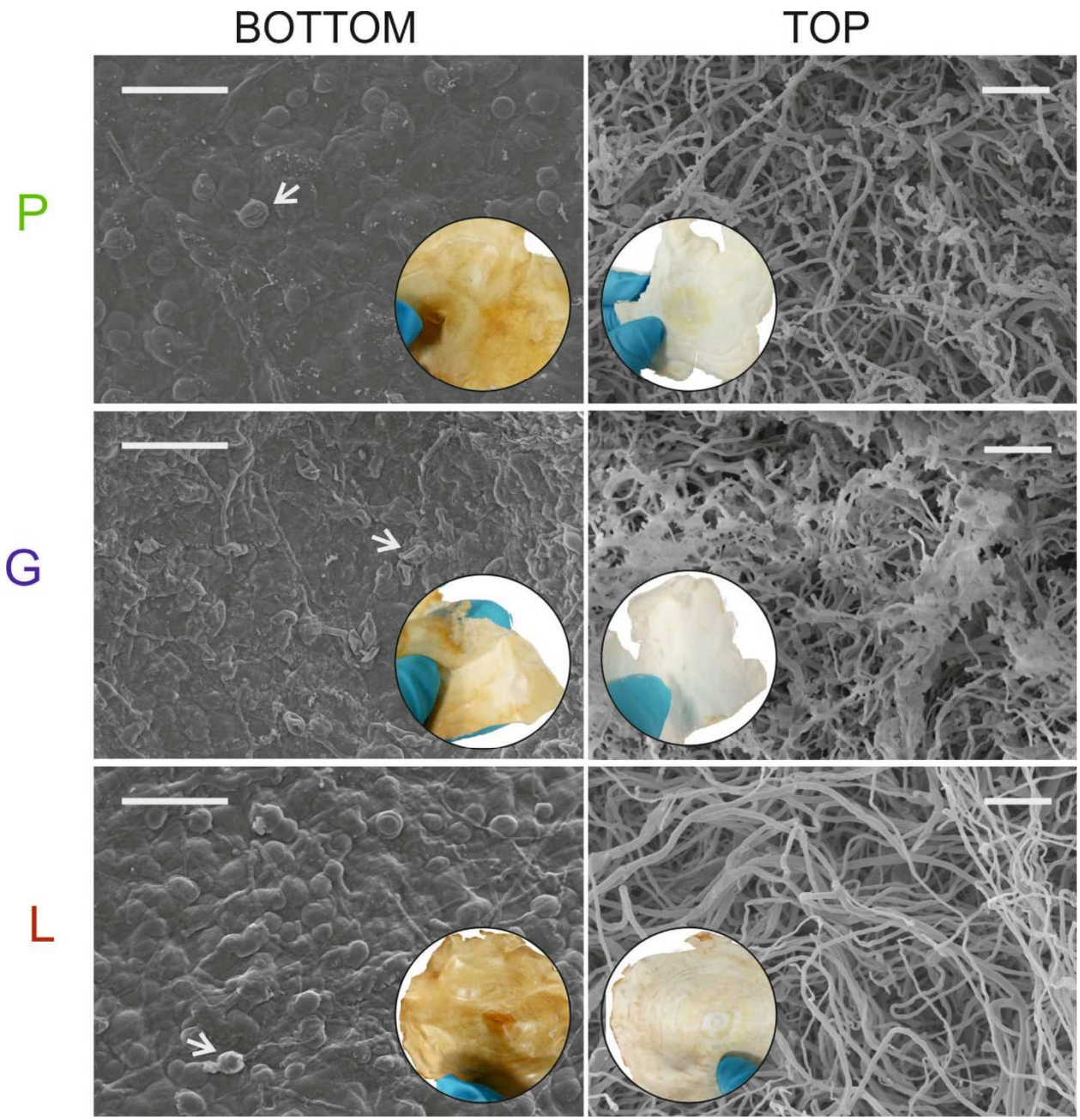

Figure 1.1. Photo-camera pictures (circular insets) and SEM images (main squares) of mycelia grown in three different media (P: only PDB, G: PDB and D-glucose, L: PDB and alkali lignin) for 20 days. Scale bar is 50 μm for SEM images on the left and 10 μm for SEM images on the right. Arrows indicate spores.

As described in the Introduction, mycelial growth occurs only by apical extension, prompted by accumulation of vesicles carrying nutrients and compounds necessary to cell wall building. Branching, on the other hand, occurs laterally in hyphae, seldom from the apex (Webster and Weber). Numerous works have tried to correlate apical extension/branching ratio with nutrients consumption in the medium. Generally, they report that hyphal extension is more employed when mycelia need to expand in the medium in search for fresh resources, while branching enables full utilization of already colonized medium. However, it is also observed that branching can be reduced under favorable nutrition conditions to maintain extension rate (Prosser and Tough). Hyphal morphology shown by L-samples can represent the latter case, where hyphae linearly expand in the medium with a constant rate. On the other hand, branching assures full exploitation of polysaccharide-rich substrate in the other two conditions. Indeed, in media P and G, growth occurs at different spots in the starting phases, which merge in a single, large colony after approximately ten days (Figure 1.2).

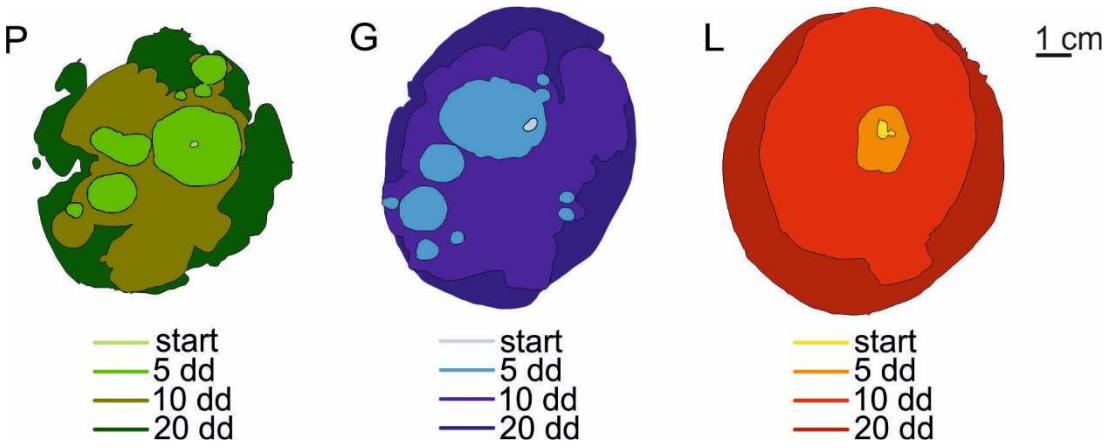

Figure 1.2. Macroscopic patterns of growth of mycelia grown in the three media (P: only PDB, G: PDB and D-glucose, L: PDB and alkali lignin), from inoculum (start) to final time.

On account of this, the rate of increase of the area occupied by the mycelium is the highest for L medium, (5.4±2.6) cm^2/day, and the lowest for P medium, (2.5±0.8) cm^2/day (Figure 1.3a). Moreover, mycelia cultured in L medium expand especially onto the surface of the feeding medium, with respect to the other media, and they reach their final weight within 3 weeks of growth (Figure 1.3b). By 21 days their weight amounts to approximately 300 mg, as for P-samples, even if the latter mycelia cover a reduced area in the plate ([73.8±1.8] cm^2 is the area for L-samples, [68.7±4.7] cm^2 for P-samples, after 21 days). *Ganoderma lucidum* grown in G medium shows the highest final weight, with a value of (506±28) mg (Figure 1.3b). The weight of all the samples in all the media seems to be stable after three weeks of growth.

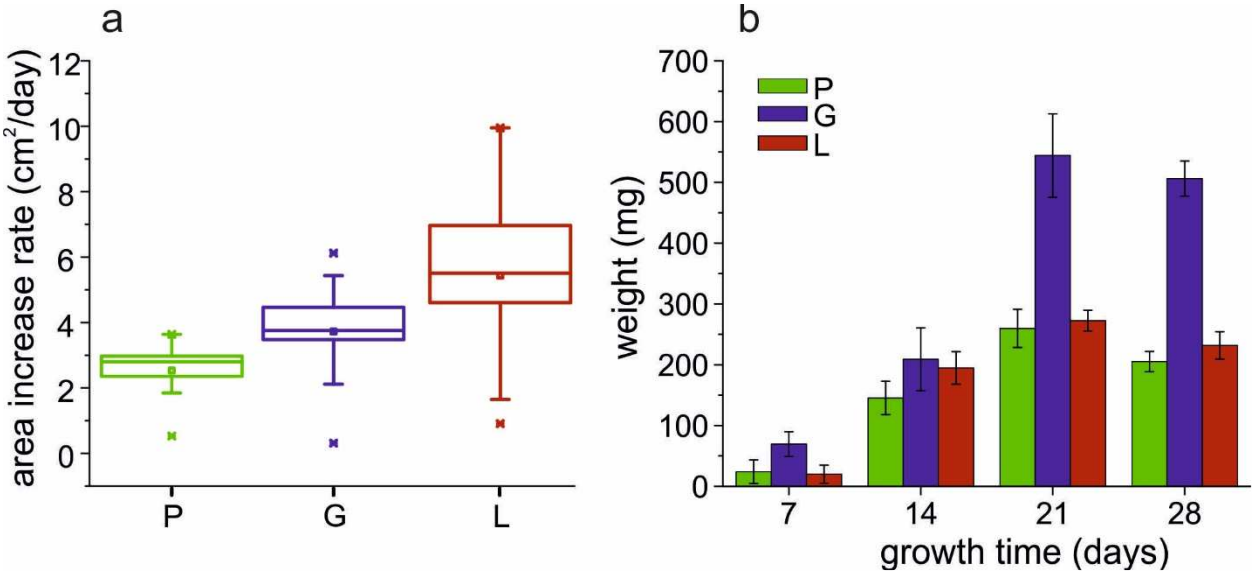

Figure 1.3. Growth trends results. a) Mycelial area increase rate for the different growing media. b) Weight trend vs. growing time in the three media.

The same considerations are valid also when comparing skeletal densities and porosities of mycelia. The highest value of density observed for samples grown in L medium ([1.535±0.005] g/cm^3) can be explained by the prevalence of surface growth, so

that all hyphae concentrate towards the surface. G- and P-samples on the other hand reach an intermediate ([1.438±0.014] g/cm^3) and the lowest ([1.342±0.021] g/cm^3) value respectively thanks to broad branching occurring more in depth in the Petri dish and more extensively in G medium, which is richer in polysaccharides. Indeed, G grown mycelia are thicker and more porous (around 85% of porosity, Figure 1.4b, curves), while L grown mycelia are thinner and less porous (around 68%, Figure 1.4b, curves). G grown mycelia also present pores of larger diameters (from 10 to 50 μm), than the other two types of samples, when analyzed both by SEM and mercury intrusion porosimetry (Figure 1.4a and 1.4b). SEM investigation of samples cross section shows that pores of larger diameter gather in specific areas, probably where mycelium starts concentrating the excess of glucose. For P and L grown mycelia, the pore size distribution is between 1 and 3 μm. Porosity has never been evaluated in detail for mycelium-based materials, but it is an alluring property opening a wide range of applications, from drug delivery to tissue engineering, as investigated in Chapter 2.

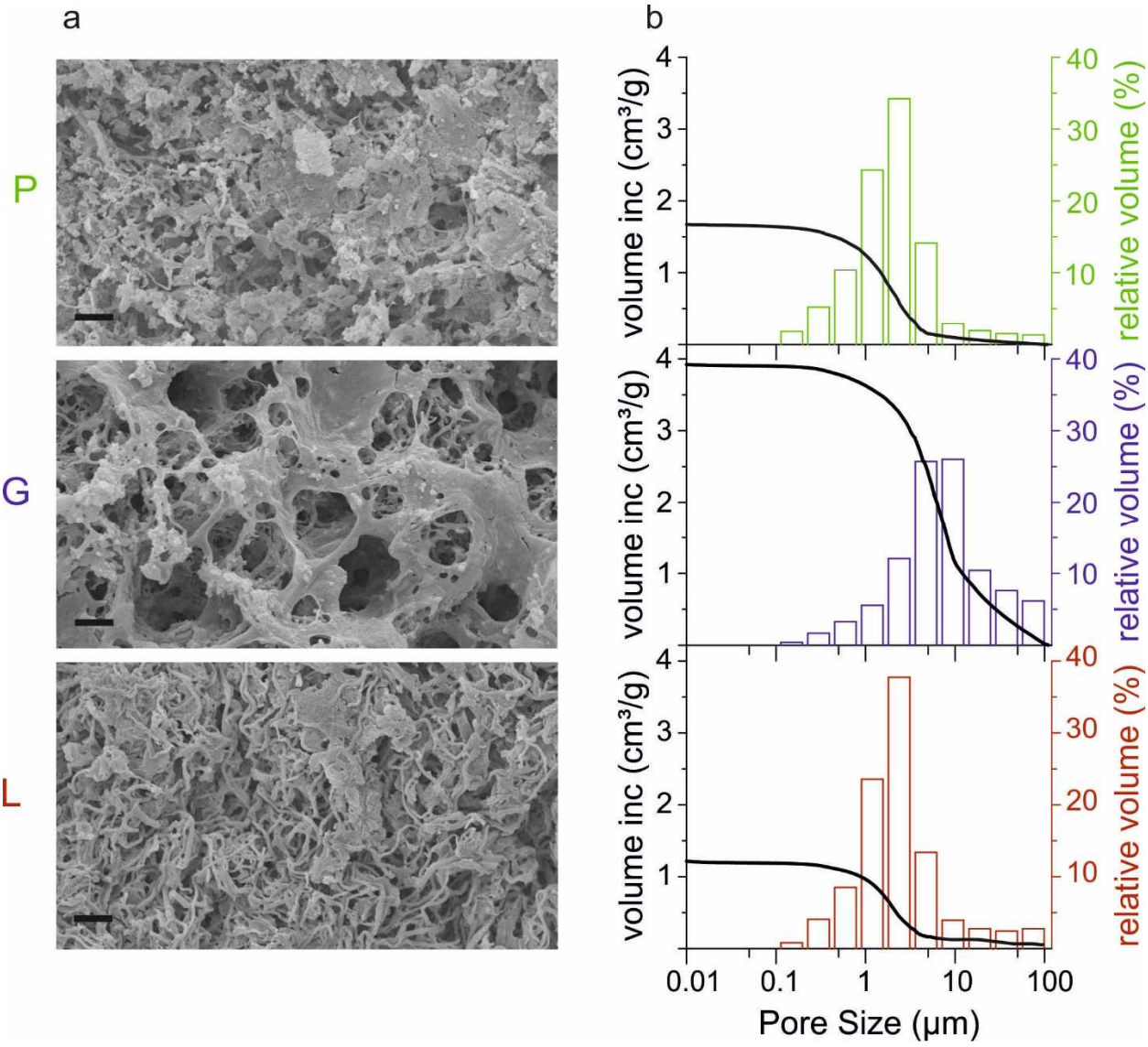

Figure 1.4. Morphological analyses. a) Cross section SEM images of mycelia which show the morphological differences between the different samples. P: only PDB, G: PDB and D-glucose, L: PDB and alkali lignin. Scale bar is 10 µm. b) Pore diameter distribution (bars) and total porosity as volume of mercury intruded (curves) measured by mercury intrusion porosimetry in mycelia grown 21 days.

Chemical characterization

The choice of growth substrates aimed also at varying the chemical composition of mycelium materials, especially with regards to chitin content. As said in the Introduction, this polysaccharide is one of the main structural components of the cell wall and it has been widely studied for applications in bioplastics and bioengineering (Shamshina et al.). Chitin synthase is primarily active at the growing apex tip of hyphae, but chitin synthesis can be enhanced also in response to stress conditions where resistance of cell wall is crucial (Feofilova). In materials science applications, chitin is also considered for the increase in hydrophobicity it causes. In this work, the introduction of alkali lignin in the medium was expected to trigger construction of a diverse cell wall with respect to the other two conditions, where only sugars concentration was varied (Branco). Chemical composition of mycelia was studied both by ATR-FTIR and elemental analysis, with comparable results. Infrared spectra only of the top sides of mycelia are shown. For the bottom sides, in fact, it is difficult to distinguish the contribution of the growth medium from that of the fungal biomass. Figure 1.5a shows some representative spectra of mycelia at different growth times and feeding substrates. Differences in relative height and contribution of each peak are analyzed for the mycelium surface grown in contact with air (top surface in Figure 1.1). In general, main absorption of polysaccharides (O-H stretching mode at ~3250 cm^{-1}, C-O stretching mode at ~1070 cm^{-1}, C-C stretching mode at ~1020 cm^{-1}, and C-H bending mode at ~895 cm^{-1}), lipids (asymmetric and symmetric CH_2 stretching modes at 2935 and 2880 cm^{-1}, respectively, and C=O stretching mode at ~1720 cm^{-1}), proteins (amide I and II at ~1645 and ~1545 cm^{-1}, respectively), and chitin (C-H bending mode at ~1375 cm^{-1}) are identified (Haneef et al.; Wu et al.). Intensity of peak

related to C-C stretching mode at ~1020 cm^{-1} is taken as reference for calculation of contributions of the different biopolymers to the composition of the cell wall. This peak is in fact quite constant across the samples and generally attributable to polysaccharides. In particular, three infrared ratios, namely chitin/polysaccharides, protein/polysaccharides, and lipid/polysaccharides were calculated from the ratio of the intensities of the C-H bending mode at ~1375 cm^{-1}, amide I at ~1645 cm^{-1}, and asymmetric CH_2 stretching mode at 2935 cm^{-1}, respectively, with the reference peak. Ratios are shown in Figure 1.5b. For PDB, the ratios are almost duplicated from 14 to 28 days, indicating a relative increase of chitin, proteins, and lipids with respect to polysaccharides in two weeks of development. On the other hand, for G and L feeding substrates, the increase of the ratios is less intense in the same period. However, while for G substrate the ratios show constant low values, high ratios are calculated for L substrate for all growth periods. The same values are reached by the mycelia grown in the P medium only after 28 days of growth. These differences can be related to a wide variety of factors. First, the increase or decrease of specific metabolic pathways is a function of the acquired nutrients. Thus, D-glucose from G substrate can be directly involved in carbohydrate metabolism, increasing the polysaccharide amount in the cell wall and keeping low the above described infrared band ratios (Holligan and Jennings). In contrast, alkali lignin from L substrate can be depolymerized into lignin monomers and, from there, participate in many other biochemical routes, steadily keeping high the production of chitin, protein, and lipids with respect to other polysaccharides (Brink et al.; Mäkelä et al.). Moreover, the strategy chosen for hyphal network development, either in an extensive or in a more branched manner, determines differential composition of cell

wall. As observed above, L-samples seem to develop according to the former strategy, differently from the other two conditions.

Results from elemental analysis are shown in Figure 1.5c, where the C, O, and N percentages of the samples at different days and feeding substrates are reported. In general, it is observed that C % (with values between 42 and 53%) increases with the growth time, while O % (ranged between 36 and 50%) decreases and N % (that shows values of 0.9-2.3%) is constant or slightly decreased. Despite the similar trends overall, N/C ratio is the lowest for G samples, being 0.019±0.002, while it is 0.038±0.001 for P samples and 0.025±0.008 for L ones. Elemental analysis thus confirms the differences in polysaccharides and protein production already observed by infrared band ratios.

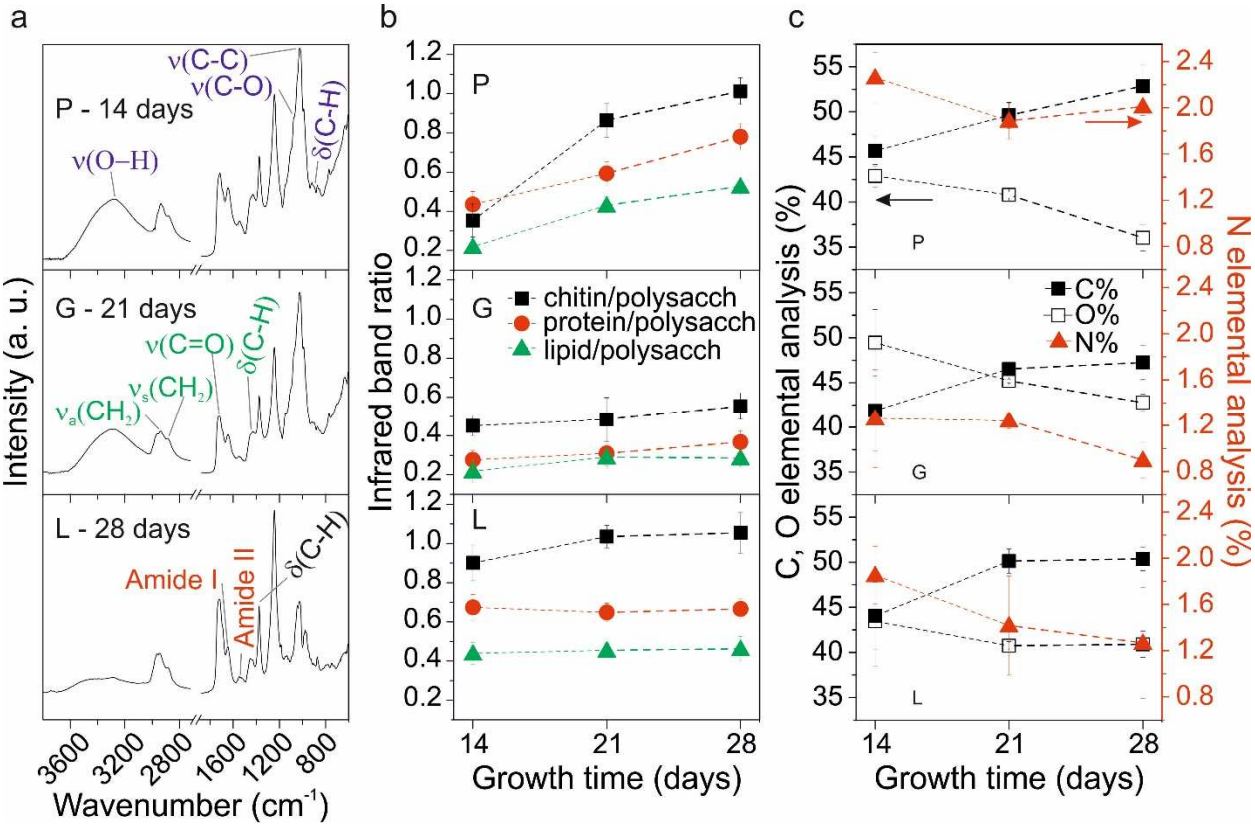

Figure 1.5. a) ATR-FTIR spectra of mycelium samples growth on a P substrate after 14 days, G substrate after 21 days, and L substrate after 28 days. Main assignations of polysaccharides (blue), lipids (green), proteins (red), and chitin (black) are included. b) infrared band ratios between chitin and polysaccharides (black squares), protein and polysaccharides (red circles), and lipid and polysaccharides (green triangles) of the spectra from samples growth in different substrates (P, G, and L ones) at different days (14, 21, and 28 days). c) carbon (black filled square), oxygen (black unfilled square), and nitrogen (red filled triangle) percentages determined by elemental analysis of samples growth in different substrates (P, G, and L ones) at different days (14, 21, and 28 days).

Hydrodynamic characterization

Chemical composition highly influence hydrodynamic features, which are measured both by moisture uptake and static water contact angle measurements. Mycelia grown in G medium adsorb more humidity than those grown in L and P media (Figure 1.6a). More specifically, when collected after 28 days of growth, G mycelia can adsorb moisture up to (58±10)% of their weight, while L and P adsorb only up to (31±3.0)% and (37±3.0)% respectively. The high content in polysaccharides with respect to proteins and chitin can explain these results for G-samples. Moreover, these mycelia, as shown in Figure 1.4b, have the highest porosity, which further enhances moisture uptake capabilities. On the other hand, values of water contact angle are very similar for the different mycelia samples, showing that all the samples are hydrophobic with values around 120° (Figure 1.6b). It is very likely that differences in chemical composition are not so large to determine a different behavior at the surface. Moreover, hydrophobicity depends strongly on surface roughness and on the air gaps present on the surface, which cannot be very

different given the highly porous structure of all the samples (Mazzon et al.; Zahid et al.). Moisture adsorption is more informative in this sense since it does not regard only the surface but the overall material.

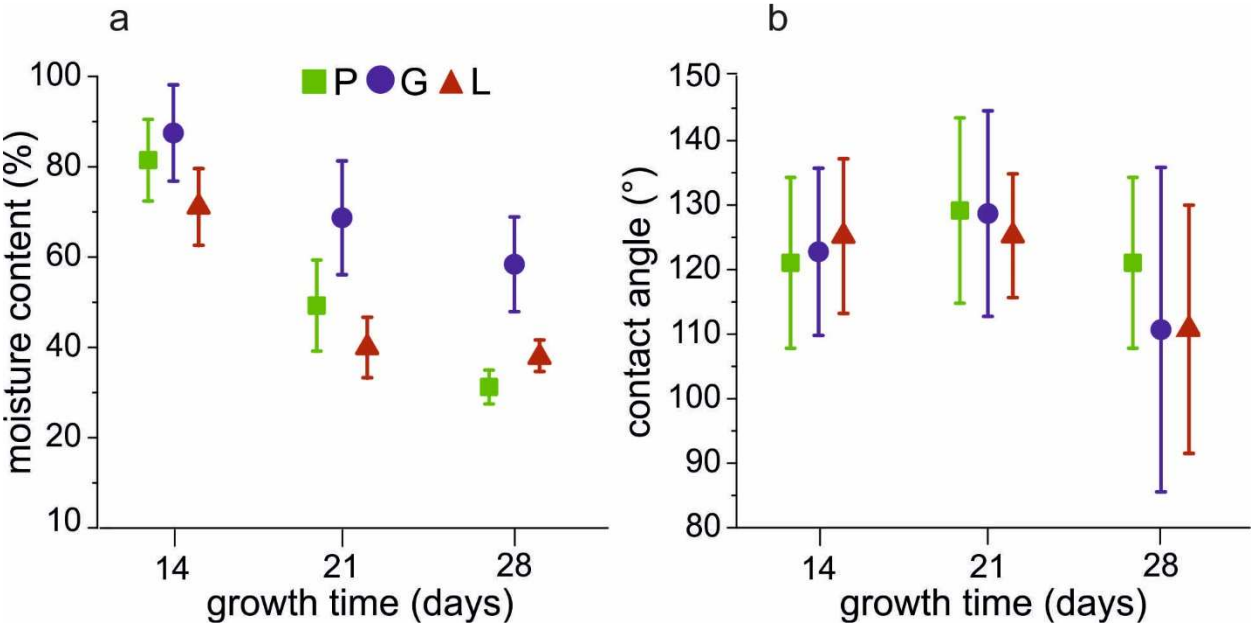

Figure 1.6. Hydrodynamic properties. a) Moisture content of samples kept 24 hours in water humidity chamber. Samples have been collected after 14, 21 or 28 days of growth. b) Static water contact angle measured on mycelial surface grown in contact with air. Samples have been collected after 14, 21 or 28 days of growth.

Mechanical characterization

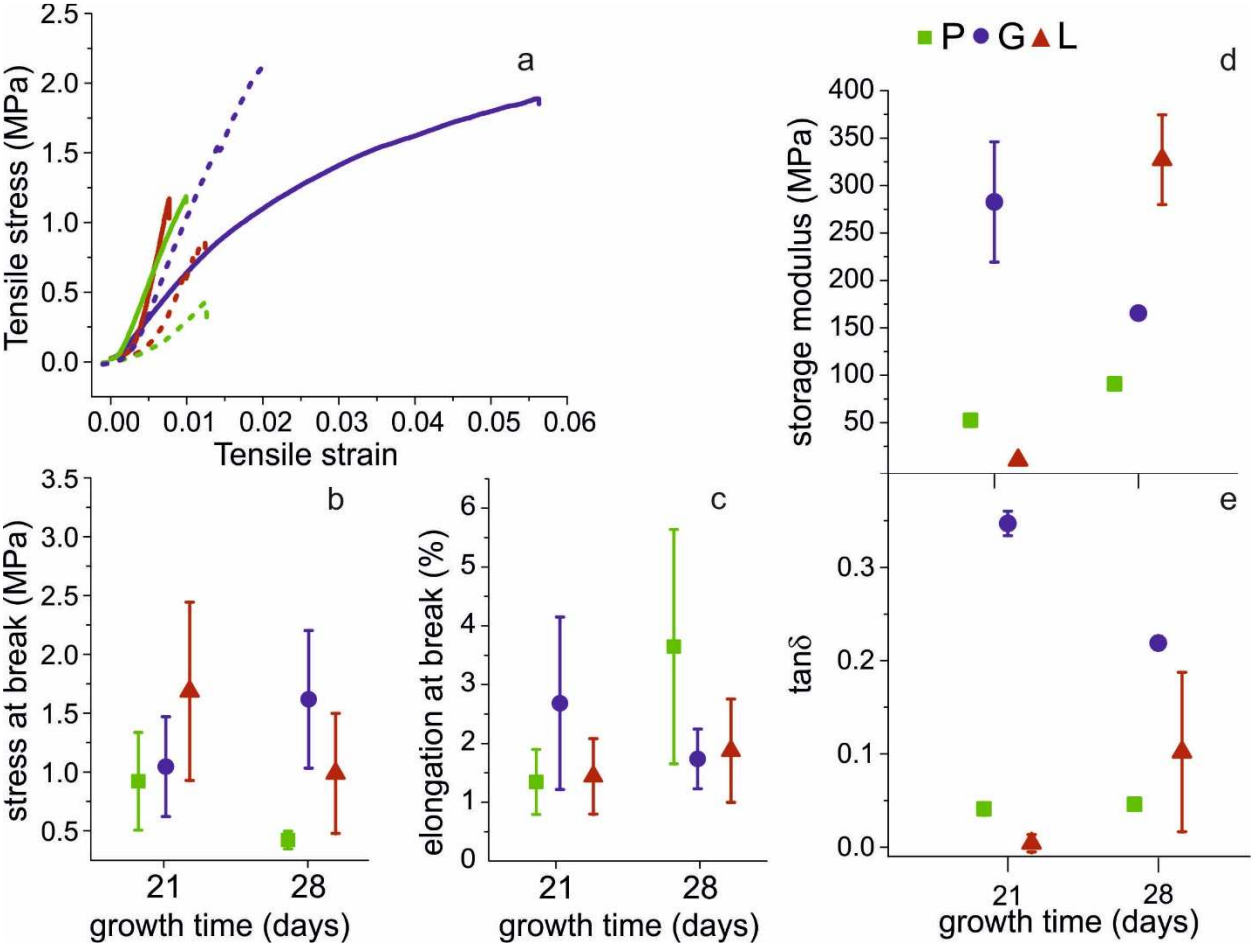

Figure 1.7. Mechanical properties. a) Stress strains curves of different samples collected after 21 (straight lines) and 28 days (dotted lines), analyzed by tensile test. b,c) Average stress and elongation at break of different samples collected after 21 and 28 days, from tensile test curves. d,e) Storage modulus and tanδ calculated by DMA on mycelia collected after 21 or 28 days of growth.

Mechanical properties were characterized both by tensile tests and DMA (Figure 1.7). As fibrous materials, in fact, mycelial response to stress is complex to analyze due to

random orientation of the fibers with respect to the force. Moreover, mycelia are structurally inhomogeneous in terms of fiber distribution and orientation, and thickness, which adds a stochastic character to the mechanical response, especially for large deformations and at the onset of the fracture. For these reasons, tensile test commonly used for mechanical characterization did not provide reproducible results, as it can be observed from the diversity of curves which can be obtained for the same sample (Figure 1.7 a). Large error bars make difficult to compare results in different conditions and among samples (Figure 1.7b and c). Dynamic mechanical analysis, on the other hand, allows to get reliable data, as the strain applied is much lower (<0.1% with respect to a tensile test), so that local inhomogeneity and fibers orientation do not affect the overall response. The results obtained with DMA (Figure 1.7d and e), despite the large scatter, can be correlated with the external and touch appearance of the mycelia. After 21 days of growth, G grown mycelia have the highest storage modulus and the highest energy dissipation: their tanδ is indeed ten times higher (0.347±0.013) than for P (0.041±0.006) mycelia and 100 times higher than for L (0.004±0.009) ones. Storage modulus correlates with the ability of the material to store deformation in an elastic manner, while tanδ is related to its damping (Jacob et al.), so G mycelia appear very ductile at this time of growth. However, when collected after 28 days of growth, they start becoming more brittle since their storage modulus diminishes from (612±48) MPa to (283±63) MPa and their tanδ to 0.219±0.007. On the other hand, P mycelia keep more or less the same mechanical characteristics regardless the harvesting time, while L mycelia increase their storage modulus 30 times up to a value of (327±47) MPa when collected after 28 days with respect to previous harvesting. However, they are still more brittle than G mycelia since they have a lower

tanδ. These results suggest that mechanical properties are strongly influenced by the time of collection and they must be evaluated for each condition. G samples in particular are the only ones that become more brittle with the time. This could be due to the stronger influence that humidity has on them. Water indeed has a plasticizing effect and moisture capacity is the highest for mycelia grown with more glucose, as reported in Figure 1.6a. This capacity diminishes with the time even for G samples, influencing also the mechanical properties as observed. Overall, the apparent mechanical properties are worse than those of other common biopolymers produced by microorganisms, as polyhydroxyalkanotes (Ten et al.) or bacterial cellulose (Ullah et al.; Khattak et al.), which are reported to have Young's modulus in the order of GPa (Jariyasakoolroj et al.). This is mainly due to the fact that mycelia are not bulky polymers but low density networks: indeed, mechanical measurements performed by AFM on single fibers yield much larger values (Haneef et al.). Moreover, the chemical composition is not homogeneous but composed of different macromolecules that cannot form very ordered structures. These are the less promising properties for pure mycelia materials.

Conclusions

In this Chapter, development and tuning of the properties of pure *Ganoderma lucidum* mycelium were studied when slightly varying the components of the feeding substrate. It was observed that even small modifications in the composition of a common fungal growth medium determine significant changes in various mycelium properties. In particular, *Ganoderma lucidum* mycelia were cultured on PDB, a standard fungal medium (P medium), and on PDB with the addition of either D-glucose or lignin (G and L media, respectively), and their structural, chemical, hydrodynamic and mechanical properties were compared. The surface area of mycelia grown in L medium expands faster and the final material appears denser with elongated filamentous features, while G-grown mycelia are the most porous and thick. As expected, G-grown mycelia were also more hygroscopic than the rest, absorbing more humidity in a vapor-saturated atmosphere. Water contact angle was comparable for all the mycelia indicating the development of hydrophobic materials. Mechanical properties, even if hard to evaluate considering the inhomogeneous structure of the mycelia, were also affected by the substrate composition and influenced especially by the amount of glucose, which turns the mycelia more ductile.

Chapter 2

Ganoderma lucidum and *Pleurotus ostreatus* as Potential Self-Growing Biomedical Scaffolds

Introduction

Cells in organs and tissues receive functional and structural support from the extracellular matrix (ECM), composed several macromolecules (Frantz et al.). ECMs differ in terms of porosity, mechanical properties and biochemical cues for attachment, depending on the types of cells they support and the tissues in which they reside (Stevens and George). In particular, carboxyl, sulfate and hydroxyl groups exhibited by glycosaminoglycans and the aminoacidic RGD motifs presented on ECM proteins (*i.e.* collagen and fibronectin) are the main responsible for cell attachment (Rozario and DeSimone).

In the last decades, several efforts have been made in the field of tissue engineering to mimic this diversity and achieve specific tools to support the regeneration of human tissues. In particular, functional bioscaffolds should have a suitable surface chemistry and topography to promote cell adhesion; they should be porous, to allow cell communication and exchange of oxygen and nutrients; they should have good mechanical properties to ensure cell attachment and growth; they should be biodegradable in many applications (Biswal). Techniques such as electrospinning, freeze-drying and 3D printing have been

developed and extensively employed to produce materials with these characteristics. These top-down approaches allow a fine and accurate fabrication of suitable porous structures but require the use of solvents and sophisticated instrumentation (Doostmohammadi et al.; Subbiah and Guldberg; Y. Zhang et al.). The introduction of biopolymers has ensured a big step forward in the production of functional and smart scaffolds. Biodegradable polymers, such as PCL and PLA, succeeded in matching the requirements for biodegradable materials with prolonged life-time and mechanical resistance inside the body. On the other hand, the chemical moieties available in these polymers are not capable to actively promote cellular interactions and proper functionalization must be performed in additional steps (D. Mondal et al.). Polysaccharides and protein polymers, such as chitin (Campuzano and Pelling), alginate (A. Mondal et al.), hyaluronic acid (Tolg et al.), silk (Lee et al.), and keratin (Giuri et al.) display biochemical guidance for cell migration, overcoming the lack of targeting for the attachment, typical of the aforementioned synthetic polymers. However these polymers are usually complex to extract or process (Suarato et al.).

In this Chapter, mycelia are proposed as bioscaffolds thanks to the combination of their structural (porous) and chemical, hydrodynamic and mechanical properties. As reported in the Introduction (table 0.2), mycelium based materials developed up to now, never employed both the structure and the properties of mycelia. The novelty of this work stays in the exploitation of both these features in the final material and in the application in the biomedical field. Up to now, solely extracts from mycelia have been used in medical applications and tested for biocompatibility. Indeed, several fungal strains, including *Ganoderma lucidum*, are reported to secrete bioactive polysaccharides, which can be

extracted either from the growth medium or directly from the mycelial network using strong bases or acids (Wasser). Membranes produced by simply casting these extracts, such as the Saccachitin reported by (Su et al.) and the Rhizochitin produced by (Chien et al.), showed positive effects on fibroblast and keratinocytes growth. Here, mycelia from *Ganoderma lucidum* and *Pleurotus ostreatus* are directly employed as a valid and sustainable alternative for the controlled fabrication of all-natural, self-grown and low-cost biomedical scaffolds.

Objectives

In this chapter, mycelia of *Ganoderma lucidum* and *Pleurotus ostreatus* are tested as suitable bioscaffolds for fibroblasts growth. After growth in PDB, they are analyzed for their morphological, chemical, hydrodynamic and mechanical properties. A proper inactivation of mycelia to ensure sterility of these materials is also studied. Biocompatibility is tested on Human Dermal Adult Fibroblasts (HDFAs) by two assays, to analyze possible production of cytotoxic compounds by the mycelia in cell medium and achievability of contact by the cells with the mycelia themselves. Direct plating of HDFAs on *Pleurotus ostreatus* mycelia, functionalized or not with Fibronectin (FN), is also performed.

Materials and Methods

Strain, media and growth conditions

Ganoderma lucidum DSM9621 and *Pleurotus ostreatus* DSM11191 active cultures were purchased from DSMZ (Germany) and maintained in 100 mm Petri dish with Potato Dextrose Broth (PDB, Merck) as growth medium, transferring the culture to fresh medium every 30 days. A piece of 20 day-grown mycelium was inoculated in 100 mm Petri dishes containing 30 mL of PDB at 24 g/L in water. Media were autoclaved before use, at 120 °C for 20 minutes by SYSTEC-VX 40. Mycelia were incubated in a climatic chamber (Memmert, HPP 260) at 27°C and 78% water humidity, in the dark.

Material preparation

After 20 days of growth, when the whole surface of the plate was covered, mycelium was collected, cleaned from the substrate with a spatula and clean deionized water. Mycelia were then either dried for 15 hours at 50°C in an oven or autoclaved at 120 °C for 20 minutes. Autoclaved mycelia were then dried under a laminar fume hood and illuminated for approximately 100 minutes with UV light.

SEM and TEM analyses

Mycelia were fixed in a solution of 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 hours at room temperature. After several washes in the same buffer, the samples were post-fixed in 1% osmium tetroxide in MQ water for 2 hours and washed with MQ water. Mycelia were subsequently dehydrated with a series of 10-minute incubations in rising

concentrations of ethanol in water solutions (from 30% to 100%), 1:1 ethanol:hexamethyldisilazane (HMDS, Sigma-Aldrich) and 100% HMDS and dried overnight in air. Finally, the samples were sputtered with a 10 nm gold layer and analysed using a JEOL JSM-6490LA Scanning Electron Microscope (SEM) equipped with a tungsten filament and operating at 10KV of accelerating voltage.

For TEM analysis, after fixation with glutaraldehyde as the previous description, mycelia were post-fixed in 1% osmium tetroxide in MQ water for 2 hours and stained overnight at 4 °C in an aqueous 0.5% uranyl acetate solution. After several washes in MQ, the samples were dehydrated in a graded ethanol series and embedded in SPURR resin. Sections of about 70 nm were cut with a diamond knife on a Leica EM UC6 ultramicrotome. Transmission electron microscopy (TEM) images were collected with a Jeol JEM 1011 (Jeol, Japan) electron microscope and recorded with a 2 Mp charge-coupled device camera (Gatan Orius)

Density and porosity

Skeletal density was measured by helium pycnometry Thermoscientific Pycnomatic Evo with a 44 cm^3 chamber. Measurements were performed at 20 °C. The real (or skeletal) density is the sample mass referred to the sample volume, excluding all pores and void volumes but considering “closed” pores (*i.e.* cavities within the material that cannot be reached by any gas). Skeletal density was measured by detecting the change in pressure due to the volume of helium that is displaced by the sample within the sealed and pressure-equilibrated chamber. Helium is a tiny atom that can permeate even extremely narrow pores in a solid, thus permitting the determination of the real volume

occupied by that solid. The ratio of the dried mass and its volume gives the real density of the material under test as a result. Ten measurements were averaged for each sample. Porosity was determined by mercury intrusion porosimetry (MIP) performed with Pascal 140 Evo and Pascal 240 Evo mercury porosimeters (Thermo Scientific). In this technique, the sample is entirely embedded by mercury. The pressure is then increased so that mercury starts entering in the pores. The total porosity (estimated by MIP) is related to the volume of mercury totally intruded at the end of the measure. The porosity, in fact, is expressed as the ratio between the pore volume (inner cavities volume) and the external sample volume, while the pore size distribution depends on the volume of mercury intruded at each pressure range. The pressure of mercury intrusion was set at 0.0136 MPa and continuously increased up to 200 MPa, with a rate of 6–14 MPa min^{-1}. The contact angle of mercury with the samples and the surface tension of pure mercury were assumed to be 140° and 0.48 N m^{-1}, respectively. Washburn equation was used to calculate the pore size from the applied pressure, assuming that the pores are of cylindrical shape.

Chemical analysis

Infrared spectra of samples were obtained with an attenuated total reflection (ATR) accessory (MIRacle ATR, PIKE Technologies) coupled to a Fourier transform infrared spectrophotometer FTIR spectrometer (Vertex 70v FT-IR, Bruker). All spectra were recorded in the range from 3800 to 600 cm^{-1}, with 4 cm^{-1} resolution, accumulating 64 scans. The samples were gently placed on a spot of ATR accessory and slowly pressed, with the part grown in contact with the substrate (named “bottom”) on the ATR crystal. To

ensure the reproducibility of the obtained spectra, three samples of each type were measured. Spectra analysis was performed with Origin pro 2016 software.

Hydrodynamic characterization

A contact angle (CA) goniometer (DataPhysics OCAH 200) was used for static water contact angle measurements at room temperature. Five µL droplets of water were deposited on the corresponding surfaces and side-view images of the drops were captured after 60 s. CA were automatically calculated by fitting the captured drop shape. Up to 15 contact angle measurements were carried out on every sample at random locations and their average values and standard deviation were reported. Contact angle was measured after a conditioning in either a dry or a humid environment (*i.e.* conditioned for 24 hours at 100% RH). For the water uptake, dry samples were weighed on a sensitive electronic balance and then placed in different humidity chambers. Samples were dried by conditioning in a desiccator for 24 hours, weighed, and then transferred in 100% humidity conditions for additional 24 hours, before being weighed further. The amount of adsorbed water was calculated based on the initial dry weight.

Mechanical characterization

Samples were cut into 20 × 35 mm^2 rectangles and tested at room temperature, after 24 hours conditioning at 100% RH. Tensile stress curves were obtained by a dual column universal testing machine (Instron 3365): samples were mounted on the machine clamps and deformed at a rate of 1 mm/min until failure. The Young's modulus E, ultimate tensile

strength UTS and elongation at fracture were extracted from the stress-strain curves. Storage modulus E' and tanδ (*i.e.* the ratio between loss and storage moduli, representing the relative energy dissipation) were also measured with a Q800 DMA testing machine (TA Instruments), in uniaxial tensile mode, applying a sinusoidal deformation with an amplitude of 20 μm, and frequencies of 7, 10 and 16 Hz.

Mycelia extract characterization

In vitro biocompatibility

Primary human dermal fibroblasts (HDFa, Thermo Fisher Scientific) were used as a cellular model to investigate the biocompatibility of the grown mycelia. After being cultured in T75 flasks in the presence of Medium 106 supplemented with LSGS Kit (Thermo Fisher Scientific), cells were seeded onto 24-well plates at a density of 7000 cells/cm^2 and let attach overnight in an incubator at 37°C and with 5% CO_2. *P. ostreatus* and *G. lucidum* extracts were prepared as following. Autoclaved mycelia were cut in pieces of about 20 mg and sterilized under the UV light for 20 minutes (10 minutes per side). To remove the excess of PDB from the fungi matrices, the pieces were immersed in sterile potassium phosphate buffer (PBS, pH 7.4, Gibco) and incubated at 37 °C for 24 hours. A second washing was performed with fresh PBS for additional 24 hours. Afterwards, each 20-mg mycelia piece was incubated with 1 mL of Medium 106 for the following 24 hours and the resulting stock solutions were used to prepare the tested dilutions (1:2, 1:3 1:4 1:20, 1:40, 1:100). Attached HFDa cells (at passages 4-6) were treated with mycelia extracts for 24 hours, while cells incubated in normal Medium 106 + LSGS were considered as controls.

MTS assay (CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay, Promega) was conducted to determine cell viability. Briefly, all samples were incubated in fresh culture media (500 µL) and 25 µL of reagent were added to each well. After 3.5 hours of incubation, absorbance readings at 490 nm were recorded. Three independent experiments were carried out in triplicates. A Student's *t*-test, assuming unequal variances, was carried out, considering a $p < 0.01$ value.

To further assess the biocompatibility of the mycelia matrices, a semi-contact assay was performed. Primary human fibroblasts (at passages 4-6) were seeded onto 24-well plates at density of 7000 cells/cm^2 and let attach overnight. *P. ostreatus* and *G. lucidum* were cut in small pieces (with weights ranging from 1 to 5 mg) and washed and sterilized as previously described. The next morning, fresh culture medium (1 mL) was replenished and a piece of mycelium was gently immersed in each well and let float for additional 24 hours. Afterwards, the matrices were carefully removed, paying attention not to perturb the layer of attached cells at the bottom of the well, and the MTS assay was carried out.

In order to visualize the morphology of the fibroblasts subjected to the various treatments, cells were plated onto glass coverslips at a density of 5000 cells/cm^2 and treated as described above. Afterwards, cells were washed with pre-warmed PBS and fixed with 3.7% paraformaldehyde for 20 minutes. A DAPI solution (2.5 µg/mL) was used to stain the cell nuclei (15 minutes in the dark). To allow the actin fibers staining, fibroblasts were permeabilized with 0.3% Triton X-100 (8 minutes), prior to incubation in Alexa Fluor 488 Phalloidin (Thermo Fisher Scientific, 1:100 dilutions in PBS) for 20 minutes in the dark. The prepared coverslips were then mounted onto glass slides with Fluoromont-G and imaged with a confocal microscope Nikon A1.

Cell plating onto the P. ostreatus matrices

With the aim of investigating the mycelia suitability as substrates for cell attachment and growth, human primary fibroblasts were used as a challenging platform. Briefly, mycelia matrices were cut into round pieces (1 cm in diameter) and sterilized/washed, as reported in the previous paragraph. The substrates were placed at the bottom of a 24-well plate and kept firm with sterile PDMS rings (outer diameter = 1.5 cm, inner diameter = 0.8 cm). Some of the matrices were incubated with 400 μL of fibronectin (20 μg/mL, Fibronectin Human Protein, Thermo Fisher Scientific) for 1 hour at 37 °C, while other matrices were left uncoated and incubated in sterile PBS. After fibronectin/PBS removal, the matrices were dried for 2 hours under a sterile hood and seeded with cells at a density of 5000 cells/cm^2. After 48 hours of culture, samples were fixed in 3.7% paraformaldehyde. In order to partially block the autofluorescence signal of the fungal substrate, all the staining solutions (DAPI and Alexa Fluor 546 Phalloidin) were prepared in 1% Bovine Serum Albumin and the samples were processed as described above. The stained and glass-mounted substrates were imaged with a confocal microscope Nikon A1, equipped with a 560 nm laser. Images were acquired with both PMT and spectral detector (spectral acquisition from 541 nm to 679 nm, grating resolution of 6 nm). The image analysis (spectral unmixing, ROI definition and spectral profiling) was carried out with ImageJ (https://imagej.nih.gov).

SEM imaging of the P.ostreatus cell scaffolds

P. ostreatus matrices, prepared as above-mentioned and seeded with HDFa cells for 48 hours, were treated as reported above and observed by JEOL JSM-6490LA Scanning

Electron Microscope equipped with a tungsten filament and operating at 10 kV of accelerating voltage.

High resolution UPLC-Mass spectrometry

The samples were dried under nitrogen and then reconstituted in 10% acetonitrile in water. Five microliters of these samples were then injected in an Acquity UPLC liquid chromatography system coupled with a Synapt G2 QToF high-resolution mass spectrometers (both from Waters, Milford, MA, USA). The analytes were then separated on a BEH (2.1 X 100 mm) reversed-phase column (Waters) using a linear gradient of acetonitrile in water (5 to 100%). The eluting compounds were analyzed by high-resolution mass spectrometry in both positive and negative ion electrospray modes. Leucine Enkephalin reference standard was used as lock-mass to achieve a mass accuracy below 5 ppm. Metabolites were tentatively identified by interrogating the publicly available HMDB (Human Metabolome Database) and LipidMaps reference databases.

Results and Discussion

Growth, harvesting and inactivation of mycelia

The fungal mycelia were grown in potato dextrose broth (PDB) in 10 mm diameter Petri dishes at 27°C and 78% R.H. Mycelia were harvested as soon as they had completely covered dish surface (approximately after 20 days) to collect fully developed hyphal network, but to prevent aging of mycelia. Fungal mats were cleaned from substrate and autoclaved (Figure 2.1 a-d). In Chapter 1, mycelia inactivation was obtained just by heating overnight at 50°C, following the procedure usually employed for mycelium-based materials (Elsacker et al.; Lelivelt et al.). Since biomedical applications are considered in this work, any possibility of mycelium regrowth in biological conditions has to be excluded. Moreover, heath-resistance of *Ganoderma lucidum* has been reported (X. Zhang et al.), differently from *Pleurotus ostreatus* (Yan et al.). For this reason, a control experiment was performed in which pieces of oven-dried (50°C for 15 hours) and autoclaved mycelia were placed back in contact with PDB. Occasional regrowth of oven-dried *Ganoderma* mycelia was observed, differently from *Pleurotus* samples and from all the autoclaved mycelia (Figure 2.1 f,h). Autoclave did not damage the structural organization of hyphal networks (cell wall) but it affected only the biological inner cell organization, as observed by TEM images. In particular, after the oven treatment (Figure 2.1 m,n), the inner cell organization was comparable to the control (Figure 2.1 i,l). After autoclaving (Figure 2.1 o,p), the hyphal cells content drastically changed: large white areas appeared inside the cells, probably due to plasmolysis, and vacuoles formed as a consequence of the heat and pressure shocks (Pera and Callieri). However, the main structural component, the cell wall (CW), was still intact in both mycelia, even if it slightly shrunk in *Pleurotus*. The cell

diameter was comparable between oven-dried and autoclaved mycelia, suggesting that cell shrinkage, usually reported for fungi undergone strong thermal treatment, did not occur in this case (Qiu et al.). Given these results, the autoclave was chosen as post-growth treatment.

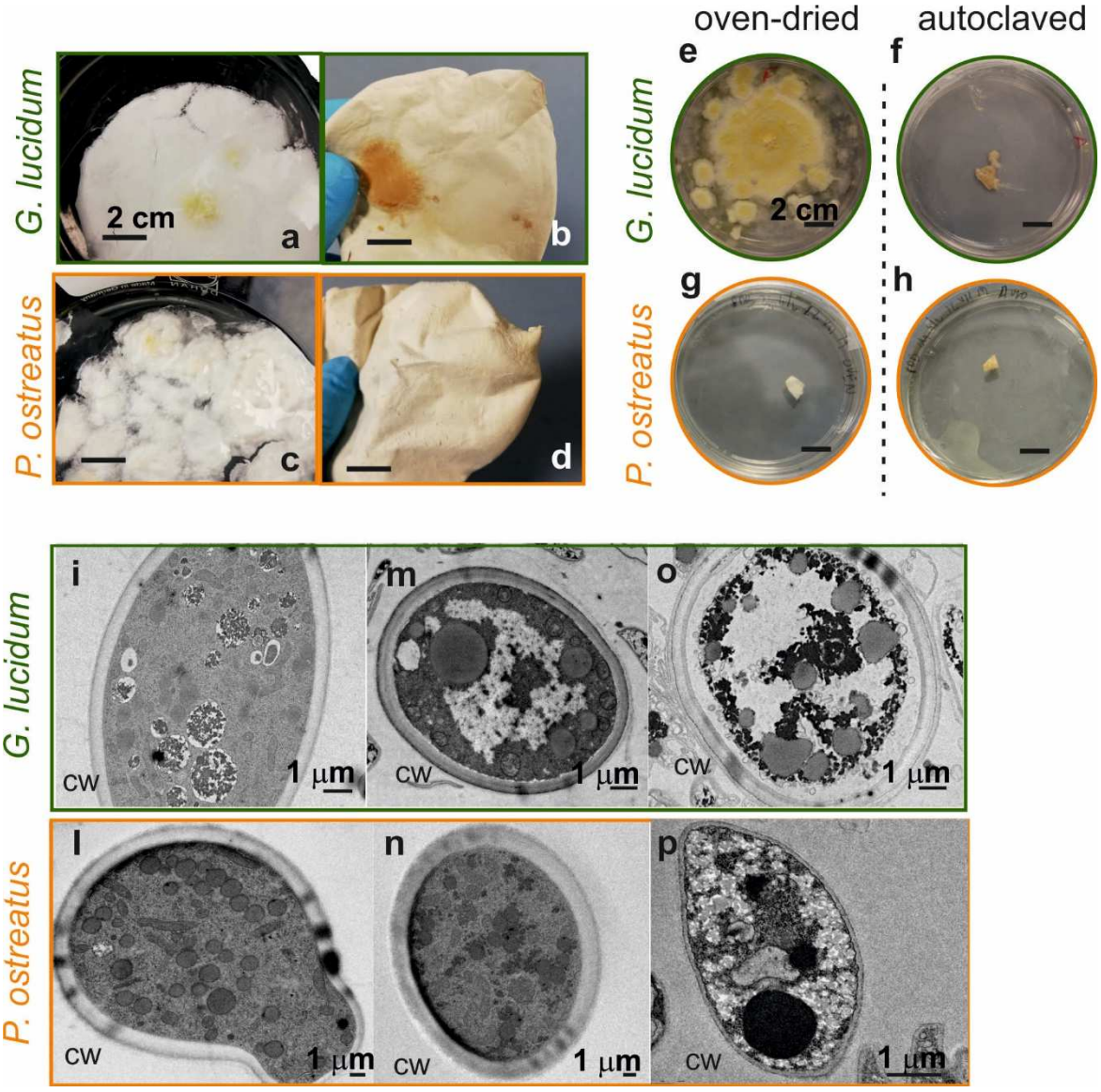

Figure 2.1. Effect of autoclave on mycelium materials. (a-d) Photographs showing the macroscopic aspect of the two types of mycelia under study after their growth without further treatment (a,c), and (b,d) after autoclave. (e-h) "Re-growth" experiments with

oven-dried and autoclaved mycelia (i-p) TEM images of mycelia as grown (i,l), dried in oven (m,n) and autoclaved (o,p), *CW=cell wall*.

Morphological characterization

Hyphal structure was investigated by scanning electron microscopy (SEM) and hyphae diameters were calculated (Figure 2.2k-n). The same morphological differences already observed in previous works from *Smart Materials* group were observed (Haneef et al.): fibers are randomly oriented in both strains, but in *Ganoderma lucidum* two kinds of hyphal structures are noticeable, a *tube-like* short one, and a long smoother one, defined as *thread-like*, while *Pleurotus ostreatus* mycelia are only composed by the latter kind. Distribution of hyphal diameters is comparable to what previously reported for oven-dried mycelia, too: *Pleurotus* hyphae are larger, measuring on average (1.5 ± 0.4) μm, while *Ganoderma* thread-like ones average at (0.7 ± 0.2) μm (Figure 2.2o).

Porosity is a key parameter in scaffold design since it greatly affects cell migration, oxygen regulation and nutrient exchange (Bružauskaitė et al.). Mycelial porosity was measured by mercury intrusion porosimetry (MIP, see Experimental Methods section), preceded by skeletal density measurements by helium picnometry (Figure 2.3a-b). Picnometry results are compared to those obtained by mercury intrusion to avoid false positives given by pore opening under the high pressures characteristic of MIP. In MIP, in particular, determination of total porosity depends on the whole amount of mercury volume intruded (black curve in Figure 2.3 a,b), while information on different pore size is calculated from the amount of volume intruded at each pressure (bars in Figure 2.3 a,b).

Pleurotus 3D network results denser ([1.48 ± 0.03] g/cm^3) than *Ganoderma* ([1.34 ± 0.02] g/cm^3), and it is also characterized by a higher over-all porosity (85%) than that of its counterpart (68%). The pore size distribution shows that the majority of mercury is intruded in pores between 1 and 5 µm for *Ganoderma*, while in holes between 7 and 20 µm for *Pleurotus*.

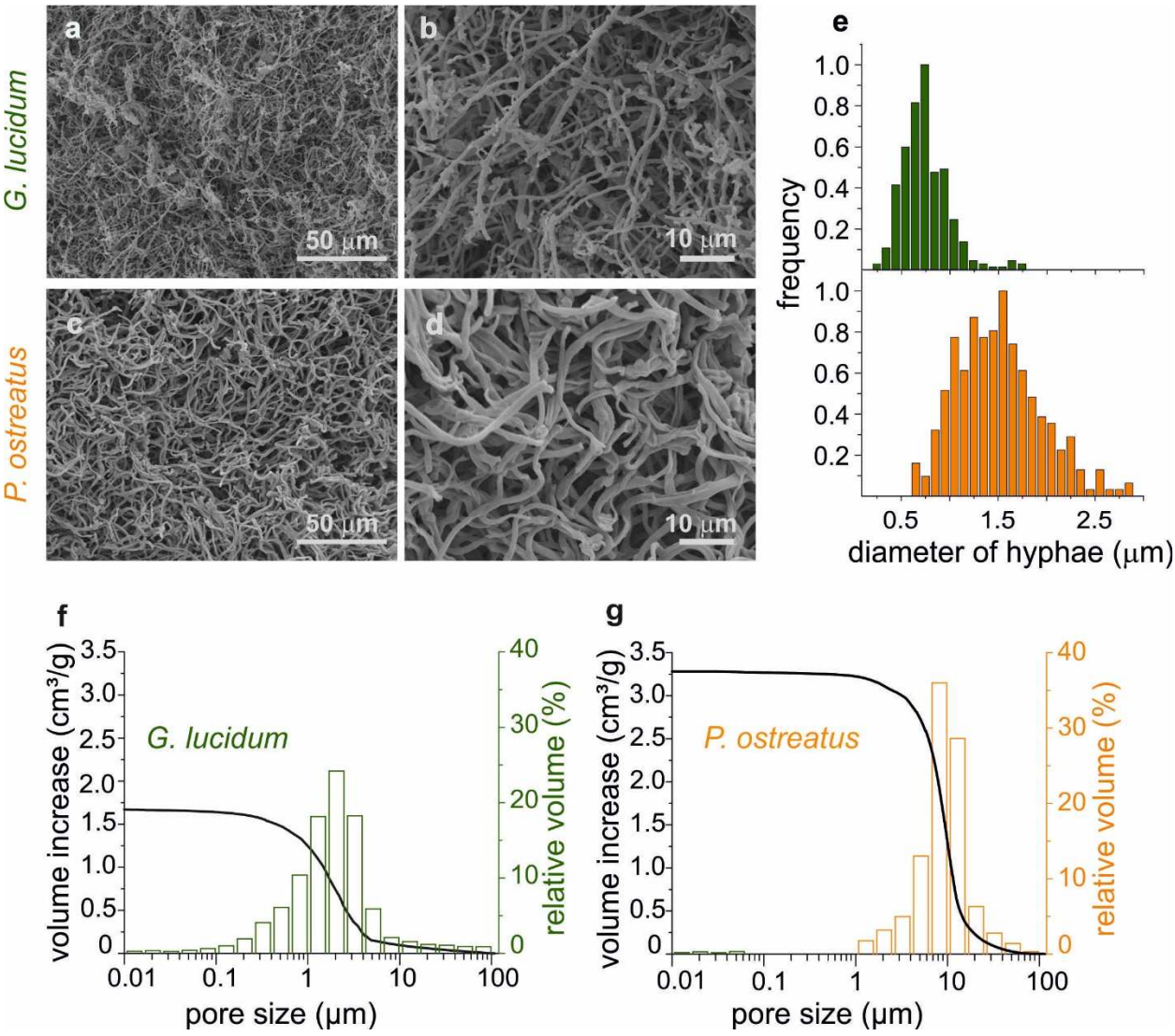

Figure 2.2. Morphology. (a-d) SEM images of autoclaved mycelia; (e) distribution of hyphal diameters as calculated from the SEM images of the autoclaved mycelia; (f,g) Pore diameter distribution (bars) and total porosity (curves) measured by MIP.

Chemical composition of mycelia

Usually, polymers and biomaterials employed for scaffold biofabrication do not naturally display the chemical functionalities necessary for cell attachment, which must be added to the scaffold surface in a second step. For example, cellulose is phosphorylated to improve its biocompatibility, while polymeric electrospun fibers are enriched by sulfated compounds (Edgar et al.). Mycelia are interesting materials for the development of bio-scaffolds because they naturally display all chemical groups on the surface of their cell wall (Bueno and Silva). ATR-FTIR analysis (Figure 2.3a) of the self-grown materials confirms this hypothesis since hydroxyl, carboxyl and amide groups are detected in both strains. Polysaccharides, lipids and proteins compose the fungal cell wall and change according to the conditions of growth and the strain (Crowther et al.). The main differences in the ATR-FTIR spectra between the two strains under study concern the area corresponding to the amide bond stretching. *Ganoderma* mycelium shows one peak at 1715 cm^{-1} (amide I stretching area) and two peaks at 1375 and 1240 cm^{-1} (amide III stretching range), which are not visible in the *Pleurotus* spectrum. On the other hand, this latter presents two peaks at 1620 and 1540 cm^{-1} (amide II stretching range), differently from the *Ganoderma* spectrum, where only one peak at 1450 cm^{-1} is visible in the same region. Differences in these ranges can be related to variations in chitin content, which

broadly influences the mycelial properties (*i.e.* hydrophobicity and mechanical resistance) (Feofilova).

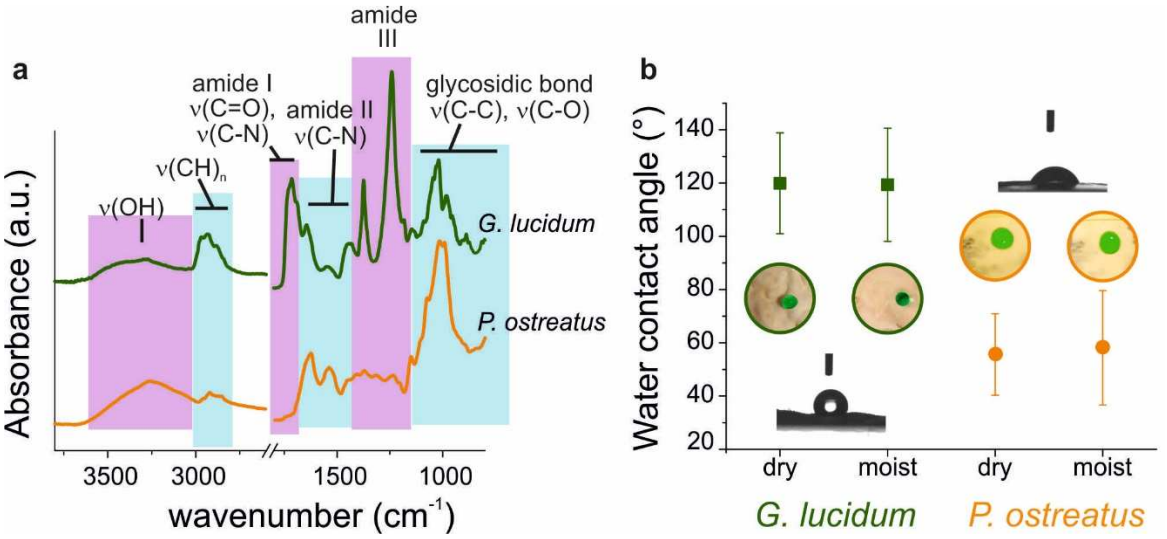

Figure 2.3. Chemical and hydrodynamical features of mycelia. (a) ATR-FTIR spectra of the two mycelia; (b) Contact angle of dry and moist mycelia. Inset: colored water drops on mycelium pieces of both strains, either dry or moist. The drops volume is 5 µL.

Hydrodynamic characterization

The hydrodynamic behavior of the developed mycelia was characterized through the static water contact angle (CA) and the ability to adsorb water moisture in a saturated water atmosphere. Contact angle was measured after conditioning for 24 hours the mycelium substrates both in a dry and a humid environment, since applications in a water-containing medium are being considered in this work (Figure 1.3b). *Ganoderma* displays a stable CA of ≈119° under both conditions. At the same time, *Ganoderma* mycelia present a limited capability of moisture adsorption, since only a (30 ± 1)% weight increase is recorded after placing the material in a humid chamber. On the other hand, *Pleurotus*

mycelia exhibit lower CA values in both conditions, *i.e.* CA is (56 ± 15)° for dry mycelia and (58 ± 21)° for moist ones. These mycelia are more prone to adsorb moisture, with values reaching up to an increase of (68 ± 6)% of their weight after 24 hours in a humid chamber.

The differences in chemical composition and porosity reported above can explain the diverse hydrodynamic behavior, especially in terms of moisture uptake. Moreover, the resulting water contact angle strongly depends on the entire surface topography (Mazzon et al.).

Mechanical characterization

Mechanical properties are a key feature of materials intended for tissue engineering, since sufficient support should be granted to cell growth and proliferation in the scaffold (Biswal). As shown in Chapter 1, mechanical properties of mycelia can be difficult to characterize due to their fibrous nature and to the ability to respond only to soft stresses (Islam et al.). Mechanical properties were measured anyway both by tensile test and Dynamic Mechanical Analysis (DMA) since prior conditioning in a humid atmosphere (100% RH) could ameliorate them (figure 1.4). Water in fact, as observed in Chapter 1, seems to exert a plasticizing effect on mycelia. Moreover, even if single measurements own high bar errors, still it could be possible to observe differences between the two strains. *Ganoderma lucidum* shows a higher Young's modulus (26.8 ± 11.7) MPa and a smaller elongation (6.6 ± 0.8)%, with respect to the *Pleurotus ostreatus,* that presents a Young's modulus of (3.2 ± 0.1) MPa and an elongation of (10.7 ± 3.8)%. Instead, the recorded stresses at break were only slightly different, *i.e.* (2.9 ± 1.2) MPa for *Ganoderma*

and (0.7 ± 0.3) MPa for *Pleurotus*. Considering these results and the calculated tan-delta values, *Ganoderma* mycelium appears stiffer, while *Pleurotus* results more ductile.

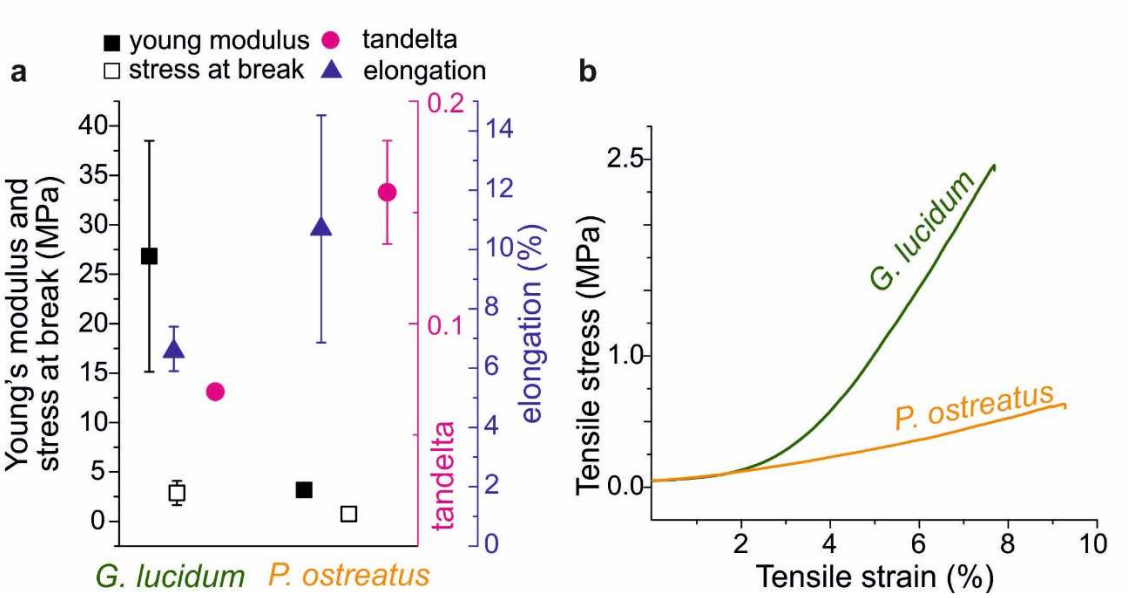

Figure 1.4. Mechanical features of mycelia. (a) Young modulus, stress at break and elongation results after the tensile test; tanδ values after DMA analysis. (b) Tensile test curves of the two mycelia under study.

Primary human dermal fibroblasts response to fungal mycelia

Biocompatibility assays

As shown above, mycelia seem to own all the properties required for an effective bioscaffold, *i.e.* a surface with topographical features, all the chemical groups necessary for cell attachment and a porous structure. A preliminary biocompatibility investigation was conducted following the ISO10993-5 standard test, which assessed the cyto-toxicity effects of a cell culture medium extract of the whole mycelium materials. Experiments were performed with primary human dermal fibroblast adult cells (HDFa) (Kossyvaki et

al.). Two different assays were performed. First, the viability of cells grown in the presence of the mycelium cell culture medium extracts was assessed by MTS assay (Figure 2.5a). In addition, a "semi-contact" experiment was carried out (Figure 2.5b), where fibroblasts attached to the bottom of 24-wells plates were cultured in the presence of a piece of mycelium kept floating on top of them. From the experiments performed with *Pleurotus ostreatus*, a maximum viability was recorded for each condition tested (Figure 2.5a-b, full orange bars). Moreover, these fibroblasts displayed the same morphology of the control cells (supplemented with culture medium), as noticeable via actin staining and confocal microscopy (Figure 2.5c-d). Thus, mycelium from *Pleurotus ostreatus* appeared a promising substratum for tissue engineering applications. On the contrary, the preliminary assays conducted in the presence of *Ganoderma lucidum* revealed a different trend: in the "extract" experiments, the cyto-compatibility slightly increased with respect to the control samples for high dilutions (1:200 and 1:40 mycelia extract/culture medium, v/v) and then remarkably dropped in the presence of more concentrated extracts (reaching a viability of [6.0 ± 0.2]% when diluting 1:2, Figure 2.5a, empty green bars). This might suggest a slight stimulating effect at low *Ganoderma lucidum* concentrations, but further investigations are required.

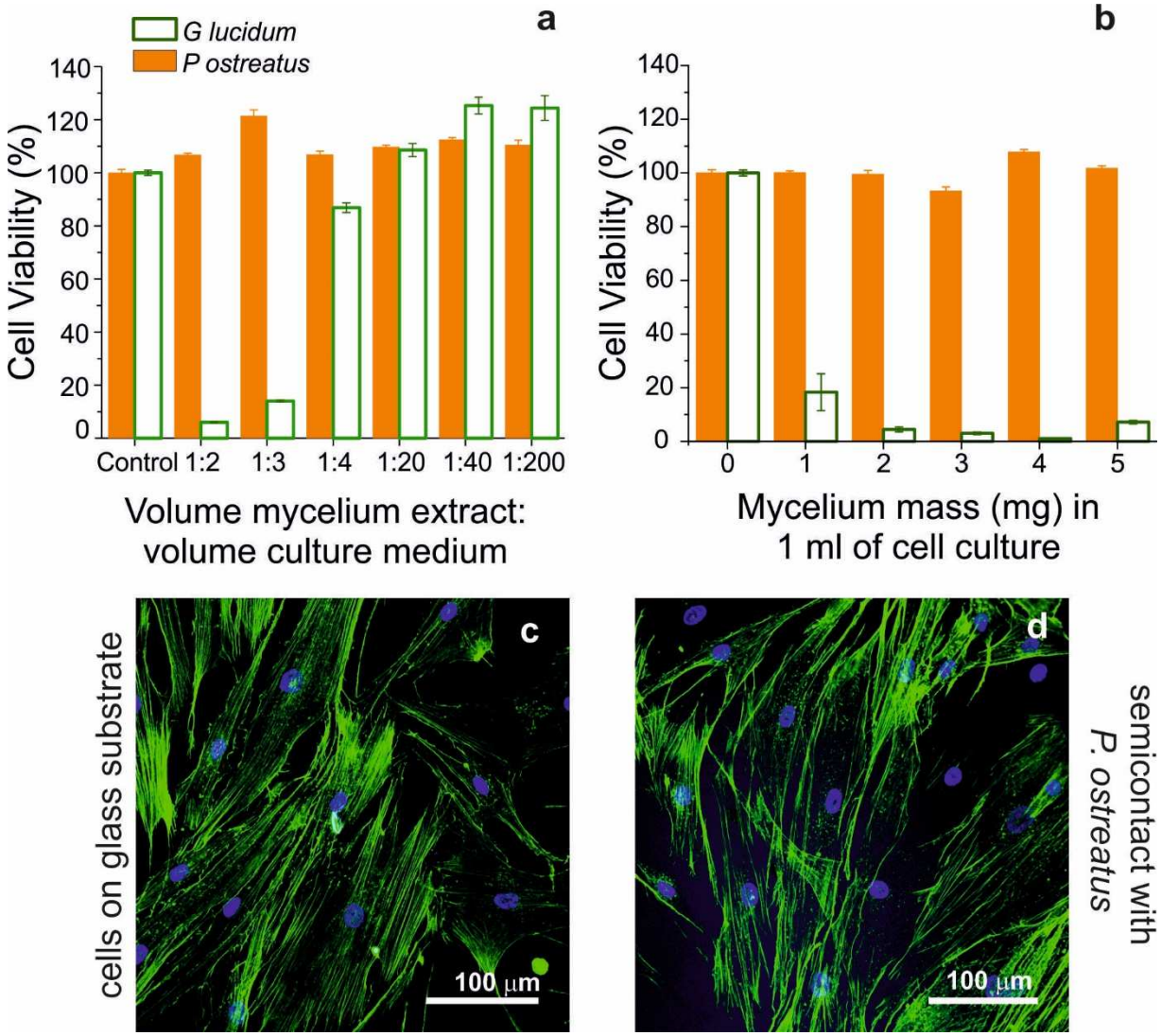

Figure 2.5. Biocompatibility towards HDFA cells. (a,b). Primary human fibroblast (HDFa cells) viability in the presence of mycelia extract (a) or semi-contact (b) as measured by MTS assay after 24 hours. The reported values indicate the dilutions prepared starting from a stock solution of 20 mg of mycelium in 1 mL of Medium 106 containing growth factors. (c,d). Confocal images of control cells plated onto glass substrates and treated with Medium 106 containing growth factors (c), or in semi-contact with a 5 mg-piece of *P. ostreatus* (d).

When pieces of *Ganoderma lucidum* were directly placed in the culture wells, however, fibroblasts did not survive. To gain some insights on a possible explanation for this outcome, extracts from the two strains were analyzed by high-resolution UPLC-MS (ultra-performance liquid chromatography – mass spectrometry). This experiment revealed a significant amount of a *Ganoderma* metabolite even after two 24 hour-washing steps with sterile PBS. This metabolite (Ganoderic acid V, a highly oxygenated lanostane-type triterpenoid, Figure 2.6) is known to have apoptotic and cytotoxic activities (Radwan et al.; You et al.). The toxic glucoside oleandrin was also detected in the extract, probably causing the fibroblasts death (Frese et al.). On the other hand, the same analysis on *Pleurotus* showed that hydroxylated fatty acids are the major metabolites (Figure 2.7), which resulted harmless for the primary cells.

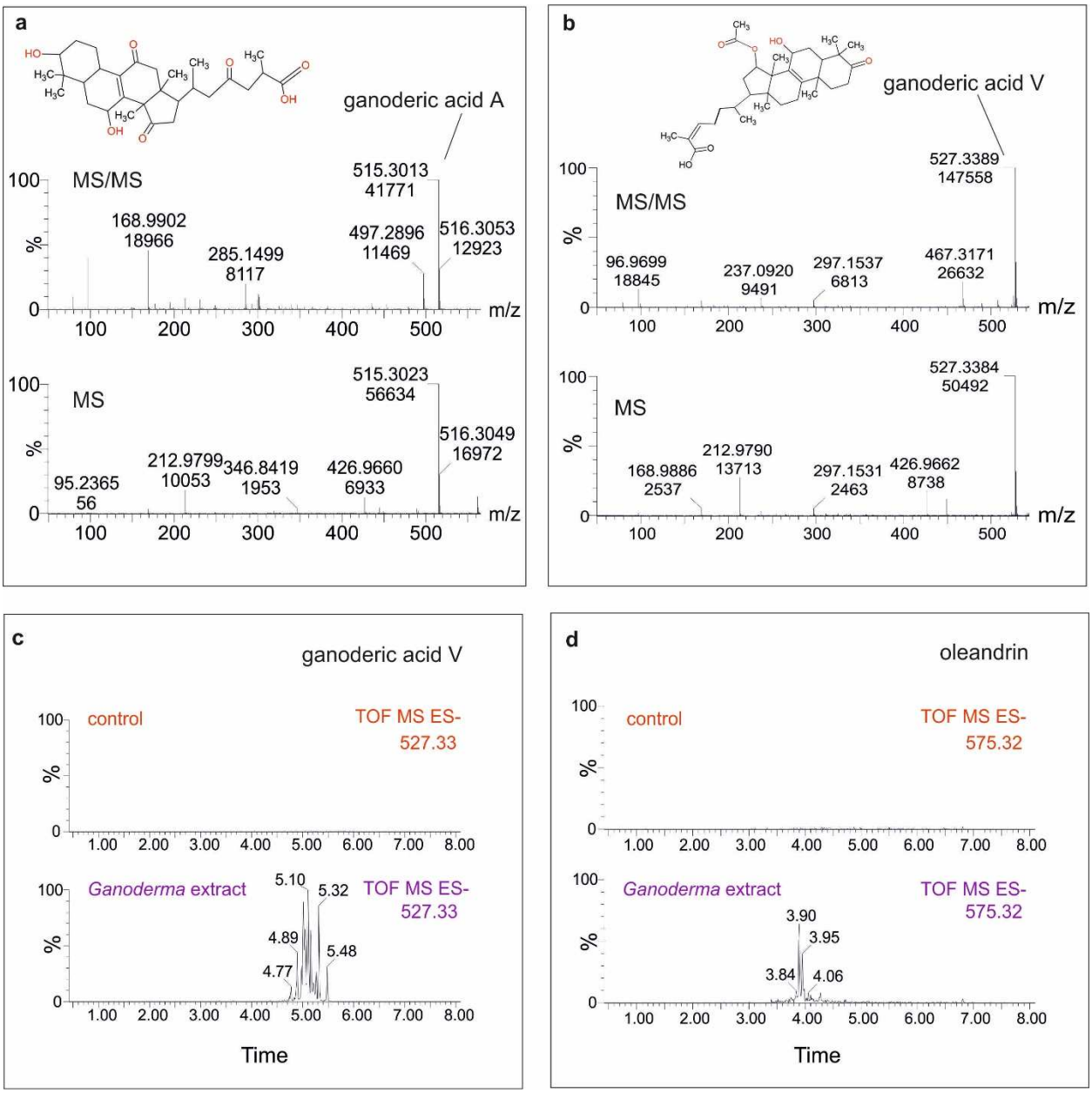

Figure 2.6. Results of UPLC-MS analysis of extracts and washing PBS from *Ganoderma lucidum.* TOF MS spectra of ganoderic acid A (a), used as standard, and ganoderic acid V (b), effectively detected in the extracts. Traces for extracted ions m/z 527.33 (ganoderic acid V, c) and 575.32 (oleandrin, d).

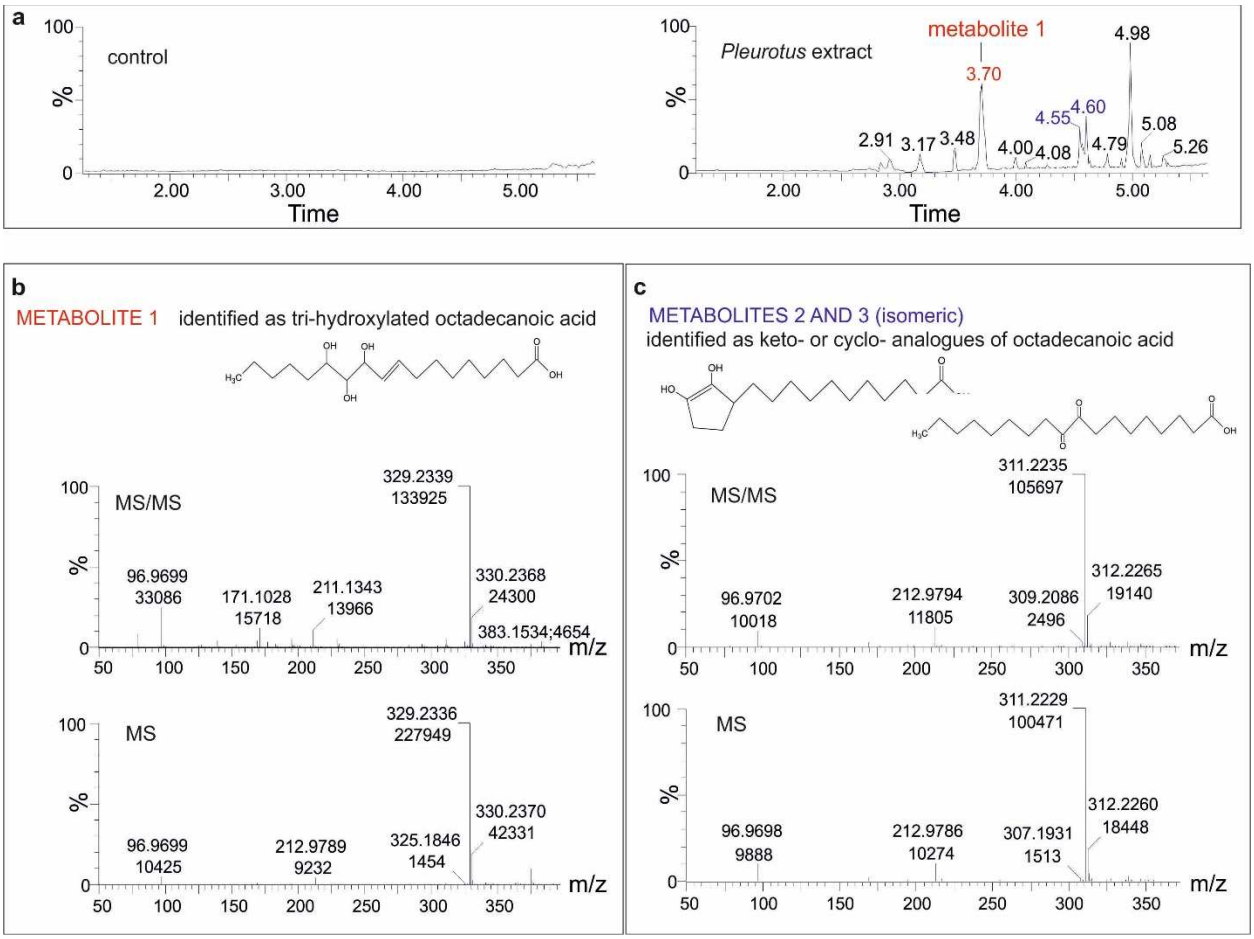

Figure 2.7. Results of UPLC-MS analysis of extracts and washing PBS from Pleurotus ostreatus. UPLC chromatograms (a) and MS spectra of metabolite 1 (b) and metabolites 2 and 3 (c) .

Direct growth of primary human fibroblasts on *Pleurotus ostreatus* mycelia

Considering the preliminary biocompatibility outcomes, primary fibroblasts attachment was tested only onto the mycelia from *Pleurotus ostreatus*. Experiments were conducted either onto Fibronectin (FN) coated substrates (Figure 2.8d,f,g,h) or onto uncoated mycelia (Figure 2.8i,j), to observe if the presence of this ECM protein known to mediate cell-substrate interaction could be necessary to achieve cell adhesion. As observed by

confocal and SEM microscopy, cell attachment was successful, with primary fibroblasts adhering to the corrugated, wavy, 3D mycelium matrix, and presenting a healthy morphology comparable to that observed on FN-coated control glass slides (Figure 2.8a,c,e). Interestingly, the SEM investigation revealed the presence of cytoplasmic filaments (filopodia, indicated by yellow arrows in Figure 2.8 f,h,j) protruding from the leading edges of the cells. This phenomenon is more evident for the cells plated on the *Pleurotus ostreatus* scaffolds, both on the FN-coated and the non-functionalized ones, as the filaments appeared anchored to the hyphae. As expected, a denser sheet of adherent fibroblasts was observed with the FN surface functionalization, indicating that the ECM protein coating is useful but not essential to promote primary fibroblasts attachment onto *Pleurotus ostreatus* substrates.

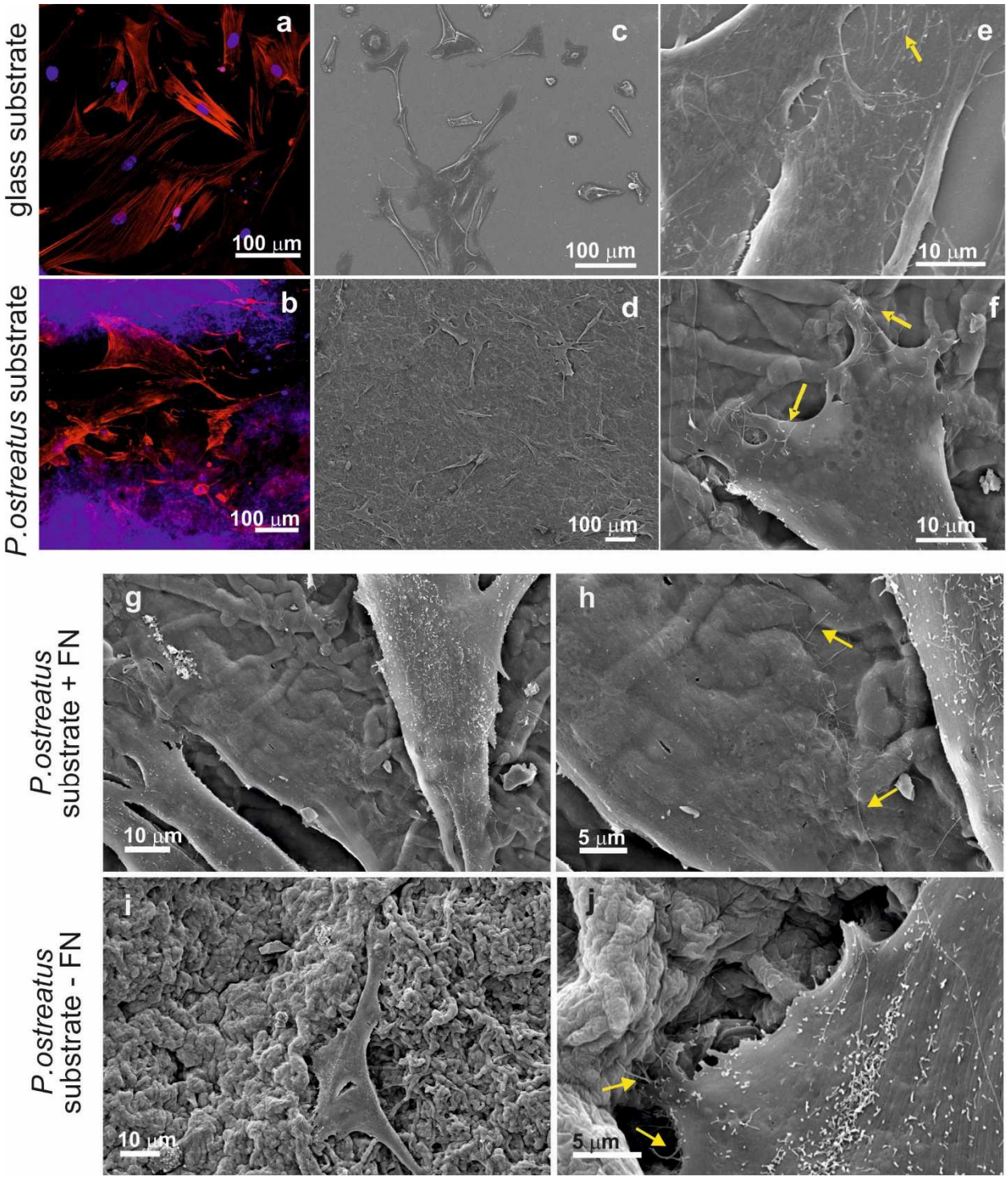
glass substrate
P.ostreatus substrate
P.ostreatus substrate + FN
P.ostreatus substrate - FN
a
b
c
d
e
f
g
h
i
j
100 μm
100 μm
100 μm
100 μm
10 μm
10 μm
10 μm
5 μm
10 μm
5 μm

Figure 2.8 (a-f). Confocal (a) and SEM micrographs (c,e) of HDFa cells grown for 48 h onto a FN-coated glass coverslips. Confocal (b) and SEM micrographs (d,f) of HDFa cells grown for 48 h onto a FN-coated *P. ostreatus* substrate. Nuclei are stained with DAPI (visible in blue), while the actin fibers are stained with Alexa Fluor Phalloidin 488 (highlighted in green). Filopodia extending from the cytoplasm of the attached cells are indicated with yellow arrows. (g-j) Effect of the scaffold coating onto primary cell growth. HDFa cells are seeded onto *P. ostreatus* scaffolds, either coated with fibronectin (g,h) or uncoated (i,j). Filopodia extending out of the attached cells are highlighted with yellow arrows.

Conclusions

Mycelia of filamentous, not pathogenic fungi having a spontaneously-formed, porous, tridimensional biopolymeric network can constitute self-growing, all-natural biocomposite scaffolds for cellular growth. For the first time, mycelium from *Pleurotus ostreatus* was directly used as scaffold for the growth of cells, determining attachment of primary human fibroblasts with excellent viability and morphology comparable with that observed in the control samples. A simple and fast autoclaving process was the only treatment performed onto the self-grown composite biomaterial, serving the double purpose of completely inactivate the fungal spores and sterilize the scaffold via a standardized method. Results obtained with another filamentous fungus, *Ganoderma lucidum,* showed that complete inactivation of the hyphal cells is necessary but not enough to grant biocompatibility, as its water extract contained picomolar concentrations of organic acids (Ganoderic acid V and Oleandrin), which resulted detrimental for the HDFa cells survival.

Conclusions

In this Thesis, mycelia from *Ganoderma lucidum* and *Pleurotus ostreatus* are considered for their application as pure mycelium materials. The numerous properties of mycelia, from their ability to grow on a wide variety of substrates, even waste ones, to their porous structure, displaying numerous chemical compounds including chitin, widely employed in materials science, make mycelia interesting candidates as petroleum-based plastics alternatives. Several groups have developed mycelial composites for use as EPS substitutes or as thermal and acoustic insulator, but no one has focused on pure mycelium materials.

In Chapter 1, it is demonstrated that it is possible to finely tune morphological, chemical and hydrodynamic properties of the *Ganoderma lucidum* mycelium materials by small changes in the substrate of growth. In particular, the strain was grown on a standard fungal medium, Potato Dextrose Broth (PDB), and on PDB with additional D-glucose or alkali lignin. The latter, in particular, triggers a faster growth of mycelia, mainly on the surface of the medium, with a denser hyphal network, richer in chitin. On the other hand, mycelia grown in the presence of additional glucose display larger pores and are more porous in general. They are also more prone to uptake water in high moisture conditions, even if they continue to show a high surface contact angle.

In Chapter 2, the possibility to tune features like chemical surface composition and porosity is exploited in the field of tissue engineering, where the goal is the reproduction of porous, chemically defined Extracellular Matrix (ECM). Differently from other biodegradable polymers and bioplastics, mycelia spontaneously grow in a porous

structure that shows all the chemical groups required for cell attachment. In particular, biocompatibility was successfully tested with Human Dermal Adult Fibroblasts (HDFAs) on *Pleurotus ostreatus,* at the point that it was possible to directly plate these cells on the mycelium, covered or not with Fibronectin.

The results presented in this Thesis open a wide range of possibilities for the use of mycelia as advanced, nanostructured materials. On the one hand, variables as fungal strain, substrate of growth, growth conditions and post-processing parameters can be further tuned and investigated for their effect on mycelium materials properties. On the other hand, results shown in Chapter 2 open a new field of investigation to be carried on through cell and *in-vivo* tests. Moreover, other strains, especially those belonging to Zygomycetes, known to produce the highly biocompatible biopolymer chitosan, can be investigated for this application. Tuning of mycelium properties can be the starting point for the development of bioscaffolds mimicking different tissues and organs.

This thesis shows that mycelia can be successfully employed as low-cost, sustainable materials for applications at the nanoscale. In perspective, this field of application can benefit from results already obtained in numerous works exploiting Fungi, from bioremediation to pharmaceutics, since all these functions are naturally merged in the mycelium itself.

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