

Biophysical characteristics and cellular studies of polyhydroxyoctanoate (PHO) - biodegradable and biocompatible polymer for biomedical applications.



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Oświadczenie

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1. Abbreviations

AFM - Atomic force microscopy	PGA - Poly (glycolic acid) (Polyglycolide)
BNH - Brinell hardness number	PHA - Polyhydroxyalkanoates
CAGR - Compound Annual Growth Rate	PHB - Polyhydroxybutyrate
DCM - Dichloromethane	PHO - Poly(3-hydroxy) octanoate
DMA - dynamic mechanical analysis	PHO _{Ac} - PHO casted from acetone
DMF- Dimethylformamide	PHO _{Chl} - PHO casted from chloroform
ECM - Extracellular matrix	PHO _{EtAc} - PHO casted from ethyl acetate
FAD - Flavin adenine dinucleotide	PI - Propidium iodide
FDA – Fluorescein diacetate	PLA - Poly lactic acid (Polylactide)
FDA* - Food and Drug Administration	PLA _{Ac} - PLA casted from acetone
FID - Flame ionization detector	PLA _{Chl} - PLA casted from chloroform
FLIM - Fluorescence-lifetime imaging microscopy	PLA _{EtAc} - PLA casted from ethyl acetate
FLIP - Fluorescence loss in photobleaching	PLGA - Poly-lactide- co-glycolide
FRAP - Fluorescence recovery after photobleaching	PLLA - Poly (L-lactic acid)
FS - Force spectroscopy	PMMA – Polymethylmethacrylate
FT-IR - Fourier transform infrared	PP - Polypropylene
GC - Gas chromatography	Ra - Average roughness
HHx - Hydroxyhexanoate	RMS - Root mean square
IF - Intermediate filaments	ROP - Ring opening polymerization
IR - Infrared	SD – standard deviation
LA - Lactic acid	TGA - Thermogravimetric analysis
LDPE - Low density polyethylene	VDH - Vickers diamond hardness
MF - Micro filaments (Actin filaments)	vdW – van der Waals
MPE - Multi-photon excitation	XRD - X-ray diffraction
MSM - Minimal salt medium	
MT - Microtubules	
NA - Numerical aperture	
NAD - Nicotinamide adenine dinucleotide	
PALS - Positron annihilation lifetime spectroscopy	
PAM - Polyacrylamide	
PAS - Positron annihilation spectroscopy	
PE - Polyethylene	

2. Abstract

2.1. English

Growing interest in novel materials and shrinking supplies of fossil fuels cause that materials of natural origin are attracting more and more attention of scientists and industry. Due to their features such as biointegrity and biocompatibility, they can not only displace traditional polymers, but also find completely new applications in biomedical sciences and industry. The growing demand for this type of polymers has resulted in intensive development of research and production companies dealing with novel materials. Thanks to the completely natural origin bacterially derived polyhydroxyalkanoates polymers are promising materials to suit the above purpose. One of their characteristics is the biocompatibility with the mammalian cells. Therefore, the polymer can be used to manufacture scaffolds for tissue regeneration, starting from wound dressings constructions and ending with endo-prostheses or bone scaffolds coatings.

The presented work focuses on the study of a representative medium chain length elastomeric polyhydroxyalkanoate polymer – poly(3-hydroxyoctanoate) (PHO). In order to characterize the influence of different solvents used for biomaterial preparations (solvent casting of PHO films) three solvents, namely ethyl acetate, acetone and chloroform, were used to prepare thin films. A comparison of the physicochemical features changes over time in these differently casted films was investigated against polylactide (PLA) controls – a quite deeply researched and well-known material. Tests included nanoindentation and scratch testing of the material, the obtained results were compared with AFM data. The Young Modulus values are consistent for both methods and comply with literature. Further, the work compares also the impact of PHO substratum on mammalian cells physiology and morphology with common polyacrylamide (PAM) and PLA substrates. Techniques such as double staining with fluorescent diacetate (FDA) and propidium iodide (PI) cytotoxicity assessment and long-term microscopic studies revealed high biocompatibility level of polymer and no toxic impact on Mouse Embryonic Fibroblast cells. Further research focused on cytoskeletal analysis of cells employing confocal microscopy. The received data indicates the influence of polymer on the structure and morphology of the cytoskeleton relative to the reference materials. Additionally, both the shape of the cells and the structure of actin filaments suggest a high level of cell

adhesion to the substrate (compared with a glass controls). Due to the considerable commercialization potential of the PHA polymers, the author also conducted an analysis of the global biopolymer market as well as considered the application and commercialization perspectives of the PHO based final products. Current research and trends indicate that PHA are used not only in the narrow field of medical products and bioengineering but also in widely understood everyday objects such as packaging, shopping bags, electronic casings or even home furnishings and appliances. Owing to their chemical structure, PHAs can be subjected to simple modifications that can change both their biological and chemical properties, as well as their physical properties such as hydrophobicity or hardness. These features make polyhydroxyalkanoates versatile polymers, which applications and commercial use can be dictated by the needs of the market and not by limitations posed by the material itself.

Thanks to employment of advanced research methods like nanoindentation, high resolution confocal microscopy combined with complex data analysis it was possible not only to precisely elucidate physical properties of material, but also its impact on living mammalian cells. The obtained results indicate that PHO can map the mechanical properties of popular polymers such as polyethylene (PE) or polystyrene (PS) at the same time presenting full biocompatibility with living cells. A quantitative analysis was also applied to determine that PHO exerts impact on both cells' morphology, cytoskeleton architecture and migration velocity.

2.2. Polish

Rosnące zainteresowanie nowymi materiałami i kurczące się zasoby paliw kopalnych powodują, że materiały pochodzenia naturalnego przyciągają coraz więcej zainteresowania zarówno naukowców jak i przemysłu. Dzięki swoim właściwościom, takim jak biodegradowalność i biokompatybilność, mogą one nie tylko zastąpić tradycyjne polimery, ale także znaleźć zupełnie nowe zastosowania w naukach biomedycznych i medycynie. Rosnące zapotrzebowanie na tego typu polimery spowodowało intensywny rozwój firm badawczych i produkcyjnych zajmujących się nowymi materiałami. Dzięki całkowicie naturalnym pochodzącym od bakterii polihydroksyalkanianom (PHA) polimery z nich wykonane są obiecującymi materiałami spełniającymi oczekiwania stawiane im przez dzisiejszy przemysł, medycynę oraz wpisują się w światowe trendy ochrony środowiska i redukcji emisji gazów cieplarnianych. Jedną z cech polihydroksyalkanianów jest ich biokompatybilność z komórkami ssaków. Dlatego polimer ten można stosować do wytwarzania produktów medycznych służących regeneracji tkanek, poczynając od konstrukcji opatrunków na rany a kończąc na endo-protezach lub rusztowaniach wspomagających regenerację uszkodzonej tkanki kostnej.

Prezentowane w pracy badania koncentrują się na przedstawicielu średnio łańcuchowych polihydroksyalkanianów - poli(3-hydroksyoktanie) (PHO). W celu scharakteryzowania wpływu różnych rozpuszczalników stosowanych do przygotowania biomateriału (produkcja folii PHO) do przygotowania cienkich warstw zastosowano trzy różne rozpuszczalniki, mianowicie octan etylu, aceton i chloroform. Porównanie zmian właściwości fizykochemicznych w czasie dla tych różnie prefabrykowanych filmów zostało zbadane i porównane z dobrze znanym i szeroko opisywanym w literaturze polilaktydem (PLA). Badania materiałowe obejmowały nanoindentację i testy typu „*Scratch testing*” polimerów. Uzyskane wyniki porównano z danymi otrzymanymi techniką AFM. Wartości modułu Younga są spójne dla obu metod i zgodne z literaturą. W pracy porównano również wpływ podłoża PHO na fizjologię i morfologię komórek ssaków (Mysich embrionalnych fibroblastów – MEF 3T3). Jako materiałów porównawczych użyto podłoży poliakryloamidowych (PAM) oraz wykonanych z PLA. Techniki, takie jak testy cytotoksyczności oparte na podwójnym barwieniu dwuctanem fluoresceiny oraz jodkiem propidyny oraz długoterminowe badania mikroskopowe wykazały wysoką biokompatybilność polimeru i brak jego negatywnego wpływu na komórki fibroblastów. Dalsze badania dotyczyły analizy cytoszkieletu komórek. Obserwacje prowadzono przy użyciu technik mikroskopii konfokalnej i fluorescencyjnej. Otrzymane dane wskazują na wyraźny wpływ rodzaju podłoża na strukturę i morfologię cytoszkieletu.

Dodatkowo, zarówno kształt komórek, jak i struktura filamentów aktynowych sugerują wysoki poziom adhezji komórek do podłoża w przypadku obydwóch biopolimerów. Ze względu na duży potencjał komercyjny polimerów typu PHA, autor przeprowadził również analizę globalnego rynku biopolimerów, a także rozpatrzył perspektywy zastosowania i wdrożenia do produkcji gotowych produktów wytwarzanych z PHO. Aktualne badania i trendy wskazują, że polihydroksyalkaniany są wykorzystywane nie tylko w wąskim zakresie produktów medycznych i w bioinżynierii tkankowej, ale również w produkcji szeroko rozumianych przedmiotów codziennego użytku, takich jak opakowania, torby na zakupy, obudowy urządzeń elektronicznych czy nawet meble i sprzęt gospodarstwa domowego. Ze względu na swoją strukturę chemiczną, PHA można łatwo poddawać modyfikacjom, które mogą zmieniać zarówno ich właściwości biologiczne, chemiczne, jak i fizyczne, takie jak hydrofobowość lub twardość. Cechy te sprawiają, że możliwa jest produkcja uniwersalnych polimerów opartych na polihydroksyalkanianach, które mogą posiadać cechy dopasowane do konkretnych potrzeb rynku, a ograniczenia powodowane przez właściwości surowego materiału są mocno zredukowane.

Dzięki zastosowaniu zaawansowanych technik eksperymentalnych takich jak nanoindentacja, wysokorozdzielcza mikroskopia fluorescencyjna i konfokalna w połączeniu ze złożoną analizą danych było możliwe nie tylko wyznaczenie właściwości fizycznych materiału ale także określenie jego wpływu na żywe komórki ssące. Otrzymane wyniki pomiarowe wskazują, że PHO dzięki swoim właściwościom fizycznym, może zastąpić popularne obecnie na rynku tradycyjne polimery jak polietylen czy polistyren i równocześnie wykazuje pełną biogodność z żywymi komórkami. Analiza ilościowa zastosowana w pracy pokazała, że materiał ten wywiera wpływ zarówno na morfologię komórek, kształt cytoszkieletu jak i na prędkość migracji.

3. Acknowledgments



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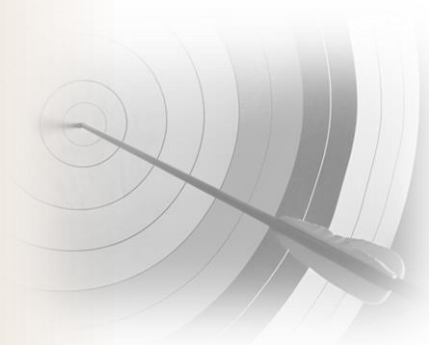


Pragnę podziękować wszystkim osobom jakie przyczyniły się do powstania tej pracy oraz wspierały mnie w czasie jej pisania. Przede wszystkim chciałbym podziękować moim promotorom dr. hab. Zenonowi Rajfurowi, dzięki któremu mogłem przez czas trwania studiów doktoranckich stać się częścią wspaniałego zespołu badawczego oraz móc korzystać z aparatury badawczej na najwyższym światowym poziomie, oraz dr. Maciejowi Guzikowi, który zdecydował się zaangażować mnie w realizację swojego projektu, dzięki czemu miałem okazję zgłębić i rozszerzyć swoją wiedzę oraz poznać wielu niesamowitych ludzi.

Chciałbym też podziękować członkom Zakładu Biofizyki Molekularnej i Międzyfazowej dzięki których dobrej radzie i wsparciu byłem w stanie zrealizować zamierzone w pracy cele. Szczególnie chciałbym podziękować Darii Solarz oraz Karolinie Feliksiak, które często kosztem swojego wolnego czasu pomagały mi w przygotowaniu i przeprowadzeniu eksperymentów, a przy tym bez mrugnięcia okiem wysłuchiwały moich narzekań i pomagały w rozwiązywaniu problemów.

Największe podziękowania kieruję do mojej rodziny - Mamy i Cioci, bliskich i przyjaciół, którzy zaakceptowali moje zaangażowanie w realizację zadań badawczych i chroniczny brak czasu na spotkania czy rozmowy. Bez waszego wsparcia, cierpliwości i wyrozumiałości praca ta by nie powstała.

4. Aim of the research project



The purpose of research project presented in this thesis is to assess the usefulness of a representative of medium length chain polyhydroxyalkanoates (mcl-PHAs) – polyhydroxyoctanoate in field of medical applications.

In the first part of this work author is presenting global trends and social approach to widely understood biomaterials. An assessment of economic factors affecting the popularity of biomaterials alongside with a forecast regarding their potential future areas of applications. Due to the high commercialization potential, the world trends on biopolymers were analyzed, to draw a bigger picture of their usability in current global markets. The potential role of biopolymers, especially polyhydroxyalkanoates in the circular economy model was discussed (Chapter 8).

Due to the main topic of the work focusing on PHO applications in medicine it was important to thoroughly examine material's physical and biological properties. Physical tests included nanoindentation and scratch testing of the polymer, the obtained results were compared with AFM data. To be suitable for biomedical applications, the material must not be toxic to mammalian cells. Techniques such as FDA/PI double staining cytotoxicity assessment and long-term microscopic studies evaluated biocompatibility level of polymer and its impact on Mouse Embryonic Fibroblast cells (MEFs). Further, the work compares the impact of PHO on mammalian cells physiology and morphology with commonly used polyacrylamide (PMA) and well characterized PLA substrates. Additionally, research focused on cytoskeletal analysis of cells employing confocal and fluorescent microscopy techniques. Learning its physical and biological properties along with their comparison with another, well-described biopolymer representative – polylactic acid (PLA)²⁻⁴, aims to identify areas of potential medical and industrial applications for new material. Detailed knowledge of the biopolymer properties and clear delineation of paths and areas of its industrial and medical applications will allow for effective commercialization of the project and transferring basic research to the industrial, applicable level.

5. Introduction



5.1. Petrochemical plastics

Traditional plastics can be used in a wide variety of products and have displaced other materials, such as wood, metal, or glass. They can be formed into polyesters for use in fabrics and textiles, polyvinylidene chloride for food packaging, and polycarbonates for eyeglasses and compact discs, among thousands of other uses. In the last few decades, crude oil-based plastics became common in every area of life. Their popularity is best seen in the exponential increase in polymer production in recent years (Fig. 1). Durability and low fabrication costs have created plastics as nearly irreplaceable material in modern society. Despite the decrease in demand for plastics, due to the global financial crisis over 330 million tons were produced worldwide in 2016. In Europe the production quantity remains constant since the beginning of the 21st century and amounts about 60 million tons every year⁵ (European Commission, 2011).

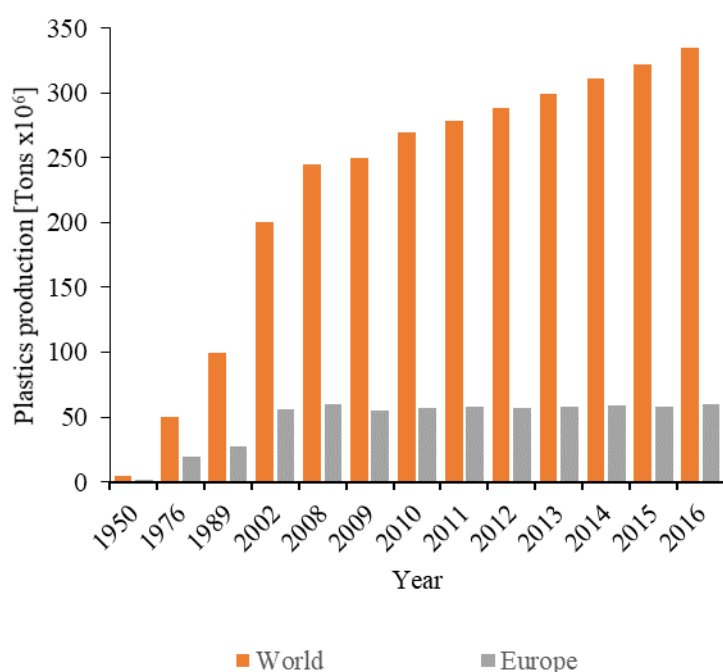


Fig. 1 Global plastic production from 1950 to 2016. In 2016, world plastics production totaled around 335 million metric tons⁵.

Plastics are a family of hundreds of different materials with a wide variety of properties. They are designed to meet the needs of each single application in the most efficient manner. Currently, most plastic materials are derived from fossil feedstocks such as natural gas, oil or

coal. However, it is important to stress that only 4 to 6% of all the oil and gas used in Europe is employed in the production of plastic materials⁶.

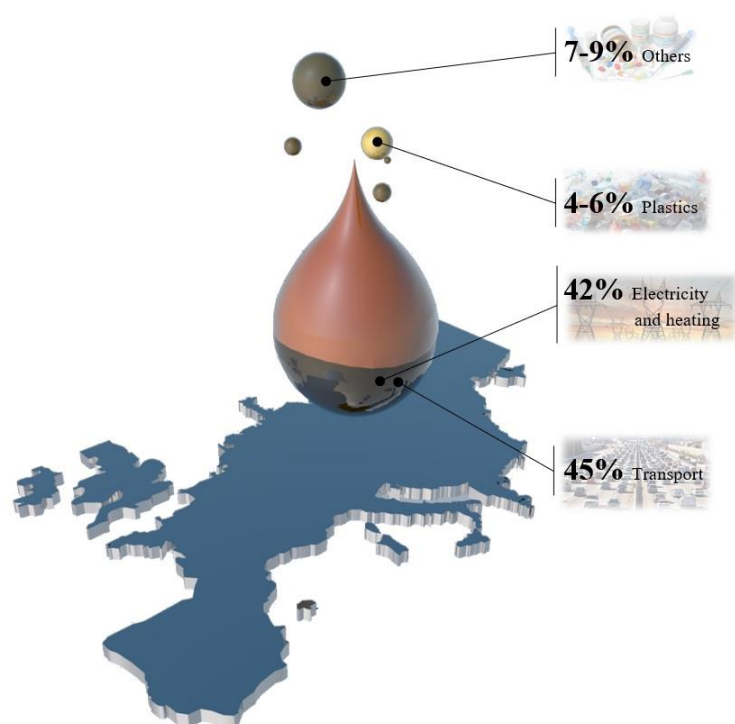


Fig. 2 Current distribution of oil consumption in various life and industry areas in Europe. The plastics production is allocated between 4 and 6% of total consumption. Almost 90% of oil is absorbed by the transport, energy consumption and heating.

The growing demand for cheap and durable plastics leads to a fast-growing global waste problem. Many different technologies are still being developed to address the plastic waste issue. Many of recycling technologies focus on energy recovery or mechanical recycling. Energy recovery closes the multimillion-year life cycle of plastics through their thermal decomposition to CO₂ or liquid fuel, which can be converted to CO₂ through combustion. Mechanical recycling has a disadvantage of aggravating the material mechanical properties which means that this process is not possible to be carried out indefinitely. The coal life cycle is presented below (Fig. 3), the rates of exchanges between individual stages are marked. It can be clearly seen that the application of bio-polymers, such as polyhydroxyalkanoates, eliminates the long-lasting composting process and prevents the materials accumulation on the landfill.

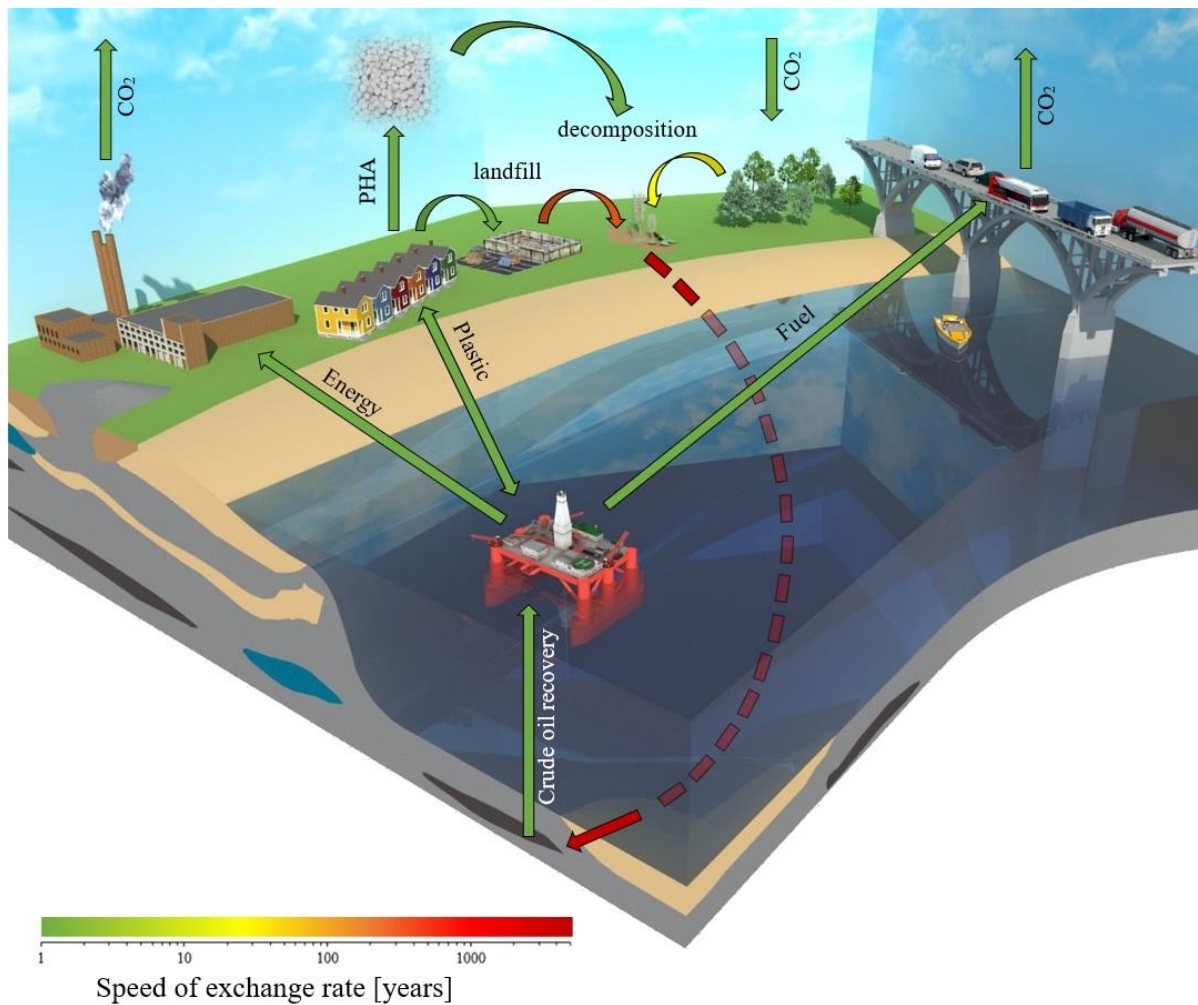


Fig. 3 Carbon life cycle. Mineralization of organic matter into fossil fuels is a very slow process. Crude oil is quickly recovered and can be easily converted into fuels or plastics. The plastic wastes are produced at a very fast rate and their mineralization subsequently takes hundreds of years. Plastic waste can serve as a microbial fermentation substrate, which is converted to PHA, thus capturing the CO₂. Biopolymers undergoes natural decomposition, which results in a much slower CO₂ release to the atmosphere.

5.2. Plastic waste



Petrochemical plastics are recalcitrant in nature and wide scale of biodegradation in case of conventional polymers of petrochemical origin has never been demonstrated. The multi-million tones levels of plastic manufacturing and usage coupled with disposal and lack of recovery has led to serious waste management issues. In 2014, out of over 180 million tons of garbage discharged in USA almost one fifth were plastics⁵ (Fig. 4). The share of plastics on the landfills is systematically growing. Less than 10% of that plastic waste was recovered for recycling leaving 30.92 million tons of plastic for landfill or incineration in 2014.

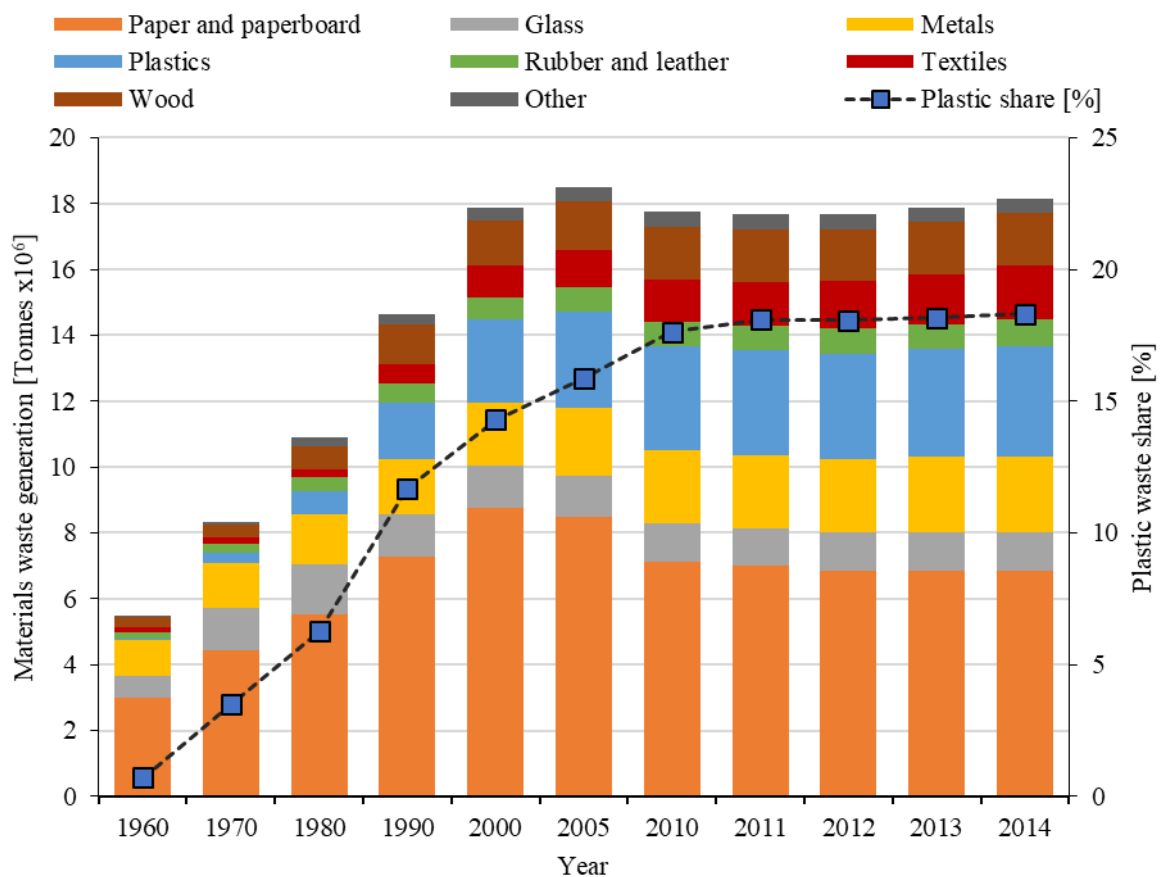


Fig. 4 The amount of selected materials generated in the municipal waste stream in the United States between 1960 and 2014. The share of plastic garbage in the entire amount is also indicated on the graph⁵.

Although the Europe situation is improving, in many countries, landfill is still the first or second option of treatment for plastic post-consumer waste. In countries such as Switzerland, Austria or Germany virtually no plastic waste is being disposed of at landfill. Over 70% of plastic waste

is managed in these countries through energy recovery, the remaining part is being materially recycled. New EU member states, as well as Poland, Spain and Greece, are trailing far behind with less than 40 % of plastic waste being recovered⁶.

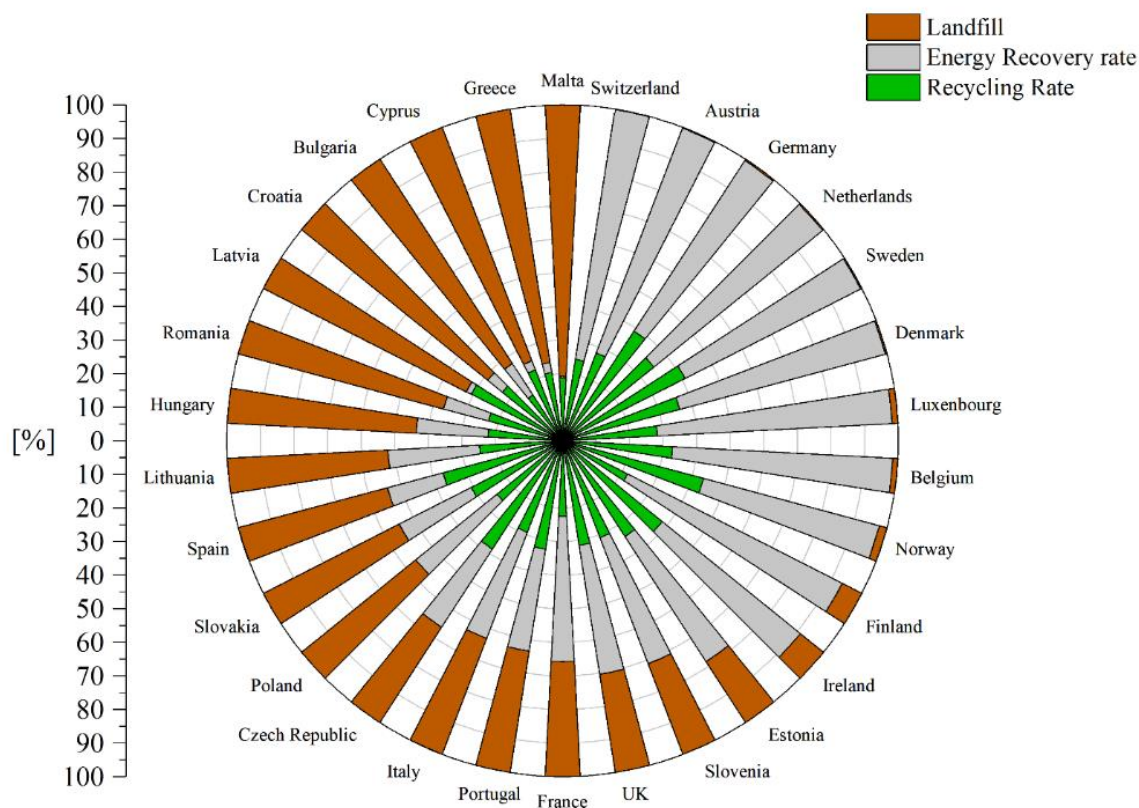


Fig. 5 Plastic post-consumer waste rates of recycling, energy recovery and landfill per country in 2016⁶

The difference in recovery and recycling rates among European countries is large. The existing regulations have a significant importance for waste management in individual countries. In some countries specific legislation exists (Switzerland, Austria, Germany, Netherlands), while in others plastic producers and farmers voluntarily try to achieve recycling goals (UK, Belgium, France.). In the worst case are countries where no legislation or restrictions are implemented (Poland, Bulgaria, Lithuania, Romania). The state of legislation in a given region clearly translates into the amount of plastics that can be found in landfills (Fig. 6) and are not being recycled in any way.



Fig. 6 Plastics post-consumer waste landfill rate across Europe⁶

5.3. From traditional plastics to biopolymers



Biopolymers are the types of polymers that are produced by living organisms. This includes plants of many types, such as corn and soybeans, but can also come from different types of trees and even some bacteria⁷. They are polymeric biomolecules and can be classified depending on the polymers structure and monomeric unit used to form the molecule⁸:

- Polynucleotides like DNA or RNA – composed of 13 or more monomers
- Polypeptides – short amino acid polymers
- Polysaccharides – linear carbohydrate structures

depending on their origin⁹:

- Polyesters (PHAs, PLA)
- Proteins (Silk, Collagen, Gelatin Soy, Zein, Gluten, Albumin etc.)
- Polysaccharides:
 - Bacterial (Xanthan, Dextran)
 - Fungal (Pollulan, Elsinan)
 - Algal, Plant (Starch, Cellulose)
 - Animal (Chitin, Hyaluronic Acid)
- Lipids (Acetoglycerides, Waxes)
- Polyphenols (Lignin, Tannin)
- Other polymers (Natural rubber, PGGA, polymers from fats and oils)

The first man-made bioplastic a material called *Parkesine* produced by Alexander Parkes who exhibited it at the 1862 Great International Exhibition in London. It was a biological material derived from cellulose⁹. *Celluloid* is a biomaterial originating from cellulose and alcoholised camphor, introduced by John Wesley Hyatt as an alternative for the ivory in billiard balls in 1868. Pure cellulose polymer was not sufficiently strong enough until the supplement of camphor, a by-product of the laurel tree. Celluloid found its major application as the first flexible photographic film. John Wesley Hyatt produced celluloid in a strip format for movie film applications¹⁰.

After cellulose nitrate, formaldehyde was the next substance to change the technology of plastics. Around 1897 technology turned to casein plastics (milk protein mixed with formaldehyde) what effected in production of *Galalith* and *Erinoid*, two initial products to be found in the trade.

In 1899, a British Patent no. 16,275 (*"Phenol-formaldehyde resins for using an ebonite substitute in electrical insulation"*) was established by Arthur Smith. It was the first attempt for formaldehyde resin processing. In 1907, Leo Hendrik Baekeland upgraded phenol-formaldehyde reaction performances and invented the first entirely synthetic resin named Bakelite.

5.4. Biomaterials



The investigation of biopolymers has been reserved to biochemists and molecular biologists for over half of a century. Nevertheless, during the last decade, the soft matter physics, biophysics, chemists, and material scientists have been seized to this research field¹¹. The current interest in developing novel materials has motivated an increasing need for biological and medical studies for a variety of clinical applications. The concept of biomaterials is one of the most important concepts generated by the need of application of materials science in the medical field¹². A biomaterial can be defined as a material used to make devices replacing a part or a function of the organism in a safe, reliable, economic, and physiologically acceptable manner¹³. According to this definition a knowledge in a number of different disciplines have to interact and connect with each other in order to properly and efficiently develop and use biomaterials in bioengineering or medicine¹⁴. Originally, the selection of materials used as medical implants was limited to those already available and well known. Early implantable materials include metals such as gold that were used in dentistry over 2,000 years ago¹⁵. The term “biomaterials” was first introduced in the 1950’s.

Parallel in the same time, fueled by the industrial expansions of polymer synthesis, the employment of synthetic polymers for biomedical applications was initiated. The first polymer material used in medicine were polymethylmethacrylate (PMMA), which found application in dentistry in the 1930s and cellulose acetate used in dialysis tubing in the 1940s¹⁶. Naturally occurring materials such as collagen have also been used as biomaterials. However, in nearly every case, materials were adopted from other areas of science and technology without substantial redesign for medical use¹⁵. Those early implants and medical devices made from the available industrially used materials were oftentimes incompatible with the host tissues, caused recoils and rarely fulfilled their task.

The role of possible causes was attributed to factors like plasticizers, un-polymerized reactants, and degradation products. All this led to the development of research areas focusing on exploration of polymer features for biomedical applications and biocompatibility testing. Rapid advances in immunology and better understanding of the possible foreign body reaction caused the development of first generation biomaterials in 1960’s and 1970’s¹⁶. These materials were designed with a main objective to match the mechanical properties of the replaced tissue, and by preventing protein adsorption and cell adhesion, to reduce the possible immune response

and rejection^{12,17}. The next stage of development took place in the early 1980s, research shifted from materials exclusively exhibiting bio-inert tissue response to substances actively interacting with their environment. The second-generation biomaterials were specifically designed to be “bioactive” by eliciting specific and desired cellular responses, like cell adhesion, proliferation and differentiation into a specific cell type¹⁸. An additional advance in the second generation materials was the development of biodegradability which allowed controllable chemical disintegration into non-toxic degradation products, which can be metabolized or directly eliminated from the organism¹⁹. Whereas second-generation biomaterials were designed to be either resorbable or bioactive, the next generation of biomaterials is combining these two properties, with the aim of developing materials that, once implanted, will help the body to heal itself. Third-generation biomaterials are being designed to stimulate specific cellular responses at the molecular level. Third-generation bioactive glasses and macroporous foams are being designed to activate genes that stimulate regeneration of living tissues. A cellular and molecular basis for development of third-generation biomaterials provides the scientific foundation for molecular design of scaffolds for tissue engineering and for in situ tissue regeneration and repair, with minimally invasive surgery. There are important economic advantages to each of these new approaches that may aid in solving the problems of caring for an aging population¹⁸. Over the years, a significant increase in the share of biopolymers in biomaterials production has been observed. This share is constantly growing, and biopolymers are slowly replacing traditional petroleum-based polymers and materials commonly available and used in other industrial fields. A schematic illustration showing the process of changes taking place on the biomaterials market is shown on Fig. 7.

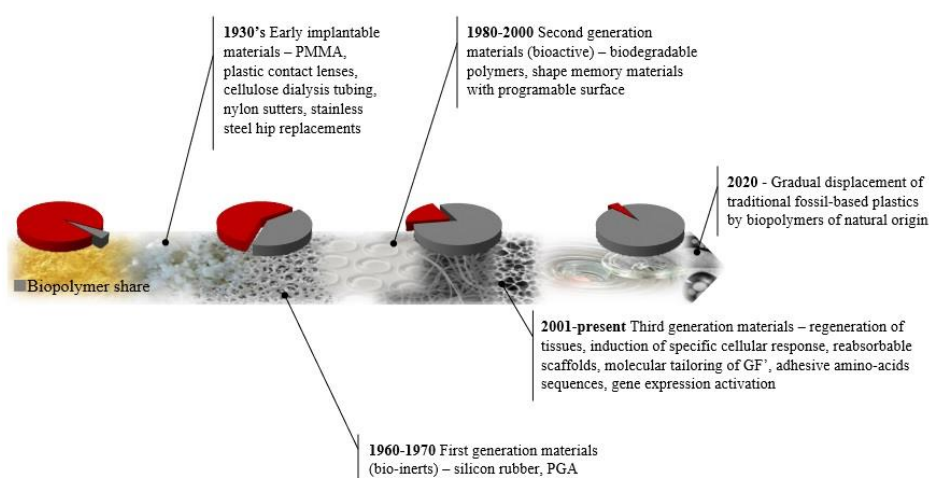


Fig. 7 Schematic representation of biopolymers participation in biomaterials market evolution^{17, 18}.

5.5. Biodegradable polymers – sources of origin

The term biodegradation is associated with materials that could be broken down by natural processes either through chemical mechanisms without enzymes contribution (hydrolysis) or enzymatic driven mechanism¹⁴. It is a two stage process with regards to polymers, the first phase is a fragmentation of the polymeric chain into lower molecular mass species, the second one relies on bio assimilation of the polymer fragments by microorganisms and their mineralization²⁰. This basic property of materials depends on many factors, and one of them is the origin of the polymer.

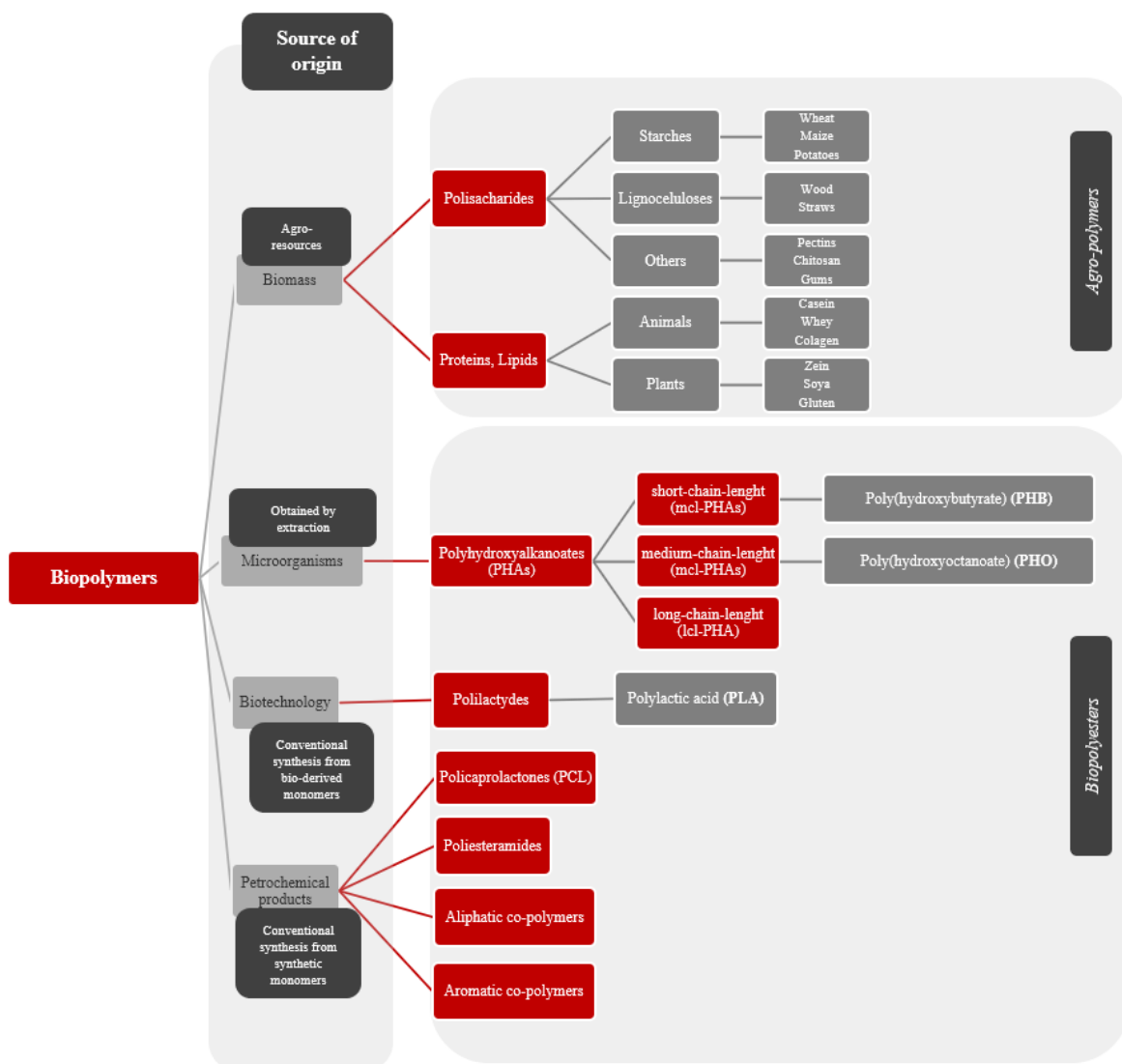


Fig. 8 Biopolymers classification by source of origin, Petroleum based polymers are included on this graph, because selected plastics exhibit biocompatibility and are biodegradable²¹.

5.5.1. “Green polymers” originating from petroleum resources

These are synthetic polymers containing hydrolysable functional groups, like ester, amide and urethane, or polymers with carbon backbones, in which additives like antioxidants can be added²⁰. Because of fact that petroleum-based polymers do not present full biodegradability a modification relying on enriching them with additives is necessary. Recent research activity on biodegradable synthetic polymers is focused on designing polymers with degradable backbones, *e.g.* polyanhydrides, polycarbonates, polylactones. Other method to enhance the biodegradability is the introduction of additives into the polymer chains which by reaction with UV light will induce degradation by photo-oxidation. Light - induced polymer degradation, or photodegradation, includes the physical and chemical changes caused by irradiation of polymers with ultraviolet or visible light. In order to be effective, light must be absorbed by the substrate (polymeric system)²². Polyolefins are resistant to hydrolysis, to oxidation and to biodegradation due to photoinitiators and stabilizers. They can be made oxo-degradable by use of pro-oxidant additives. These additives are based on metal combinations, such as Mn^{2+}/Mn^{3+} . The polyolefin will then degrade by a free radical chain reaction²⁰.

5.5.2. Biopolymers derived from Renewable Resources

Due to extensive use in industry and agriculture, synthetic polymers are essential for modern material sciences. Most synthetic polymers based on fossil fuel resources are incompatible with the natural environment, since they cannot be directly incorporated into the natural recycling system. It is possible to produce conventional polymers from natural resources (*i.e.* polyethylene (PE) from ethanol), but a better solution is widening use of naturally occurring biopolymers, traditional polymers, even when produced from renewable resources, do not present full biodegradability and contribute to the environment degradation. Much effort has been undertaken to examine the properties of environmentally friendly polymers originating from natural, renewable to expand their practical applications²³. Moreover, natural polymers perform a diverse set of functions in their native setting. For example, polysaccharides mediate transmembrane and intracellular communication and also can act as storage materials or as catalysts²⁴. These biological polymers can be obtained from microbial, plant or animal sources.

5.5.2.1. Agropolymers

Various polymers obtained from products or by-products of agricultural origin are proposed for the formulation of biodegradable materials or edible films. These polymers

(polysaccharides, proteins and lipids, or polyesters) can be used in various forms. The materials obtained from agropolymers are fully renewable and biodegradable. They are non-toxic to the soil and the environment, and they can be even edible when food-grade ingredients are used to prepare the material²⁴. Polysaccharides are a class of biopolymers created from simple sugar monomers. Those used in commerce and industry are isolates from terrestrial and marine plants or are principally the exogenous metabolites of some bacteria²⁵. The monomers (monosaccharides) are linked together by O-glycosidic bonds that can be made to any of the hydroxyl groups of a monosaccharide, conferring polysaccharides the ability to form both linear and branched polymers.

Two processing pathways can be distinguished for agropolymers²⁴:

- Dry process – thermoplastic extrusion, based on biopolymers thermoplastic properties
- Solvent process – based on drying of the film-forming solution

Since agro-polymers are relatively costly to produce, actual application areas are limited to special niches with environmental considerations. Loose-fill packaging and compost bags are the two major end uses, creating 90% of the global demand. New applications in agriculture and food packaging are emerging. Edible films and coatings provide an easy means of structurally strengthening certain foods, reducing particle clustering and improving visual and tactile features on product surfaces. Edible films can also be used to package components or additives that are to be dissolved in hot water or food mixes, and also act as an additional parameter for improving overall food quality and stability²⁶. A wide spectrum of applications can be found in medicine where these polymers can be used for the production of surgical sutures, gauzes, implants or biodegradable wound dressings^{24,27}.

5.5.2.2. Microbial polymers

There is a wide group of polymers produced by microorganisms that can find applications in many industry branches. Protein polymers are custom-designed material-forming proteins. They are made by living cells in the same way as other proteins but behave like synthetic molecules with special controllable characteristics²⁸. Nanocellulose based biopolymers has generated a great deal of interest as a source of nanometer-sized reinforcement, because of its good mechanical properties. In the last few years, nanocellulose have also attracted much attention due to environmental concerns²⁸. One of the intensely studied and widely used group of biopolymers of bacterial origin are polyhydroxyalkanoates (PHAs)²⁹. They are stored inside the bacteria in the form of granules, which are easy to recover and

purify³⁰. This group of polyesters is produced by a wide variety of microorganisms as an internal carbon and energy storage compounds, as part of their survival mechanism³¹. Polyhydroxyalkanoates represent a class of optically active biodegradable polyesters³². The first reported PHA, a homopolymer of PHB, was discovered by Lemoigne in 1926. It took nearly 60 years for the first medium chain length PHA (mcl-PHA) to be characterized and described³³. Bacterially synthesized polyhydroxyalkanoates (PHAs) have attract much attention because they can be produced from a variety of renewable resources, are fully biodegradable and highly biocompatible materials³⁴. This intriguing biological polyester initially attracted the attention of microbiologists and managed to keep many polymer scientists occupied over the second half of the last century. Concerted multidisciplinary scientific approaches have been directed to the elucidation of various aspects of PHA³⁵. The identification of hydroxyacid (HA) units other than poly-3-hydroxybutarate (P3HB) in microbial PHA proved to have major impact on the research and commercial interest for this bacterial reserve polymer. While the homopolymer of P(3HB) is a brittle material with limited applications, the incorporation of a second monomer unit into P(3HB) can significantly enhance its useful properties. This finding is therefore highlighted as a landmark which signifies the beginning of the second developmental stage of research on PHA³⁶. The last stage of development in research concerning bacterial PHA involved the cloning and characterization of genes engaged in the biosynthesis of reserve polyesters. The onset of the molecular biology revolution during the late 1970s provided new tools for biological research, which were successfully used to decipher genetic information and to understand further the principles behind PHA biosynthesis at the genetic level³⁷.

5.5.2.3. Polymers derived from monomers produced in biotechnological processes

The development of synthetic polymers using monomers from natural resources provides a new direction to develop biodegradable polymers from renewable resources. One of the most promising polymers in this regard is polylactic acid (PLA)³⁸. PLA is obtained through the chemical polymerization of enantiopure lactic acid produced from fermentation process. The cost of producing PLA is lower than PHAs and its uses in the plastic market have increased based on the development of additives to improve its properties³⁹. PLA can be prepared by direct polycondensation of lactic acid. However, it is usually difficult to obtain high molecular weight polymer by polycondensation. Each polymerization step generates one molecule of water, which degrades the forming polymer chain and results in low molecular weight PLA. To obtain a high molecular weight water has to be removed during the polycondensation process

(see 5.7.2.1). PLA is biocompatible and bioresorbable and can find application in a number of biomedical products such as sutures, implants, bone scaffolds, drug delivery systems, and in tissue engineering. PLA can also be employed in the preparation of bioplastics, useful in production of packaging, compost bags and everyday objects².

5.6. Biopolymers – application and perspectives

Sustainability, industrial ecology, eco-efficiency, and green chemistry are guiding the development of the next generation of materials, products, and processes⁴⁰. The same durability properties which make plastics ideal for many applications such as in packaging, building materials and commodities, as well as in hygiene products, can lead to waste-disposal problems in the case of traditional petroleum-derived plastics, as these materials are not sustainably produced, in most cases not readily biodegradable and because of their resistance to microbial degradation, they accumulate in the environment. These facts have helped to stimulate interest in biopolymers and in particular biodegradable biopolymers²⁰.

Biopolymers represent one of the leading sectors for bio-based products and their expected growth is foreseen to be significant within the coming years⁴¹. Due to the growing demand for energy and dwindling fossil fuel resources the global economy is aiming to replace currently known energy sources by greener, bio-based and sustainably produced equivalents. The trend also concerns the market for materials for which fossil fuels are also used and their production is very energy-consuming. Polymers of natural origin represent features that are unique among conventional fossil-based plastics. The market for biomaterials will grow, and the products themselves will find applications not only in niche areas where traditional polymers do not apply, but also will slowly replace them from the business^{17,42–44}. Another advantage of biomaterials is the fact that they can be produced from a range of currently underused or unused environmental resources such as biomass, post processed industrial waste streams (food, wood and biodiesel industries effluents) or wastewaters. Due to their chemical structure, these substances can be easily modified both by attaching functional groups and by creating physical mixtures. This means that their functionality is virtually unlimited and depends only on the modifications applied. Subjecting the material to enzymatic or chemical modifications allows their use in all branches of industry, both the production of ordinary packaging as well as specialized biomedical products such as implants or scaffolds. Biopolymers as biomaterials and matrices in tissue engineering offer important options in control of structure, morphology and chemistry or material functions such as mechanical properties in gel, fiber and porous scaffold formats⁴⁵. For example, xanthan gum was the first biopolymer to be produced at the industrial scale and still is one of the most significant on the market. Its worldwide annual production in

2012 was approximately 100,000 metric tons with a market price of 3 to 5 US\$ per kg⁴⁶. Bio-based polymers still hold a tiny fraction of the total global plastic market, they share less than 1% of the total market. As indicated by ISI Web of Sciences (Fig. 9A) and Thomas Innovations (Fig. 9B), there is a tremendous increase in the number of publication citations on bio-based polymers and applications in recent years⁴⁴.

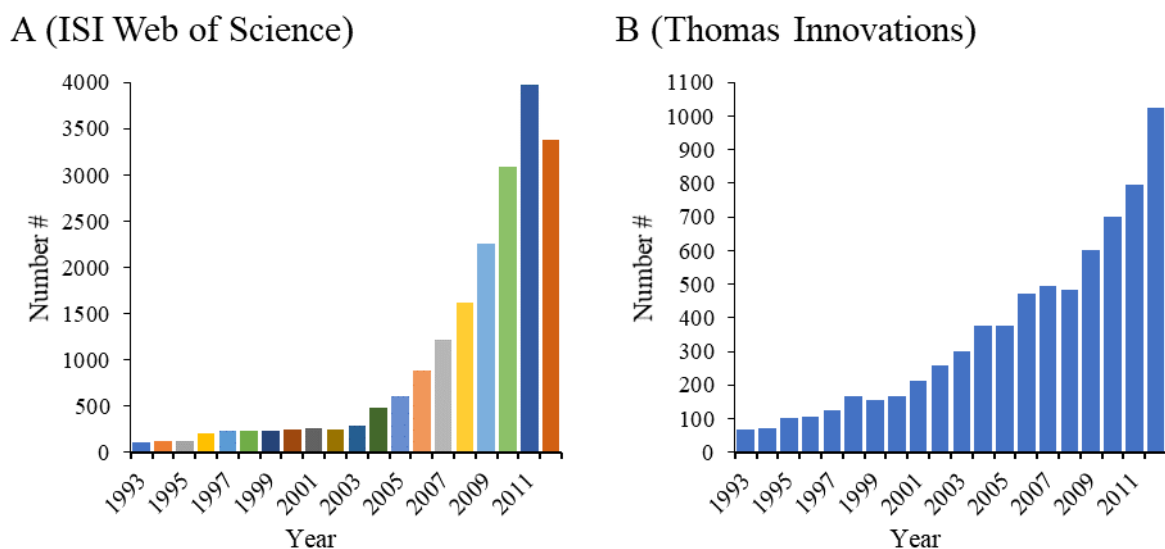


Fig. 9 Citation trends of (A) publications and (B) patents on bio-based polymers in recent years⁴⁴.

One of the extensively studied class of biodegradable polymers are aliphatic polyesters. The reason for this is their diversity and synthetic versatility. A large variety of monomers can be used in the production process. Various routes leading to the development of synthetic polyesters exist and have been recently reviewed and tested²⁰. Low molecular weight polymers can be obtained by polycondensation, whereas ring opening polymerization leads to high molecular polymers. Most biodegradable polyesters are product of ring opening polymerization of six or seven membered lactones⁴⁷. There is also a large group of polyesters naturally occurring in the environment like caoutchouc (India rubber) or cellulose. Some of microorganisms like bacteria present ability of producing and storing biopolymers (i.e. PHAs).

5.6.1. Polyhydroxyalkanoates – biopolymers produced by naturally occurring microorganisms

PHAs are excellent biocompatible materials due to the lack of toxicity with human tissue or blood⁴⁸. *In vivo* tests have shown that these polyesters are biocompatible to osteoblastic, epithelial and neuronal cells^{49,50}. Other engaging feature of PHAs is their full biodegradability. Even though PHAs have proved their usefulness due to their desirable mechanical properties,

intrinsic biodegradability and biocompatibility, the high price is limiting their everyday life application significantly. Production of PHAs using agro-industrial residues offers an alternative use of low-cost feedstock to produce materials with appropriate physicochemical properties offering a broad range of applications⁴¹. Currently, biopolymers based on PHA account for around 2.5% of the market (Fig. 11).

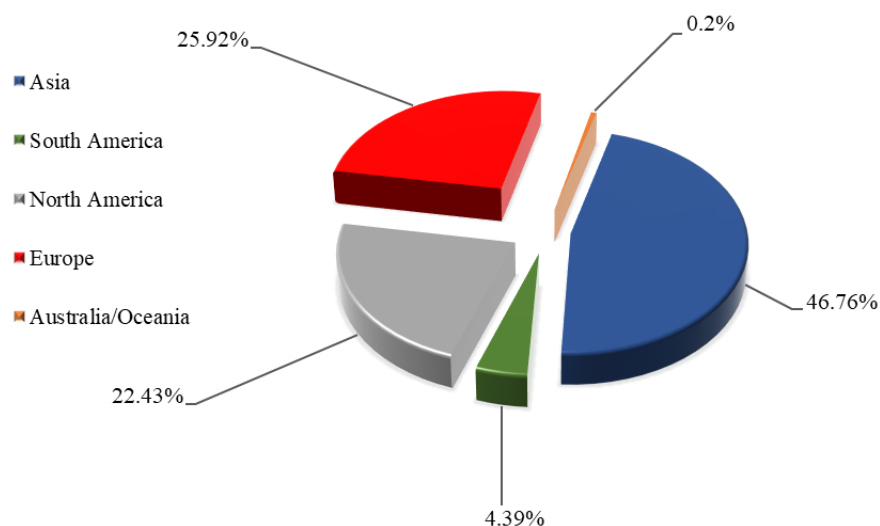


Fig. 10 Biopolymers global production capacities by Region. (European Bioplastics, nova-Institute, 2016)

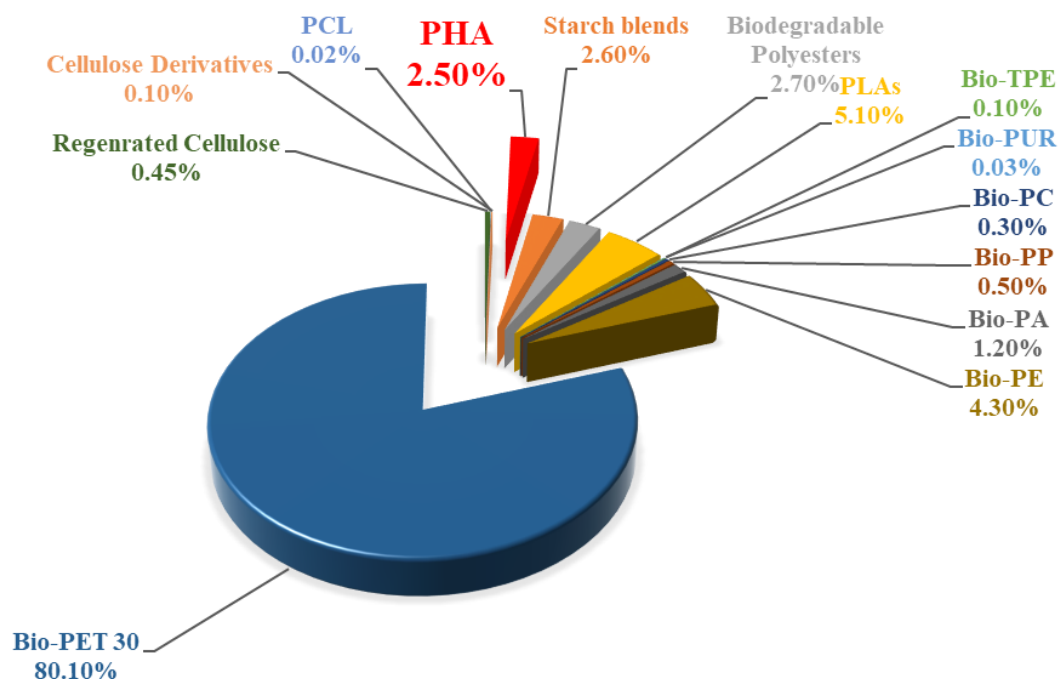


Fig. 11 Material share of biopolymer production capacity.
(European bioplastic| Institute for Bioplastics and Biocomposites)

5.7. Biopolymers – future material for medicine and biomedical sciences



5.7.1. Elastic substrates' significance for biological sciences

Despite considerable progress in understanding chemical mechanisms in interactions between cells, and the environment, such as signaling pathways and molecular structures, the influence of mechanical properties of the environment on the behavior of cells, tissues or entire organs remains faintly understood field in cellular research⁵¹. It is confirmed that all cells undergo complicated mechanical affections with their environment. These interactions are the physical basis for numerous physiological phenomena like cell migration, wound healing, embryonic development or tumor metastasis⁵². The significance of relationship between the cell physiology and its surroundings was not revealed until the 1950s⁵¹. Numerous studies conducted in scientific centers around the world has confirmed that many diseases, dysfunctions and pathological occurrences of an living organism have their basis in alterations in mechanical properties of a given tissue^{51,52}. The mechanical properties of the extracellular matrix have also been found to be crucial in the regulation in cell proliferation, differentiation and growth. The parameter that particularly affects the behavior of cells is flexibility of the substrate. In addition, it has been proven that often the progression of a cancer disease is closely related to the change in the elasticity of a given tissue^{51,52,53,54}. In Table 1 the chosen tissues of a given species along with the values of their elasticity modules are presented.

Type of tissue	Animal	Elastic Modulus	Testing method
Articular cartilage	Bovine	950kPa	Compression
Skeletal muscle	Rat	100kPa	Tension
Carotid artery	Mouse	90kPa	Perfusion
Spinal cord	Human	89kPa	Tension
Cardiac muscle	Mouse	20-150kPa	Tension
Lung	Guinea pig	5-6kPa	Tension
Kidney	Swine	2.5kPa	Rheology
Liver	Human	640Pa	Compression
Brain	Swine	260-490Pa	Indentation
Thyroid cancer	Human	45kPa	Compression
Fat	Human	17Pa	Indentation

Table 1. Tissues of a given species along with the values of their elasticity modules and testing methodology⁵¹.

To collect information about processes affected by flexibility of the cell's environment, the forces exerted by the cell as well as their response should be thoroughly investigated. An effective and authoritative method to gather all desired data are flexible cellular substrates. One of the commonly used materials in cellular research are polymer hydrogels. They have also found applications in biomedicine and pharmacy, allowing, e.g., monitoring the delivery and release of drugs⁵⁵. One of the types of polymer hydrogels are polyacrylamide flexible substrates. In polyacrylamide gels water acts like dispersed phase, while as a gelling phase various polymer are used. They are formed by the polymerization of acrylamide crosslinked with bisacrylamide. For the reaction to occur, the presence of polymerization catalyst - TEMED, as well as the reaction initiator – persulfate ammonium is required⁵⁶. A tidy feature of elastic polyacrylamide hydrogel is isotropy and elasticity, which gives the opportunity to study cellular tractions and migration, cytoskeleton architecture and other parameters on the entire surface of the substrate. A very important feature of polyacrylamide substrate is its transparency, which allows optical microscopy studies. The pores present in the polymerized gel do not exceed 100 nm, which prevents cells from migrating deeper into the substrate. An additional advantage of polyacrylamide gels is that their surface may be easily functionalized with various extracellular matrix proteins. This feature allows to create conditions close to physiological with simultaneous manipulation of mechanical and chemical properties of the substrate. It creates comprehensive research possibilities and allows to simulate the

physiological behavior of cells from different regions of the organism⁵⁷. Proper execution of this type of substrate is accompanied by a difficult, multi-stage preparation process. In addition, they include ingredients that are not neutral for cultured cells. Cells do not show willingness for adhesion to the substrate without additional surface functionalization with proteins like fibronectin or FBS^{54,55,58,59}.

Due to the rapid advances in cellular and material research, it is important to look for novel materials that can serve in cellular studies and as biomedical substance with unique features. New biocompatible materials can facilitate cellular research as well as perfectly serve as a raw material for the production of medical articles such as wound dressings, implants or scaffoldings for bone tissue.

5.7.2. Polylactide – biodegradable and bioactive polyester

Polylactide (PLA) is one of the most innovative materials being actively investigated for a wide range of industrial applications. This biodegradable and biocompatible polymer is a linear aliphatic thermoplastic polyester. Polylactic acid (PLA) polymers are synthesized and obtained from renewable agricultural resources⁶⁰. PLA is an attractive choice for applications involving human interface because of its biocompatibility and biodegradability⁶¹. By considering the production at large industrial scale, PLA is a relatively new polymer because the first full-scale plant capable to produce 140,000 metric tons per year was started in Blair, Nebraska, USA in 2002⁶². The bio-based plastics market is projected to increase to about 3.45 million tons by 2020, in which PLA alone is estimated to account for about 800,000 tons⁶³.

5.7.2.1. Polymer synthesis

PLA can be obtained by chemical synthesis in three different reaction routes (Fig. 15)^{2,64}. Polymers can derive directly from lactic acid (LA) and be obtained by polycondensation (Fig. 12). Those are usually referred to as poly (lactic acid). This process needs to be carried out at less than 200°C; above that temperature, the entropically favored lactide monomer is generated^{65,4}. Polycondensation is polymer formation process by linking monomers together, accompanied by elimination of byproducts (e.g., water and alcohols). In case of PLA, polycondensation of lactic acid by connecting carboxyl and hydroxyl groups produces water byproduct simultaneously. Due to the difficulty in removing byproducts completely from the highly viscous reaction mixture, polymer produced through direct polycondensation is usually of low molecular weight⁶⁶.

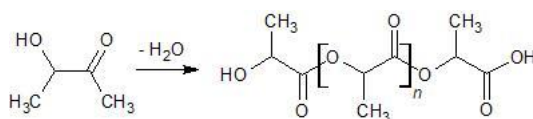


Fig. 12 Obtaining PLA by polycondensation directly from lactic acid.

In 1932, a new method named ring opening polymerization (ROP) was firstly demonstrated by Carothers⁶⁷. In the recent decade, polycondensation techniques have been improved with more efficient synthesis methods. Several modified methods have been reported to produce PLA with high molecular weight successfully⁶⁶. Ring-opening polymerization is a propagation process of cyclic monomers initiated by different ions. As the reactive center of proliferation, the terminal end of a polymer classifies the mechanism into anionic ROP, cationic ROP and radical ROP⁶⁸. PLA preparation from lactide by ring opening polymerization (ROP) is presented on Fig. 13. Materials obtained with the second route of synthesis are popularly called polylactides. Both types are commonly referred to as PLA, and this abbreviation is current in the scientific publications⁶⁹. Ring-opening polymerization (ROP) of lactide is a preferred route to prepare PLA because of the higher controllability of the polymerization process².

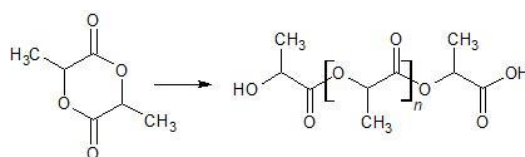


Fig. 13 Production of PLA by ring opening polymerization (ROP) method.

Because the lactide molecule has two chiral carbon atoms in its structure, three stereoisomers of lactide (Fig. 14) can be generated. L-, D- and meso-lactide are the final product of the reaction⁶⁹.

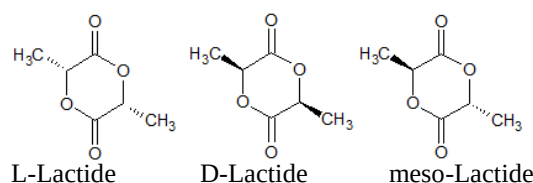


Fig. 14 Stereoisomers of lactide⁶⁹.

Comparing with polycondensation, polymerization of lactide can produce polymer with wider range of molecular weight by controlling the purity of lactide and synthesis conditions, without chain coupling agent or azeotropic system⁶⁶.

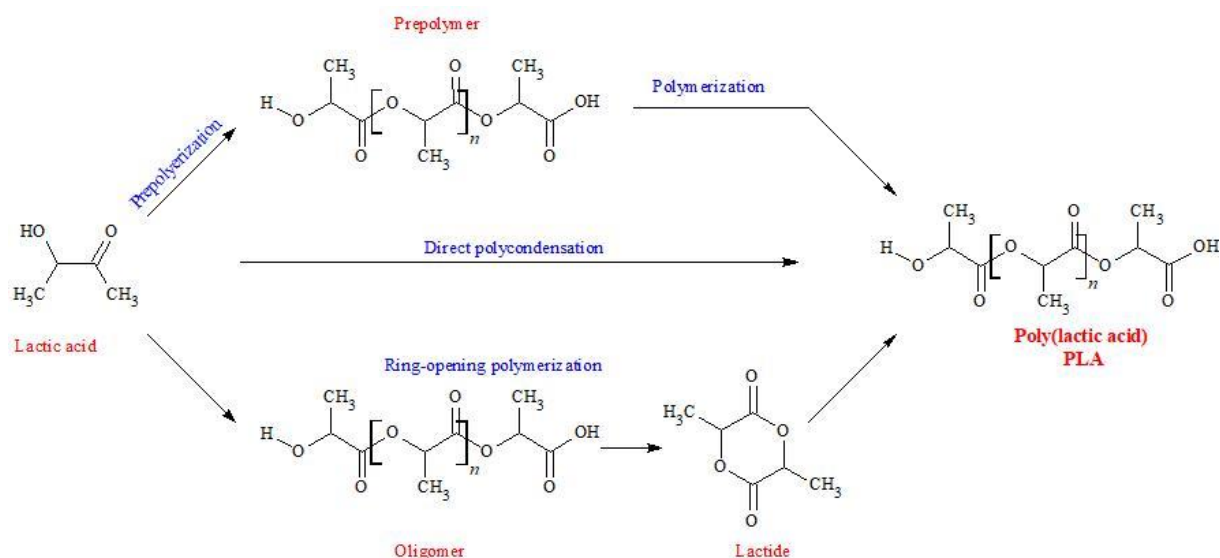


Fig. 15 Routes of poly (lactic acid) (PLA) synthesis from lactic acid.

5.7.2.2. PLA microbial production

Poly lactide can be chemically synthesized in a cheap and efficient way but to maintain the high reaction velocity the chemo-process must be carried out via harmful metal catalysts with high reaction what leads to production of chemical residues that are subject to health and safety concerns. The bio-process for PLA production is thus preferable to overcome this problem. Polylactic acid is naturally produced by a wide spectrum of microbes including bacteria, yeast and filamentous fungi. In general, bacteria ferment C5 and C6 sugars to lactic acid by either homo- or hetero-fermentative mode. Among bacterial strains lactic acid producing bacteria is reserved specifically for the lactic acid bacteria (LAB), which include *Lactobacillus*, *Bacillus*, *Pediococcus*, *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Tetragenococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Weissella*, *Streptococcus* and *Lactococcus*⁷⁰.

Lactic acid bacteria are categorized as homo- and hetero-fermentative strains. Homo-fermentative lactic acid bacteria contain the gene expressing a mechanism of converting one molecule of glucose into two molecules of LA generating two molecules of ATP. Hetero-fermentative bacteria use the phosphoketolase pathway to convert one molecule of xylose to one LA molecule as a byproduct one ethanol or acetic acid molecule is formed⁷⁰.

Molecular biology, as a tool to create more efficient process, comes with aid to chemical polymerization. Therefore, with its help scientists created a synthetic pathway in order to synthesize PLA *in vivo*. In the PLA biosynthesis pathway, the engineered propionyl-CoA transferase (PCT) converts lactate into lactyl-CoA. The lactyl-CoA is subsequently polymerized by the engineered PHA synthase (PhaC) to form PLA⁷¹.

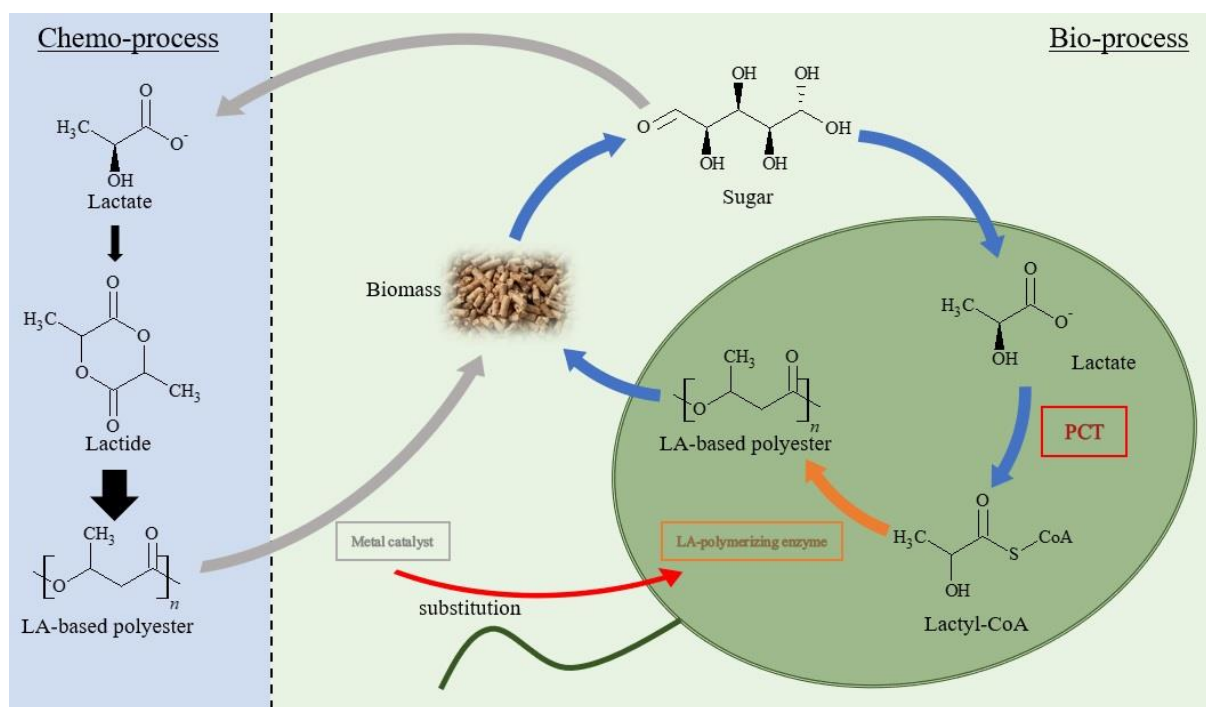


Fig. 16 Microbial production of LA-based polyester. PLA can be synthesized during chemo-process where the copolymerization of LA with other monomer units is performed. In microbial bio-process, the LA monomer substrate is recognized as a CoA form (LA-CoA) by LA-polymerizing enzyme.

5.7.2.3. PLA production from raw materials

Lactic acid is traditionally produced from glucose, lactose, dairy plant wastes (skim milk, whey, paneer whey), starch (potato, cassava, wheat, rice and sorghum), molasses and glycerol originating from biodiesel industry. However, the use of traditional raw materials competes with the supply of foods and feeds increasing the production cost. Therefore, producing LA using less expensive carbon sources has now become a focus of LA production⁷⁰. Plant biomass is the most abundant renewable resource on earth. Plant cell wall contains cellulose, hemicellulose and lignin. Starch is a carbohydrate polymer which cannot be directly utilized by most microbes for metabolism. The addition of amylases is usually mandatory to release fermentable sugars (glucose) from starch. Algal biomass is rich in carbohydrates and proteins which could be a good source for producing LA. Lack of lignin in algal biomass is an additional advantage over plant biomass. Dairy plant wastes have been used for LA production by for a long time. Due to its poor nutritional status wastes such as whey, must be enriched with nitrogen sources such as yeast extract, peptone, soy flour or corn steep liquor to significantly improve LA production efficiency. Nitrogen is the building block for living cells and is essential for the synthesis of amino acids, purines, pyrimidines, some carbohydrates and lipids, co-factors and other nitrogen containing substances⁷².

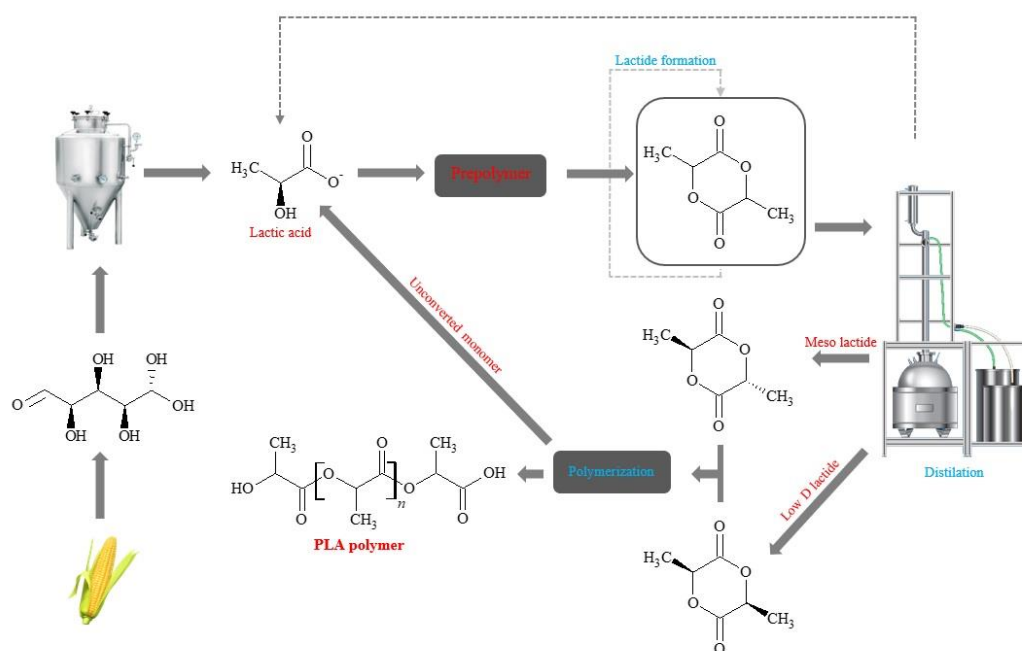


Fig. 17 PLA production from renewable plant resource. The plant is fermented and distilled to produce lactic acid. Lactic acid is then converted into PLA resin through a series of processes, such as refining, dehydration, polymerization, high-temperature de-polymerization and polymerization.

5.7.2.4. PLA physical properties

PLA boasts high mechanical strength and Young modulus value, but it has low impact toughness, low use temperature, and a relatively small window for processability. In addition, PLA has a high hydrophobicity and low degradation rate, which may be undesirable for certain biomedical applications⁶¹. Below in Table 2 selected physical properties of PLA have been collected.

Physical properties of PLA	
Crystallinity	0% (PDLA)-37%(PLLA)
Glass transition temperature T_g	50-64°C
Melting temperature T_m	145-168°C
Tensile strenght	28-50 Mpa
Young's modulus	1.2-3.0 GPa
Elongation at break	2-6%

Table 2. Selected physical properties of PLA polymer⁶¹.

5.7.2.5. PLA applications

PLA is an attractive choice for applications involving human interface because of its complete biodegradability and biocompatibility⁶¹. Some of the mechanical properties of polymer like tensile resistance makes it an ideal candidate for load-bearing applications, however low impact toughness, limits its usability in mechanically intense applications such as implants or bone grafts⁶⁰. Beside medical applications, PLA is a growing alternative as a “green” food packaging polymer. New applications have been claimed in the field of fresh products, where thermoformed PLA containers are used in retail markets for fruits, vegetables, and salads. The market capacity of these products packaged in PLA is virtually unlimited.

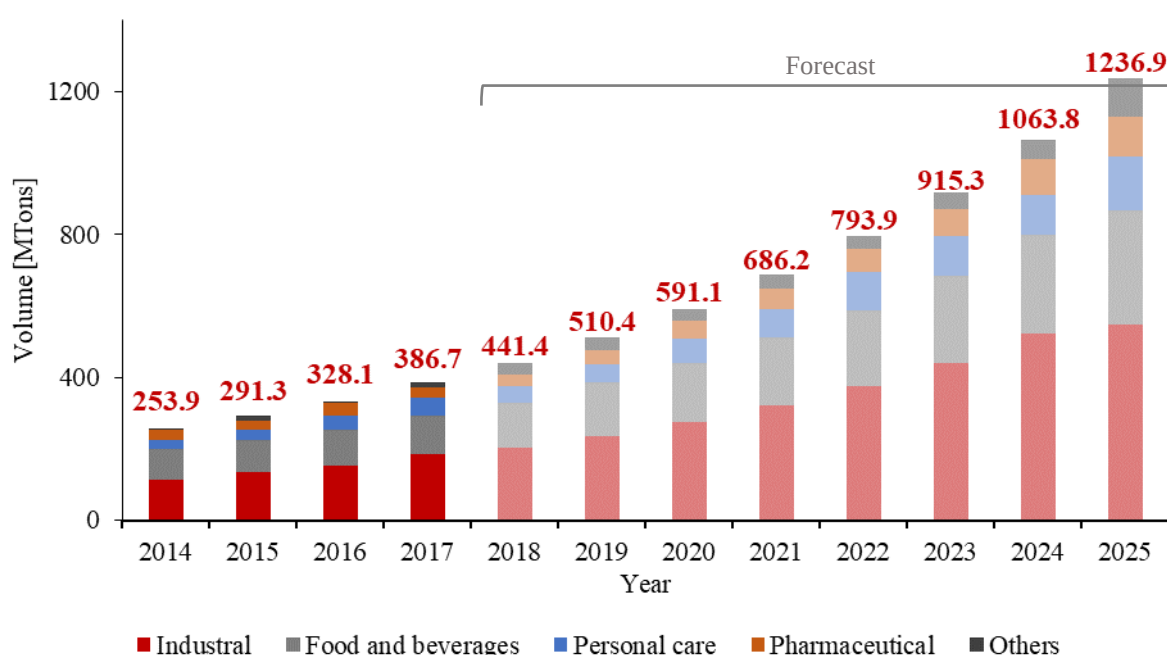


Fig. 18 U.S. PLA market volume by application, 2014 – 2025⁷³.

Commercialized PLA products demonstrate this fact that PLA is not being used solely because of its degradability, nor because it is made from renewable resources; it is being used because it functions very well and provides excellent properties at a competitive price. There are many commercialized PLA products in today's market and their variety and consumption are increasing rapidly.

5.7.3. Polyhydroxyalkanoates (PHAs) – biocompatible materials of bacterial origin

Production of PHAs from various microorganisms including wild-type and engineered bacteria has been extensively investigated⁷⁴. PHAs are non-toxic materials which biodegradation leads to products that are harmless for the natural environment⁷⁵. Due to the

diverse chemical structure they are highly versatile and present a broad range of physical and chemical properties. Short chain PHA (scl-PHA) has properties similar to conventional plastics such as polypropylene or polyethylene, while mcl-PHA has elastomeric properties²⁷. Therefore, they are a wholesome alternative to synthetic petrochemical polymers in a broad variety of applications. Initially PHAs were used in packaging industry. They served as a stock material for cosmetic bottles, shopping bags and food containers⁷⁶.

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates (HAs) synthesized and stored in the cell cytoplasm as water insoluble inclusions by various microorganisms^{34,35}, as light refracting granules inside the cell (Fig. 20). This accumulation in granules separates the polymer from the cell lumen and, consequently, the osmotic pressure of the cell is not changed extensively. The number of granules per cell seems to be strain specific⁷⁷. A scheme of PHA granule^{77,78} is shown in Fig. 19.

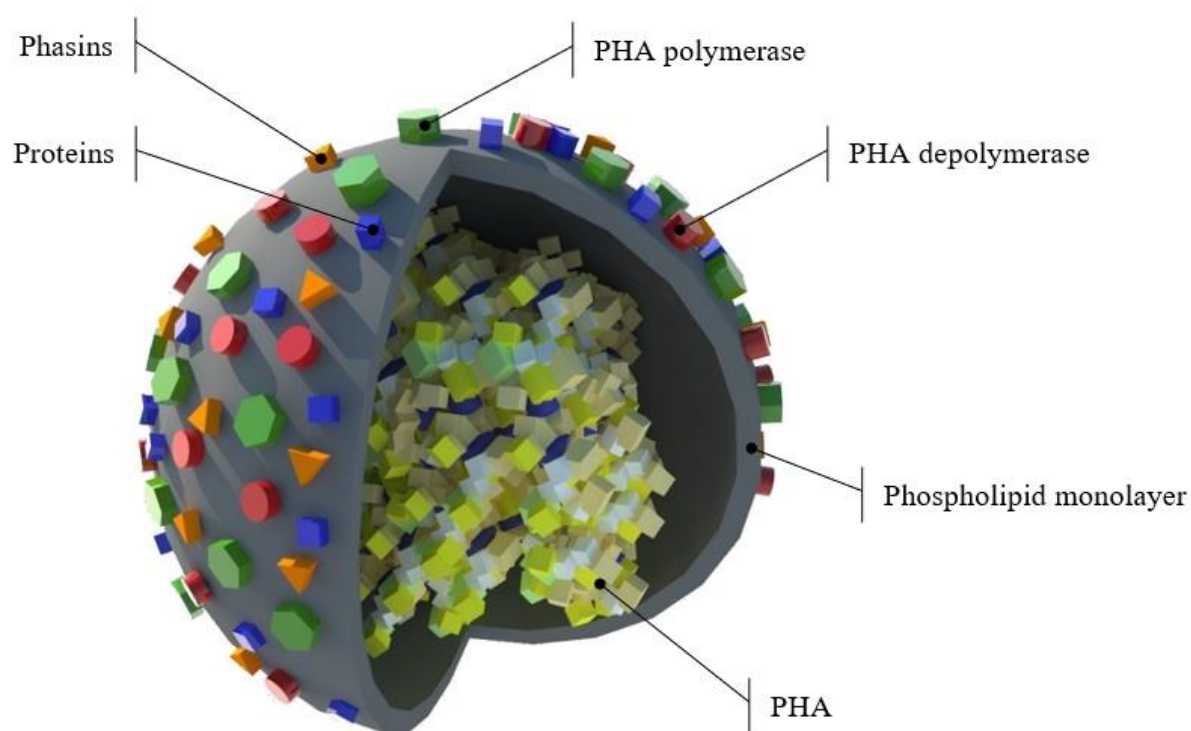


Fig. 19 Scheme of PHA granule. The core consists of PHA and is covered with lipid monolayer with integrated proteins. The proteins integrated in the monolayer are PHA polymerase, PHA depolymerase, phasins (structural proteins) and small fraction of proteins of unrecognized function.

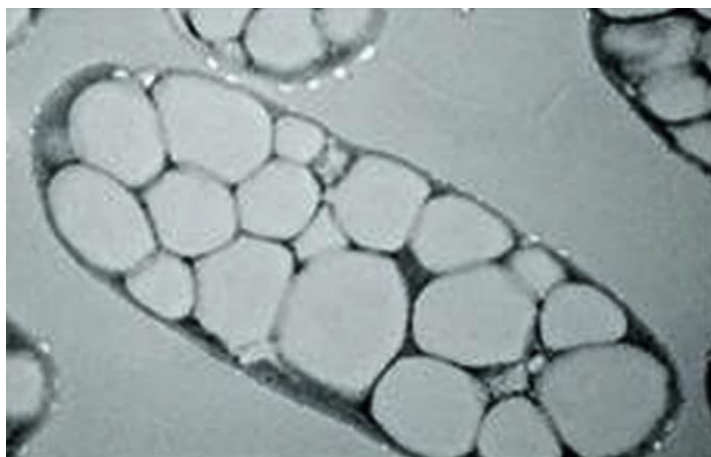


Fig. 20 Polymer producing cells filled with PHA granules⁷⁴.

Polyhydroxyalkanoates are gaining increasing attention in the biopolymer market due to their promising properties such as high biodegradability in different environments and processing versatility. These biogenic polyesters represent a promising replacement for fossil fuel-based thermoplastics⁴⁹ due to their structural diversity and close analogy to common plastics⁷⁹. More than 150 HAs have been marked as PHAs components, they provide various mechanical properties (i.e. solid crystalline polymer or elastic rubber) depending on the incorporated monomer units in this thermoplastic materials³⁴.

PHAs represent a class of optically active biodegradable polyesters accumulated by numerous bacteria as discrete intracellular granules⁸⁰ or as a net like extracellular structures⁸¹ to levels as high as 90% of the cell dry weight under conditions of nutrient stress⁷⁹. PHAs serve as a carbon and energy reserve material for organisms using them as substrate³². Poly(3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules, their chemical structure is linear in a head to tail formation of (*R*)-3-hydroxyalkanoic acids with the carboxyl group of one monomer forming an ester bond with the hydroxyl group of the neighboring monomer⁸². The chemical structure of PHAs monomer is shown in Fig. 21.

PHAs can be divided into different categories depending on the chosen criteria. When the length of a single monomer chain is considered PHAs can be categorized as short (C3–C5; scl-PHA), medium (C6–C14; mcl-PHA) and long (>C14; lcl-PHA).

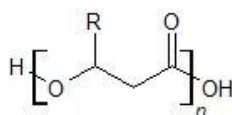


Fig. 21 Chemical structure of single PHA monomer.

When in a single PHA molecule monomers of different length are incorporated a mixed copolymer can be formed i.e. as a copolymer consisting of (R)-3-hydroxybutyrate and (R)-3-hydroxyhexanoate (PHBHx)⁸³. Thanks to the possibility of R group substitution in PHAs, with the use of various functional groups such as ester, branched olefinic, cyano, amino, nitroso, nitrophenoxy, chloro, carboxylic and phenyl, a plastic with pre-set chemical properties or biological activity can be produced⁷⁸.

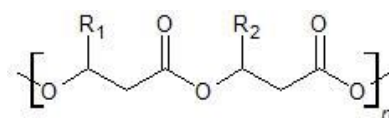


Fig. 22 Chemical structure of PHAs, where R1, R2 are various side chains

PHAs are containing only the R-enantiomer, which means they are a source of chiral molecules⁸⁴. The presence of two groups (hydroxyl and carboxyl) makes the monomer relatively susceptible to modification^{85–87}.

Criterion	Classification	
Source of origin	Biosynthetic	■ Natural - produced by microorganisms from metabolites ■ Semisynthetic - precursors that cannot be synthesized by bacterium addition required ■ Synthetic - polyesters that can only be obtained by chemical synthesis
	Chemical	■ Containing aliphatic fatty acid derivatives (with functional groups in the monomers) ■ Containing aromatic fatty acid derivatives ■ Containing both aliphatic and aromatic fatty acid derivatives
Monomer size	■ Short-chain length (C3-C5) - scl-PHAs ■ Medium-chain length (C6-C14) - mclPHAs ■ Long-chain length (>C14) - lcl-PHAs	
Type of monomers in polymer	■ Homopolymer - single monomer type present ■ Heteropolymer (copolymer) - more than one monomer type present	

Table 3. Classification of Polyhydroxyalkanoates³³(see Fig. 8).

5.7.3.1. PHAs accumulation genetics and regulation

Poly-(R)-hydroxyalkanoic acids (PHA) are a group of storage compounds of carbon and energy that are accumulated during unbalanced growth by many bacteria⁸⁸. The scl-PHA biosynthesis genes are organized on a gene cluster *phaCAB* that codes for three proteins: *PhaA* (β -ketothiolase), *PhaB* (NADPH dependent acetoacetyl CoA dehydrogenase) and *PhaC* (PHB Synthase). It has not been established how these gene products directly contribute to scl-PHAs accumulation in bacteria. There are nine enzymes presumably taking part in intracellular scl-PHAs degradation. The clear majority of them are depolymerases

(*PhaZ1-PhaZ7*) the rest is represented by two hydrolases *PhaY1* and *PhaY2*. The PHA granule surface is covered with proteins of many classes (Fig. 19) including phasins which are a class of low molecular weight proteins structurally associated with PHA grain⁸⁹. These proteins prevent granules merging can regulate the size and amount of PHA granules in the cell. It has also been proved that phasins positively affect PHB synthesis throughout the period of PHB production⁹⁰. There are 4 genes coding the phasins proteins but the main PHA inclusion binding phasin gene is *PhaP1* which protective role was revealed in PHB synthesizing *E. coli* bacteria⁹¹. The intracellular synthesis of scl-PHAs can be regulated at both enzymatic and transcriptional level. Acetyl-CoA cytoplasmic concentration regulates the polymers accumulation. The biosynthesis rate is controlled by 3-ketothiolase (*PhaA*) and acetoactyl-CoA reductase (*PhaB*). Elevated *PhaA* and *PhaB* activity results in granule size increase, the number of granules depends on activity level of *PhaC*. The expression of *PhaP* can be regulated transcriptionally by *PhaR* regulation gene. When the conditions are not conducive for PHA accumulation *PhaR* binds with the promoter region of *PhaP* thus repressing gene transcription. After the occurrence of circumstances favorable for polymer accumulation *PhaR* binds to the PHA granule enabling the *PhaP* transcription. During the following polymer accumulation stages PHA degradative enzymes (*PhaZ*) begin mobilizing granules, *PhaR* rebinds to the promoter region of *PhaP* and the gene transcription is repressed once again⁹². *PhaR* can control the amount of repressor present in cells by binding to its own promoter region. Thanks to these regulation mechanisms, the required *PhaP* concentration is always maintained to ensure the efficient polymer synthesis to take place. Other genes homologous to *PhaR* have been reported in different PHA accumulating bacteria, where they also act as phasin synthesis regulators⁹³.

The mcl-PHA accumulation is regulated by two polymerases encoded by *PhaC1* and *PhaC2* genes. These genes are separated on the chromosome by the *PhaZ* gene, which codes for the PHA degradation enzyme that mobilizes PHA to release carbon on restoration of the limiting nutrient. *PhaD*, located next to *PhaC2*, is a gene responsible for PHA accumulation and synthesis regulation through activation of *Pf* and *Pi* promoters.

reaction couples two acetyl-CoA molecules forming acetoacetyl-CoA while 3-ketothiolase (PhaA) acts as a catalyst. In further stage the NADPH dependent acetoacetyl-CoA dehydrogenase (PhaB) mediates in (R)-(3)-hydroxybutyl-CoA formation by reduction of acetoacetyl-CoA. To obtain a final product which will be a scl-PHA polymer PHA polymerase (PhaC) must initiate the polymerization process. Selected bacteria strains are capable of scl-PHA co-polymers accumulation, some bacteria were found to accumulate a ter-polymer consisting of (R)-hydroxybutyrate, (R)-hydroxyvalerate and (R)-hydroxyhexanoate monomer units.

Pseudomonas putida has a variety of potential uses in bioremediation and biosynthesis of biodegradable plastics. *P. putida* is able to utilize a wide range of carbon sources⁹⁶. *Pseudomonas putida* strains are known to produce large amounts of mcl-PHAs under nutrient limitation. *P. putida* KT 2440 is the best characterized strain and its genome has been fully sequenced. This strain is metabolically versatile and is able to utilize a wide range of materials including: glycerol, sugars (such as glucose), small organic acids (such as citrate), and fatty acids as carbon sources⁹⁷. Carbon limited exponential feeding of nonanoic acid or other related substrates to cultures of *P. putida* KT2440 is a simple and effective method of producing mcl-PHAs, during this process nitrogen limitation is unnecessary⁹⁸.

a. β -oxidation

In β -oxidation cyclic process fatty acids are broken down and metabolized. Initially the fatty acids are degraded to acetyl-CoA which is afterwards fed into the TCA cycle or directly consumed in biosynthesis. During one β -oxidation cycle acetyl-CoA, NADH and FADH₂ are produced. To provide more ATP NADH and FADH₂ can undergo oxidation via electron transport chain. The fatty acyl-CoA shortened by two carbons can enter another reaction cycle. The fatty acids metabolism consists of catabolic processes during which energy and primary metabolites are generated, and anabolic processes which lead to creation of biologically important molecules. The first step of β -oxidation is catalyzed by *FadDs*, which are long chain fatty acyl-CoA synthetases associated with inner cell membrane. To activate *FadD*, an outer membrane bound protein - *FadL* is required (Fig. 24-1). Next, the acyl-CoA thioesters enter the β -oxidation cycle where they are subject to acyl-CoA dehydrogenase (*FadE*) activity (Fig. 24-2). As a result, 2-trans-enoyl-CoA is created and subsequently converted to (S)-3-hydroxyacyl-CoA by enoyl-CoA hydratase (*FabB*). At the next stage (S)-3-hydroxyacyl-CoA is oxidized to 3-ketoacyl-CoA by (S)-3-hydroxyacyl dehydrogenase (Fig. 24-3). The last

reaction step in the β -oxidation pathway is the catalyzation of acetyl-CoA by ketoacyl-CoA thiolase resulting in receiving acyl-CoA shortened by two carbons (Fig. 24-5)⁸².

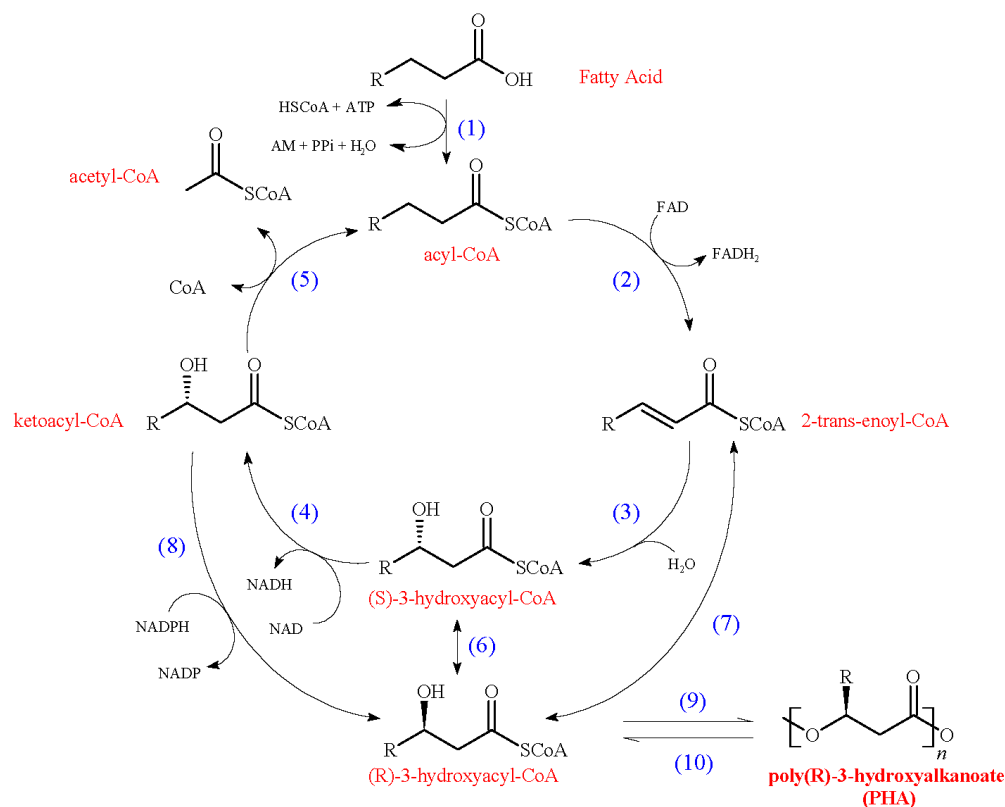


Fig. 24 Biosynthetic pathway for mcl-PHA from fatty acids.

- (1) fatty acid degradation by acyl-CoA synthase (*FadD*);
- (2) acyl-CoA dehydrogenase (*FadE*) transforms acyl-CoA into 2-trans-enoyl-CoA;
- (3) 2-trans-enoyl-CoA conversion into (S)-3-hydroxyacyl-CoA by enoyl-CoA hydratase (*FabB*);
- (4) (S)-3-hydroxyacyl-CoA oxidation to 3-ketoacyl-CoA by NAD dependent (S)-3-hydroxyacyl-CoA dehydrogenase (*FadB*);
- (5) ketoacyl-CoA thiolase (*FadA*) catalyzes 3-ketoacyl-CoA to acyl-CoA;
- (6) 3-hydroxyacyl-CoA epimerase converts (S)-3-hydroxyacyl-CoA to (R)-3-hydroxyacyl-CoA;
- (7) Two molecules of trans-enoyl-CoA are transformed into (R)-3-hydroxyacyl-CoA by (R)-specific enoyl-CoA hydratase (*PhaJ*), reaction may occur bi-directionally;
- (8) 3-ketoacyl-CoA undergoes reduction catalyzed NADPH dependent 3-ketoacyl-CoA reductase (*FabG*) and (R)-3-hydroxyacyl-CoA is obtained;
- (9) PHA polymerase (*PhaC*) and (10) PHA depolymerase (*PhaZ*) participate successively in the mclPHA polymer production and degradation reaction.

In the retrieval of PHA precursor (R)-3-hydroxyacyl-CoAs from the β -oxidation cycle three enzymes are involved. (S)-3-hydroxyacyl-CoA can be converted to (R)-3-hydroxyacyl-CoA by 3-Hydroxyacyl-CoA epimerase (Fig. 24-6). (R)-specific enoyl-CoA hydratase (*PhaJ*) mediates

the transformation of two molecules of trans-enoyl-CoA into (R)-3-hydroxyacyl-CoA (Fig. 24-7). This reaction is fully reversible. The third way to obtain (R)-3-hydroxyacyl-CoA is the reduction of 3-ketoacyl-CoA by NADPH dependent 3-ketoacyl-CoA reductase (*FabG*) (Fig. 24-8). Two enzymes with opposite action are responsible for the polymerization and depolymerization of mcl PHAs - PHA polymerase (*PhaC*) and PHA depolymerase (*PhaZ*). Other PHAs accumulation pathways known are described in supplementary materials (13. *Alternative PHAs accumulation pathways*).

5.7.3.3. PHAs applications

a. Non-medical applications

Initially PHAs were used for packaging purposes in the manufacture of cosmetic bottles, shopping bags, containers food products. Biodegradability enabled PHAs to find application in high end designer sanitary and bathroom commodities purchased by environmentally aware customers. Enclosures and cases for electronic devices such as mobile phones, notebooks and other consumer electronic goods is another major area in which PHAs potential has been noticed by suppliers⁹⁹. A very interesting application of PHA is their use as starting chemicals (synthons) for other chemicals taking advantage of their uniform chirality¹⁰⁰. The ester bond can be easily split either chemically with acid or base¹⁰¹, enzymatically¹⁰² or through in vivo degradation under specific conditions¹⁰⁰.

b. Medical applications

One of the alluring features of PHA is its biodegradability³⁴. It is property of PHA that has gained the biopolymer interest not only as a potential alternative to traditional petrochemical plastics, but also opened new areas of application for it³¹. One of greatest advantages that PHAs possess over other biodegradable polymers is their ability to degrade under both aerobic and anaerobic conditions. By biodegradation the set of chemical reactions through which macromolecules are subdivided into smaller molecules by different microorganisms is understood. PHA can also be degraded by thermal means or enzymatic hydrolysis⁷⁹. The biodegradation process of a PHA polymer depends on its chemical structure (especially presence of functional groups and hydrophilicity-hydrophobicity balance) and physicochemical properties (mainly crystallinity and morphological properties and porosity)¹⁰³. PHAs are excellent materials for medicine due to the lack of toxicity with human tissue or blood¹⁰⁴. *In vivo* tests have shown that these polyesters are biocompatible to osteoblastic, epithelial and neuronal

cells^{105,106}. PHAs, especially PHB, PHBV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)), P4HB, PHO and PHBHx are present in biomedical sciences especially in tissue engineering. They are widely used as bone plates, osteosynthetic materials, surgical sutures, controlled drug release systems, bone platin, cardiovascular patches, vein valves, nerve guides, tendon repair devices, wound dressings and in many others fields^{31,107}. The first FDA approved PHA for clinical application is the TephaFLEX®, which is absorbable suture prepared from P4HB¹⁰⁸. In recent years a novel nanobiotechnological approach has been also undertaken. It employs proteins intrinsically binding the PHA granules as tags to immobilize recombinant proteins of interest and design functional nanocarriers for wide range of applications¹⁰⁹. Modifications of PHA chemical composition allows to control the biodegradation rates¹¹⁰ and extends PHA potential to be used in controlled release drug delivery systems⁷⁷. The chiral aromatic and aliphatic monomers that PHA is composed of present a distinct potential as drug synthons²⁹. Due to the asymmetric carbon in the beta position (Fig. 22), PHAs are optically active polymers containing only the (R)-enantiomer⁹⁵. The (R)-3-hydroxyalkanoic acids provide chiral compounds for pharmaceutical products like vitamins, pheromones and antibiotics synthesis¹¹¹. (R)-3-hydroxybutyrate has been already used in the synthesis of the antibiotic thienamycin and captopril, a drug used to treat high blood pressure¹¹². PHA's biocompatibility with mammalian systems, including the human body¹¹³, numerous forms of PHA are being studied for use as drug delivery devices⁷⁷.

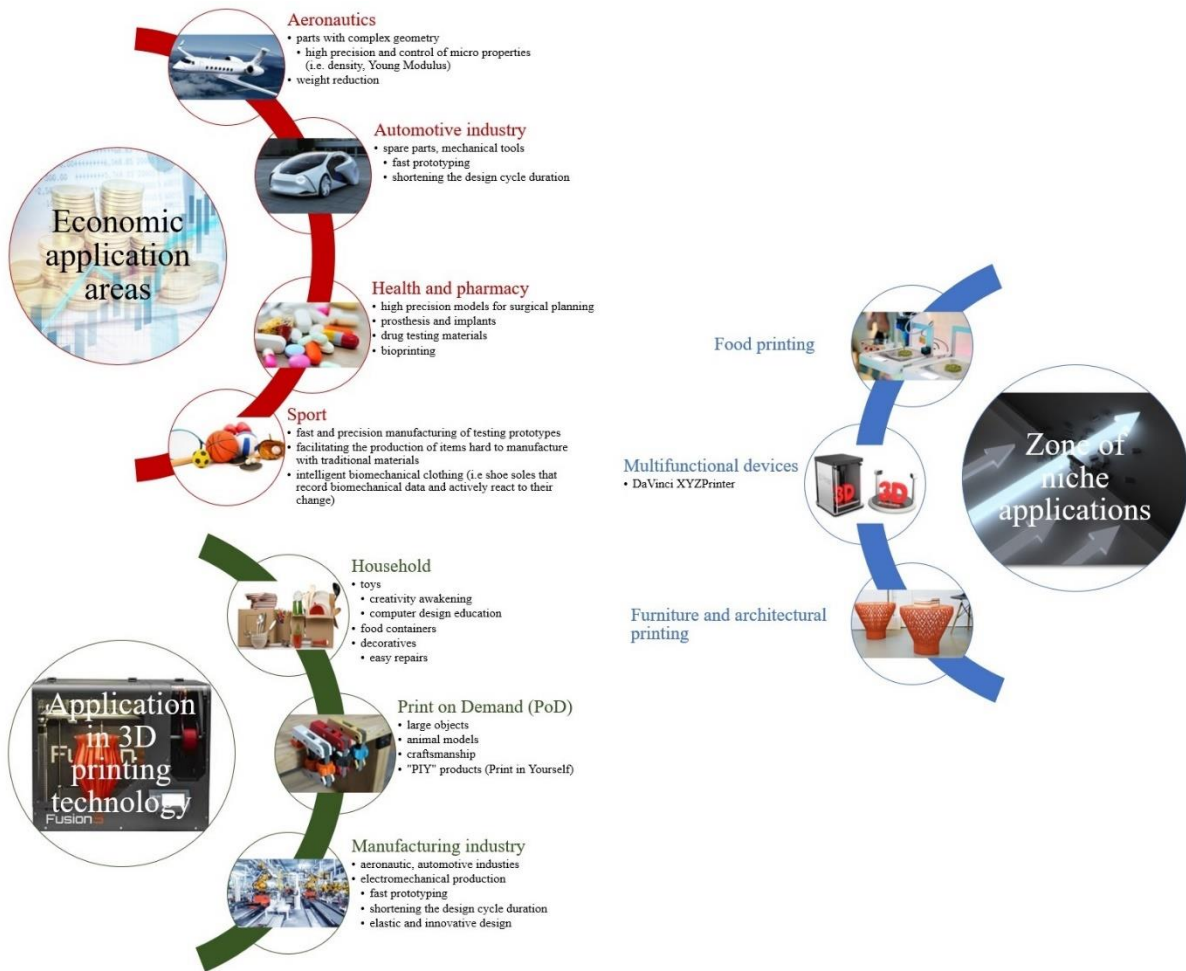
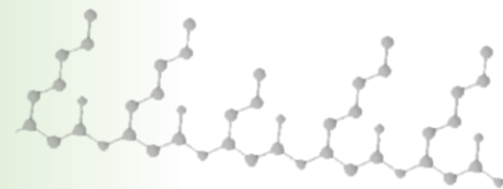


Fig. 25 Areas of potential PHAs applications.

5.8. Polyhydroxyoctanoate



5.8.1. Material characteristics

PHO is a biodegradable polymer of bacterial origin^{76,98}. It belongs to medium-chain length polyhydroxyalkanoates (mcl-PHAs) group^{30,79}. It fully degrades under industrial composting conditions within 60 days, the degradation through anaerobic digestion or home composting does not occur so quickly and takes over 6 months¹¹⁴. The conducted research revealed that the polymer has physical properties similar to polypropylene which indicates a wide field of its potential applications in the future¹. Mcl-PHAs are accumulated intracellularly mainly by *Pseudomonas* sp. under unbalanced growth conditions of excess carbon source and one or more other nutrients in limiting amounts¹¹⁵.

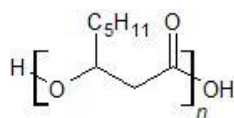


Fig. 26 Chemical structure of single PHO monomer.

PHO has been selected as a potential material for biomedical applications due to physical properties such as Young's modulus or elastomeric nature of the material. In addition, thanks to its thermoplastic properties, it can be easily shaped and processed.

In the table below, selected physicochemical properties of biopolymer were collected¹. A detailed discussion of the physicochemical measurements results can be found in chapter 7.1.1 and a description of particular techniques used to determine the parameters can be found in chapter 13.

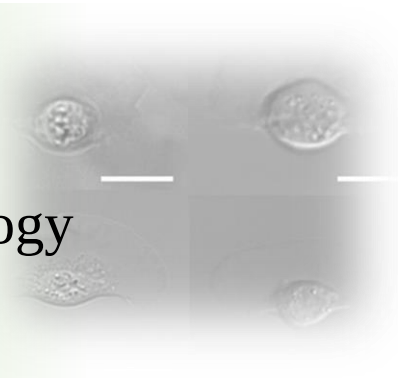
Parameter	T _g [°C]	T _m [°C]	ΔH _m [J/g]	X _c [%]	T _{onset} [°C]	M _n [kDa]	M _w [kDa]	PDI	Young Modulus [MPa]
Value	-35.4	52.8	18.6	36.7	278.5	73.0	137.0	1.88	12.6-25.4
Parameter	Intensity of o-Ps annihilation [%]		Lifetime of o-Ps [ns]		Free volume holes [nm]		Free volume radius [nm]		RMS surface roughness [nm]
Value	27-31		2.8		17.9		0.35		11.5-30.9

Table 4 Structural, topographical, and mechanical characteristics of polyhydroxyoctanoate polymer¹.

5.8.2. Polyhydroxyoctanoate applications

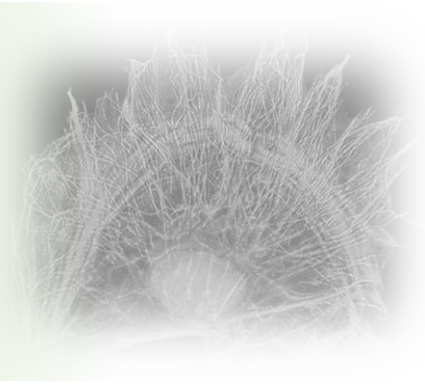
PHAs, especially PHO, PHB, PHBV and P4HB, are commonly used in tissue engineering. They are widely used as bone plates, osteosynthetic materials and surgical sutures ^{31,116}. Functionalized PHO polymeric foams can find a direct use in implantology and dermatology. They can be optimized for the specific needs – incrustated enzymatically with a single drug or a mixture of several different medicaments in order to suit personalized patient's needs. Additionally, to their functionalization, the materials can not only support growth of the tissues by providing matrix but also by nourishing the growing cells with (R)-3-hydroxyacids.

5.9. Substrate properties as a cell physiology altering factor



Mechanical and chemical properties of the environment influence the structure and morphology of living cells. The ability of an eukaryotic cell to resist deformation during motility, to change shape and to maintain intracellular transport comes from the presence of the cytoskeleton of an interconnected network of filamentous polymers and regulatory proteins located in the cell body^{52,54,117}. Cells, cultured on substrates with different elasticity, undergo a number of intricate mechanical interactions mediated by focal adhesions and other adhesive structures^{52,54}. It is known that certain migrating cells (i.e. Fibroblasts) respond to substrate rigidity by orienting movement towards substrates with higher Young modulus value¹¹⁸. Actin and tubulin are the key cytoskeletal components that underpin main cellular processes. Highly versatile, dynamic polymers capable of arranging cytoplasmic organelles and intracellular compartments are being formed, establishing cell polarity and generating pushing and contractile forces resulting in cell movement and migration¹¹⁹. Cellular structures and functions plainly depend on its microenvironment what can lead to a distinctive association between cellular properties and the adjoining surroundings¹²⁰. Sensing of the environment is possible due to the interactions of extracellular matrix (ECM)-associated protein networks with transmembrane proteins that can affect the cytoskeleton allowing transfer and transformation of mechanical stimuli. The cells also are able to sense and respond to the substrate chemical properties like substratum coating¹¹⁸.

5.10. Cytoskeleton



A cell's shape, internal organization, and functional polarity are provided by a three-dimensional filamentous protein network that consists of three distinct, yet interconnected, filament systems: intermediate filaments (IF), actin-containing microfilaments (MF) and microtubules (MT), called the cytoskeleton^{121,122}. Basic properties of every filament type present in the cell are summarized in Table 5. The cytoskeleton carries out three broad functions: it spatially organizes the contents of the cell; it connects the cell physically and biochemically to the external environment; and it generates coordinated forces that enable the cell to move and change shape¹¹⁷. The basic properties of individual elements of the cytoskeleton are summarized in Table 5. The normal functioning of a cell requires constant interaction with its environment. Signals from neighboring cells or the extracellular matrix are continuously perceived by membrane bound receptors. There are also assumptions regarding that the cytoskeleton might act as an interconnected network of filamentous macromolecules, which extends over the entire cytoplasm¹²³. This means that the role of the cytoskeleton is not limited only to the cell-shaped skeleton that is responsible for its contraction and migration, but also that filaments can serve as information and metabolite routes. Cytoskeleton is involved in many cellular processes like endocytosis, cytokinesis. It plays an important role in such life necessary processes like cell division or intracellular transport^{122,124,125}.

Cytoskeletal component	Structure scheme	Diameter	Building protein
Microtubule (MT)		23-25nm	α - and β -tubulin
Intermediate filament (IF)		10nm	fibrous proteins: - vimentine - creatine - glial fibrillary acidic protein
Actin filament (MF)		6nm	actin

Table 5 Cytoskeletal structures present in a cell.

Actin and tubulin form highly versatile, dynamic polymers that are capable of organizing cytoplasmic organelles and intracellular compartments, defining cell polarity and generating

both pushing and contractile forces¹¹⁹. Intermediate filaments (IF) form a class of insoluble cytoplasmic filaments of dimensions intermediate in size between microtubules and microfilaments. They can be found in virtually all differentiated types of cells and it seems possible that IF-like proteins also are present in even the simplest eukaryote cell types¹²⁶. Intermediate filaments are dynamic, motile cytoskeletal elements. Many types of IF are now known to engage in a specific array of movements that can be closely associated with their assembly, disassembly and subcellular organization¹²¹. Spatial distribution of individual cytoskeletal structures in the cell are shown in Fig. 27.

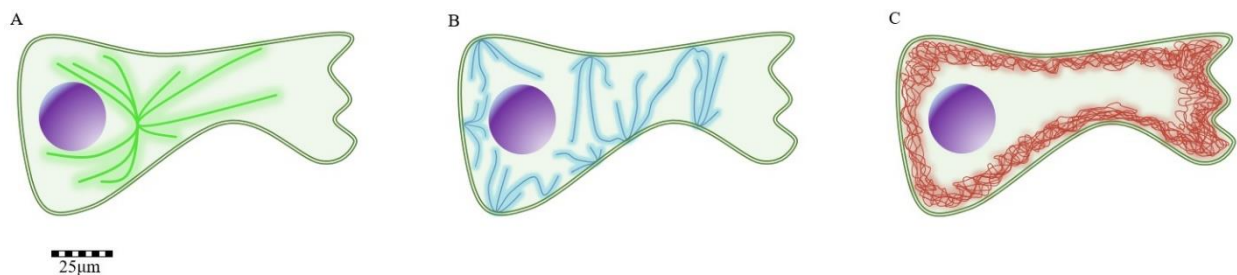


Fig. 27 Cell cytoskeletal structures (A-microtubules, B-intermediate filaments, C- actin filaments), along with their distribution in the cell¹²².

5.10.1. Microtubules

Microtubules are a filamentous assembly of protein subunits, α - and β -tubulin. They are hollow cylinders with external diameter of 25nm. Along with actin and intermediate filaments, they form the eukaryotic cytoskeleton and participate in defining cell morphology^{127,128}. Unpolymerized tubulin exists as a tight $\alpha\beta$ -tubulin heterodimer with binding sites for two GTP molecules, one exchangeable and the other not. A microtubule is a tube constructed from parallel linear polymers called protofilaments in which the heterodimers are assembled head-to-tail in a polar fashion. This polarity is related with the distinction between the plus and minus side of protofilaments^{122,128}. Monomers in adjacent protofilaments are slightly shifted so that they form a set of shallow helices. Microtubules can vary in numbers of longitudinal protofilaments (Fig. 28). When the number of protofilaments is 13, they run straight, allowing microtubule-associated motor proteins, such as dynein and kinesin to run for long distances along microtubules without switching lanes^{129,130}. If the number of protofilaments deviates from 13, the microtubular lattice must rotate slightly, so that the protofilaments wind slowly around the microtubule axis¹²⁷.

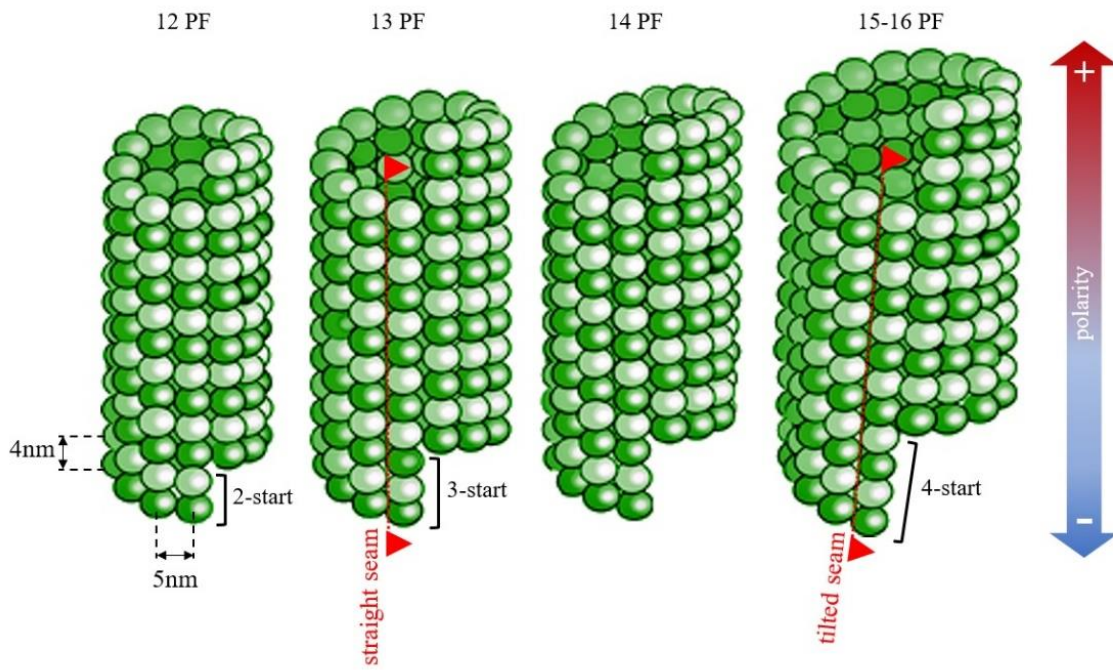


Fig. 28 Microtubules with varying numbers of longitudinal protofilaments.

5.10.2. Intermediate filaments

Intermediate filaments are a type of cytoplasmic filaments of dimensions intermediate in size between microtubules (about 25 nm) and microfilaments (about 7 nm). They can be found in virtually all types of cells¹²⁶. At least 30 keratins are described and known. Vimentin is the most widely expressed, produced by mesenchymal cell types and by a variety of transformed cell lines and tumors, representative of protein type IFs¹³¹. Unlike other cytoskeletal elements, intermediate filaments distribution in cells show no direct correlation with the position of either mitochondria or endoplasmic reticulum¹³². IF's present ability to withstand large deformations, they can be stretched several times their initial length¹³³. About 70 different genes are coding for various intermediate filament proteins. On this basis IFs can be categorized into six types among which similarities in amino acid sequence and protein structure are fundamental for the distinction¹²⁶. There are several diseases directly linked with mutations in IF genes. Intermediate filaments primary component are monomers that associate with each other and form dimeric structures. Pairs of dimers create tetrameric forms through association in an anti-parallel fashion. Connection in lateral axis between eight tetramers give origin to filaments, which by annealing to each other, end-to-end, to form intermediate filaments¹³⁴(Fig. 29).

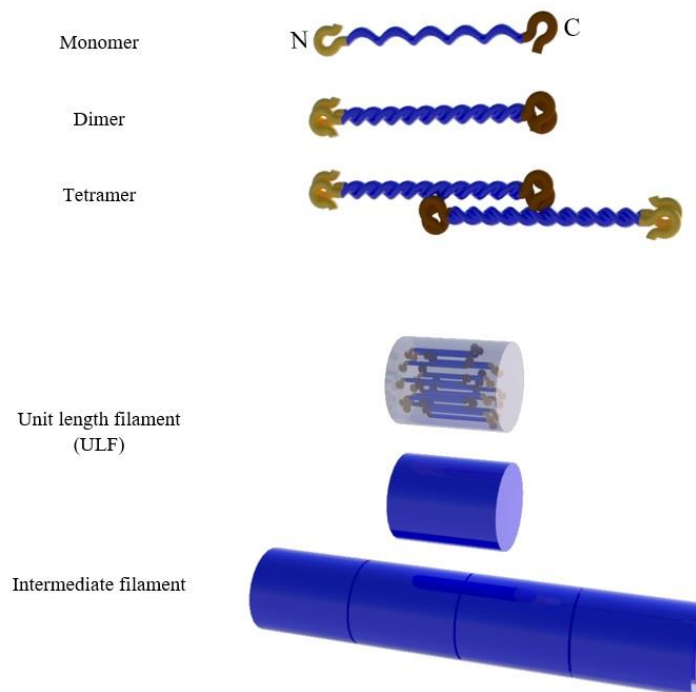


Fig. 29 Intermediate filaments assembly process¹³⁵.

5.10.3. Actin filaments

Actin is a family of globular proteins that arrange in form of microfilaments. Actin network can be found in essentially all eukaryotic cells. Actin cytoskeleton is directly associated with changes in cell shape, anchorage and motility¹³⁶. Dynamic formation of microfilaments allows it to rapidly remodel itself in response to its environmental changes. Actin takes also part in processes such as the intracellular transport of vesicles and organelles or muscular contraction at tissue level. First actin observations were conducted in 1887 by W.D. Halliburton, who extracted a protein from muscle he called "myosin-ferment"¹³⁷. Actin can be found throughout the cytoplasm in eukaryotic cells, , in muscle fibers its inherence by weight of total cellular proteins is about 20% and between 1% and 5% in other cells¹³⁸. Cellular actin occurs in two forms as monomeric globules namely G-actin and polymeric form called F-actin also described as microfilaments. The actin cytoskeleton consists of pair of parallel F-actin streaks which rotate 166° to form a double helix structure called actin fiber. Microfilaments are approximately 7 nm in diameter the helix is repeating every 37 nm¹³⁹.

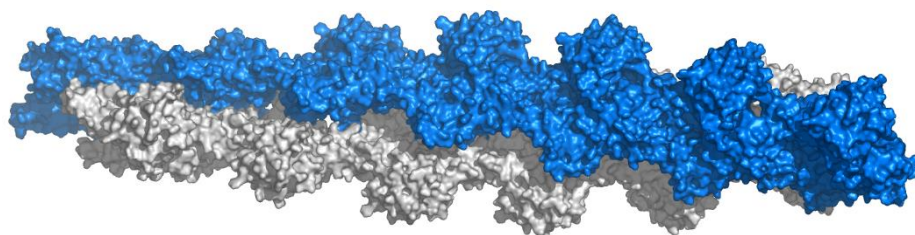


Fig. 30 F-actin; surface representation (Ken Holmes' actin filament model)¹⁴⁰.

5.11. Cell migration

Cell movement or motility is a highly dynamic phenomenon that is essential to a variety of biological processes. It is the basis for many physiological phenomena like the development of a living organism (morphogenesis), wound healing or immunological mechanisms, but its deregulation can trigger many pathological processes like cancer metastasis, chronic inflammation or neurological diseases. Cell migration is an integral part of the cell biology, embryology, immunology, and neuroscience¹⁴¹. A targeted movement of dividing cells can give rise to new tissues and organs¹⁴². There is an increasing evidence to suggest that physical parameters, including substrate rigidity, topography, and cell geometry, can play an important role in cell physiology and migration¹⁴³. Understanding cell migration which is an integrated process requires an appreciation of chemical and physical properties of multicomponent system of different intracellular structures¹⁴⁴.

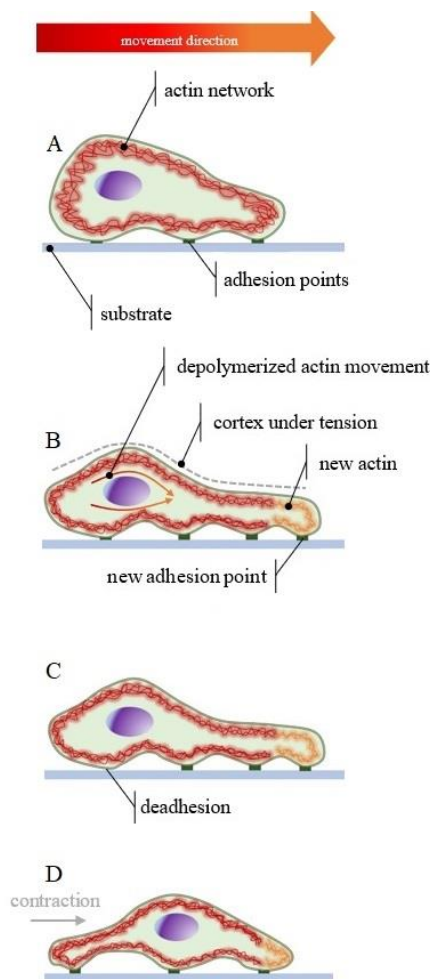


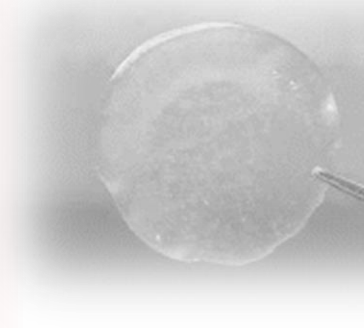
Fig. 31 The cell migration mechanism with division into individual phases¹⁴².

- A – Protrusion of leading edge,
- B – Adhesion at the leading edge,
- C – Deadhesion at the trailing edge,
- D – Movement of the cell body

6. Materials and methods



6.1. Polymer substrates preparation



6.1.1. Polyhydroxyoctanoate

6.1.1.1. PHO production

Bacterial strain of *Pseudomonas putida* KT2440 was used to produce PHO in a 5L fermenter. Bacteria was revitalized from a -80°C stock by suspending it in a fresh solid minimal salt medium (MSM)⁹⁸ supplemented with octanoic acid sodium salt (20 mM). Bacterial growth continued overnight at 30°C. A single colony was transferred into liquid MSM supplemented with sodium octanoate (20 mM). Culture was left to grow for 12-15 hours and then used as a seed for a fermentation (1:25). Decaying exponential feeding strategy of octanoic acid developed by Maclean *et. al*¹⁴⁵ was adapted to our fermentation system (Fig. 32) (5l quad, Control Flow, Poland) in order to produce PHO polymer. Following, the fermentation biomass was separated from the medium by centrifugation, frozen and lyophilized. Isolation of PHO with ethyl acetate and its purification over an activated charcoal was performed as described by Wampfler³⁰. When bacterial biopolymers are isolated for medical applications, contaminating pyrogenic compounds must be removed¹⁴⁶.

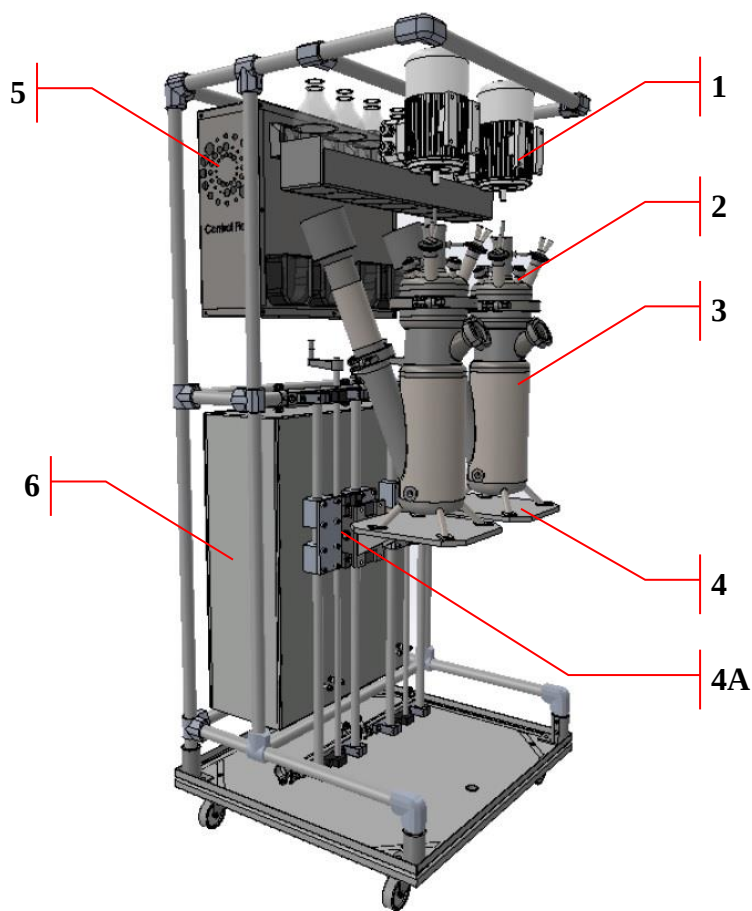


Fig. 32 A three-dimensional diagram showing the detailed construction of the reactor for conducting fermentation under controlled conditions to obtain a polymer.

- 1 - Electric motors providing agitator drive, maximum RPM 2000, generated torque 10 Nm,*
 - 2 - Reactor covers equipped with sterile ports for modification of the reaction mixture from the outside,*
 - 3 -5-liter reactors made of acid-proof steel,*
 - 4 - Mounting table - enables the attachment of the tank under the agitator, and thanks to the manual lifting and lowering mechanism (4A) allows the coupling of the agitator clutch to the motor drive shaft.*
 - 5 - Control cabinet controls a mixing module, valves and thermostats. The fermentation automatically adjusted basing on selected process formula.*
 - 6 - Computer allows data collection and sending the files via network to other computers.*
- (Copyright Control Flow Sp. z o.o.)*

6.1.1.2. Sample preparation

In the case of substrates made of biopolymer, the raw material was dissolved in three different solvents. Due to the fact that the efficiency and the polymerization process is similar for these three substances^{1,152}, the determination of their impact on both physical and biological parameters of the substrate could be conducted. Amount of 10ml of the solvent was used to dissolve 0.5g of the polyhydroxyoctanoate raw biopolymer. 18 - 21 μ l of solution was poured on the glass bottomed dish to receive a flat substrate surface of 80 - 100 μ m height.

6.1.2. Polyacrylamide (PAM)

6.1.2.1. Sample preparation

Polyacrylamide substrates of various elasticities (40kPa, 19kPa, 8kPa) were prepared to study the morphology of MEF cells, deposited on these substrates. The main advantage of this material is that it is easy to precisely control the stiffness of the substrate by only changing the chemical composition of the mixture¹⁴⁸. In addition, it allows for reproducible preparation of substrates with desired properties⁵³. Preparation of polymeric film includes a number of important steps. The first step is the glass activation on which the polymer substrate was poured. For every glass bottomed dish, 0.8ml of silane (3-aminopropyltrimethoxysilane) (Sigma Aldrich) was used. After 10 minutes, the silane solution was removed and replaced with glutaraldehyde (Sigma Aldrich). After 30 minutes, the dishes were emptied and then rinsed three times with distilled water. A mixture of polymers of a given elasticity was prepared. 0.5ml of a solution containing bis acrylamide (BIS), acryl (ACRYL) and 10mM HEPES were prepared in a suitable quantitative ratio corresponding to the desired substrate elasticity (Table 6). Then, 0.1g of ammonium persulfate (APS) was added to 1 ml of distilled water and then thoroughly mixed. 50 μ l of APS solution was added to the previously prepared polyacrylamide gel mixture and 5 μ L of TEMED (Sigma Aldrich) to initiate the polymerization process.

Expected Young Modulus [kPa]	BIS [μ l]	ACRYL [μ l]	HEPES [μ l]
8	37,5	37,5	425
19	66	100	334
40	105	100	280

Table 6. Composition of substrate components with expected Young Modulus ^{56,149}.

The next step of substrate preparation was to pour of 20 - 23 μ l of polymer gel onto a prepared glass bottomed dish. Polyacrylamide drop was enclosed with 18mm diameter cover slide to maintain a smooth and homogeneous surface¹⁵⁰. After one-hour polymerization the substrate was submerged in phosphate buffer solution (PBS) containing of 8g of NaCl, 0,2g KCl, 1,44g Na₂HPO₄ and 0,24g KH₂PO₄ dissolved in 800ml of distilled water, and the cover slide was carefully removed. To maintain the desired stiffness of the polymer and remove all toxic unbound residues of acryl and bisacrylamide, the polymerized mixture was rinsed one more time with PBS solution. Before being suitable for cell culturing the surface of the polymer substrate has to be activated⁵³. To perform this task a solution of sulfo-SANPAH combined with UV light ($\lambda \sim 365$ nm) was applied to the material¹⁵⁰. The last stage was coating the gel with 300 μ l of FBS or fibronectin (Thermofisher) solution to functionalize it with the proteins necessary to form the extracellular matrix^{58,151}. The process of activating the polyacrylamide substrate for cell culture needs is shown in Fig. 33.

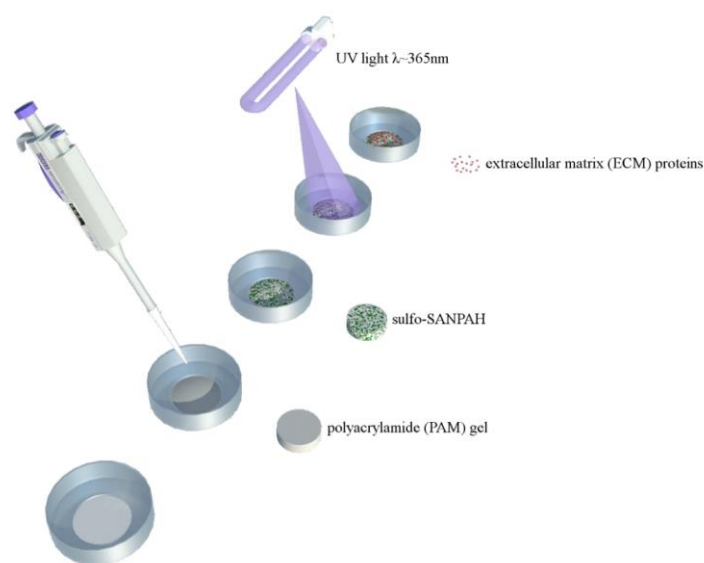


Fig. 33 Activation process of polyacrylamide cell substrates on round glass bottomed dish⁵⁷.

6.1.3. Polylactic acid (PLA)

Polylactic acid (PLA) was used in this study as a material from the biopolyesters group which biological^{7,15,69} and physical^{20,62,152} properties are already widely described in the literature. The experimental results for PLA were compared with those obtained for PHO and PAM. Due to the well-defined and developed applications of PLA in different industrial branches^{62,65} and medicine^{16,17,61,153}, a direct comparison of its properties with PHO will allow to determine potential application areas for medium chain length polyhydroxyalkanoate.

6.1.3.1. Sample preparation

The raw material (PLA – commercial grade) was dissolved in three different solvents (ethyl acetate, chloroform and acetone). Amount of 10ml of the solvent was used to dissolve 0.5g of the raw polymer. Solutions were casted on round bottomed dishes. 18 - 21µl of solution was poured on the glass to receive a flat polymer surface of 80 - 100µm height. This value covers the range at which the influence of the glass hardness of the substrate on cells is minimized¹⁴³, on the other hand it enables the observation of cells using a 40x magnification lens - in this case the "working distance" parameter is a limitation of maximal substrate height.

6.2. Cell cultures



6.2.1. Mouse embryonic fibroblasts (MEFs)

For the experimental studies mouse embryonic fibroblast cell line (MEF 3T3) was used. This cell type was selected because fibroblasts are primary tissue component and are involved in wound healing processes^{149,154}. This cell type is crucial in processes such as wound contraction, breaking down the fibrin clot, creating new extra cellular matrix and structures to support other cells taking part in wound healing¹⁵⁵. Fibroblast can be found in healing wound from the late inflammatory stage until the full epithelization¹⁵⁶.

Cells were grown in plastic culture dishes under sterile conditions in an incubator (Thermo Scientific 8000 Series WJ) maintaining constant environment (37°C, 5% CO₂). The medium used was DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) (Sigma Aldrich®). The cell culture was split using a standard passage procedure when the confluence reached approximately 80%. Cells used in the study were after third, but not beyond ninth passage¹⁵⁷.

6.3. Experimental methods



6.3.1. Fluorescence microscopy

6.3.1.1. Introduction

Fluorescence microscopy mainly bases on observing light originating from the visible range, although advanced detection systems such as charge coupled device (CCD) cameras are available to detect fluorescent light in the ultraviolet (UV) and infrared (IR) range. A fluorescent molecule is called fluorochrome and its properties are based on a phenomenon of absorbing a photon of light of a particular wavelength (Fig. 34- λ_1) and, after an interval, emitting part of that energy in the form of a photon of a different, longer wavelength (Fig. 34- λ_2). The time gap between absorption and emission is called the fluorescence lifetime and also can carry a portion of information about the investigated system (Fluorescence-lifetime imaging microscopy - FLIM). Energy absorption by the fluorochrome is the result of interactions between the oscillating electric field vector of the light wave and the electrons in the fluorochrome. For a certain electron a specified amount of energy is required to allow the transition process to occur. Because photon energy is related with wavelength ($E = h\frac{c}{\lambda}$), each fluorochrome is capable of generating fluorescence only while excited with light certain wavelength. This makes the fluorescence process highly specific¹⁵⁸. The absorption and emission of photons by a fluorochrome can be illustrated using a Jablonski energy diagram (Fig. 34), introduced in 1953 by Polish physicist Aleksander Jabłoński.

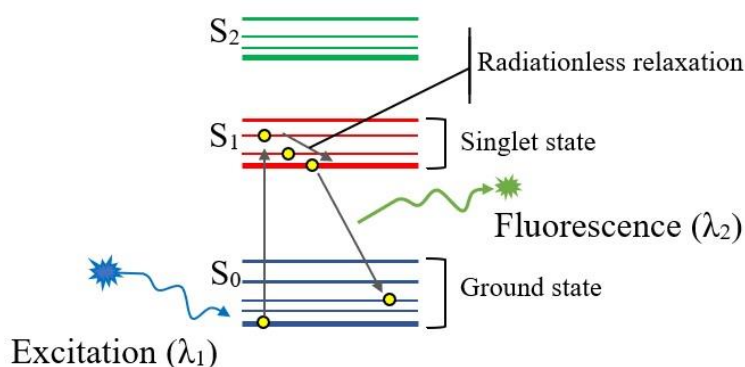


Fig. 34. Jablonski energy diagram of excitation and fluorescence.

An additional information characterizing a fluorochrome is its quantum yield (6-1). This parameter specifies the ratio of the number of emitted to the number of absorbed photons and is a simple measure of fluorescent efficiency of a particular fluorochrome:

$$Q_Y(\phi) = \frac{\text{emitted photons}}{\text{absorbed photons}} = \frac{k_f}{(k_f + k_{nr})}$$

6-1

where Q_Y is the quantum yield, k_f is the rate constant for fluorescence decay, and k_{nr} is the sum of rate constants for nonradiative effects (e.g., internal conversion and quenching). A perfect emitter would have the quantum yield of value 1.0 which means that every photon of longer wavelength absorbed causes emission of a single photon with a shorter λ . In most situations, nonradiative phenomena such as heating or photobleaching reduce the energy, leading to quantum yields below ideal value. Another important, fluorochrome parameter is the molar extinction coefficient (ϵ). It expresses the quantity of light absorbed as a function of the path length and the concentration of the fluorochrome:

$$\epsilon = \frac{A}{cl}$$

6-2

where ϵ is molar extinction coefficient, A is absorbance, c is the molar concentration of absorbing molecules and l stands for the optic path length. It is usually established at a path length of 1 cm and at a one molar concentration.

Fluorophore	Solution	Quantum Yield (QY)
Alexa Fluor 488	Water	0.92
Alexa Fluor 568	Water	0.69
Cy5	PBS	0.90
Fluorescein isothiocyanate (FITC)	Water	0.94
Fluorescein	Water	0.79
Rhodamine B	Water	0.31
Tetramethyl rhodamine	Water	0.20
Texas Red	Water	0.90
Tryptophan	Water	0.14

Table 7. Quantum yields for several commonly used fluorophores¹⁵⁹.

6.3.1.2. Factors limiting fluorescent emission

Fluorochromes with the highest possible quantum yield should be used for fluorescent or confocal microscopy. The probability of emission strongly depends on the closest surroundings of the fluorochrome. Every contact between fluorescent molecule and any neighboring particle able to accept transferred energy can lower the fluorescence efficiency, so the fluorescence event does not occur in the system. The loss of energy caused by transfer to surrounding particles is known as vibrational relaxation. It often occurs in fluids or gasses because in these conditions, the probability of solvent molecules-fluorochrome collision is relatively high. These interactions do not change the shape of the emission spectrum, only the intensity is reduced, which can be observed by decrease of the spectrum amplitude. Another way for the energy to escape from the system are photobleaching or phosphorescence. These phenomena are examples of nonfluorescent radiation, and they occur when a molecule enters a triplet state.

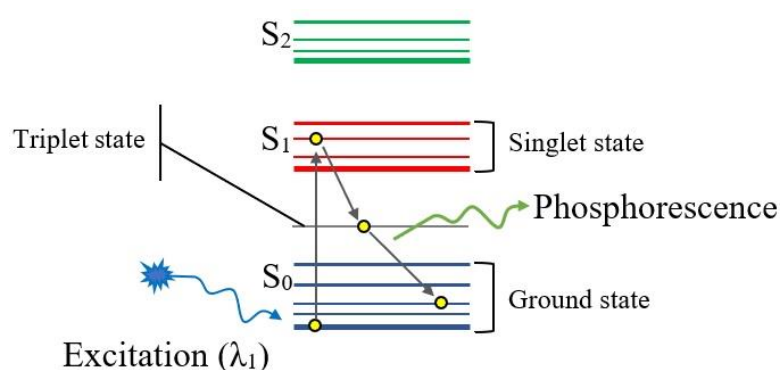


Fig. 35. Energy diagram for phosphorescence process.

In this process the energy absorbed by a substance is released relatively slowly in the form of light. This mechanism is used for "glow-in-the-dark" materials which are "charged" by light exposure.

Photobleaching is a critical concept in fluorescence microscopy. Despite its negative activity in some processes, it can be useful in other techniques in which the loss of fluorescence is a desired phenomenon. Fluorescence loss in photobleaching (FLIP) and the related methodology of recovery after photobleaching (FRAP) are techniques for observing the movement of intracellular materials through photobleaching of fluorescence^{159,160}.

6.3.1.3. Multiphoton excitation

Multi-photon excitation (MPE) microscopy plays a important role among microscopic methods used for biological matter studies¹⁶¹. It was first mentioned in a work written by Maria Göppert Mayer almost 80 years ago¹⁶². In most common cases, a fluorescence event involves absorption of a single photon of relatively high energy and results in the emission of

a single photon of distinctly lower energy. The “single” nature of the event results from the fact that absorption must occur rapidly and fast. If the photons concentration is high enough, the probability of two or more photons transferring their energy simultaneously, or almost simultaneously, to a fluorochrome increases. Such an event is known as multiphoton excitation¹⁵⁸.

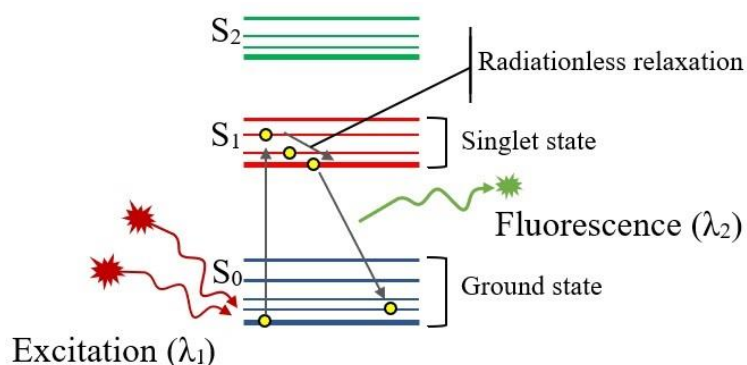


Fig. 36. Energy diagram for multi photon excitation and fluorescence.

The energy transfer from multiple photons is additive, it results in the transfer of enough amount of energy for a fluorochrome molecule to raise from the resting S_0 state to the excited S_1 state (Fig. 36). Multiphoton excitation of fluorescent molecule is a nonlinear process. In a multiphoton event the emitted photon energy is higher than the energy of excitation photons although it is lower than the sum of the excitation photons energies. If a fluorophore is normally excited by light with a wavelength of 315nm, it can also be excited by absorbing two photons that are around 630nm each^{158,161}. Additionally, the probability of two or more photon excitation decreases rapidly away from the focal point of a light beam, because the photon concentration reaches it maximum in this spot (large numbers of photons packed into a small space). Therefore, in a multiphoton microscopy, the excited sample volume is limited to the area where the laser beam is focused. Fluorochrome molecules located away from the focal point remain in their ground state and the excitation does not occur. The spatial restriction of the excitation and the fact that longer wavelengths can be used to induce fluorescence are major advantages of this technique in fluorescence imaging of living material^{158,161,163}.

6.3.1.4. Optical basics of fluorescence microscopy

In standard transmitted light microscopy, a beam of light is focused onto the specimen by a condenser located below the sample in upright microscopes (Fig. 37A) or over the microscope stage in an inverted device (Fig. 37B).

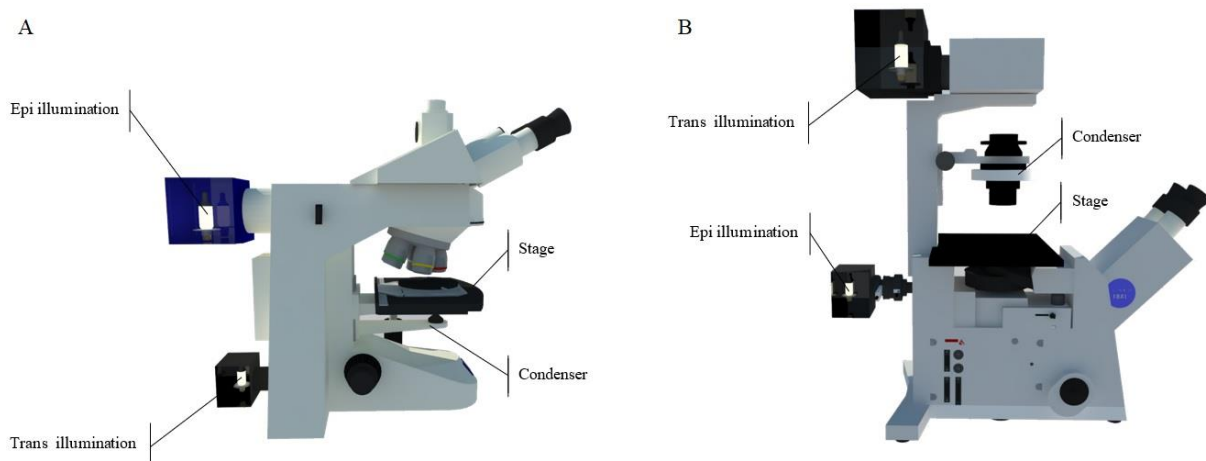


Fig. 37. Trans and epi illumination in an upright (A) and inverted microscope (B).

When using trans illumination, the specimen image is created as a result of the light rays passing through the sample and then being collected by the objective lens and transferred directly to the imaging device. This design can be technically used in fluorescence techniques but there are significant difficulties in separating the excitation light ray from the one emitted by the sample. In fluorescence microscopy only, the emitted light should be captured and imaged, all other wavelength signals are undesirable and can degrade the final image quality. It is much easier to maintain the separation between emitted and excitation light when they are traveling in the opposite directions (Fig. 38B). For this reason, most fluorescence microscopes are designed to use epi illumination to light up the sample. In this construction, the excitation light is focused onto the specimen by the objective lens rather than by a condenser as it is implemented in trans illumination microscope design. Like in trans illumination setup the objective lens is also collecting and focusing the light emitted by the sample.

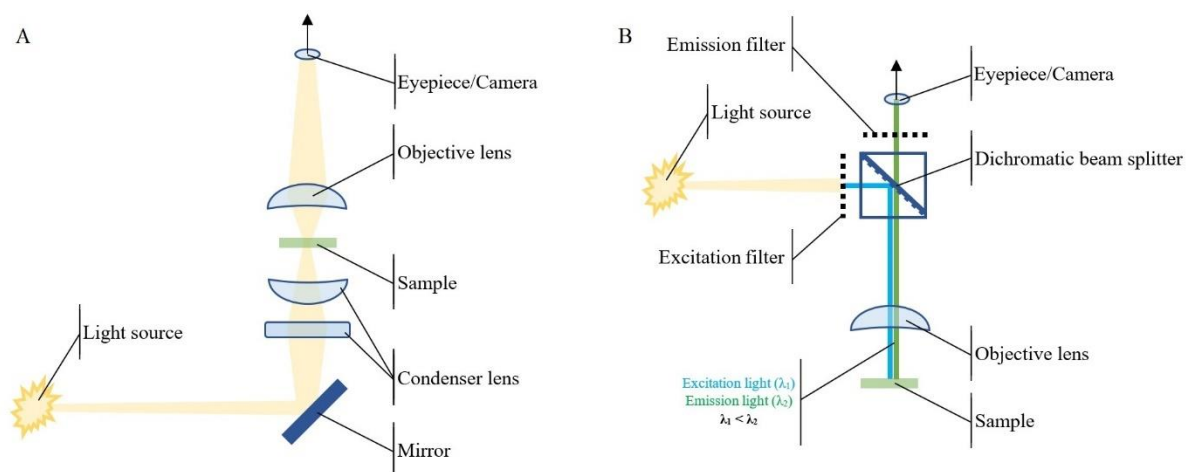


Fig. 38. Optical path in epi illumination (A) and trans illumination (B) microscope designs.

The presented optical system is based on an upright microscope, but both configurations are successfully used in inverted systems.

When the specimen is illuminated with light with appropriate wavelength in the imaging path two components are present. One of them is part of the excitation light beam reflected from the sample or the glass and a light beam of higher wavelength emitted by the fluorophores inherent in the fluorescent specimen. To obtain an eligible image, only the beam originating from the fluorescence process is important, the remaining light part is minimized with use of specially designed emission filters. Such epifluorescence strategy can be used in both widefield and confocal fluorescence microscopy¹⁵⁸.

6.3.1.5. Objective lens

All components located on the optical path are important to obtain a high-quality microscopic image. The objective lens may be the most important element because the image parameters such as resolution, final magnification, field of view depend on the performance and quality of the objective. If a poor quality or improper objective lens is used for imaging, the final microscopic picture can present parameters below expectations and some of the desired information can be lost. During every microscope employing experiment it is important to understand the optical characteristics of available objectives and how each of these parameters may affect the collection and interpretation of the final image. All information concerning the objective parameters and information about the type of imaging for which it is optimized and designed is permanently applied on the objective barrel. Information concerning type of lens, magnification, numerical aperture (NA), focal length, immersion application, and the cover glass thickness correction are the key factors provided by

every objective lens manufacturer and crucial for designing and planning a successful experiment.

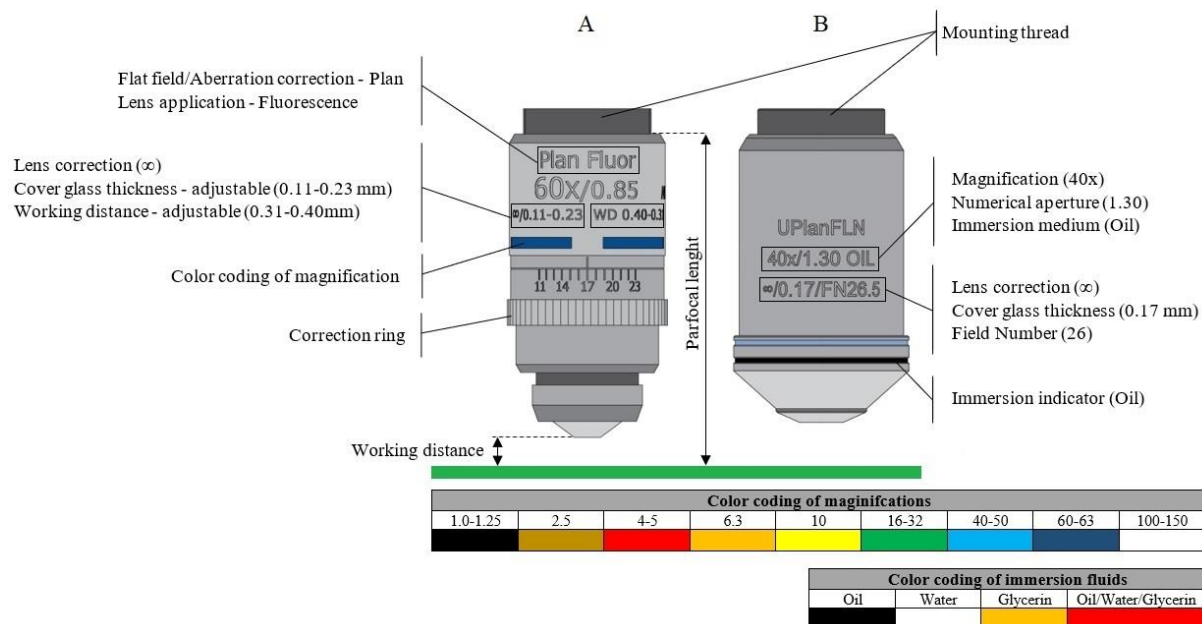


Fig. 39. Exemplary markings on two types of objective lenses,
A – dry and B – oil immersive objective.

A scheme of color coding for magnifications and immersion fluids, used by leading manufacturers, is also presented (Thorlabs Inc.).

Plan objectives are flat-field objectives corrected to eliminate curvature of the lens, which may result in loss of focus in peripheral regions of image. In objectives with low magnifications plan lenses are often not being produced. The microscope software should be then used to zoom the image and limit the acquisition area to the central field of the objective where no aberration occurs. The task of *immersion lenses* is reducing the refractive index mismatch between all the components present in the optical path. This is important in trans illumination fluorescence microscopy because both excitation and excited photons from the specimen have pass through the same glass components within the light path. Oil-immersion objectives have a high numerical aperture value (over 1.3), which also provides a high-resolution imaging. Water immersion objectives are designed to match the refractive index of the biological matter. This type of immersion allows deep tissues imaging, but the numerical aperture lenses is significantly lower that for oil immersion optics. Multi-immersion objectives are also available and can be used for various mounting media. *Numerical aperture (NA)* is an indicator of both the light gathering capacity and resolution of a lens. The bigger the NA increases, the lens capability of collecting photons also rises, and the

resolution the picture provided by the lens is also elevated. The numerical aperture value can be determined, as shown in the equation:

$$NA = n \sin \theta$$

6-3

where n is the medium refractive index and θ is half angle of light cone entering the objective. One of the main limitations in fluorescent imaging is weak emission from the sample which results in acquisition of inconsiderable number of photons by the objective. Lenses with high NA value are essential for high-resolution fluorescent imaging, because as mentioned before numerical aperture has also influence on maximal image resolution provided by lens. This dependence is shown in the equation:

$$d = 0.61\lambda/NA$$

6-4

where d is the resolution and λ is wavelength of light collected by the lens.

Depth of field (Z) refers to the distance along the z -axis of the optical path within the sample remains in focus. Depth of field is highly dependent on the NA, imaging light wavelength (λ) and the medium between the lens and sample refractive index (n):

$$Z = \frac{n\lambda}{NA^2} + \frac{n}{M \cdot NA} e$$

6-5

As the numerical aperture increases, the Z value rapidly decreases (Fig. 40) which often creates significant restrictions in high-resolution confocal experiments, where the required use of high-NA oil-immersion objective limits the maximum depth from which image planes can be collected.

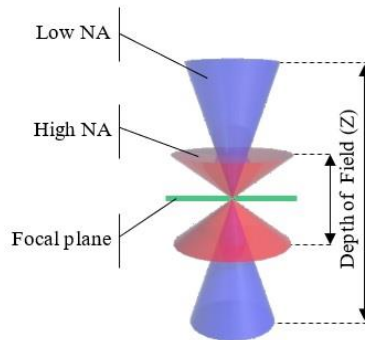


Fig. 40. Dependence between NA value and depth of field range (Z).

Depth of focus (DOF) refers to the thickness of image plane, or depth of the area around an object in an image, which is in sharp focus at the same time. Depth of focus depends on numerical aperture and magnification of the objective, under some conditions, high NA setups can offer a larger focus depth value, even though the depth of field is smaller. Human eyes focus adjustment can differ individually therefore perception of the focal depth is individual. To designate the depth of focus the Berek formula is used, because it gives a result that is converging with values obtained through experiments¹⁶⁴:

$$DOF = \frac{\omega \times 2.5 \cdot 10^5}{NA \times M} + \frac{\lambda}{2(NA)^2}$$

6-6

Where ω is the resolving power of eyes, M stands for the total magnification and λ indicates the wavelength. In the case of a video camera used for image grabbing, the focal depth will vary according to number of pixels of CCD.

The lens *working distance (WD)* is the maximum distance between the objective and the specimen in which the sample remains in focus. Low magnification objective lenses and water immersion lenses typically have a long working distance, while oil immersion lenses with high magnifications have very short working distance.

Objective parameter	Magnification	Immersion	NA	WD
Objective manufacturer/ model				
Zeiss / Apochromat 10x/045 M27	10x	-	0.45	2.00mm
Zeiss / A-Plan 20x/0.45	20x	-	0.45	0.51mm
Zeiss / A-Plan 40x/0.65 M27	40x	-	0.65	0.45mm
Zeiss / N-Apochromat 20x/0.5 M27	20x	Water	0.50	2.60mm
Zeiss / Fluor 40x 1.30 Oil M27	40x	Oil	1.30	0.16mm
Zeiss / N-Achroplan 63x/0.85 M27	63x	-	0.85	0.20mm
Zeiss / C-Plan Apochromat 63x 1.4 Oil DIC	63x	Oil	1.4	0.14mm
Zeiss / N-Achroplan 100x/1.25 Oil CG=0 M27	100x	Oil	1.25	0.45mm
Zeiss / Plan-Fluar 100x 1.49 Oil M27	100x	Oil	1.49	0.08mm
Zeiss / EC Epiplan-Apochromat 150x/0.95 M27	150x	-	0.95	0.23mm
Olympus / Plan Achromat N 4x/0.10	4x	-	0.10	18.50mm
Olympus / Plan Achromat N 20x/0.40	20x	-	0.40	0.60mm
Olympus / Plan Fluorite Oil 40x/1.3	40x	Oil	1.30	0.20mm
Nikon / Plan APO Lambda 2x	2x	-	0.1	8.50mm
Nikon / Plan APO Lambda 4x	4x	-	0.2	20.00mm
Nikon / Plan Fluor 40x Oil	40x	Oil	1.3	0.20mm
Nikon /CFI Plan Apo 10xC Glyc	10x	Glycerine	0.5	5.50mm
Nikon / CFI Plan Apo NCG 100x	100x	Oil	1.4	0.17mm

Table 8. Comparison of the parameters of selected objective lenses from leading manufacturers.

Nearly all new optical microscopes are equipped with *infinity-corrected optics*. In infinity-corrected objectives, light leaves the objective as parallel rays which do not come to a focal point to form an image until a tube lens brings the light to a focal point. The advantage of this feature, especially in objectives used in fluorescence microscopy, is that multiple components like filters beam splitters and dichroic mirrors can be placed in the optical path without affecting the parallelism of the light rays. In non -corrected lens, light is focused to an image after passing through the back focal plane of the objective which requires correction considering every component within of the optical path, thus preventing light diffraction as light passes through filter cubes, beam splitters, and every other component present in the microscope optics. If the correction is not executed correctly several image aberrations can occur blurring the final image.

6.3.2. Confocal microscopy

Confocal microscopy is a major advance upon normal light microscopy, allowing not only to visualize deep into cells and tissues, but also to create three dimensional images and to follow specific cellular reactions over extended periods of time¹⁶⁰. The invention of the confocal microscope (first patented by Marvin Minsky US Patent #3013467¹⁶⁵) did not arouse enthusiasm or interest in the research community at that time. To show the real potential innovative system had to wait almost 20 years, until the development of other accompanying technologies reached the right level. The first commercial instruments became available in the late 1980's. Confocal microscopes from the beginning of production were characterized by high resolution, considerable sensitivity and good optical properties but it was the software created real restrictions for researchers. A rendering of a medium sized stack of images into a 3D reconstruction took over an hour. The last two decades have been full of innovations in the field of both optical microscopy and the digital techniques, programming and algorithms, and this has made confocal microscopy a powerful tool in the hands of scientists from many fields. Currently, several types of confocal microscopes with different design, construction and image grabbing mode can be found on market. Among the commercially available types of microscopes, one can distinguish laser scanning microscopes which are using a finely focused laser spot to scan the specimen. The three-dimensional image is created with use of a pinhole to capture the light coming only from the selected plane. The alternative approach is presented in spinning-disk systems, where the image is generated by the simultaneous illumination of multiple spots in the object plane. Each spot is generated by a series of micro-lens or pinhole

apertures in a rapidly spinning disk. The Nipkow disk confocal microscope uses a special disk ("*Nipkow disk*") to scan through several thousands of pinholes across the specimen. The pinholes geometry design allows to remove the out of focus light, and the system itself allows to perform very fast scans. Slit scanning microscope is equipped with a slit instead of a pinhole which serves the same purpose of removing light coming outside the focused area. The most advanced and innovative technique is multi-photon microscopy. In this method far-red laser is used to scan across the sample and generate fluorescence. The dyes used for sample staining in this technique are excited by longer wavelengths than usual. Light coming out of focus is removed by the fact that the excitation light intensity is sufficient enough only on the focal plane^{158,160}.

6.3.3. Nanoindentation

Investigation of the mechanical properties of polymers not only allows to reveal regularities of the deformation and to obtain rheological characteristics of the material but also provides information about microscopic mechanisms that determine the response of a polymer to external conditions in different physical states¹⁶⁶.

Indentation testing is a simple method that consists essentially of touching the material of interest whose mechanical properties such as elastic modulus and hardness are unknown with another material whose properties are well known. The technique has its origins in Mohs' hardness scale (1822) in which materials that are able to leave a permanent scratch in another were ranked harder material. Diamond assigned the maximum value of 10 on the scale¹⁶⁷. The technique relies on a continuous measurement of depth of penetration with increasing load¹⁶⁸. In conventional indentation tests the measurement of the size of a residual plastic impression in the specimen as a function of the indenter load is the basis for the experiment. In a test in which a very low value load is applied, the size residual impression is often too small to obtain a direct and reliable measure using optical techniques¹⁶⁷. The advantage of nanoindentation techniques lies in measuring the depth of penetration as the function of applied load. This requires thorough knowledge of the geometry of the indenter and thus the use of a device made with micrometer precision. Detailed description of indenter types and geometry can be found in supplementary materials.

6.3.3.1. Load-Displacement curves

The basis of nanoindentation tests lies in extracting values of material elastic modulus and hardness by precise reading of indenter load and depth of penetration. In a standard test, load

and depth of penetration are registered as load is applied from zero to a fixed maximum and then back to zero value. The depth of penetration combined with known geometry of the indenter provides an indirect measure of the contact area at maximum load. Knowledge of this value allows to estimate the mean contact pressure, and thus the specimen hardness. When load persists, the material attempts to regain its original shape. This process is limited or completely stopped because plastic deformation occurs. In almost any case however, some degree of shape recovery takes place due to the relaxation of elastic strains within the material. An analysis of the initial portion of elastic unloading response gives an information about the elastic modulus of indented material. The form of the load-displacement curves for the most types of indenters are very similar (Fig. 41).

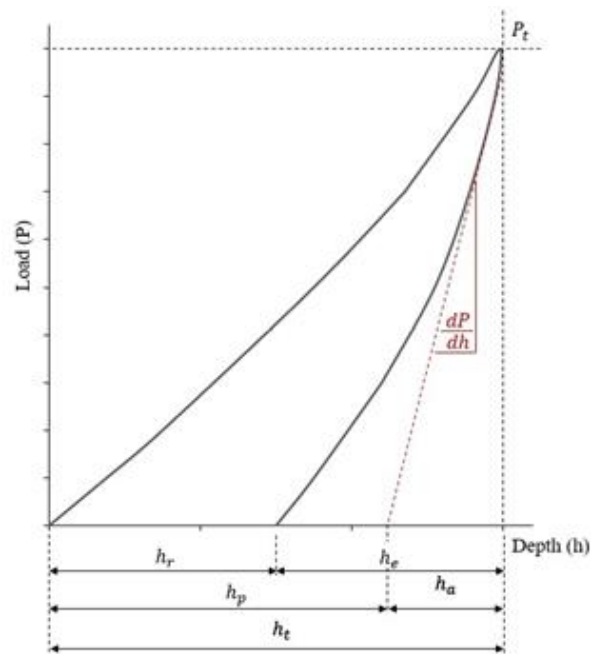


Fig. 41 Typical load-displacement curve from a nanoindentation experiment.

The maximum load was set to P_t value, h_t stands for the indentation depth beneath specimen surface, h_p represents the depth of contact circle. Slope of elastic unloading (dP/dh) allows the elastic modulus and specimen hardness to be calculated.

The shape of the load-displacement curve is often found to be a rich source of information, basing only on their shape, one can predefine not only what kind of material is being tested but also it provides information about non-linear events such as phase transformations, cracking, or delamination of films.

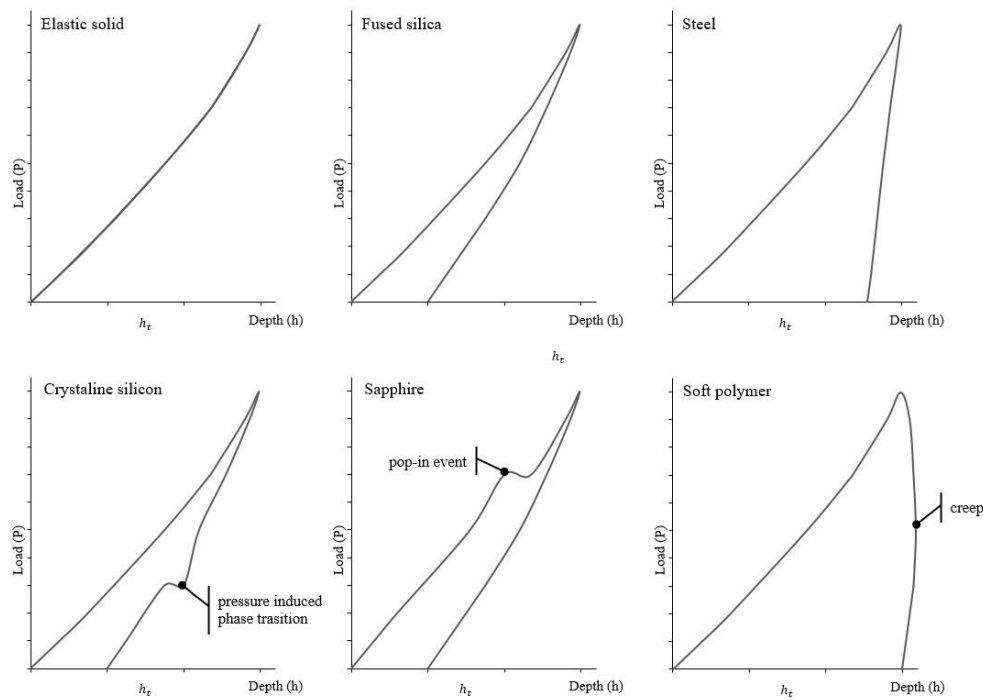


Fig. 42 Schematic load-displacement curves for different materials.

6.3.3.2. Scratch testing

The scratch resistance of fine films and protective coatings is usually defined as their ability to withstand abrasion without fracturing. A typical scratch test involves a ramped load and testing the performance to a load value at which the surface fails. Scratch testing is a routine experiment to be performed by nanoindentation instruments. In this mode of operation, a normal force (F_N) is applied to the indenter, perpendicular to the sample plane, while the specimen is moved sideways simultaneously. For this type of measurement an optional force transducer is applied to measure the friction or tangential force (F_T). In more advanced and extensive measuring setups lateral force (F_L) might also be applied^{167,169}. The friction testing diagram together with the forces operating in the system was presented in Fig. 43.

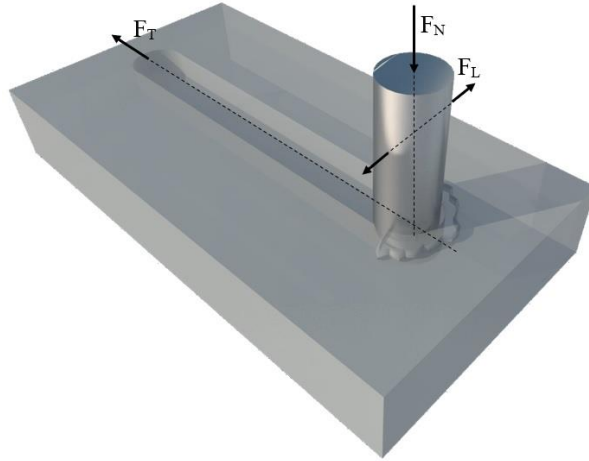


Fig. 43. Configuration of an indentation scratch test.

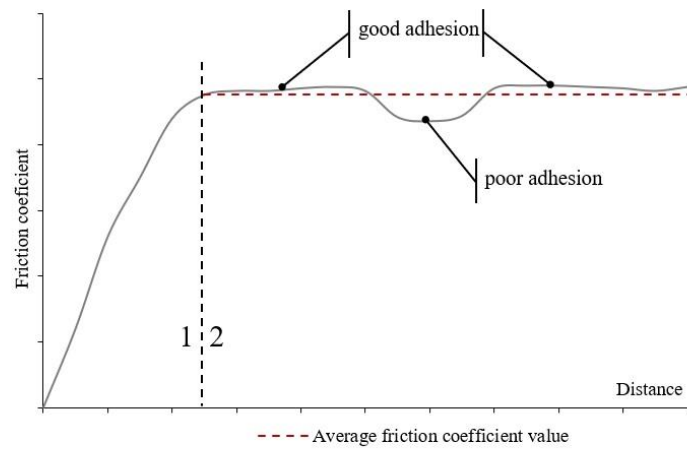
The normal force F_N can be held at a constant level or ramped up or down while the specimen is moved sideways by a tangential force F_T . In some cases, a lateral force F_L can also be applied.

The applied normal force (F_N) is ramped up while the specimen is moved in a sideways direction and the minimum force F_C at which failure occurs is an indication of scratch resistance. The detection of this critical load value can be determined using a variety of techniques such as optical microscopy, acoustic emission, and an analysis of the coefficient of friction¹⁶⁹. When the maximum value of normal force is applied the measurement of the tangential force F_T takes place (Fig. 44). The coefficient of friction (μ) can be easily calculated:

$$\mu = \frac{F_T}{F_N}$$

6-7

The use of the critical load as a measure of scratch resistance is complex because of its dependence on parameters like scratching speed, loading rate, tip radius, substrate hardness and roughness, friction coefficient, and friction force¹⁷⁰. Despite these impediments, the method allows comparative tests to be performed on different materials.



- 1 - area of force (F_N) build-up , not taken into calculating the friction coefficient
- 2 - region of friction coefficient measurement

*Fig. 44 Typical plot for scratch testing experiment.
It depicts the relationship between coefficient of friction and scratch length. A decrease in friction coefficient is an indicator of poor adhesion between sample and the indenter surface.*

Scratch testing is of closely related to the field of tribology. Not all conventional nanoindentation setups offer capability to perform this type of test, however, there is progress in this area associated with the desire of full material characterization with use of a single device.

7. Experimental results



7.1. Material characterization

7.1.1. Physicochemical analysis

The full physicochemical characteristics of the polymer were made thanks to the cooperation between several research teams, the course of the research and experimental results were published in the work entitled “*Structural, topographical and mechanical characteristics of purified polyhydroxyoctanoate (PHO) polymer*”¹. The article was published in Journal of Applied Polymer Science (Article ID: APP47192, Article DOI: 10.1002/app.47192, Internal Article ID: 15976520). The experimental works were carried out at the Jerzy Haber Institute of Catalysis and Surface Chemistry Polish Academy of Sciences, Henryk Niewodniczański Institute of Nuclear Physics, PAS and Institute of Physics, Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University. The PHO polymer was thoroughly characterized, several techniques were employed in order to describe the material. The author was responsible for partial results analysis, graphical elements, graphs and plots included in publication, as well as literature collection, creating the graphical abstract and all illustrations to be found in the final version of the publication. A broader description of both the techniques used and the material tests carried out can be found in Supplementary materials.

7.1.2. Physical properties

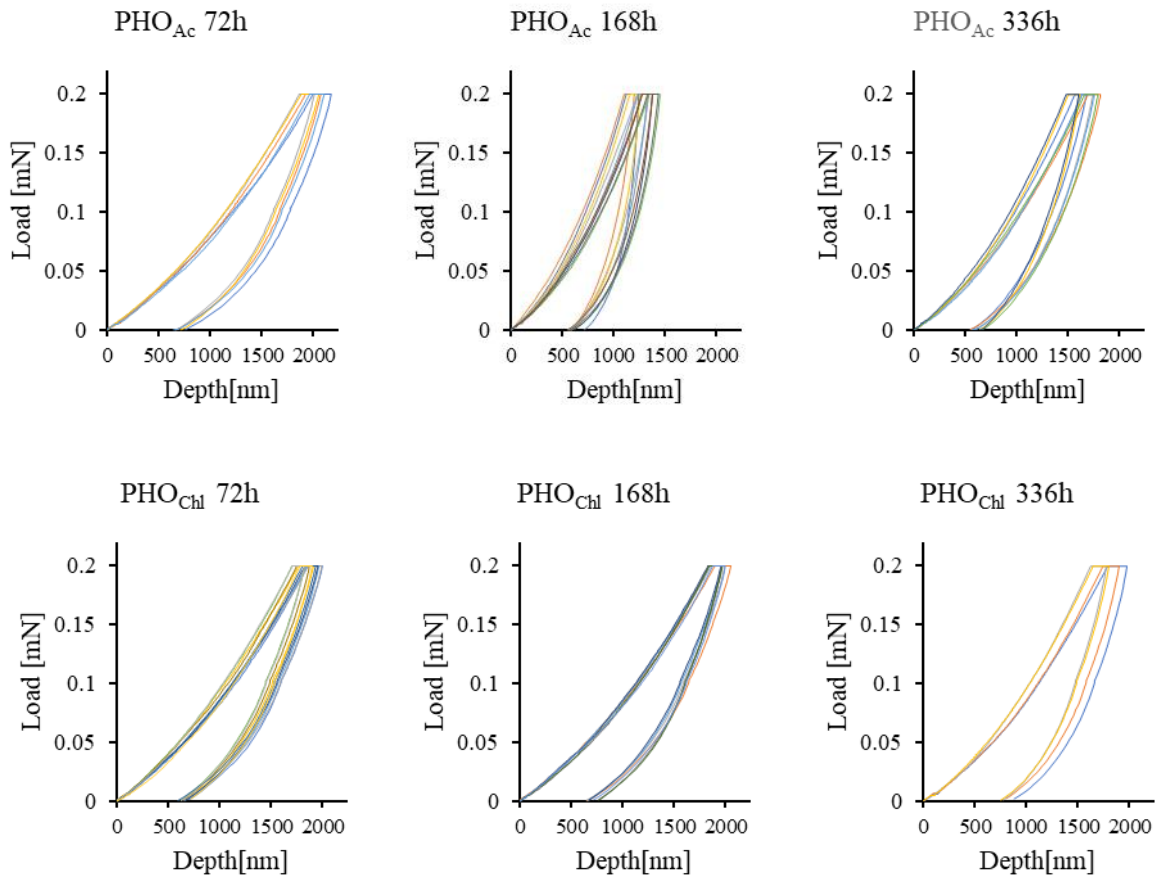
7.1.2.1. Nanoindentation – Young Modulus and hardness determination

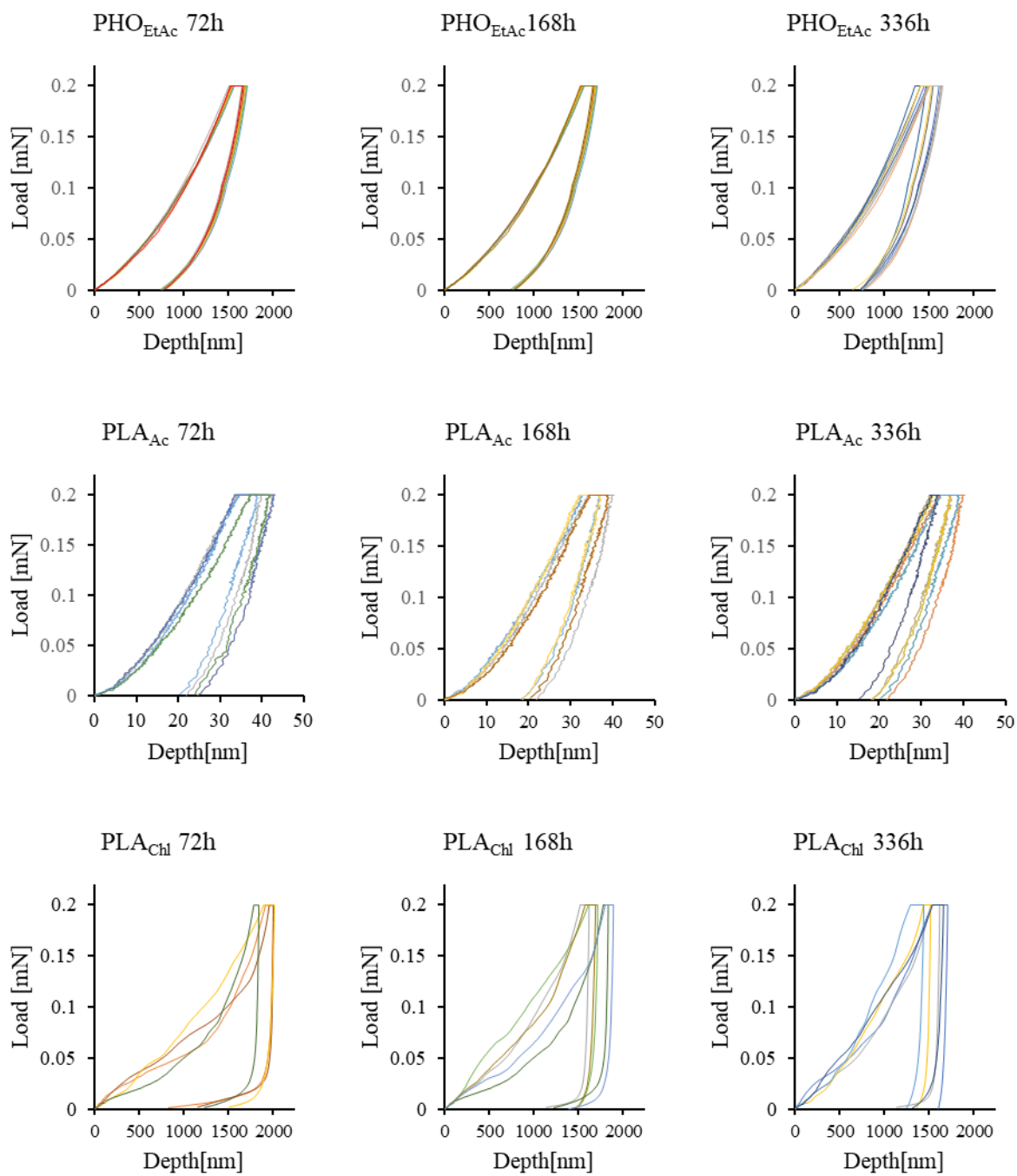
Hardness and Young modulus determination of PHO and PLA samples were held on a round microscopic slides covered with a thick (300-500µm) layer of polymer. The sample thickness was chosen in such a way that the glass substrate did not affect the measurement result during the indentation process. The microscope slide with polymer was attached to a sample holder using the same adhesive for each specimen. Micro indentation was performed on Nano Test Vantage device (Micro Materials, Wrexham, UK). The instrument used a Berkovich geometry ^{171,172} diamond indenter attached to a pendulum. The frame was loaded against samples by passing a current through a coil drawing it towards a magnet. The force with which the indenter interacted with the polymer surface depended upon the variation in voltage between the capacitor plates¹⁷³. Measurement parameters are collected in Table 9 below.

Parameter	Value
Indenter type	Berkovich
Maximum load	0.2mN
Loading rate	0.01mN/s
Unloading rate	0.04mN/s
Dwell period	30s
Number of indents per sample	10

Table 9. Basic parameters used to configure the indentation experiment.

The tests were carried out for two polymers (PHO and PLA) casted from three different solvents. In addition, tests were carried out for samples with different maturation times. Load-displacement curves for tested samples are shown below:





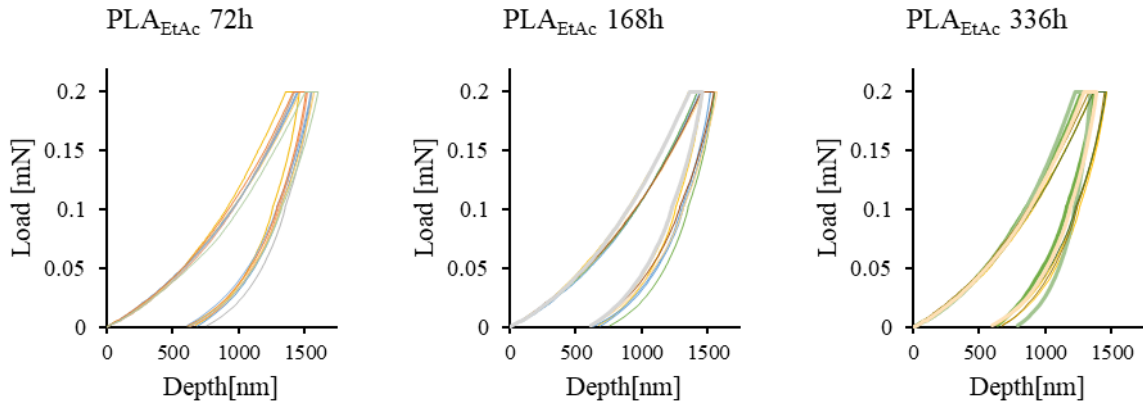
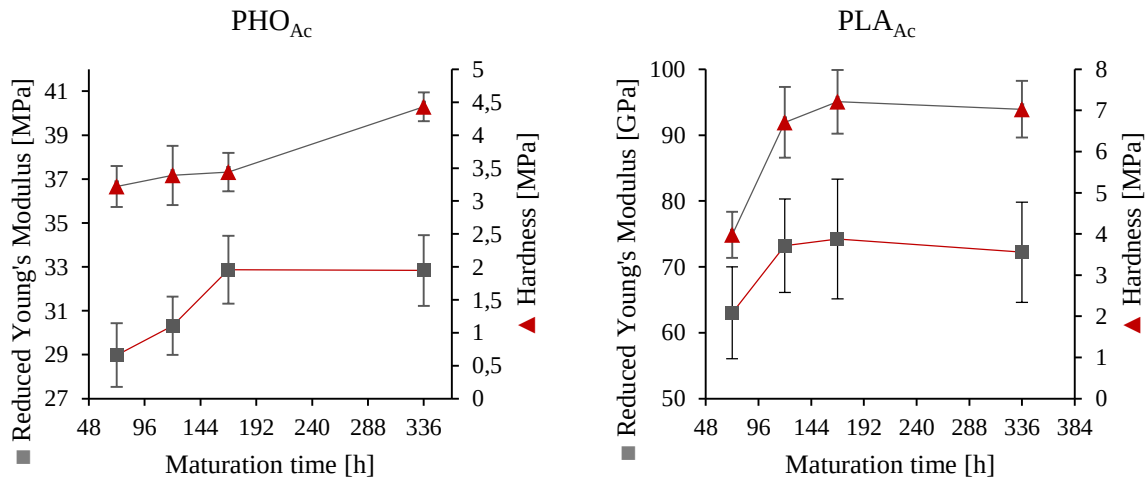


Fig. 45 Load-displacement curves for PHO and PLA in different solvents

Based on the curves shown above, it can be determined that the nature of the material corresponds to the characteristics of a polymer (Fig. 42). In all samples, a region where the indenter depth changes with a constant force value is present. It provides the information about the elastomeric nature of the polymer^{172,174,175}. A clear shift of curves depending on the time of polymer maturation can be observed which testifies increase in the Young's modulus over time. Knowing the exact indenter geometry and the load-displacement curve shape, it was possible to calculate the exact Young's modulus and hardness values for individual materials. The average values for all samples are presented on graphs below:



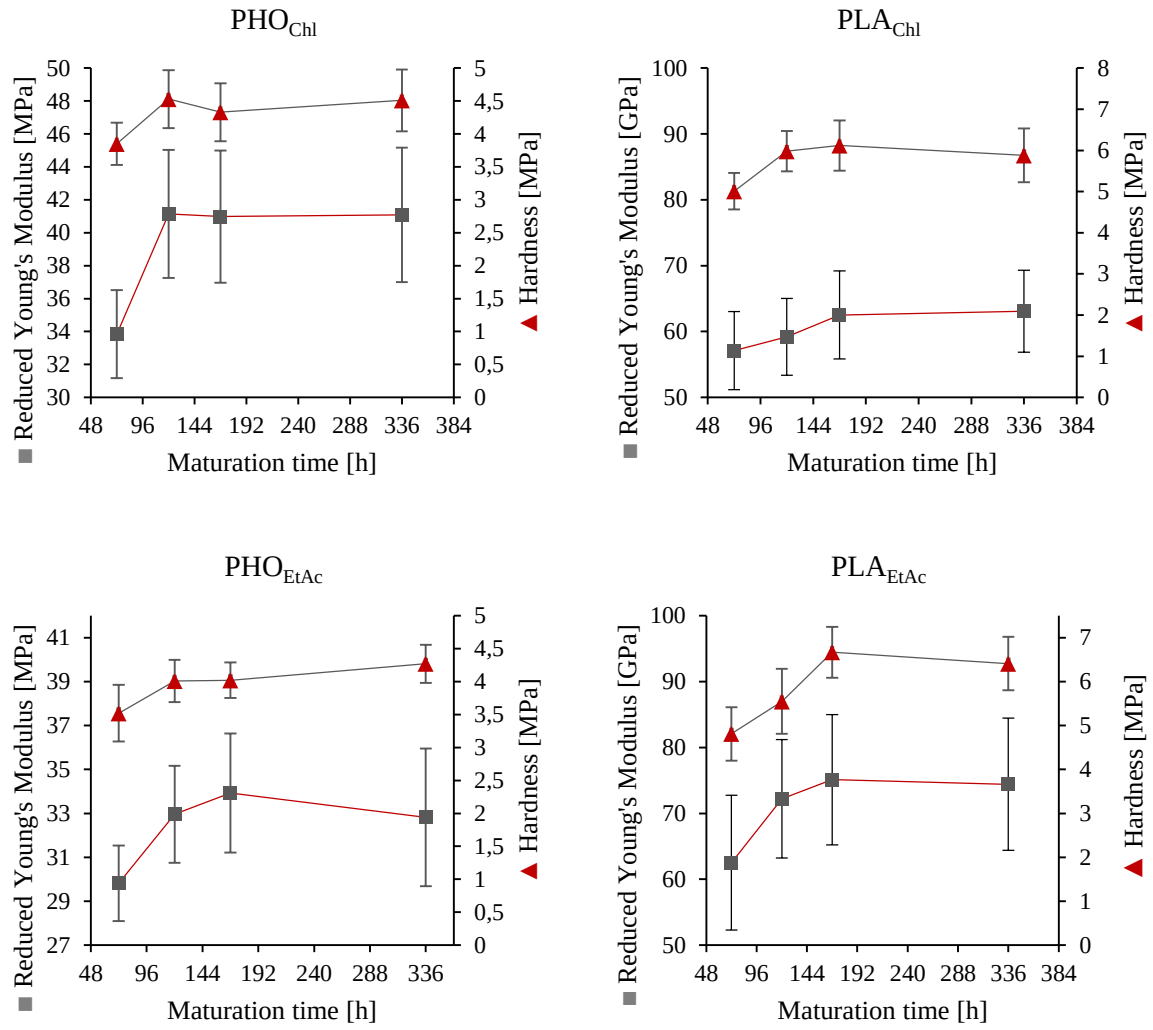


Fig. 46 Calculated Young modules for individual materials as a function of solvent drying time. The scale of Young Modulus values for PHO is in MPa, for PLA in GPa. It is distinctly shown that PLA polymer is much harder than PHO and presents similar Young Modulus values to glass^{176,177}.

The results obtained from nanoindentation are summarized in the table below:

Maturation time [h]	PHO _{Ac}				PLA _{Ac}			
	Er [MPa]	SD _{Er}	H [MPa]	SD _H	Er [MPa]	SD _{Er}	H[Mpa]	SD _H
72	28.98	1.45	3.22	0.31	63.03×10 ³	6.98×10 ³	3.98	0.56
120	30.32	1.33	3.39	0.45	73.21×10 ³	7.09×10 ³	6.71	0.86
168	32.87	1.54	3.44	0.29	74.23×10 ³	9.08×10 ³	7.21	0.77
336	32.84	1.61	4.43	0.22	72.22×10 ³	7.62×10 ³	7.03	0.69
Maturation time [h]	PHO _{Chl}				PLA _{Chl}			
	Er [MPa]	SD _{Er}	H [MPa]	SD _H	Er [MPa]	SD _{Er}	H[Mpa]	SD _H
72	33.84	2.68	3.85	0.32	57.11×10 ³	5.92×10 ³	5.01	0.44
120	41.14	3.89	4.53	0.44	59.21×10 ³	5.83×10 ³	5.98	0.49
168	40.98	4.01	4.33	0.44	62.52×10 ³	6.67×10 ³	6.12	0.61
336	41.09	4.08	4.51	0.47	63.09×10 ³	6.21×10 ³	5.88	0.65
Maturation time [h]	PHO _{EtAc}				PLA _{EtAc}			
	Er [MPa]	SD _{Er}	H [MPa]	SD _H	Er [MPa]	SD _{Er}	H[Mpa]	SD _H
72	29.81	1.72	3.52	0.43	62.51×10 ³	10.22×10 ³	4.81	0.61
120	32.96	2.21	4.01	0.32	72.21×10 ³	9.01×10 ³	5.55	0.74
168	33.93	2.71	4.02	0.27	75.11×10 ³	9.88×10 ³	6.67	0.58
336	32.82	3.13	4.27	0.29	74.42×10 ³	10.03×10 ³	6.41	0.61

Table 10 Summarized results obtained from nanoindentation technique.

The results from nanoindentation were confirmed using AFM technique. A detailed description of the AFM with measurement parameters used for the local Young modulus calculation can be found in the *Supplementary Materials section*. AFM measurements were performed for PHO samples casted from different solvents. In case of AFM tests, the dependence of Young's modulus from the time of polymerization was not checked, all polymer samples were dried for 24 hours at 50°C and then were kept for 14 days in room conditions.

Results obtained both by nanoindentation and AFM indicate that PLA presents much higher Young Modulus values than Polyhydroxyoctanoate regardless of the solvent used. A study conducted with the nanoindentation technique indicates that the solvent has no significant effect on the final Young's modulus of the material. The graphs (Fig. 46) clearly show that after the preliminary drying process the polymer changes its physical properties and the final Young modulus is reached after average 168 hours. The results obtained for both PHO^{115,178} and PLA^{4,152} are consistent with previous literature reports.

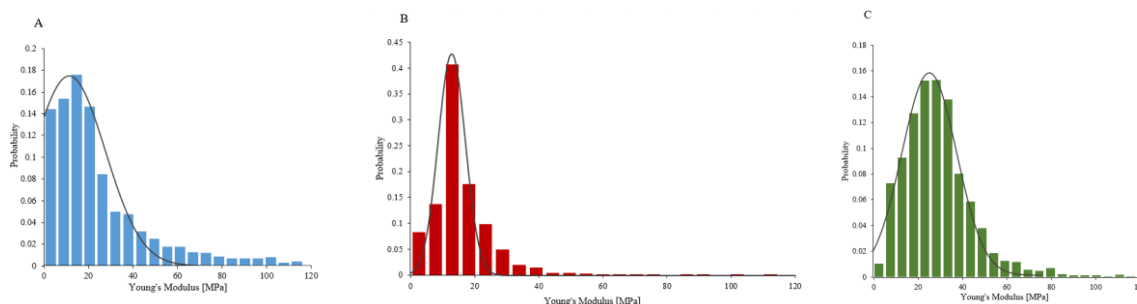


Fig. 47 AFM histograms showing the local Young's modulus distribution in PHO samples casted from acetone (A), chloroform (B) and ethyl acetate (C)¹.

In the table below, the Young's modulus values obtained with AFM for individual materials are shown:

Material	Young Modulus [MPa]
PHO_{Ac}	12.61
PHO_{Chl}	13.29
PHO_{EtAc}	25.47

Table 11 Young's modulus values obtained with AFM technique¹.

In both nanoindentation and AFM, mechanical properties are measured by indenting the material, i.e., a probe is pressed at a defined force on the sample surface in order to deform it. With knowledge of the indenter position relative to the sample, the deformation as a function of the applied force can be calculated. A suitable continuum elastic theory allows the determination of the mechanical properties of the tested material^{177,179,180}. Nanoindentation is a well-established technique to determine quantitatively the mechanical properties of materials on the nanoscale^{167,180}. In this study, the results obtained for both techniques were fairly well covered, however, it should be remembered that AFM is not a technique dedicated to Young's modulus measurements, therefore nanoindentation is a more reliable method.

7.1.2.2. Scratch Testing – friction coefficient determination

The friction testing was carried out using a fixed sapphire 1 mm diameter ball slid against a layer of polymer with a fixed load. Measurement parameters are collected in Table 12 below:

Parameter	Value
Indenter type	Sapphire ball, 1mm
Scratch length	1mm
Maximum load	50mN
Loading rate	5mN/s
Number of scratches per sample	5

Table 12. Basic parameters used for scratch testing experiment configuration.

Scratch tests were performed for PHO and PLA casted from ethyl acetate after 72 and 336h of maturation. The curves showing the experimental process are presented below:

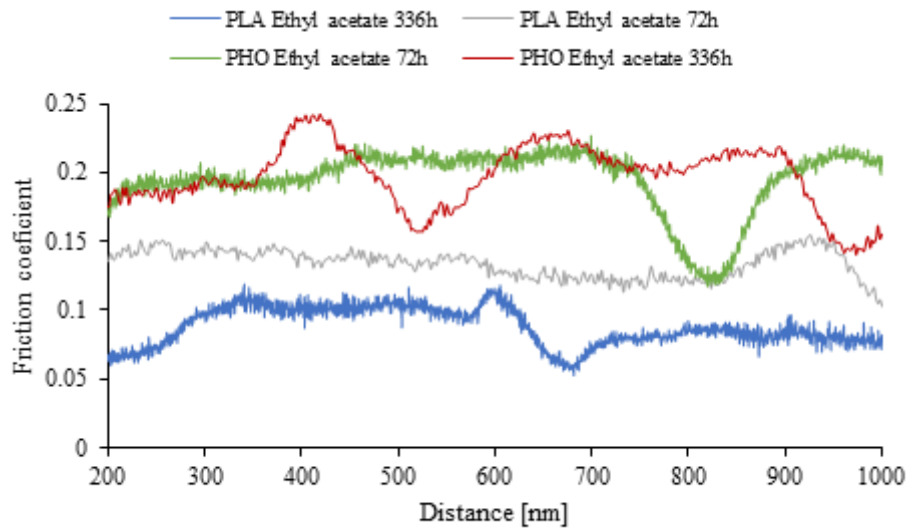


Fig. 48 Exemplary scratch testing plots for selected materials.

It is clearly visible that due to the uneven surface of polymer samples, the experimental curves do not have the model shape as shown on Fig. 44. Areas of local increase and decrease in the friction coefficient are present. Despite non-ideal experimental conditions and therefore an uneven measuring line, the differentiation in friction coefficients between PHO and PLA as well as its change over time is clearly visible. The values of individual friction coefficients obtained in the experiment are summarized in the table below.

Maturation time [h]	PHO _{EtAc}	PLA _{EtAc}
	Friction coefficient	
72	0.202±0.045	0.134±0.021
336	0.198±0.031	0.088±0.018

Table 13 Values of individual friction coefficients for selected materials.

This part of the research was aimed at assessing the PHO physical properties, comparing them with a well-known and deeply investigated and described in literature PLA polymer. In addition, knowledge of Young modulus, hardness and friction coefficient values will allow to outline the initial regions of potential bioplastic applications as well as the selection of traditional polymers that could be replaced by PHO in the future.

Below all the data obtained in this part of the research is compared with literature values for other biopolymers and traditional petroleum-based plastics.

Parameter Material	Young Modulus [MPa]		Hardness [MPa]		Friction coefficient		
	Experimental	Literature	Experimental	Literature	Experimental	Literature	
PHO	33-41	13-26 ^a	11.4-14 ^[115,178]	3.2-4.5	-	0.198	-
PLA	62-74 (×10 ³)		>3.5×10 ³ ^[4,152]	4.0-7.2	7-11 ^[152,181]	0.088	0.1-0.16 ^[152,181]
PHB	-		1.3-1.8(×10 ³) ^[182]	-	16-17 ^[182]	-	0.4-0.7 ^[183]
PE	-		28-37 ^[176,184]	-	8.5-15 ^[151,185]	-	0.2 ^[186]
PP	-		1.5-2(×10 ³) ^[176]	-	9.3-21 ^[185]	-	0.28-0.3 ^[187]
PS	-		23-33 ^[184]	-	21-27 ^[185]	-	0.5 ^[186]
PCV	-		50-160 ^[188]	-	19-21 ^[184,185,188]	-	0.15-0.33 ^[186]

^a data obtained by AFM technique

Table 14 Comparison of basic mechanical parameters for PHO, other biopolymers and traditional petroleum derived plastics.

Basing on the conducted research and analysis of the received experimental data, it can be concluded that PHO presents properties similar to most popular traditional plastics. Young's modulus values 33-41MPa indicate that PHO can mimic mechanical properties of popular polymers such as polyethylene (PE) or polystyrene (PS). The material hardness in range between 3 and 4.5MPa indicates that it can find applications where elastomeric properties are desired. Such a set of physical properties suggests that PHO not only can replace selected plastics whose production requires the usage of fossil fuels, but additionally, by combining the high Young's modulus with elastomeric properties, they can become a viable alternative to some composite materials.

7.2. Biological research



7.2.1. Cytotoxicity determination

7.2.1.1. Experimental design

In order to assess the suitability of materials in biomedical applications, it is important to evaluate their impact on living organisms. Due to the fact that PHO can be used as a material for wound dressings, mouse embryonic fibroblasts (MEFs) were selected as model cell line for cytotoxicity assays because they play an important role in wound healing processes^{154,155}. The Fluorescein diacetate/ Propidium Iodide (FDA / PI) test was used to distinguish dead and living cells. Fluorescein diacetate in a living cell is hydrolyzed by cytoplasmic esterases to fluorescein, which accumulates in the cytoplasm and leads to intense green fluorescence^{189,190}. The assay can distinguish three cell types: fluorescein-dying green (live), non-staining or weakly staining with fluorescein and non-staining PI (dead/apoptotic) and dyeing red with propidium iodide and non-fluorescein dying (dead necrotic). The mouse embryonic MEF 3T3 fibroblasts were suspended in PBS. Then, to the 0.2ml of this suspension a solution of FDA (0.02 μ g) and PI (0.6 μ g) was added. After 3 minutes of incubation at room temperature, the fluorescence was observed under a fluorescent microscope and the percentage of necrotic cells, cells in early apoptosis and live were determined.

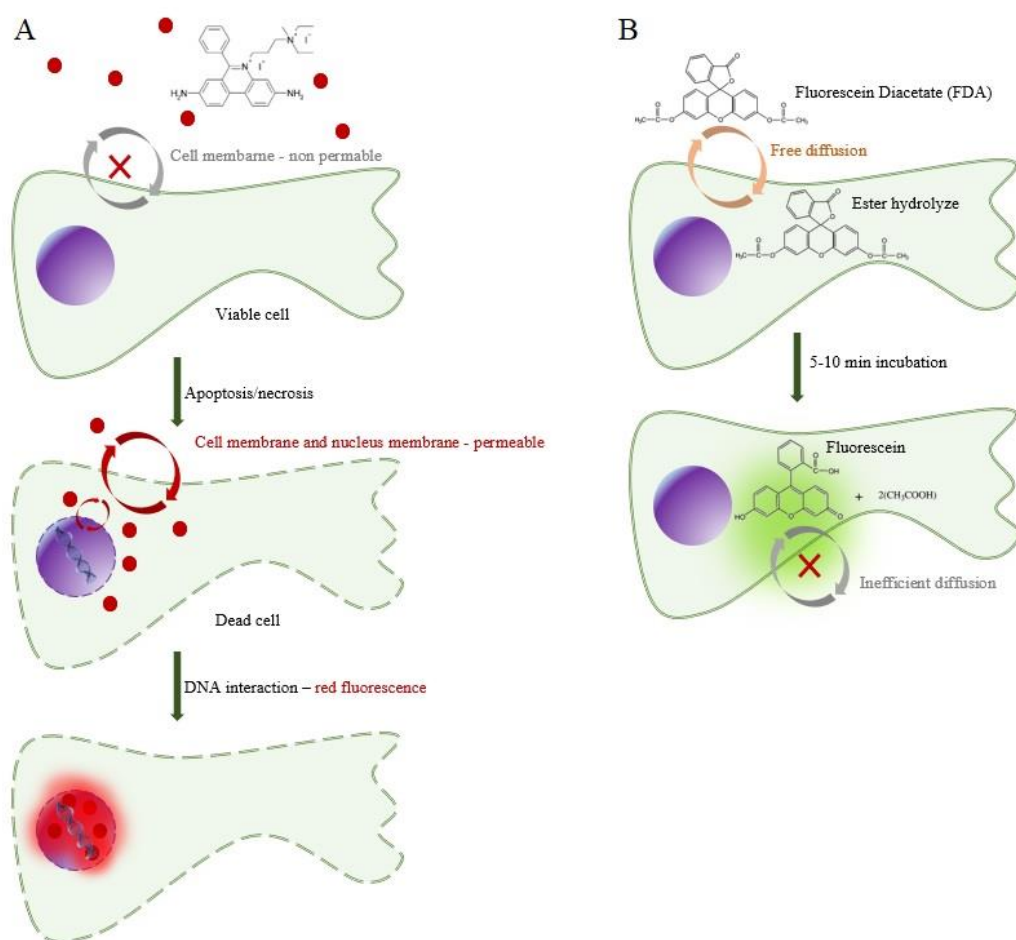


Fig. 49 The mechanism cell viability tests based on PI (A) and FDA(B). Fluorescein diacetate in a living cell is hydrolyzed by cytoplasmic esterases to fluorescein, propidium iodide (PI) penetrates the cellular and nuclear membrane, interacts with the DNA as a result a red fluorescent light is emitted.

After staining the cells, the level of viability was assessed on the basis of the microscopic image. In order to make the most accurate assessment of the impact of the polymer on living cells, a tile-scan technique was used to allow imaging of a large sample area at relatively high magnification. In the experiment, a sample image was made consisting of 121 captures of a single field of view. When using a 10x magnification lens, the final image included cells located on the 200 mm² area. Lasers with wavelengths of 488 and 561nm were used to excite fluorescent dyes, additionally a channel of bright field imaging (T-PMT) was switched on to be able to assess whether the stained object is actually a cell. The table below presents the parameters at which microscopic acquisition was carried out.

Parameter	Value	
Image size (px)	5120 x 5120	
Image size [mm]	14.17 x 14.17	
Objective	EC Plan-Neofluar 10x/0.30 M27	
Number of fluorescence channels	2	
	Channel 1	Channel 2
Laser wavelength[nm]	561	488
Excitation wavelength [nm]	561	488
Emission wavelength [nm]	657	545
Detection range [nm]	596-718	493-596

Table 15 Measurement parameters used when recording images for cytotoxicity assessment.

The cell viability tests were carried out for two biopolymers, PHO and PLA, as a reference glass substrate without any coating was used. As an additional parameter for these tests, the polymer maturation time was taken into account to check how much time does the solvent needs to evaporate enough not to affect the cells vitality.

7.2.1.2. Experimental results

On exemplary full field of view images (*Supplementary materials **Error! Reference source not found.***), one cannot see much details, despite the active T-PMT channel it is impossible to determine whether the stained object is a cell or a dust particle on the microscopic slide. In order to determine the cytotoxicity of polymers, the image was examined fragment by fragment and live and dead cells were counted. High image resolution (Table 15) allows to zoom in such level that it can be clearly determined whether the object is a stained cell and assess its condition.

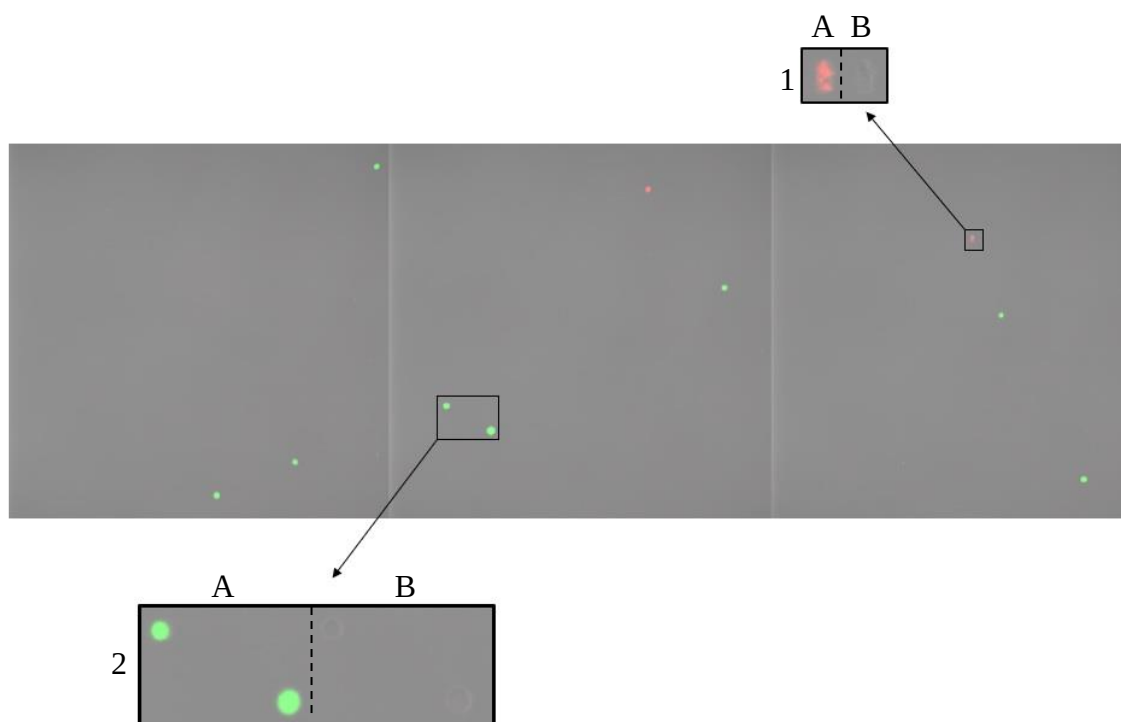


Fig. 50 A microscopic field of view containing dead and vital cells stained with propidium iodide (1) and fluorescein diacetate (2). The enlarged fields (1,2) present fragments visible with fluorescent channels switched off - the bright field technique only T-PMT (1B-2B).

The analysis was performed on total of 5 samples containing cells cultured on each material for each time step. To standardize the results for each of the samples, the number of dead cells was counted for each group of approximately 300 cells stained in green. Parameters used to calculate the cell viability for each of the materials are presented in Table 16:

Material	Time of maturation[h]*	Number individual culture dishes	Number of cells	Vital cells	Dead cells	Vitality [%]
PHO Acetone	12	5	1841	1511	330	82.07
	24	5	1719	1558	161	90.63
	48	5	1688	1541	147	91.29
	60	5	1643	1538	100	93.92
	72	5	1451	1357	105	93.52
	96	5	1414	1291	123	91.30
PHO Chloroform	12	5	1793	1434	359	79.97
	24	5	1665	1507	148	90.51
	48	5	1773	1631	142	91.99
	60	5	1669	1553	116	93.05
	72	5	1781	1676	105	94.10
	96	5	1644	1524	120	92.70
PHO Ethyl acetate	12	5	1944	1603	341	82.45
	24	5	1603	1442	161	89.96
	48	5	1530	1366	164	89.28
	60	5	1594	1482	112	92.97
	72	5	1745	1622	123	92.95
	96	5	1398	1272	126	90.98
Glass	12	5	1662	1506	156	90.63
	24	5	1521	1395	126	91.74
	48	5	1725	1587	138	92.02
	60	5	1992	1816	176	91.19
	72	5	1623	1471	152	90.63
	96	5	1765	1632	133	92.59

Table 16 Detailed results for individual tested materials.

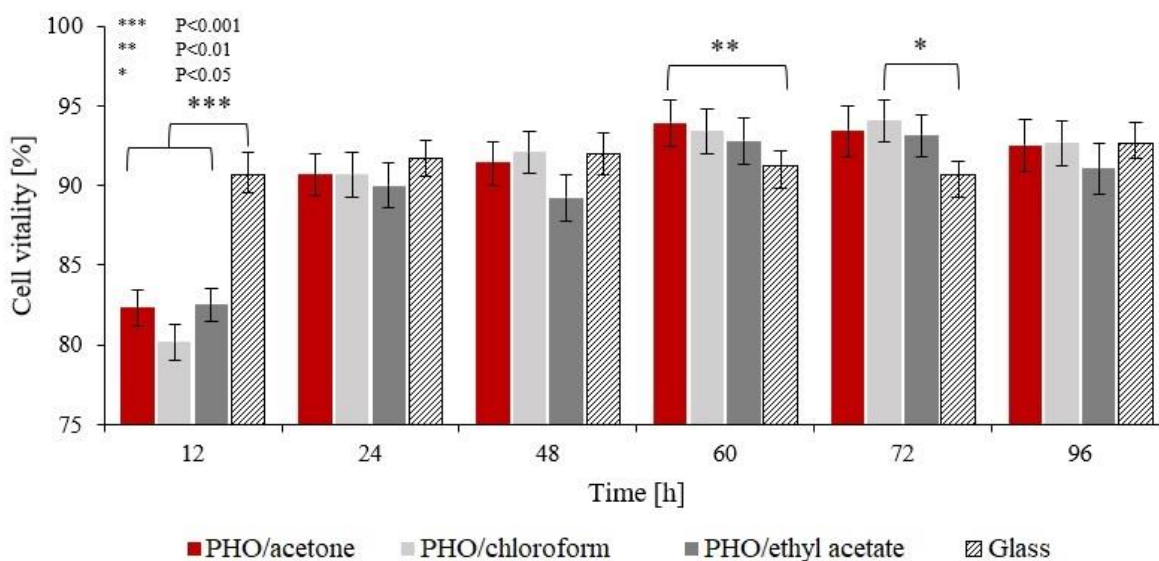


Fig. 51 Vitality rates for MEF 3T3 cells cultured on different substrates

7.2.2. Microscopic cellular morphology studies

Measurements and microscopic observations were made in fluorescent and confocal techniques. They were performed on Zeiss Axio Observer Z.1 microscope with LSM 710

confocal module. The image collection, processing, deconvolution and analysis was performed using Zeiss ZEN Black version 8.1.0.484, PALMRobo version V 4.6.0.4 and FluoRender 2.21.0 software. Single cell measurements were made using an oil immersion objective with a magnification of 40x and a numerical aperture (NA) of 1.4. The field of view was selected based on cell density, so each cell could be scanned separately. The imaging area was always in proximity to the center of the substrate surface to avoid the heterogeneity effects of the polymer and uneven coverage of ECM proteins in case of polyacrylamide substrates.

7.2.2.1. Sample preparation

Mouse embryonic fibroblasts MEF 3T3 cell line was used. This cell type was selected because fibroblasts are primary tissue component and are involved in wound healing processes¹⁴⁹. Cells were grown using the same protocol and reagents as at previous research stages.

Cells were cultured on different substrates for at least 72h. Standard breeding activities such as passage and medium exchange were maintained during this time. As an optimal cell confluence for the final, ready-to-observation sample, 30-40% was assumed. Such a value allows to observe single cells in the microscope's field of view and assured that the proximity of other cells did not affect their behavior or physiology^{191,192}. The cellular medium was removed, and 1 ml of 3.6 % formalin solution (Sigma Aldrich) was applied. After 10 minutes, the sample was three times washed with PBS and then 1 ml of TRITON X100 (Sigma Aldrich) solution (0.1%) was added to induce perforation of cell membranes. After 5 minutes, the sample was triple washed again with PBS and 20µl of fluorescent stain solution was applied. To visualize actin cytoskeleton a staining solution containing rhodamine-labeled phalloidin (Sigma Aldrich) was applied. For nucleus staining DAPI dye (Thermo Fisher Scientific) was used. Light protected samples were left in the refrigerator (5°C) until the imaging was performed.

7.2.2.2. Experimental results

Significant changes in morphology between MEF cells cultured on a reference medium (glass) and on flexible polyacrylamide substrates, covered with bovine serum or fibronectin, were observed. Similar conclusions can be drawn from earlier reports¹⁹³. Cells grown on stiff substrates were flat, characterized by the presence of visible, well-defined stress fibers, and focal adhesions. With the increase of substrate elasticity, the height of the cell actin cytoskeleton, measured from the ventral side to the highest point of the cell, increased which

was also correlated with the decrease of the area they occupy on the substrate^{52,194,195}. On elastic substrates, the cells acquired a more spherical shape, especially in the cell nucleus area. There were also significant changes in the organization of actin cytoskeleton. With the decline of substrate Young's modulus, the number of stress fibers decreased. They became thinner until almost complete disappearance in the case of the most elastic substrate (8 kPa). It has been noted that with the disappearance of stress fibers, the characteristic "granular" structure appears to be present in the cells volume, which is probably partially depolymerized/monomeric actin (G-actin)^{138,196}. In the case of cells grown on PLA substrates, their morphology was similar to the glass substrate. Cells were characterized by good flattening of the lower part and flattened cell nucleus. Cells on substrates made of PHO showed hybrid behavior combining characteristics for hard substrates and those with a lower Young's modulus. The well-flattened lower part and the cell body was accompanied by an almost round nucleus. The cell heights were characteristic for substrates with much lower Young's modulus. Parameters used for cells morphology acquisition are presented below:

Parameter	Value	
Image size (px)	1024 x 1024	
Objective	Plan-Apochromat 40x/1.4 Oil DIC M27	
Number of fluorescence channels	2	
	Channel 1	Channel 2
Laser wavelength[nm]	405	488
Excitation wavelength [nm]	405	488
Emission wavelength [nm]	455	572
Detection range [nm]	410-500	500-643

Table 17 Measurement parameters for confocal imaging of MEF 3T3 cells for the morphology assessment.

a. MEF 3T3 cells grown on glass substrate

The cells grown on glass substrate (elastic modulus around 1 GPa)¹⁹⁷ exhibited a strong tendency to flatten (Fig. 52A). The quantitative results of MEF cell height grown on glass are shown on the histogram (Fig. 55A). It can be clearly seen that the height of a vast majority of the cells was in a range from 4μm to 6μm. In the lateral projection, the flattened shape of the nucleus is clearly visible. On these cross-sections, strong proximity of the cells to the substrate can be observed, which may be a result of high adhesion forces. High resolution 3D reconstructions confirm these observations (Fig. 53).

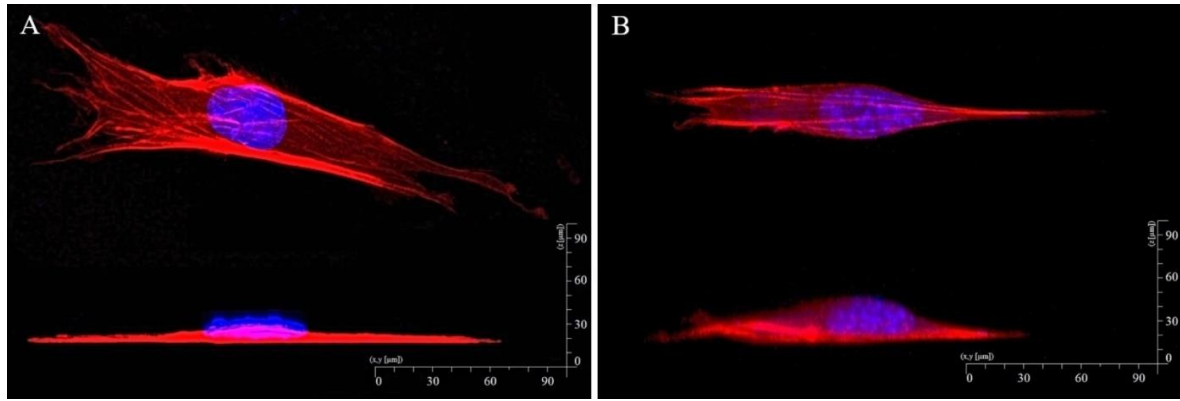


Fig. 52 Confocal images of MEF cells cultured on glass (A) and 40kPa substrate (B). Cell nucleus is stained with DAPI (blue) and cytoskeleton with Rhodamine B-phalloidin (red). Stress fibers are clearly visible in cells on both substrates but the fiber network on glass is more complex and expanded throughout the entire cell body.

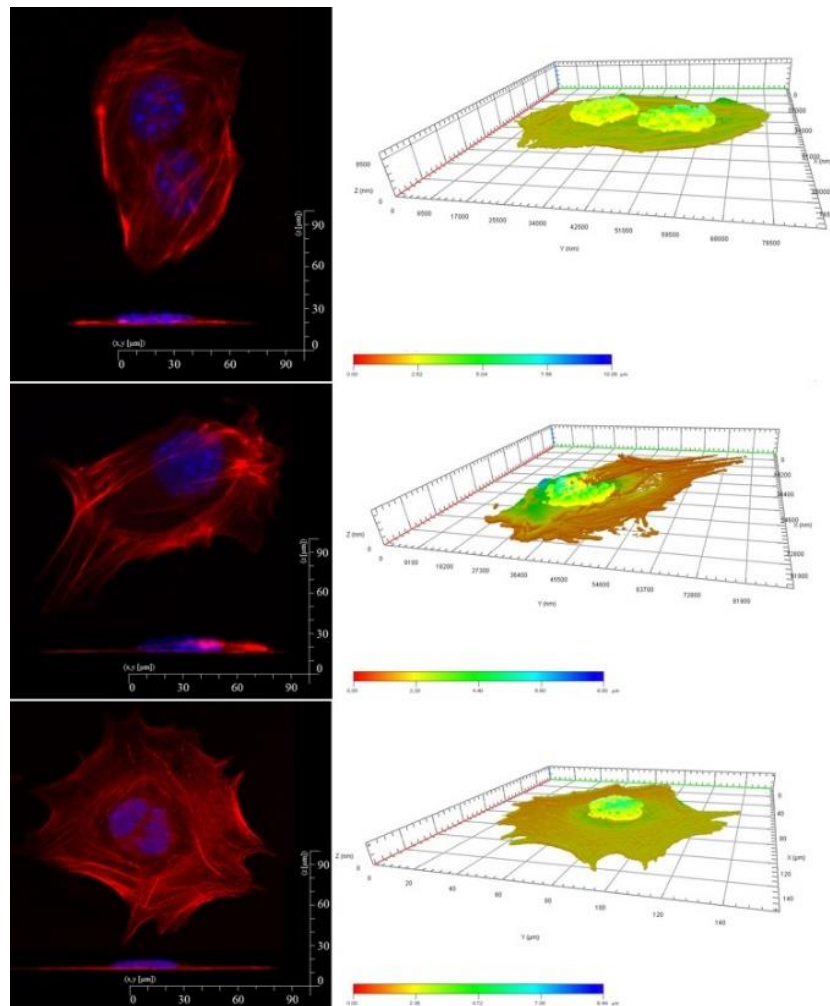


Fig. 53 Heights and morphologies of cells grown on glass presented on 3D reconstructions of images from a confocal microscope.

b. MEF 3T3 cells cultured on substrate with Young's modulus of 40kPa

Although the 40kPa elastic substrate is relatively stiff for the tested cell line, since it corresponds to the elasticity of bone rather than a soft tissue (see Table 1)⁵¹, the morphological changes compared to the glass substrate are clearly noticeable (Fig. 54). In this case, the cells were no longer so well spread on the substrate, what is shown in side view visualization (Fig. 52B). In the case of a flexible polyacrylamide substrate, differences in cells morphologies were also observed. The nucleus was clearly less flattened; it had more elliptical shape. The histogram indicates that most of cells have height between 10 and 14 μm (Fig. 55B).

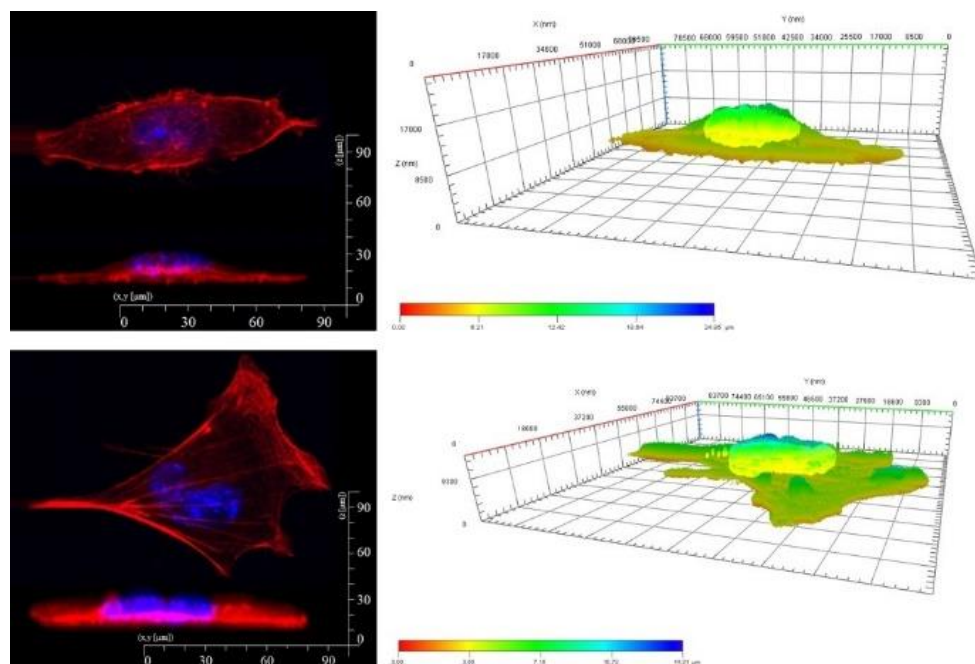


Fig. 54 3D images showing the height and cells appearance from the PAM substrate with a stiffness of 40kPa.

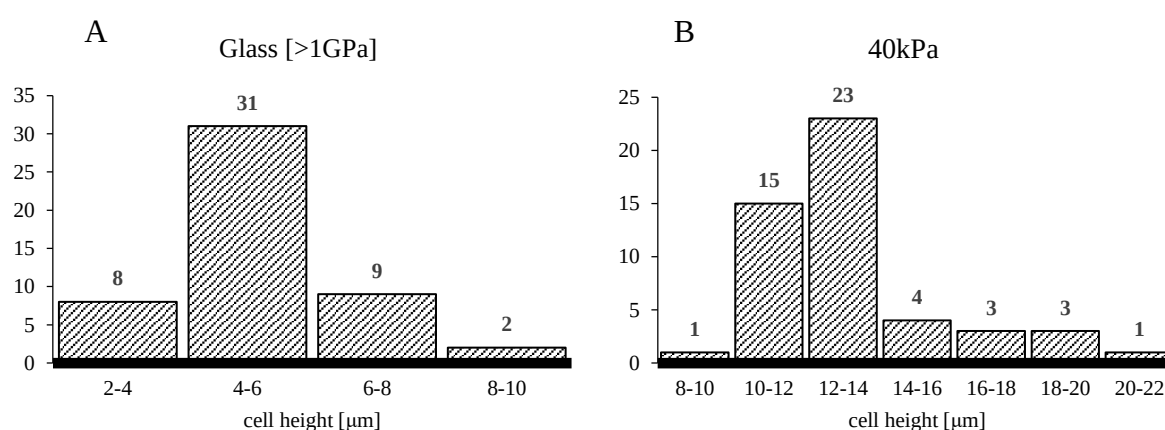


Fig. 55 Height histogram for MEF cells cultured on glass (A) and 40 kPa polyacrylamide substrate (B).

c. MEF 3T3 cells grown on substrate with Young's modulus of 19kPa

As with the 40kPa elastic substrate, MEF cells on the substrate of 19kPa coated with FBS were much less flattened than the cells grown on glass which is clearly visible in 3D reconstructions from a confocal microscope (Fig. 53, Fig. 54). This effect also appears on the histogram (Fig. 57A) where the height of nearly 60% of examined cells was between 14 and 18 μm . The cell nucleus was almost circular in shape, which is visualized on the side projections (Fig. 56A). Stress fibers were present in the cell body, but their network was not as distinct as in the case of cells on stiffer substrates.

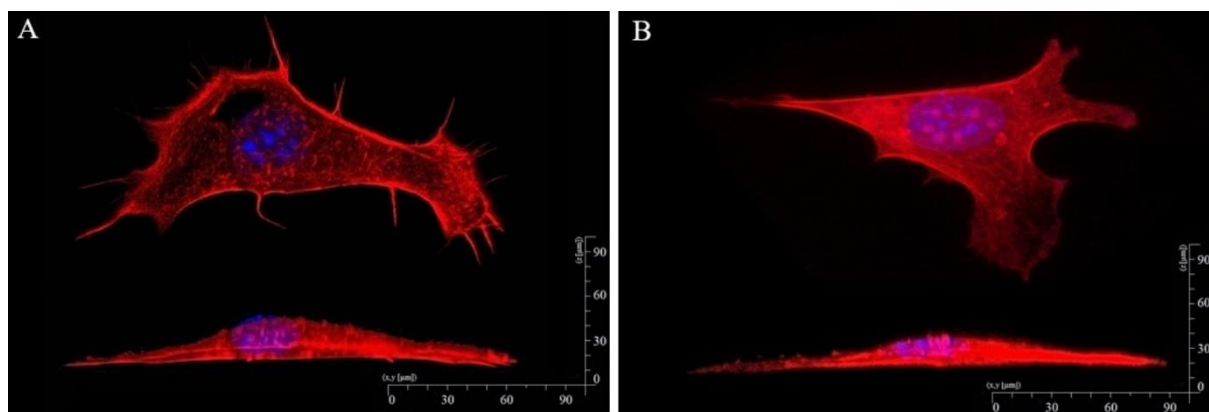


Fig. 56 Mouse embryonic fibroblast cells (MEF 3T3) cultured on 19kPa substrate, coated with FBS (A) and fibronectin (B). Cellular nucleus is stained with DAPI (blue) and cytoskeleton with Rhodamine B-phalloidin (red).

However, the fibronectin-coated substrate caused a radical change in cell shape and adhesion (Fig. 56B). Cells cultured on such substrates became similar in shape to those cultured on stiffer substrates. The height distribution of these cells were similar to that of those cells on 40 kPa substrate (Fig. 57B). These observations are confirmed by the lateral sections (Fig. 56B), indicating a stronger flattening of the nucleus and entire cell body.

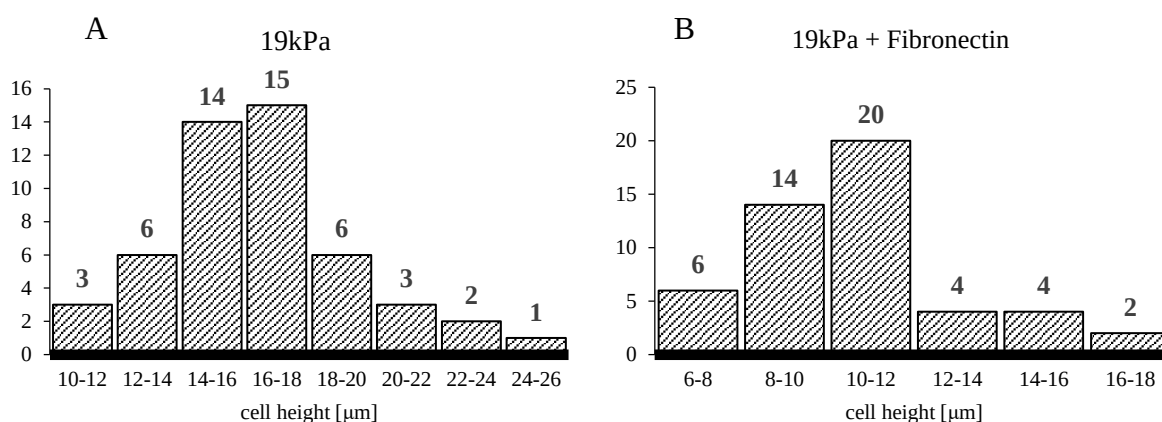


Fig. 57 Cell height histogram for MEF cells cultured on 19kPa with FBS (A) or with fibronectin covered substrate (B).

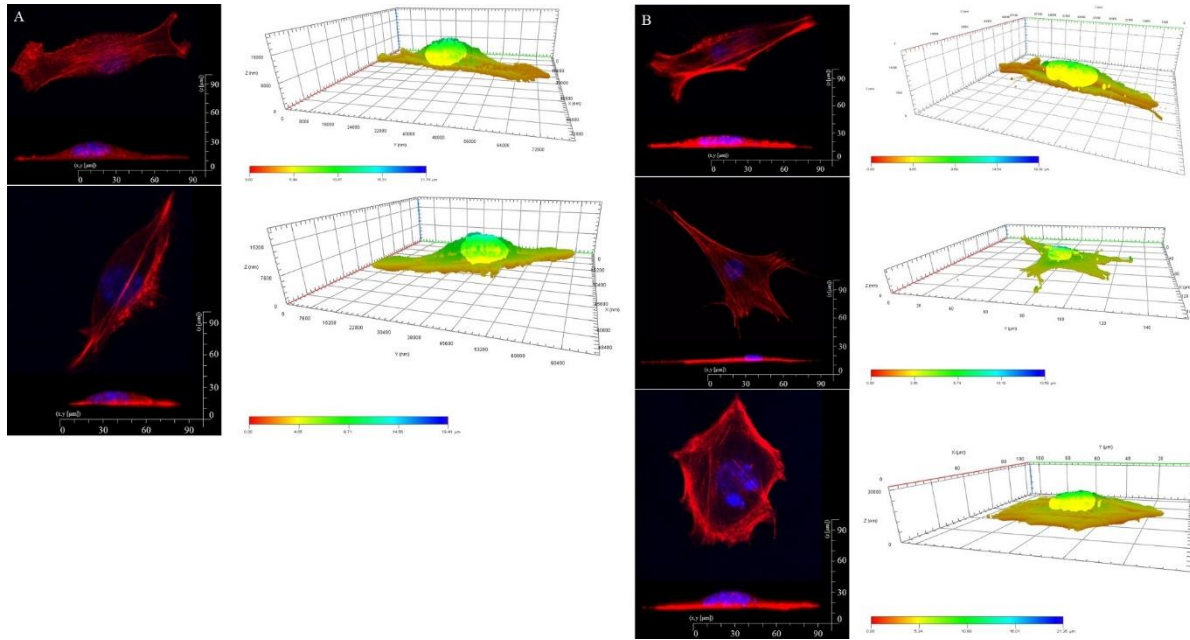


Fig. 58 3D reconstructions showing basic physiological parameters and appearance of cells cultured on substrates with Young Modulus equal to 19 kPa covered with FBS (A) and fibronectin (B).

d. MEF 3T3 cells grown on substrate with Young's modulus of 8kPa

Mouse embryonic fibroblasts on polyacrylamide substrate with Young's modulus 8kPa, are primarily characterized by their round shape (very weak flattening) and significantly weakened attachment to the substrate. The cells had, in most cases, a similar height across the entire cytoskeleton range as compared with cells cultured on stiffer substrates. The rounded nucleus region is clearly visible (Fig. 59, Fig. 61).

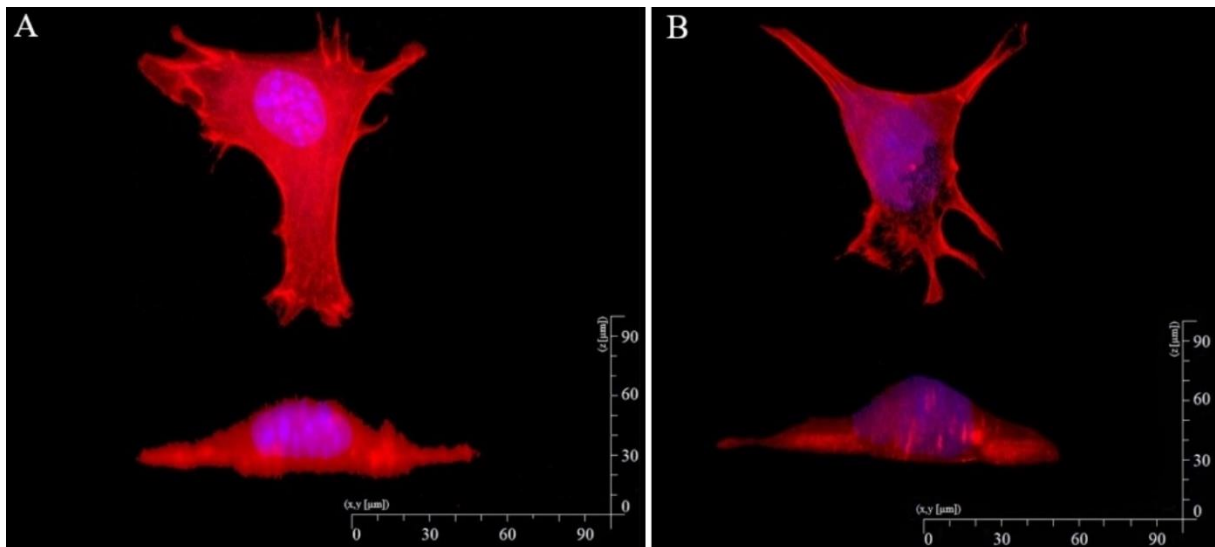


Fig. 59 Images from confocal microscope showing mouse embryonic fibroblast cell cultured on 8kPa substrate covered with FBS (A) and fibronectin (B). Cell nucleus is stained with DAPI (blue) and cytoskeleton with Rhodamine B-phalloidin (red).

Most of the cells were 18 μ m to 24 μ m height. The histogram (Fig. 60A) also shows a significant group of cells whose thickness reaches 14 μ m. Fibronectin coating reduces the mean cell height value and increases cell spreading (Fig. 60B). In this case, the largest group of cells (over 50%) reached the thickness between 10 and 12 μ m. The three-dimensional reconstruction (Fig. 61B) shows much more clearly flattened cell body and stronger cell proximity to the substratum in case of fibronectin coating.

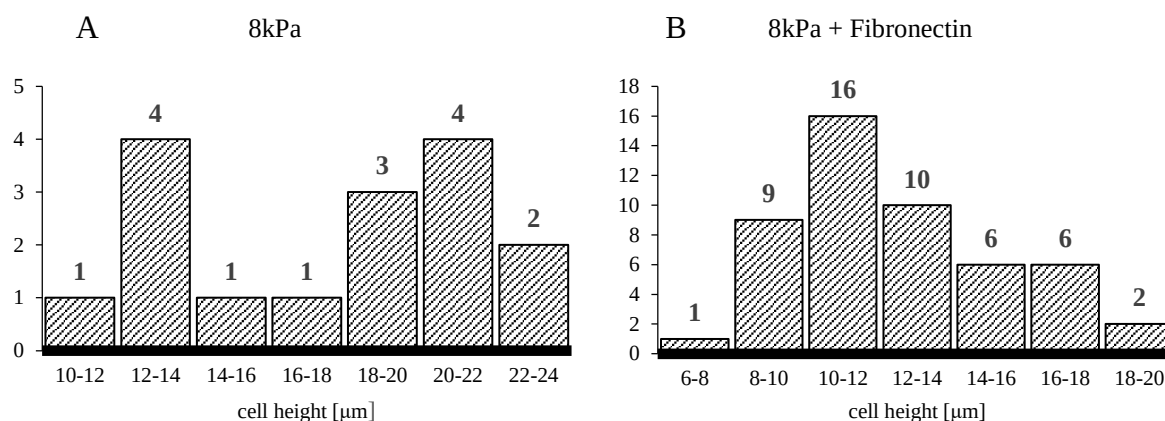


Fig. 60 Cell height distribution for MEF cells cultured on 8kPa substrate coated with FBS (A) and fibronectin (B).

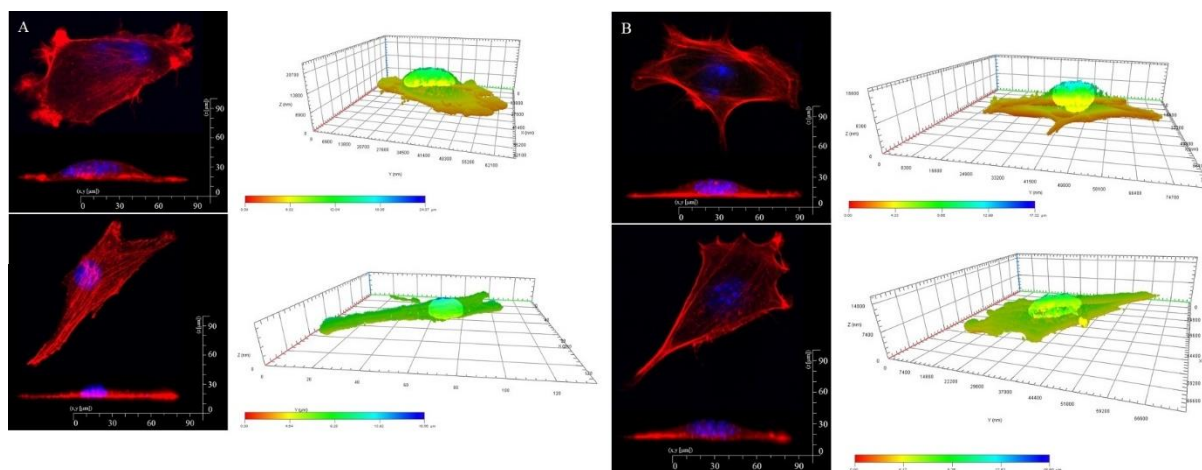


Fig. 61 3D images from confocal microscope showing differences in the appearance between cells on a 8kPa substrate coated with FBS (A) and fibronectin (B).

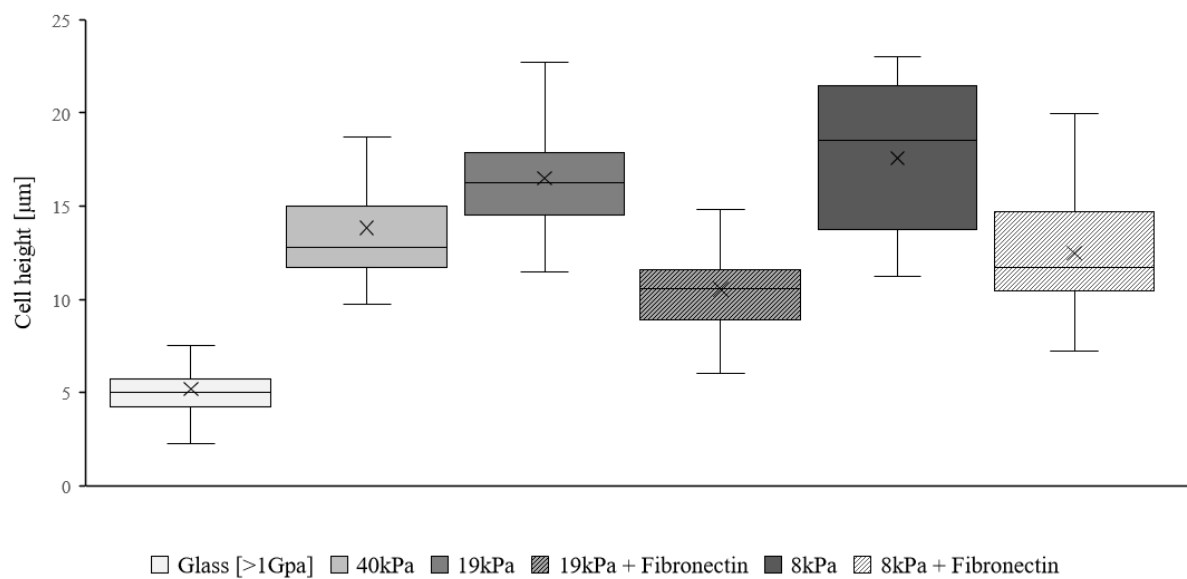


Fig. 62 Box plot containing a data set for the PAM substrates with different Young compared with glass.

A clear separation between results coming from individual substrates with different stiffness is clearly marked. In addition, low standard deviation values confirm the reliability of the observed effects. Results describing the cell height corresponding to the stiffness of the medium are consistent with previous literature reports ^{51,198,199}. Data presented on Fig. 62 are summarized in Table 18.

Substrate	Cell height [μm]			
	Min value	Max value	Average value	Standard deviation
Glass (~1GPa)	2,26	9,77	5,19	1,42 (27,4%)
40kPa	9,76	27,91	14,05	3,77 (26,8%)
19kPa	11,49	24,14	16,50	2,80 (17%)
19kPa + Fibronectin	6,04	17,11	10,55	2,31 (21,9%)
8kPa	11,23	23,03	17,57	3,87 (22%)
8kPa + Fibronectin	7,24	19,98	12,48	2,95 (23,6%)

Table 18 Collective data on MEF cell height on glass and flexible polyacrylamide substrates characterized with different stiffness coefficients.

e. MEF 3T3 cells grown on PLA substrate

To ensure that both the polymerization and solvent evaporation are completed, and the material reached its final physical parameters (Fig. 46, Fig. 51), polymeric substrates fabricated at least 14 days before the experiment were used to study cell morphology. In the case of PLA substrates, no significant differences in cell height between individual solvents were observed (Table 19). Majority of cells exhibit similar parameters to glass substrate. In case of PLA dissolved in acetone vast majority of cells (96%) were between 6 and 12 μm height. It is difficult to distinguish a narrow dominant height value due to similar results between different groups of cells (Fig. 63). In case of MEF 3T3 cells cultured on PLA substrate casted from chloroform the situation is very similar. Over 90% of measured cells are between 6 and 12 μm height. A slightly higher dominance of the cell group between 8 and 10 μm can be noticed but the differences between three major groups are at a level of single cells. No cells with heights less than 6 μm were observed, 4 of the measured cells showed heights between 12 and 14 μm . Among the cells grown on PLA dissolved in ethyl acetate, a group of cells with heights between 6 and 8 μm can be distinguished (almost 50%). There is also a significant share of cells in heights between 8 and 10 μm . Nearly 80% of cells in the experimental group present height between 6 and 10 μm .

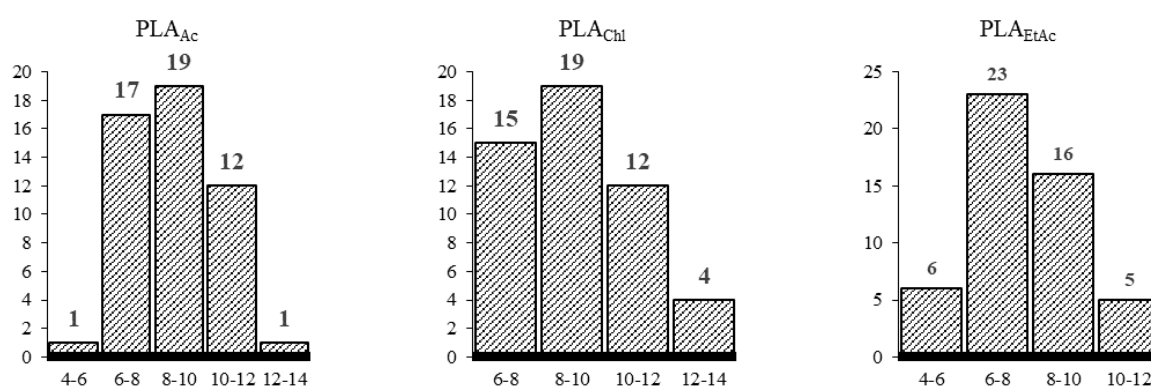


Fig. 63 Histograms showing cell height distribution for MEF cells cultured on PLA substrate.

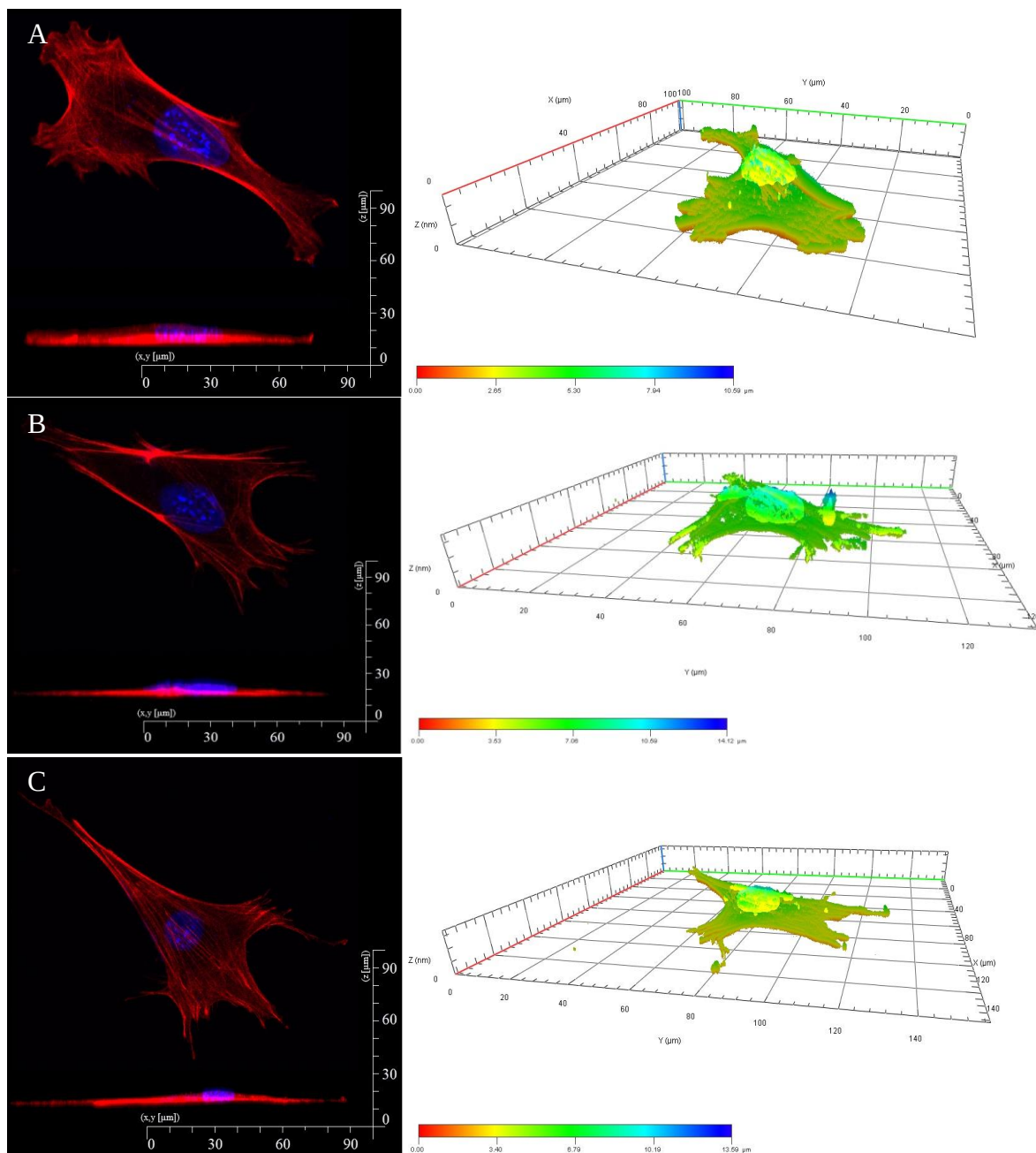


Fig. 64 3D reconstructions from confocal microscope showing morphology of cells cultured on PLA substrate casted from acetone (A), chloroform (B) and ethyl acetate (C).

Substrate	Cell height [μm]			
	Min value	Max value	Average value	Standard deviation
Glass ($\sim 1\text{GPa}$)	2,26	9,77	5,19	1,42 (27,4%)
PLA Acetone	3,98	10,01	6,58	1,59 (24,2%)
PLA Chloroform	4,21	11,07	7,05	1,75 (24,9%)
PLA Ethyl acetate	2,97	9,98	5,76	1,58 (27,5%)

Table 19 Summary table containing cell height on PLA substrates casted from different solvents.

f. MEF 3T3 cells cultured on PHO substrate

The sample preparation protocol was analogous as in the case of PLA substrates. The material was left for 14 days to complete the polymerization and solvent evaporation process. Cells on all substrates were characterized with good spreading and round shaped nucleus. In case of a PHO dissolved in acetone the majority of cells were between 8 and 10 μ m high. 90% of cells were in the range between 6-12 μ m height. Among MEF 3T3 cells cultured on PHO dissolved in chloroform almost 90% of cells were between 6 and 10 μ m high. On PHO where ethyl acetate was used as a solvent there is an evidently dominant group of fibroblast cells (68%) which height remains within the range 6-8 μ m (Fig. 65). Cells cultured on biopolymer substrates are characterized by significant height, and round shaped nucleus in combination with good flattening and adherence to the substratum. The cells retained their physiological shape, had numerous filopodia and well-formed lamellipodia, demonstrating the willingness to migrate over the substrate surface and explore their surroundings.

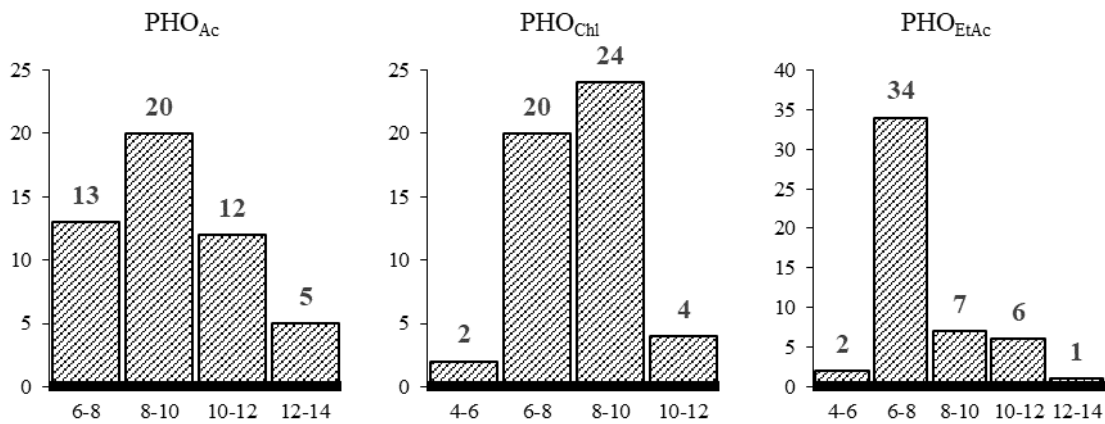


Fig. 65 Cell height distribution for MEF cells cultured on PHO substrate casted from acetone, chloroform and ethyl acetate.

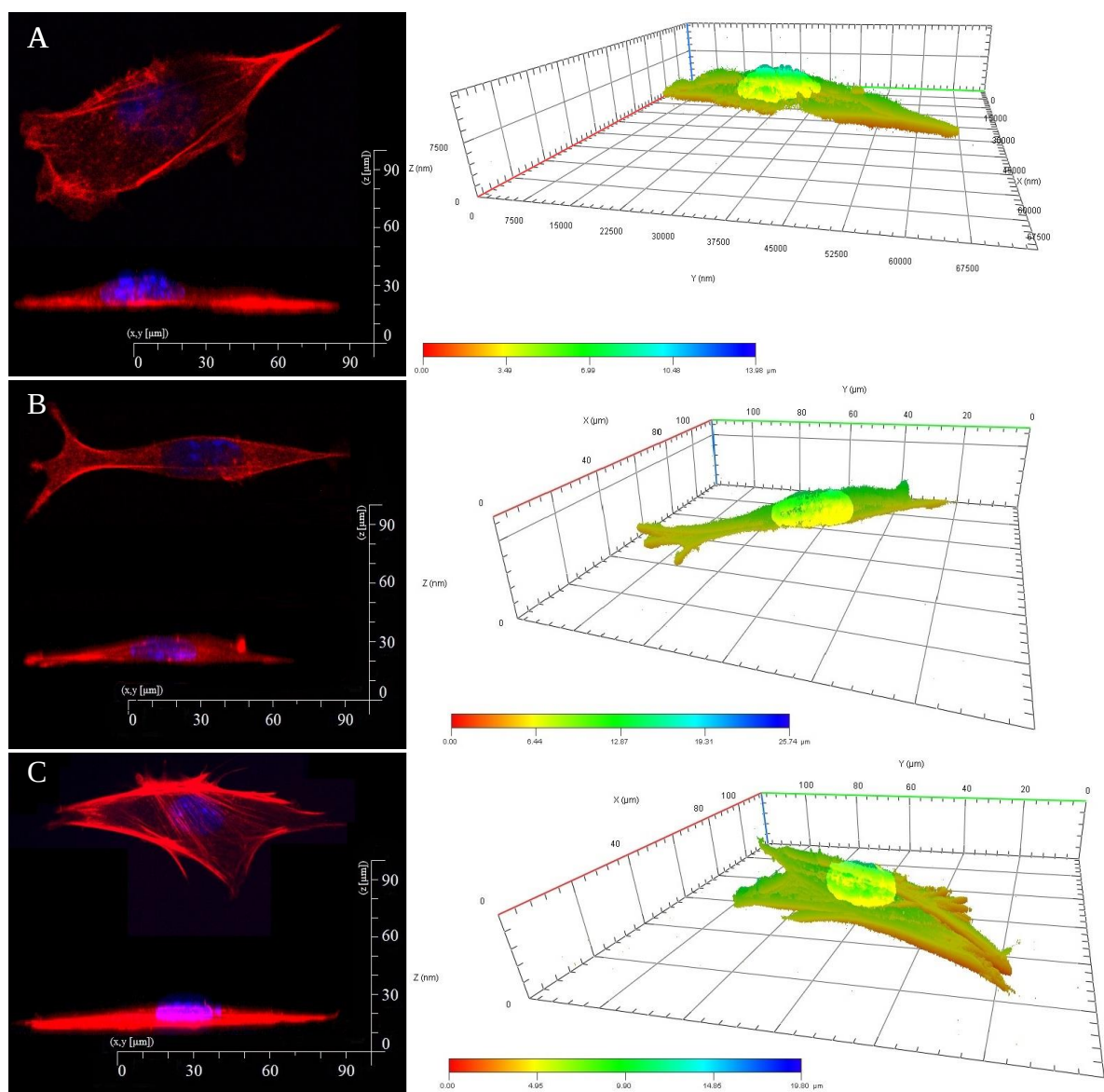


Fig. 66 Three dimensional confocal images presenting appearance of cells cultured on PHO substrate casted from acetone (A), chloroform (B) and ethyl acetate (C).

Substrate	Cell height [μm]			
	Min value	Max value	Average value	Standard deviation
Glass ($\sim 1\text{GPa}$)	2,26	9,77	5,19	1,42 (27,4%)
PHO Acetone	6,09	13,45	9,12	1,80 (19,8%)
PHO Chloroform	5,51	11,97	8,09	1,58 (19,6%)
PHO Ethyl acetate	5,98	12,58	8,14	1,72 (21,2%)

Table 20 Collective data on MEF 3T3 cell height on PHO substrates casted from different organic solvents.

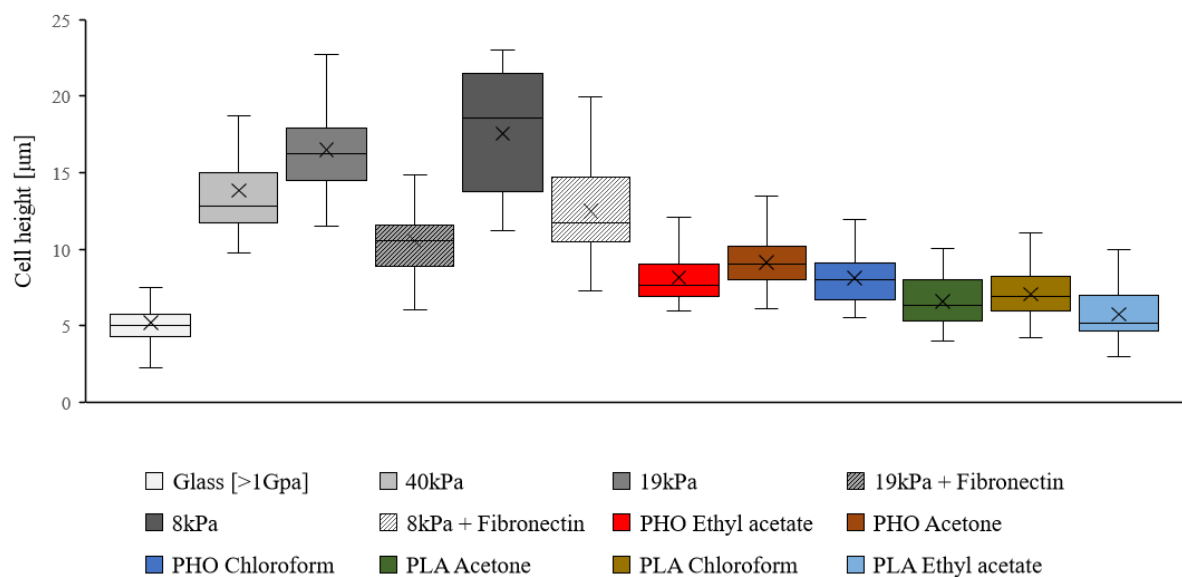


Fig. 67 Comparison of cell heights for all examined substrates.

Cells cultured on both biopolymeric materials are characterized by similar heights and well-structured filopodia and lamellipodia. In case of reference materials with high Young Modulus values like glass or PAM (40kPa) a strong flattening of the nucleus the cell body were observed. The morphologies of cells cultured on both PLA and PHO substrates resembled those characteristics for glass. Microscopic observations also show that the actin structure is similar to glass substrate. Research conducted on 3T3 fibroblasts cell culture, have confirmed the direct impact of cellular micro environmental elasticity on changes in the morphology of cells ¹⁹³. Measurements were also made for different functionalization of flexible polyacrylamide substrates with different extracellular matrix proteins (FBS or fibronectin). Analysis of the data showed that chemical modification of cellular environment also has a significant influence on the changes in cellular morphology. According to predictions, based on already existing literature ¹⁹⁸, conducted studies have shown that mouse embryonic fibroblasts change the morphology of their cytoskeleton in response to the properties of micro-environment on which they are located. The conducted research indicates similar behavior of cells for biopolymers with the exception of the phenomenon of nuclei flattening. The PLA and PHO substrates both presented Young's modulus in the order of MPa (Table 14), which puts their stiffness between the toughest PAM (40kPa) and glass (> 1GPa) substrate. In the case of PLA and PHO the height of cells grown on these materials is contained in between the height ranges characteristic for glass and PAM 40kPa substrates, but their morphology and adhesion to the substrate resembles that of glass substrate this may indicate that cells living on substrates made of

biopolymers are provided with optimal physiological conditions. This demonstrates the good biocompatibility and their suitability in cellular applications and thus confirms the observations made during cytotoxicity tests of both materials.

7.2.2.3. Migration strategies on different materials

Measurements and microscopic observations were made in fluorescent and bright field techniques. They were performed on Zeiss Axio Observer Z.1 fluorescent microscope. The image collection, processing, and analysis was performed using Zeiss ZEN Black version 8.1.0.484 and the cell tracking was executed on Cell Tracker v 1.0 Software. To precisely track all cells in the field of view an automatic vignetting and alignment were executed, and a semi-automated algorithm was selected. To identify single cells a dynamic interpolation with histogram matching setup was used. The experimental material was checked and any misidentified cells were manually corrected. To enhance the image contrast a nucleus life staining was performed for all samples which facilitated the unambiguous positioning of individual cells at each time step. In this part of the work, parameters such as migration speed, directionality and distance from point of origin traveled by cells will be determined. The subsequent images were captured in time-lapse mode which involves taking microscopic pictures with the same acquisition parameters in the same time steps. For the experiment, a time step of 5 minutes between each acquisition was selected. The individual parameters to be determined and compared between particular cell groups are presented and described below.

- speed of migration - this parameter shows directly the rate at which the cells move on certain substrate. Due to the fact that migration is the basic parameter taken into account in wounds repair analysis²⁰⁰, cell velocity is important to assess the suitability of individual materials indicated in the production of wound dressings or tissue scaffolds. The migration speed was calculated for every cell at each time step, which is shown in the graphs below (Fig. 71 - Fig. 77). In order to compare migration velocities on particular materials, the average speed for the whole experimental group was drawn and presented on a box plot (Fig. 78).
- displacement from point of origin (total displacement) - this factor shows how far from the starting point the cell arrived during the entire experiment. This allows to assess whether the MEF 3T3 cells tend to explore the entire available sample surface or whether they stick close to the starting point and often change the direction to opposite and turn back. The analysis was performed basing on coordinates of the farthest point

(X_F, Y_F) where the position of the given cell was recorded. The displacement was calculated according to the formula:

- angle from origin – information about the final angle (θ) of displacement for a single cell does not carry useful knowledge, but comparison of its value with other cells can give information about migration directionality. If the cell movement takes place in one specific direction, the angles for individual cells would be similar to each other, and the results from whole group would have a low standard deviation. When determining this parameter, the coordinates of the last point in which the cell was located were analyzed. Results were compared for individual materials and presented on Fig. 80.
- instantaneous angle speed change – this parameter gives information about migration strategy of cells grown on certain material. This parameter complements the other information about cell movement and allows for deep analysis of the migration strategies presented by MEF 3T3 cells on different substrates. Fast and rapid changes in the direction testifies a strong localized exploration of the substrate by fibroblasts, while $\theta_1 - \theta_n$ values are close to 0° or 360° indicates a linear movement and the inclination to quick position change. The effect of linear movement in one direction can be observed in the case of a strong directional stimulus such as a chemoattractant or the presence of an electric field^{201–203}.

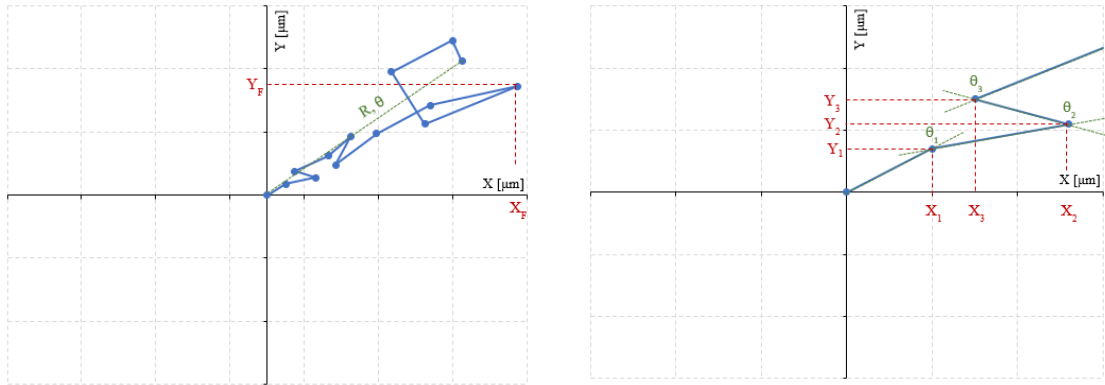


Fig. 68 Illustrative description of selected parameters which were analyzed in this experimental section.

a. Sample preparation

To evaluate the suitability of the bioplastic in the production of biomedical devices, such as dressings or scaffolds, it was necessary to assess the migration of cells on its surface. Cell culture procedures were not different from those used at earlier stages of work. In this case, intravital staining of the cell nucleus with the use of NucBlue (Thermo Fisher Scientific) reagent was used. This staining agent is based on Hoechst 33342 which is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to DNA (Fig. 69).

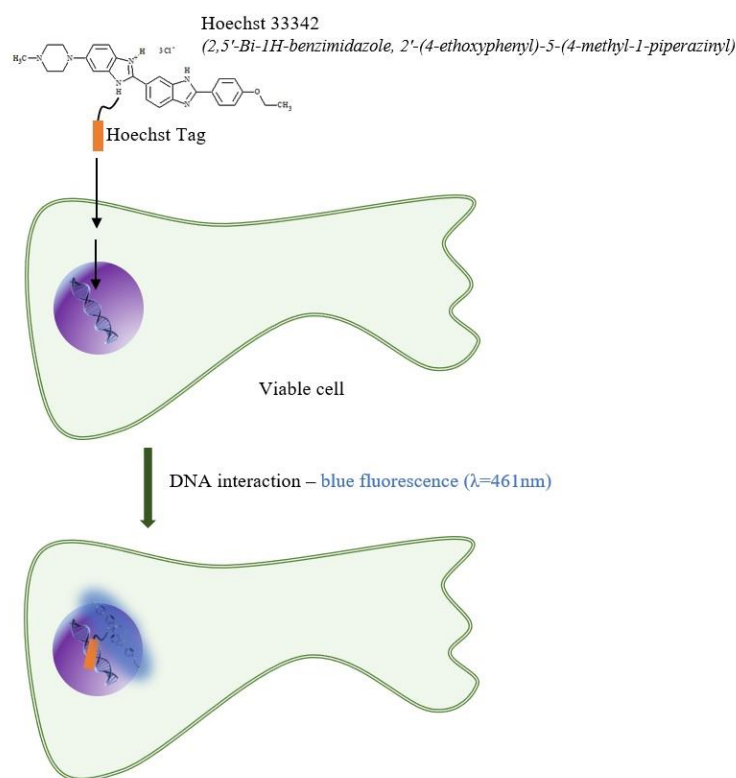


Fig. 69 Hoechst 33342 nucleus staining process.

Cell culture was treated according to manufacturer's manual. The culture medium in glass bottomed dish containing live cells and the NucBlue staining agent were mixed in recommended proportions. On every 1ml of medium two drops of stain were added. Subsequently, the cell culture was put in the incubator for about 15 min and after this time the staining of cell nuclei throughout the sample was observed under fluorescent microscope. After staining the cells, the migration was examined on the basis of the microscopic image series. To study the migration process well, the measurements lasted 24 hours with subsequent frames taken every 5 minutes. A 10x magnification lens was used to capture the time series. Observations were conducted in in bright field mode, additionally laser with wavelength of 353 nm (DAPI) was used to excite fluorescent dye, to enhance the precision of cell tracking software. In table below the acquisition parameters are presented.

Parameter	Value
Image size (px)	1388x1038
Image size (mm)	1.02x0.77
Objective	Plan-Apochromat 10x/0.45 M27
Number of fluorescence channels	1
	Channel 1
Laser wavelength[nm]	353
Excitation wavelength [nm]	353
Emission wavelength [nm]	465

Table 21 Measurement parameters used when recording image time series for migration assessment.

Below a fragment of the field of view containing combined bright field (BF) and fluorescent images is shown. Stained nuclei and cell morphology can be simultaneously observed.

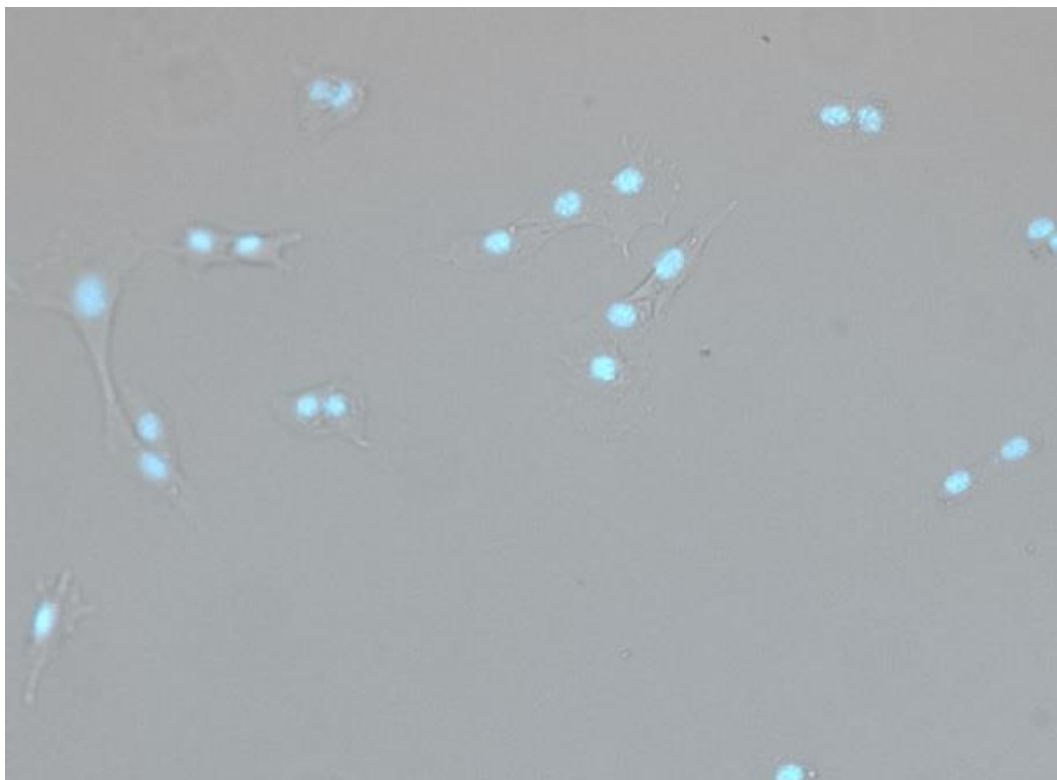


Fig. 70 Image of migrating cells presented simultaneously in BF and fluorescent modes.

b. Cells migration on glass substrate

In this group individual 16 cells were tracked and examined. For each cell average migration speed, displacement from origin and movement angle were calculated. Additionally, migration speed was determined for each time step as well as the momentary change in the angle of movement and the length of a single step. Complete data set for the entire cells group is presented in Table 22.

Parameter	<i>Average speed</i>	<i>Total displacement [μm] /</i>	<i>Angle from</i>	<i>Avg. angle</i>
<i>Cell number</i>	<i>[$\mu\text{m}/\text{min}$]</i>	<i>Avg. step/frame</i>	<i>origin [$^\circ$]</i>	<i>change/step [$^\circ$]</i>
1	0.8685	250.8038 / 4.4523	323.6316	213.5
2	0.7963	207.462 / 4.0258	93.5556	159.6471
3	0.7986	295.0976 / 4.1106	179.3696	162.2636
4	0.8572	156.6404 / 4.3152	116.7464	168.13
5	0.9416	285.2024 / 4.6552	169.0365	182.1545
6	0.6844	180.5583 / 3.4221	170.3406	164.5
7	0.9146	132.8113 / 4.6085	243.8015	181.1124
8	0.8682	318.2868 / 4.4106	100.0093	172.3261
9	0.6939	364.4405 / 3.5026	276.4711	203.9896
10	0.74919	247.9779 / 3.8015	141.5435	172.4227
11	0.9431	244.0025 / 4.8012	193.2751	161.6333
12	0.8955	337.8662 / 4.5002	114.2536	137.4242
13	1.0545	154.8974 / 5.3148	193.5926	194.7826
14	1.0031	194.6586 / 5.2361	74.9855	153.7558
15	0.6559	252.3744 / 3.3054	34.0507	169.75
16	1.0651	181.7639 / 5.4162	242.7167	175.3293
AVERAGE	0.8618	237.8028 / 4.3674	166.7106	173.2951
<i>SD</i>	<i>0.1260</i>	<i>69.4086 / 0.6523</i>	<i>75.7026</i>	<i>18.3623</i>

Table 22 Collective data of examined mouse embryonic fibroblast cells on glass substrate.

Figure shows the tracked movement of selected individual cells on glass substrate with color coding of the instantaneous migration velocity. Presentation of all examined cells on one graph made it totally unreadable, therefore individual cells were segregated due to the maximum displacement from origin. Charts for all cell's groups are included in chapter 16 (*Experimental data not included in thesis main body*). As the initial starting position for all cells the point $x = 0$, $y = 0$ was assumed to better present the mutual direction of migration of individual MEF 3T3 cells.

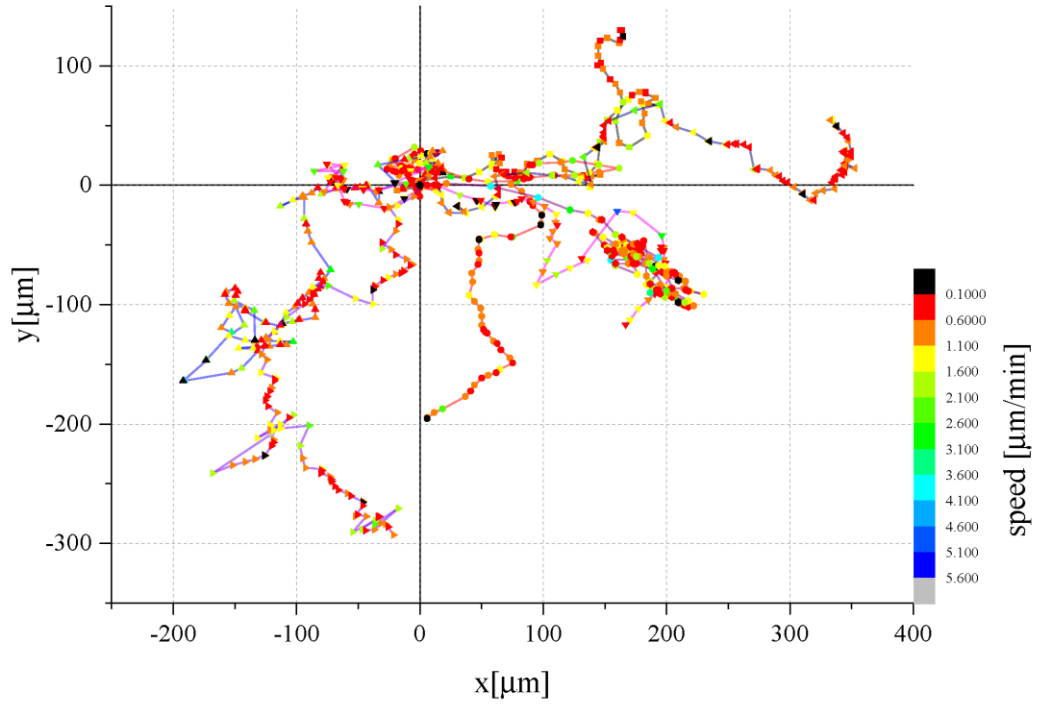


Fig. 71 Tracked movement of selected MEF 3T3 cells on glass substrate.

Cell migration speed on glass substrate calculated on the basis of experimental data, is consistent with previous literature reports^{204,205}. Both numerical data (Table 22) and plots presenting tracked cells paths (Fig. 71) show no directionality in cells movement. Low deviation in total displacement value indicates similar migration strategy in case of all examined cells. Results obtained at this stage will be subjected to further analysis and will be compared with data obtained for other substrates in the further part of the thesis.

c. Cells migration on PLA substrates

On PLA substrates, a total of 41 cells were tracked and analyzed, 13 for casted from acetone, 16 for chloroform and 12 for ethyl acetate. All collected and analyzed parameters were identical to those for glass substrate. Below the results for a group of cells grown on PLA casted from different solvents are presented.

1) PLA_{Ac}

Parameter	<i>Average speed [$\mu\text{m}/\text{min}$]</i>	<i>Total displacement [μm] / Avg. step/frame</i>	<i>Angle from origin [$^\circ$]</i>	<i>Avg. angle change/step [$^\circ$]</i>
<i>Cell number</i>				
1	0.6807	83.7971 / 3.4065	164	168.7143
2	0.5453	227.1911 / 2.7652	32.90164	205.7333
3	0.4253	168.4613 / 2.0851	73.83333	175.6364
4	0.6118	87.7258 / 3.1009	142.0244	175.4751
5	0.4882	197.5526 / 2.5075	277.2576	229.9692
6	0.3894	197.9299 / 2.0051	85.4697	152.9516
7	0.6823	184.8023 / 3.5062	231.7419	173.9355
8	0.8424	100.7282 / 4.4325	270.5882	241.2353
9	0.5279	104.0056 / 2.8352	164.4394	167.9091
10	0.4661	222.6755 / 2.3462	92.98485	168.0952
11	0.3269	92.2234 / 1.7521	218.3594	199.1964
12	0.8126	187.5522 / 4.1011	208.4394	191.3077
13	0.5291	161.3927 / 2.7131	297.8939	179.0769
AVERAGE	0.5637	155.0799 / 2.8897	173.8411	186.8643
<i>SD</i>	<i>0.1563</i>	<i>53.7723 / 0.8041</i>	<i>85.3355</i>	<i>25.8469</i>

Table 23 Experimental results for the MEF 3T3 cells migrating on PLA substrate casted from acetone.

Tracked movement of cells grown on PLA casted from acetone is presented o figure below:

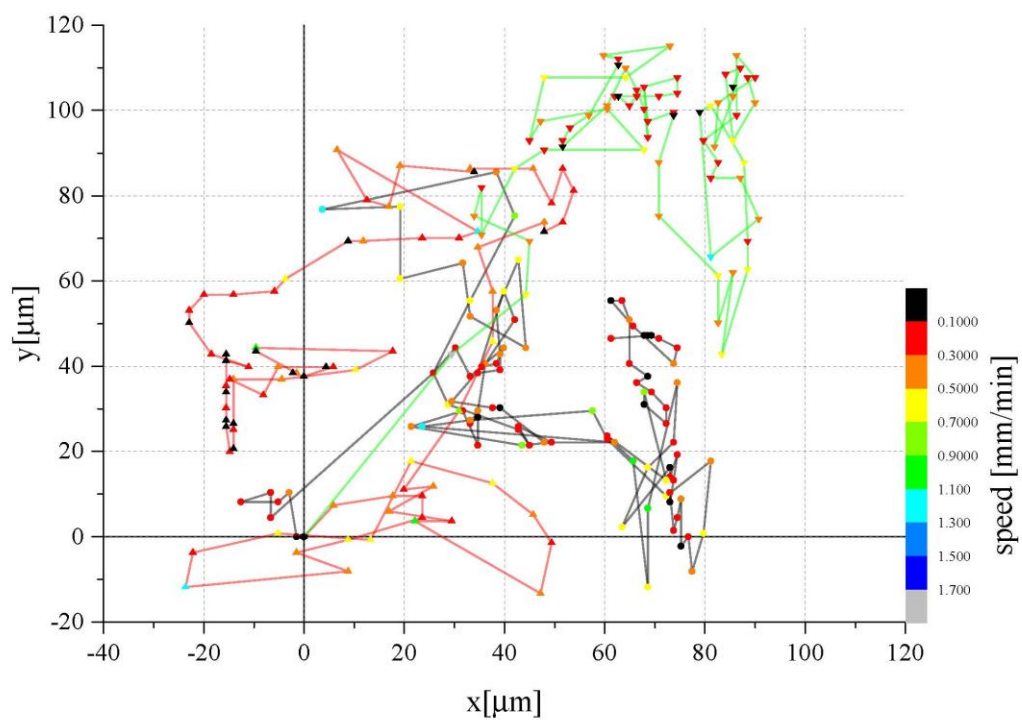


Fig. 72 Movement paths of selected fibroblast cells on PLA_{Ac} substrate.

Analogous analysis was carried out for substrates casted from other solvents (chloroform and ethyl acetate). The results are shown below:

2) PLA_{Chl}

Parameter	<i>Average speed [$\mu\text{m}/\text{min}$]</i>	<i>Total displacement [μm] / Avg. step/frame</i>	<i>Angle from origin [$^\circ$]</i>	<i>Avg. angle change/step [$^\circ$]</i>
<i>Cell number</i>				
1	0.4221	69.2876 / 2.2301	197.9685	189.9276
2	0.3819	57.9694 / 1.8995	259.1071	175.4479
3	0.4538	98.5089 / 2.3012	133.758	161.8605
4	0.5501	117.1654 / 2.8065	187.1333	212.6429
5	0.4287	53.8335 / 2.1331	275.8125	209.3293
6	0.5074	79.4837 / 2.5621	133.5606	189.6122
7	0.3271	84.4575 / 1.7091	84.64286	142.7742
8	0.1983	52.2522 / 1.0012	73.7439	152.6711
9	0.3048	70.7932 / 1.5309	56.11458	185.0109
10	0.3533	82.7558 / 1.8014	95.61458	172.9195
11	0.4367	155.4537 / 2.2193	99.04167	166.6543
12	0.4658	62.0590 / 2.3341	193.275	161.6333
13	0.3599	157.6135 / 1.8105	114.2536	137.4242
14	0.4286	143.4691 / 2.1472	53.45833	176.9529
15	0.5074	179.2195 / 2.5409	69.66879	170.5987
16	0.5112	188.0380 / 2.6552	92.98485	168.0952
AVERAGE	0.4148	103.2725 / 2.1051	132.5086	173.3472
<i>SD</i>	<i>0.0911</i>	<i>46.7684 / 0.4649</i>	<i>70.0867</i>	<i>20.8706</i>

Table 24 Experimental data for cells migrating on PLA_{Chl} substrate.

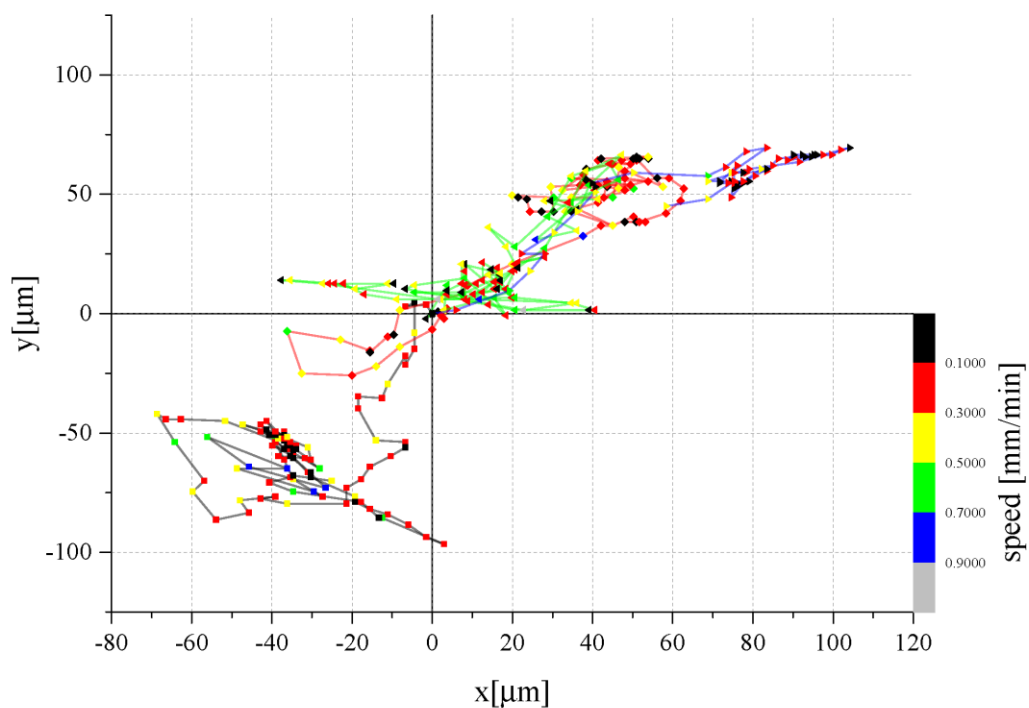


Fig. 73 Migration tracks of selected MEF 3T3 cells grown on PLA substrate casted from chloroform.

3) PLA_{EtAc}

Parameter	Average speed [μm/min]	Total displacement [μm] / Avg. step/frame	Angle from origin [°]	Avg. angle change/step [°]
Cell number				
1	0.36157	70.8825 / 1.8245	307.3231	194.7937
2	0.6984	335.6545 / 3.5012	188	179.6401
3	0.3856	166.5004 / 1.9352	233.1042	209.7263
4	0.3904	70.7403 / 1.9632	81.6354	146.3412
5	0.4585	111.7893 / 2.3314	198.2083	185.9674
6	0.4189	65.6821 / 2.1065	99.8021	182.4271
7	0.3187	56.4847 / 1.6012	77.2187	161.1954
8	0.4552	124.5734 / 2.3061	56.1146	185.0109
9	0.3639	102.7968 / 1.8205	95.6146	172.9195
10	0.4221	133.7593 / 2.1962	99.0417	166.6543
11	0.3819	74.0541 / 1.9351	91.3334	182.1311
12	0.4538	85.6398 / 2.3098	95.1591	137.3902
AVERAGE	0.4257	116.5464 / 2.1525	135.2129	175.3497
SD	0.0959	76.4049 / 0.4819	77.5242	20.1012

Table 25 Data collected for a group of cells migrating on PLA substrate casted from ethyl acetate solution.

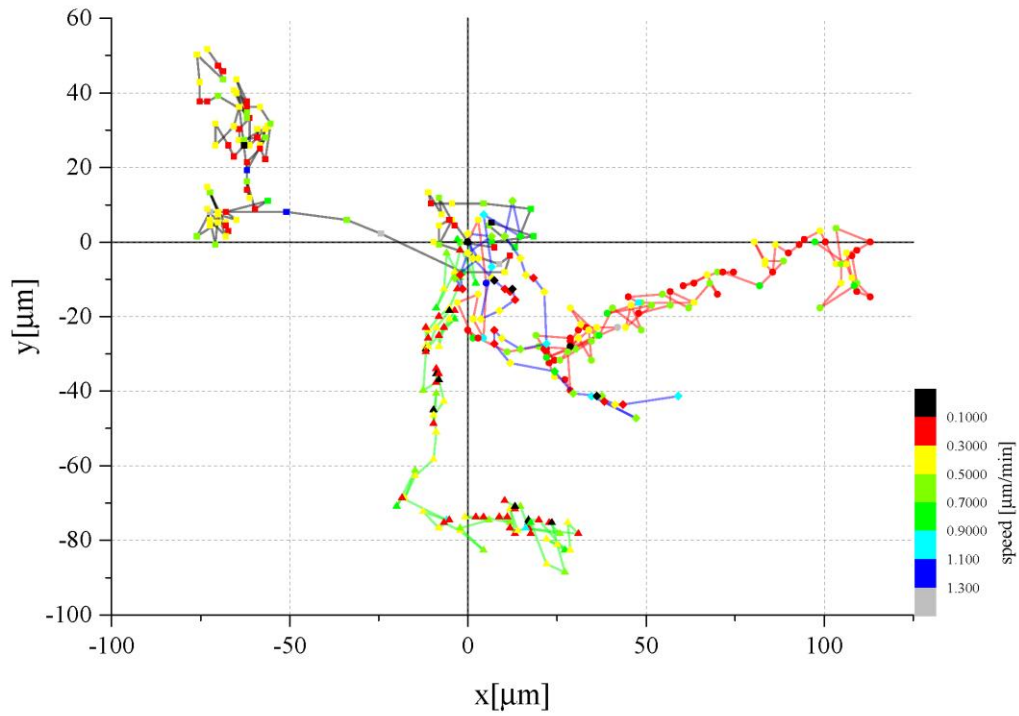


Fig. 74 Migration paths for MEF 3T3 cells migrating on PLA_{EtAc} substrate.

The migration speed on all PLA substrates is significantly lower than in case of glass. No directionality in cell movement was observed, which is confirmed by numerical data contained in the tables (Table 23 Table 25). Comparatively high SD value in total cell displacement shows that single cells present distinct migration strategies. The momentary angle change value around 180° indicates a strong local exploration of the material by migrating cells. Both the migration speed and total displacement value are distinctly lower than for glass substrate.

d. Cells migration on PHO substrates

On substrates made from PHO, a total of 51 cells were tracked and analyzed, 16 for acetone, 18 for chloroform and 17 for ethyl acetate. The data collection and analysis procedures were identical to other samples. A collective data for polymers casted from different solvents is presented:

1) PHO_{Ac}

Parameter	<i>Average speed [$\mu\text{m}/\text{min}$]</i>	<i>Total displacement [μm] / Avg. step/frame</i>	<i>Angle from origin [$^\circ$]</i>	<i>Avg. angle change/step [$^\circ$]</i>
<i>Cell number</i>				
1	0.38233	67.6589 / 1.8351	123.7083	164.5957
2	0.2437	60.0008 / 1.1063	120.8854	150.4894
3	0.3892	314.8078 / 1.7662	283.4896	177.2584
4	0.3725	154.8358 / 1.8821	162.2604	188.4375
5	0.4145	318.7998 / 2.1761	161.4896	184.7021
6	0.3534	393.5312 / 1.7705	309.2188	192.0851
7	0.43677	26.6192 / 2.1612	232.5938	189.7073
8	0.4658	225.3608 / 2.4019	43.71875	177.5543
9	0.3599	392.1441 / 1.8166	111.4583	165.3333
10	0.2607	256.6225 / 1.3517	258.4375	183.9351
11	0.3128	88.8671 / 1.5709	305.8919	201.8235
12	0.3975	315.481 / 1.9971	211.4688	188.7471
13	0.3271	148.09 / 1.7422	218.0562	181.1316
14	0.1983	447.3583 / 1.0499	273.0213	207.2375
15	0.3048	178.6829 / 1.6091	270.7292	190.5333
16	0.1436	241.3181 / 0.7259	322.1316	247.2333
AVERAGE	0.3352	226.8861 / 1.6789	213.0351	186.9253
<i>SD</i>	<i>0.0878</i>	<i>130.3571 / 0.4501</i>	<i>83.5981</i>	<i>21.3631</i>

Table 26 Data for MEF 3T3 cells migrating on substrate casted from PHO casted from acetone.

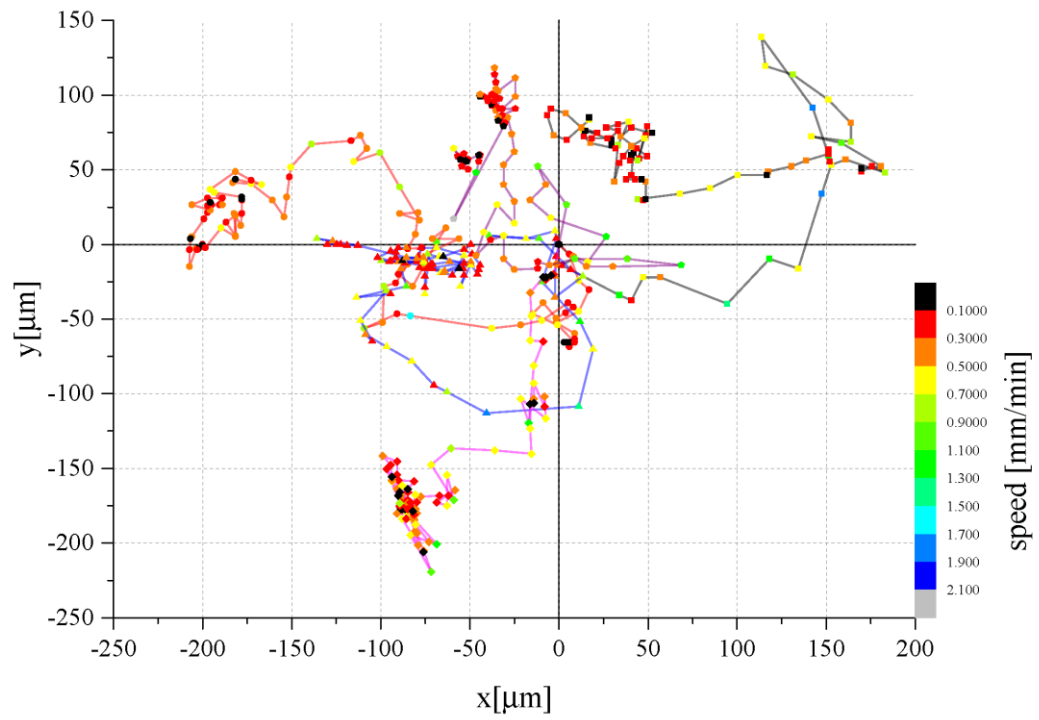


Fig. 75 Tracked migration paths of selected fibroblast cells on PHO_{Ac} substrate.

2) PHO_{Chl}

Parameter	<i>Average speed [μm/min]</i>	<i>Total displacement [μm] / Avg. step/frame</i>	<i>Angle from origin [°]</i>	<i>Avg. angle change/step [°]</i>
<i>Cell number</i>				
1	0.3383	187.1729 / 1.6588	194.1979	179.5341
2	0.3677	246.8806 / 1.9055	173.4583	178.4301
3	0.29355	264.4646 / 1.3995	203.1042	146.3667
4	0.5313	201.8211 / 2.7032	51.9375	197.0215
5	0.7537	326.4238 / 3.8112	183.125	162.6667
6	0.76336	324.1534 / 3.7996	146.7826	181.3125
7	0.2833	139.3746 / 1.4427	234.1458	172.8636
8	1.04159	247.7494 / 5.3162	187.1333	212.6429
9	0.4231	204.6603 / 2.2098	275.8125	209.3293
10	0.5659	274.5013 / 2.9321	156.6667	217.9247
11	0.5547	110.3279 / 2.5984	206.3056	202.0857
12	0.4199	199.2463 / 2.1062	76.07292	172.6707
13	0.2536	144.6649 / 1.2705	141.4896	200.4943
14	0.2451	106.4998 / 1.2335	179.5	195.6163
15	0.3139	120.8 / 1.6312	133.5606	189.6122
16	0.4067	247.5041 / 2.1084	84.6428	142.7742
17	0.30334	295.1123 / 1.5204	73.7439	152.6711
18	0.5633	124.8529 / 2.9032	174.3553	186.6716
AVERAGE	0.4722	209.2339 / 2.3639	164.2461	185.0841
<i>SD</i>	<i>0.2217</i>	<i>73.1557 / 1.0835</i>	<i>58.4540</i>	<i>22.2272</i>

Table 27 Data for cells migrating on substrate casted from PHO casted from chloroform.

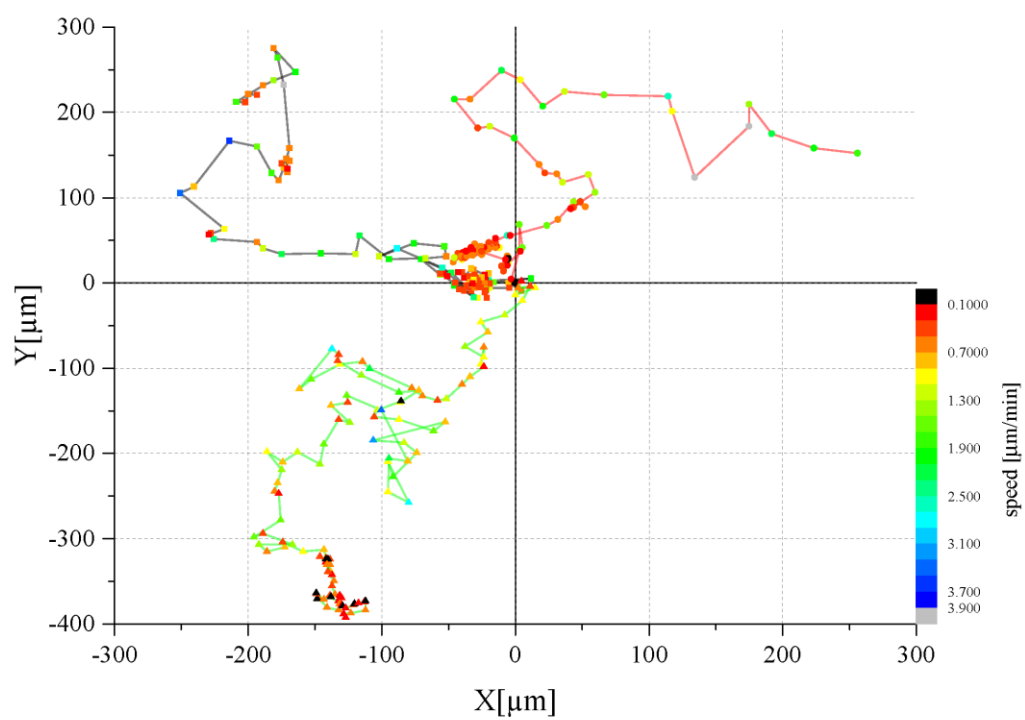


Fig. 76 Migration paths for MEF 3T3 cells cultured on PHO substrate casted from chloroform solution.

3) PHO_{EtAc}

Parameter	<i>Average speed [μm/min]</i>	<i>Total displacement [μm] / Avg. step/frame</i>	<i>Angle from origin [°]</i>	<i>Avg. angle change/step [°]</i>
<i>Cell number</i>				
1	0.3529	81.8148 / 1.6752	140.9459	189.0833
2	0.2583	203.0841 / 1.3105	53.4583	176.9529
3	0.4425	123.8873 / 2.2251	69.6688	170.5987
4	0.5573	61.7543 / 2.8013	190.2038	175.4444
5	0.6073	173.3985 / 3.0512	178.2866	189.9276
6	1.0150	177.9131 / 5.0995	194.1146	175.4479
7	0.2646	93.8305 / 1.3235	314.3115	161.8605
8	0.7031	62.8384 / 3.6158	197.9685	189.0714
9	0.8874	70.1216 / 4.4421	259.1071	201.8171
10	0.5501	157.5141 / 2.8751	133.758	171.7947
11	0.4287	58.3580 / 2.1553	160.3922	164.0601
12	0.5074	141.0275 / 2.7095	178.8535	179.9403
13	0.5112	140.9657 / 2.6554	311.664	178.5593
14	0.8157	82.9061 / 2.1097	115.8618	155.1161
15	0.7267	90.7529 / 3.6052	69.4615	230.1538
16	0.7455	70.1255 / 3.8124	219.9104	149.7021
17	0.7312	123.6561 / 3.5982	151.7447	174.3251
AVERAGE	0.5859	112.5852 / 2.8861	174.2479	178.7206
<i>SD</i>	<i>0.2177</i>	<i>46.3011 / 1.0553</i>	<i>78.2701</i>	<i>19.2482</i>

Table 28 Data collected for a group of cells migrating on PHO substrate casted from ethyl acetate.

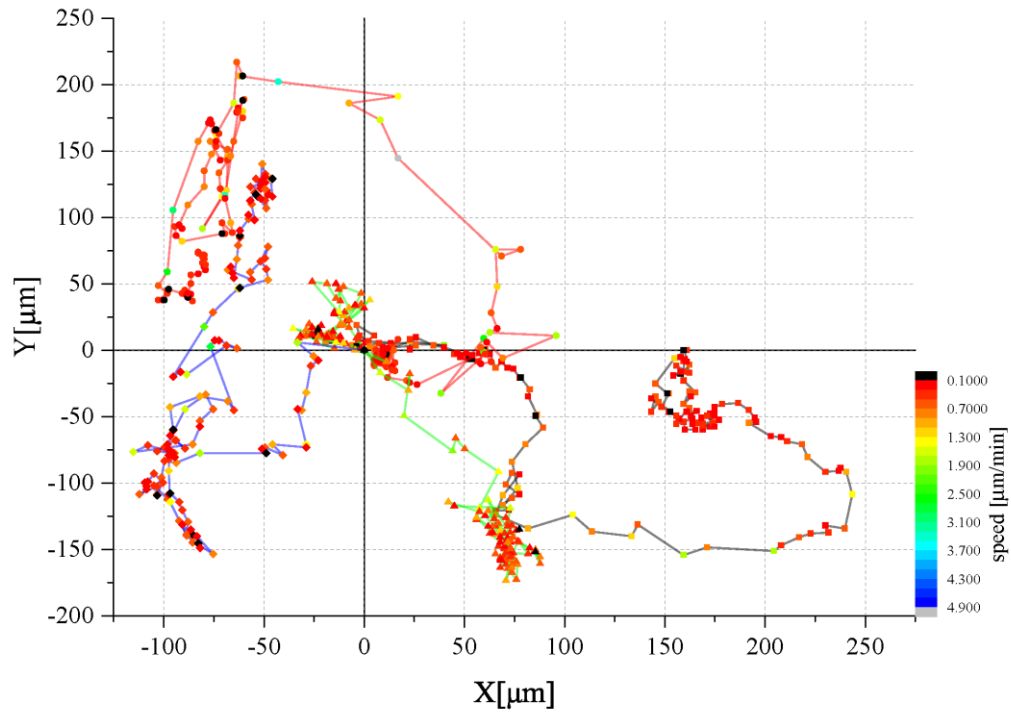


Fig. 77 Migration tracks of selected MEF 3T3 cells grown on PHO substrate dissolved in ethyl acetate.

Cells migrating on PHO biopolymer exhibited similar parameter to those grown on PLA substrate. Migration speed was visibly lower while compared to glass by 30%. High standard deviation value for “Angle of origin” parameters proves lack of directional migration, this effect is also visible in figures containing tracks of individual cells (Fig. 75, Fig. 77). A comparatively low SD value for the momentary movement angle change parameter indicates that most of cells on PHO polymer show a similar migration strategy. Momentary angle change value around 180° indicates a strong exploration of the surface of the material by the cells.

In order to be able to directly compare data for all tested materials, the obtained data were collected in diagrams below:

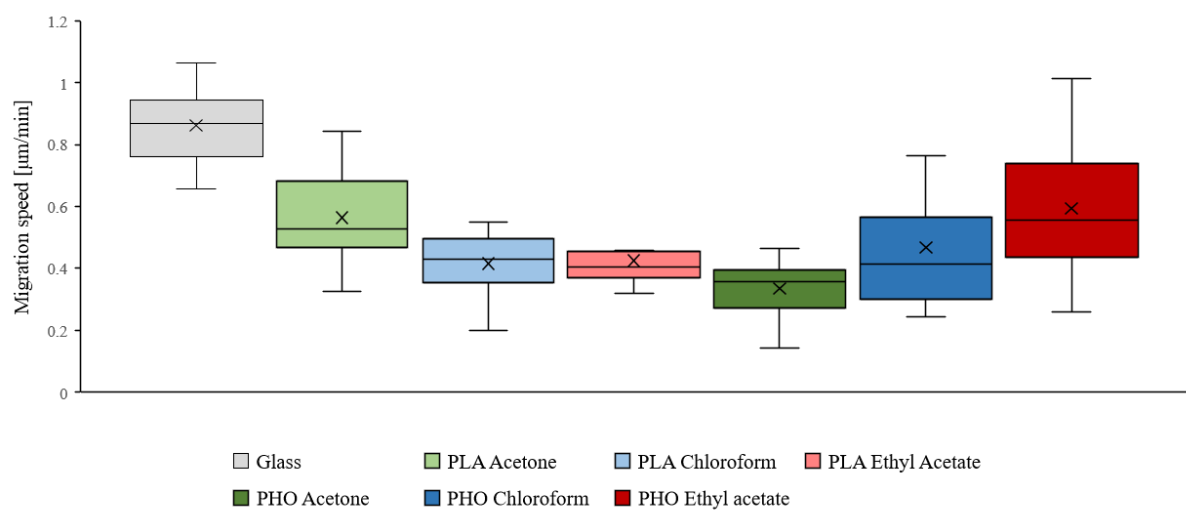


Fig. 78 Collective data showing average cell migration rates in individual experimental groups.

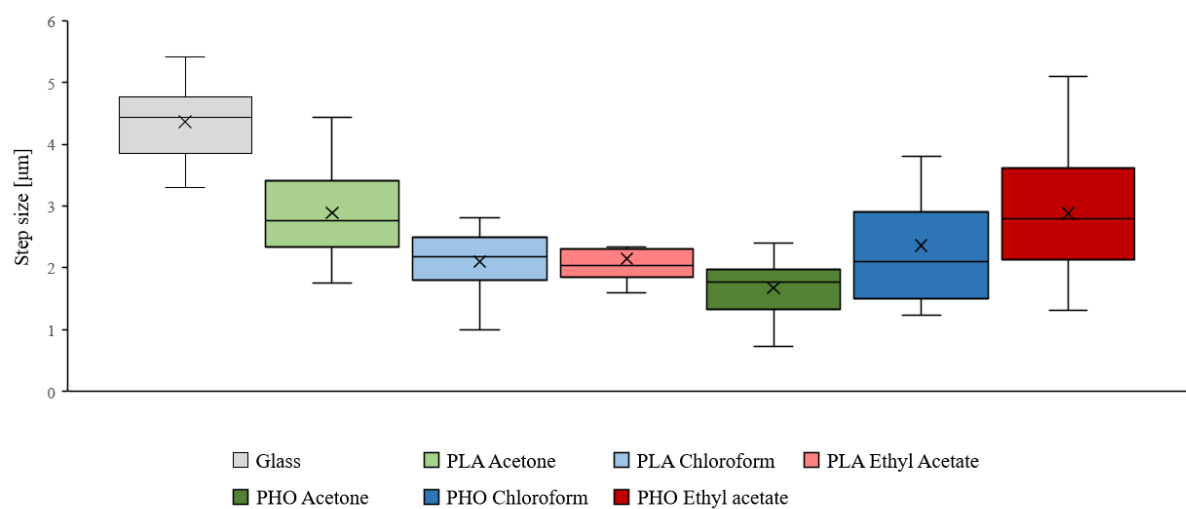


Fig. 79 Diagram presenting average cell step size for different materials.

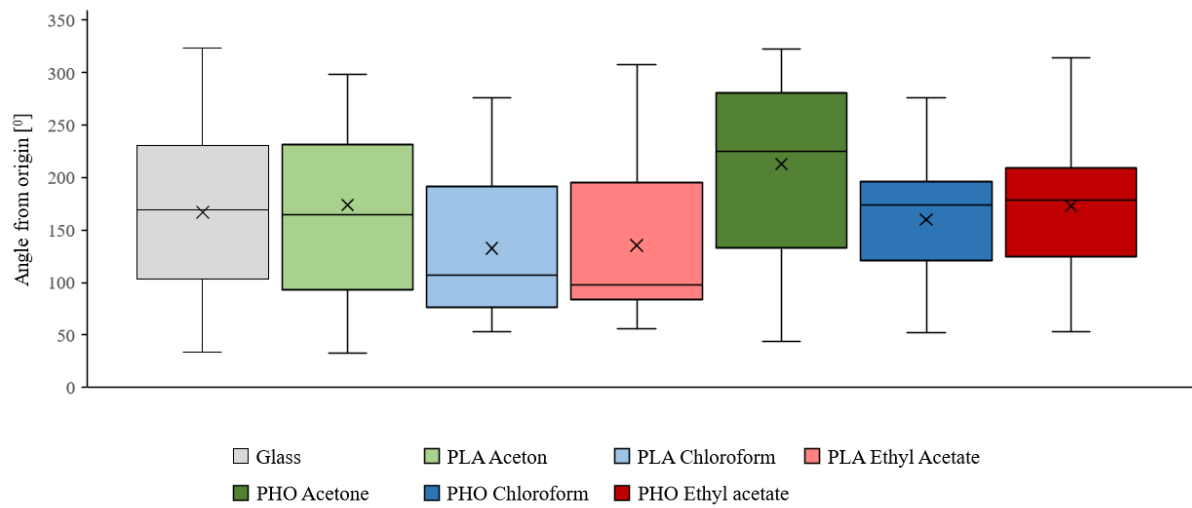
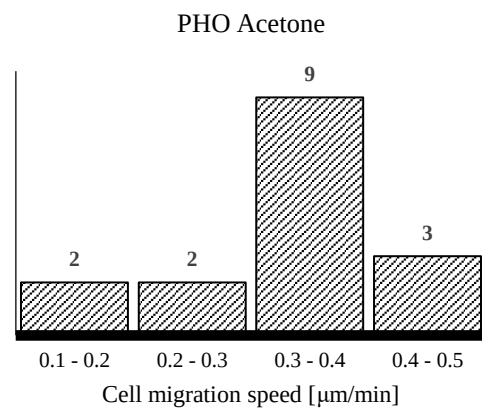
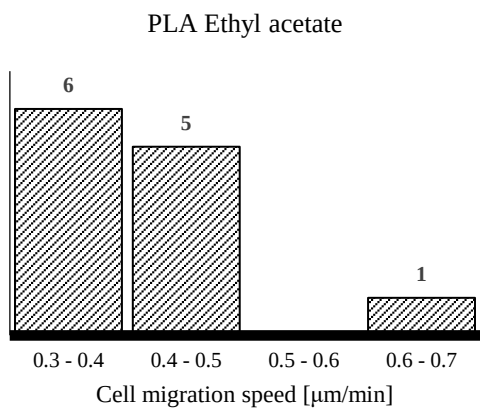
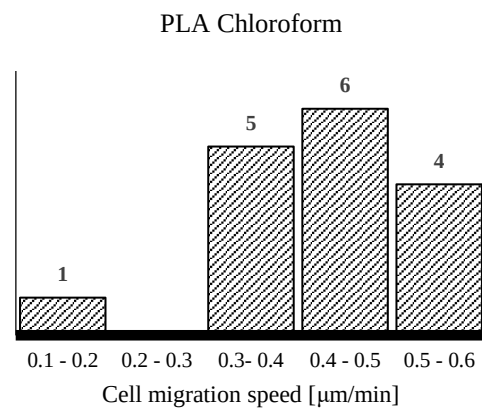
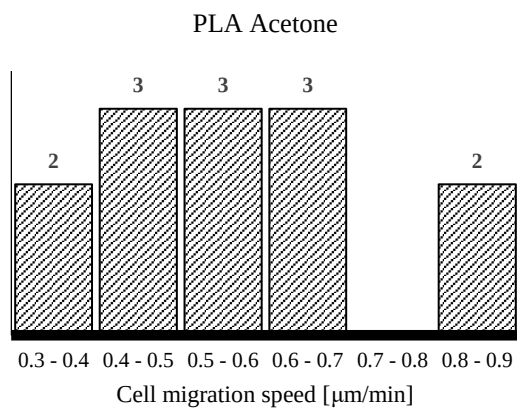
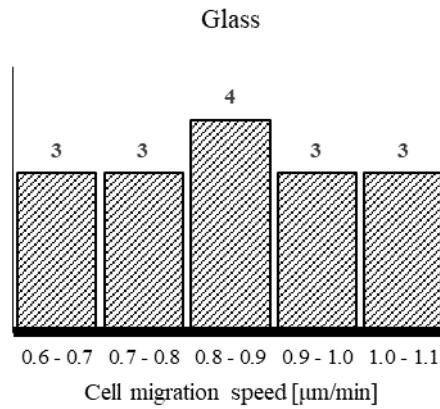


Fig. 80 Illustration of the angle that individual cells have reached from starting point at the end of the experiment. This type of observation is intended to show whether there is any directionality in the cell's movement.

Speed distribution for each experimental group has been presented on histograms to see if there was a dominant group of cells at a given migration speed on examined substrate or if the distribution was homogeneous.



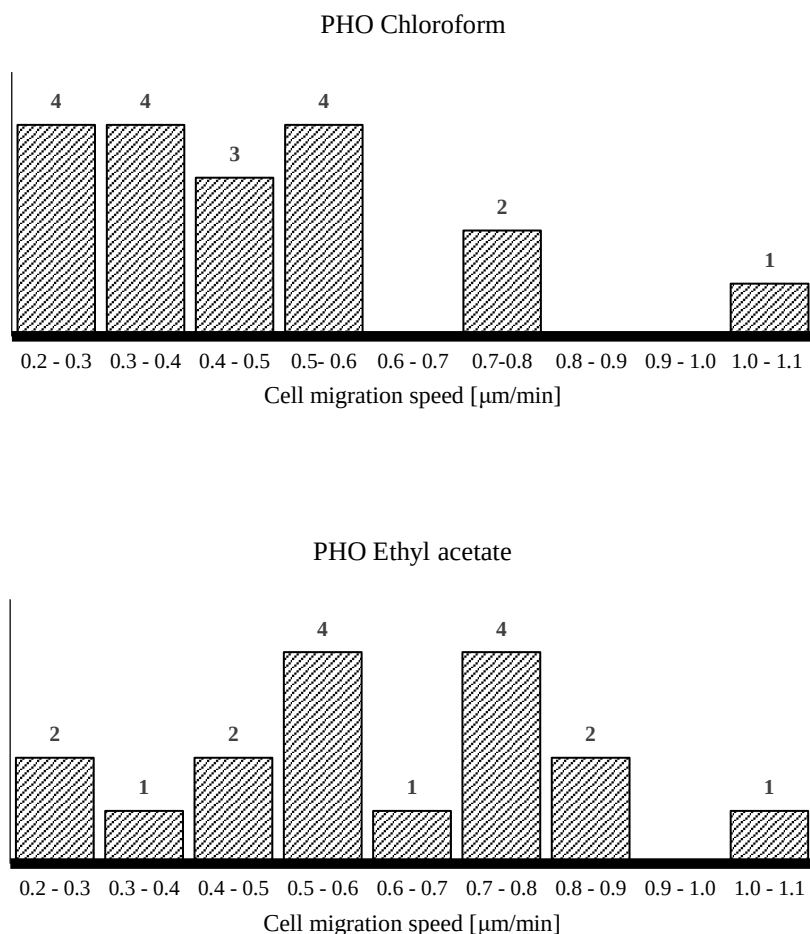


Fig. 81 Histogram plots presenting the speed distribution among cells grown on different substrates.

In case of glass substrate the speed distribution is almost ideally homogenous. For cells migrating on PLA materials the dominant group also cannot be indicated, for acetone the distribution is almost perfectly uniform, there are no cells moving at speeds between 0.7 - 0.8 $\mu\text{m}/\text{min}$. In case of PLA substrates dissolved in chloroform the majority of cells (94%) reaches speed in range 0.3-0.6 $\mu\text{m}/\text{min}$. For chloroform as solvent the picture is similar. In case of PHO substrates dissolved in acetone a clear dominance (56%) of cells reaching speeds in the range of 0.3 - 0.4 $\mu\text{m}/\text{min}$ can be noticed. For substrates casted from PHO dissolved in chloroform a homogenous distribution occurs in 0.2-0.6 $\mu\text{m}/\text{min}$ speed range, only single cell was moving at speeds exceeding 1 $\mu\text{m}/\text{min}$. In experimental group measured on PHO biopolymer dissolved in ethyl acetate cells migrating at velocities in ranges 0.5 – 0.6 $\mu\text{m}/\text{min}$ and 0.7 – 0.8 $\mu\text{m}/\text{min}$ stand out, in the remaining speed range distribution can be considered as homogenous. The speed distribution is in this case the widest. Minimal migration speed below 0.3 $\mu\text{m}/\text{min}$ and the highest exceeds 1 $\mu\text{m}/\text{min}$. The situation presented on histograms coincides with the results obtained from numerical data and exhibited on box

plots (Fig. 78). The average speed for most groups shows a real and reliable picture of experimental data and actual cell behavior.

7.2.2.4. Cytoskeletal research

In this chapter a comparison in cytoskeleton morphology in cells grown on different substrates has been made. The subject of analysis were two major cytoskeleton elements – actin and microtubular filament systems. The microscopic observations were performed on Zeiss Axio Observer Z.1 microscope with LSM 710 confocal module. The image collection, processing, deconvolution and analysis was performed using Zeiss ZEN Black version 8,1,0,484, PALMRobo version V 4.6.0.4 and FluoRender 2.21.0 software. Single cell measurements were made using an oil immersion objective with a magnification of 40x and a numerical aperture (NA) of 1.4. The field of view size and position was adjusted to cell density in the sample, so each cell could be scanned and analyzed separately. Appearance and morphology of the cytoskeleton components for cells cultured on glass and on two biopolymers (PLA and PHO) were compared.

a. Sample preparation

The cell line (MEF 3T3) and culturing parameters were identical as in previous experiments. To stain actin fibers a dye solution containing rhodamine-labeled phalloidin (Sigma Aldrich[®]) was applied. DAPI dye (Thermo Fisher Scientific) was used for nucleus staining. Because of subtle microtubular structures and therefore limited fluorescence a double raw staining was applied to visualize the microtubular network. Monoclonal Anti- α -Tubulin mouse antibody (Sigma Aldrich[®]) was used in combination with Goat anti-Mouse IgG secondary antibody with Alexa Fluor 488 fluorophore (Thermo Fisher Scientific). Light protected samples were left in the refrigerator (5°C) until the imaging was performed. For the staining completion light shielded samples were left in the refrigerator (5°C) until the imaging could be performed.

b. Actin cytoskeleton

Acquisition parameters used for actin cytoskeleton morphology analysis are presented below:

Parameter	Value	
Image size (px)	1024 x 1024	
Objective	Plan-Apochromat 40x/1.4 Oil DIC M27	
Number of fluorescence channels	2	
	Channel 1	Channel 2
Laser wavelength[nm]	405	488
Excitation wavelength [nm]	405	488
Emission wavelength [nm]	455	572
Detection range [nm]	410-500	500-643

Table 29 Measurement parameters for confocal imaging of MEF 3T3 cells for cytoskeleton analysis.

During the data analysis, the attention was focused on the arrangement of the fibers in the cell body as well as their distribution. The area occupied by actin fibers (Fig. 82) was calculated and compared between cells grown on different materials.

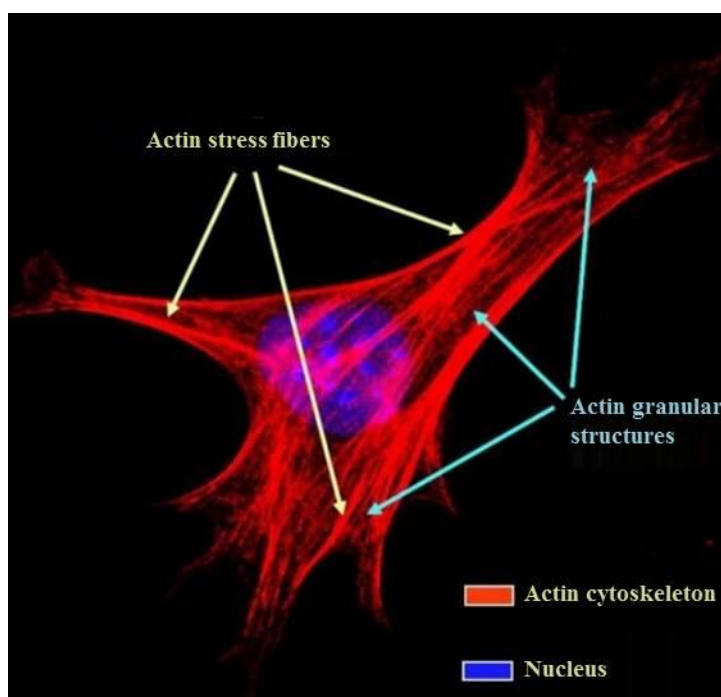


Fig. 82 . MEF cell after staining (cell nucleus- DAPI, actin cytoskeleton- rhodamine-labeled phalloidin) with marked stress fibers, actin monomers and short polymeric fragments.

Cells grown on glass substrate:

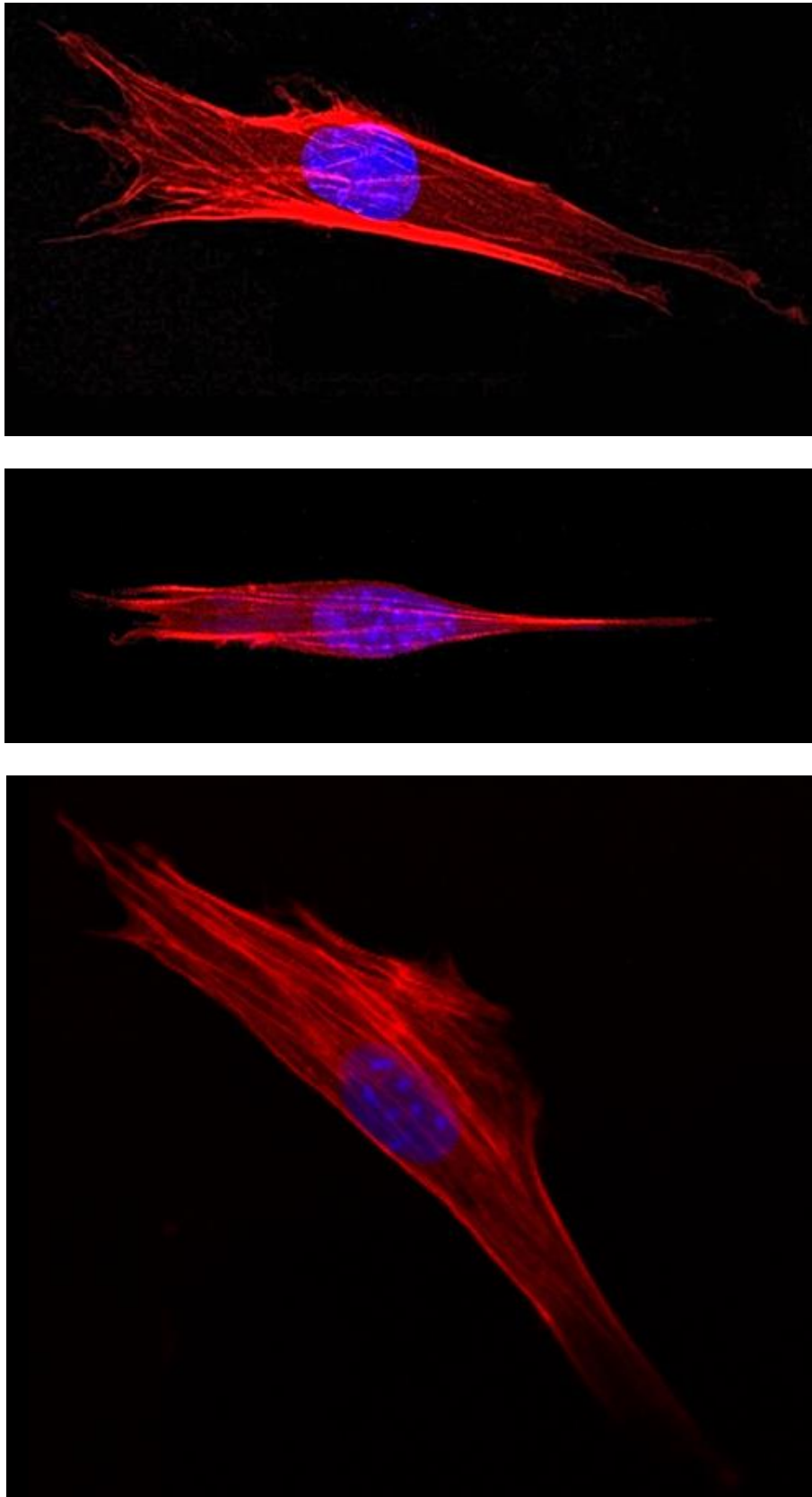


Fig. 83 Confocal imaging of MEF 3T3 cells grown on glass substrate.

Cells grown on PLA (acetone) substrate:

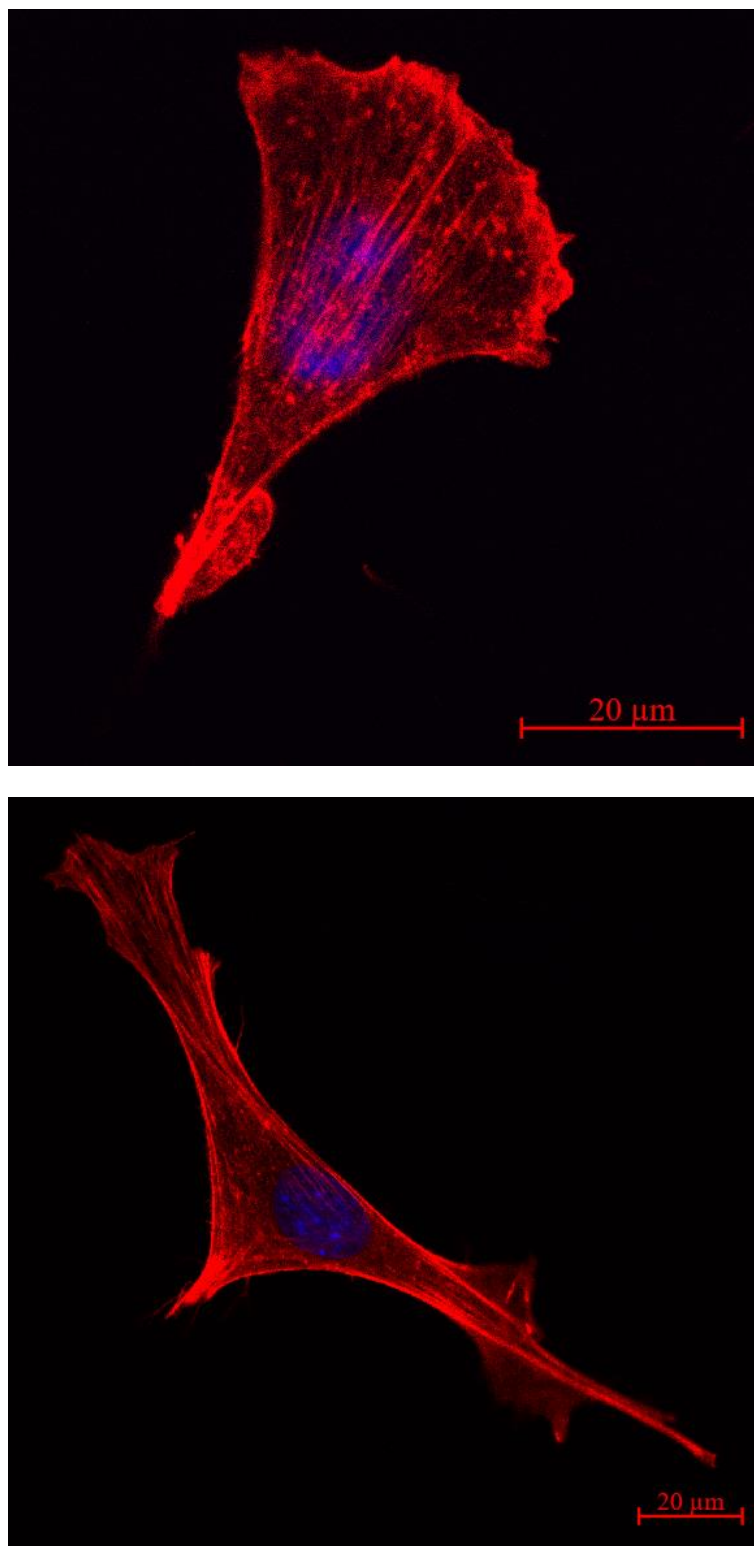


Fig. 84 Microscopic images of actin fibers in MEF 3T3 cells cultured on PLA substrate casted from acetone solution.

Cells grown on PLA (chloroform) substrate:

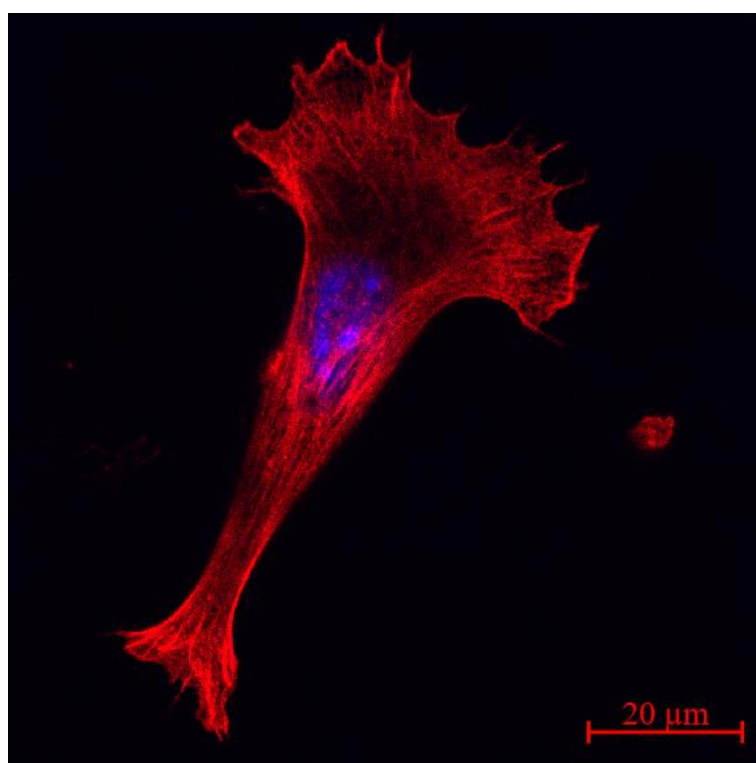
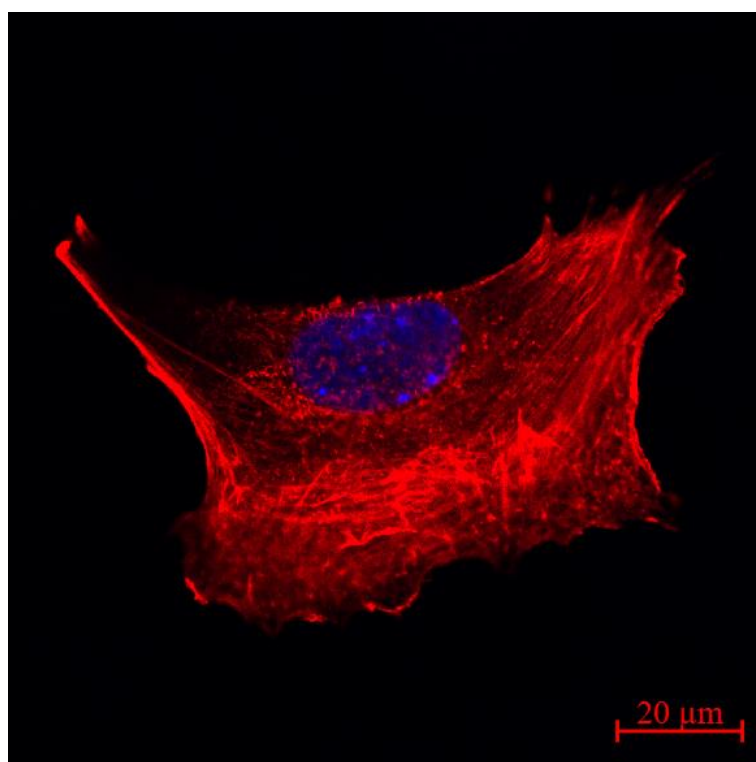


Fig. 85 Fluorescent microscopic images of stained actin cytoskeleton in mouse embryonic fibroblasts grown on substrate from PLA casted from chloroform.

Cells grown on PLA (ethyl acetate) substrate:

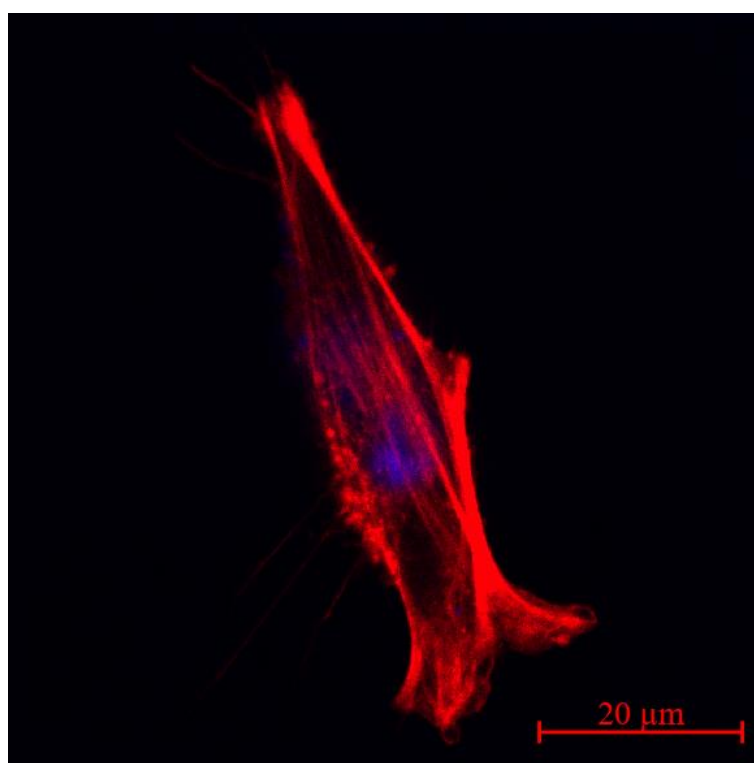
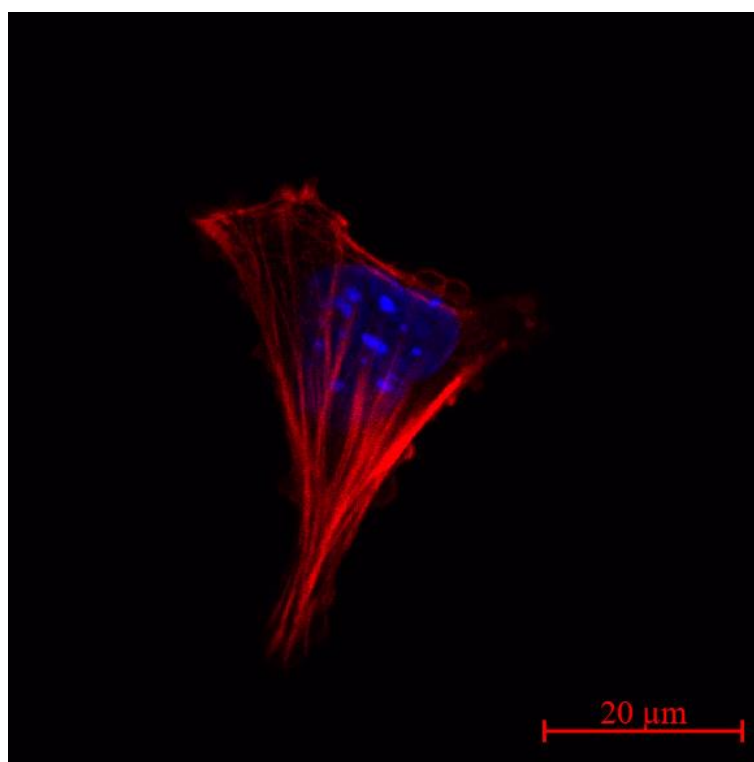


Fig. 86 Microscopic pictures presenting actin filament network in MEF cells grown on PLA_{EtAc} substrate.

Cells grown on PHO (acetone) substrate:

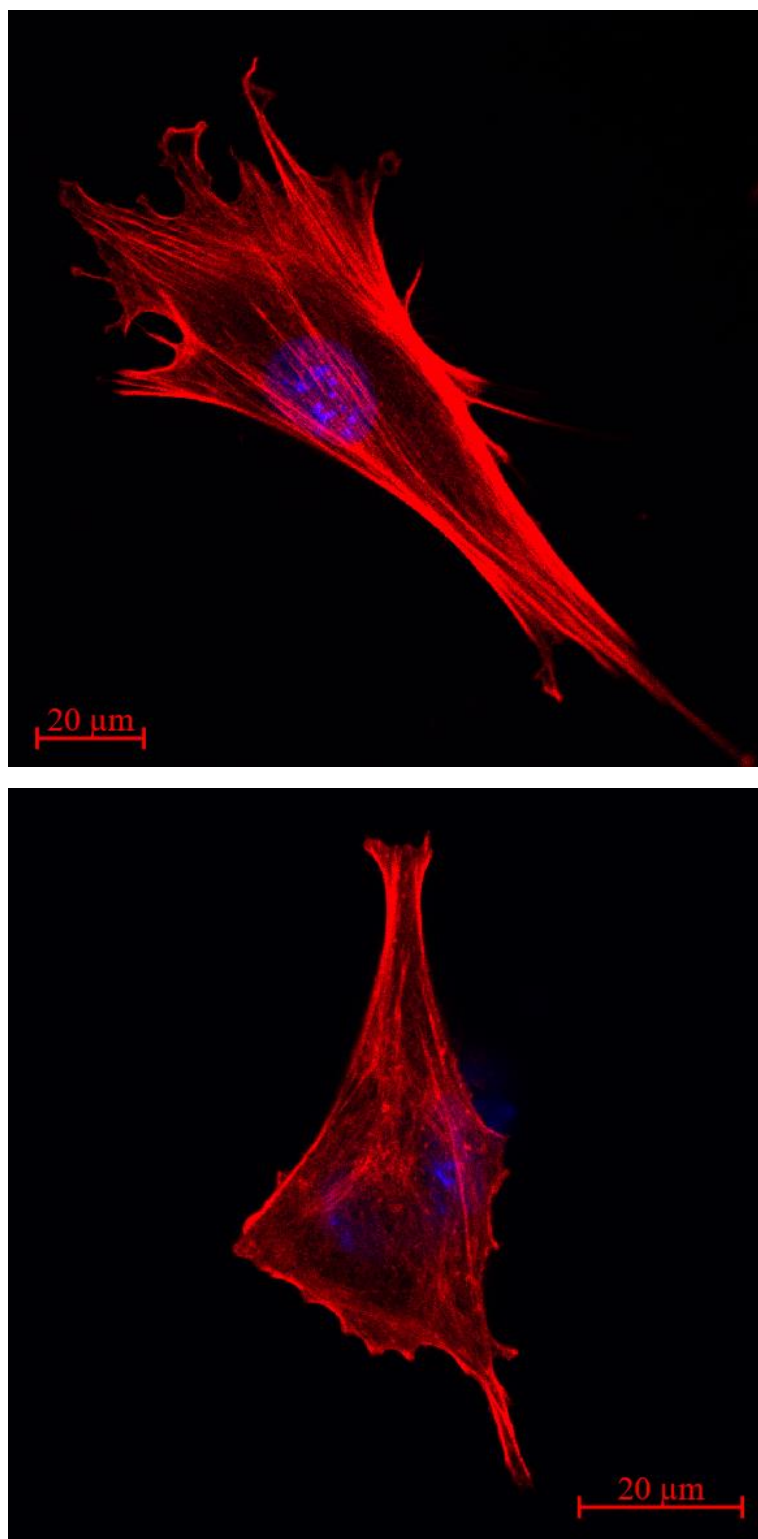


Fig. 87 Fluorescent images presenting actin filaments in MEF 3T3 cultured on PHO_{Ac} substrate.

Cells grown on PHO (chloroform) substrate:

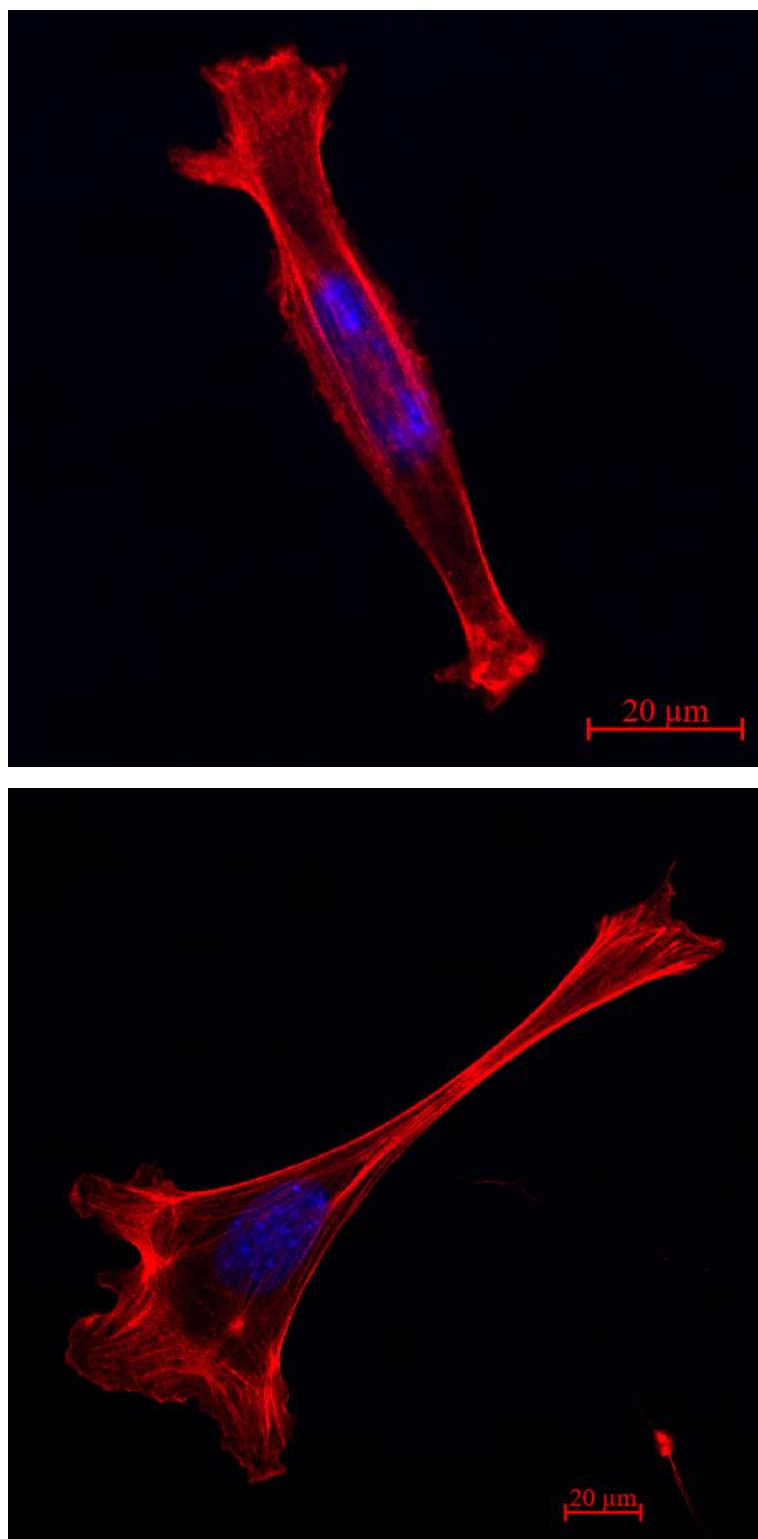


Fig. 88 Actin filaments in mouse embryonic fibroblast cells grown on PHO substrate casted from chloroform polymer solution.

Cells grown on PHO (ethyl acetate) substrate:

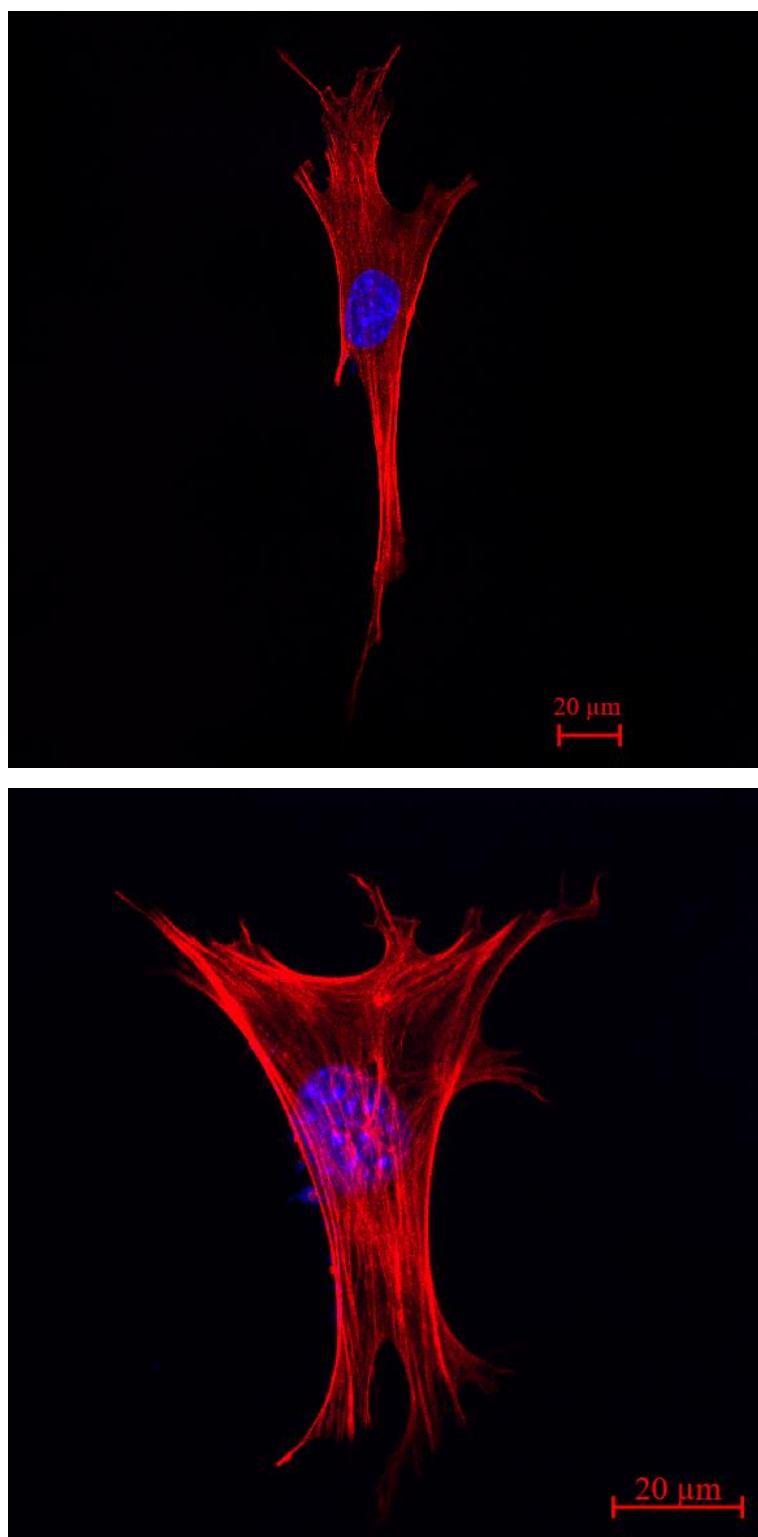


Fig. 89 Microscopic images of actin fibres in MEF cells cultured on PHO_{EtAc} substrate.

Actin cytoskeleton analysis

The cytoskeleton analysis was conducted using ImageJ (Fiji) ver. 1.51h software for both entire cell and with distinction into individual regions.

In “whole cell” approach presence of unpolymerized actin monomers makes it very difficult to adjust contrast, brightness and threshold lever for the entire region. Below the procedure for calculating the area in the entire cell occupied by the actin cytoskeleton is presented:

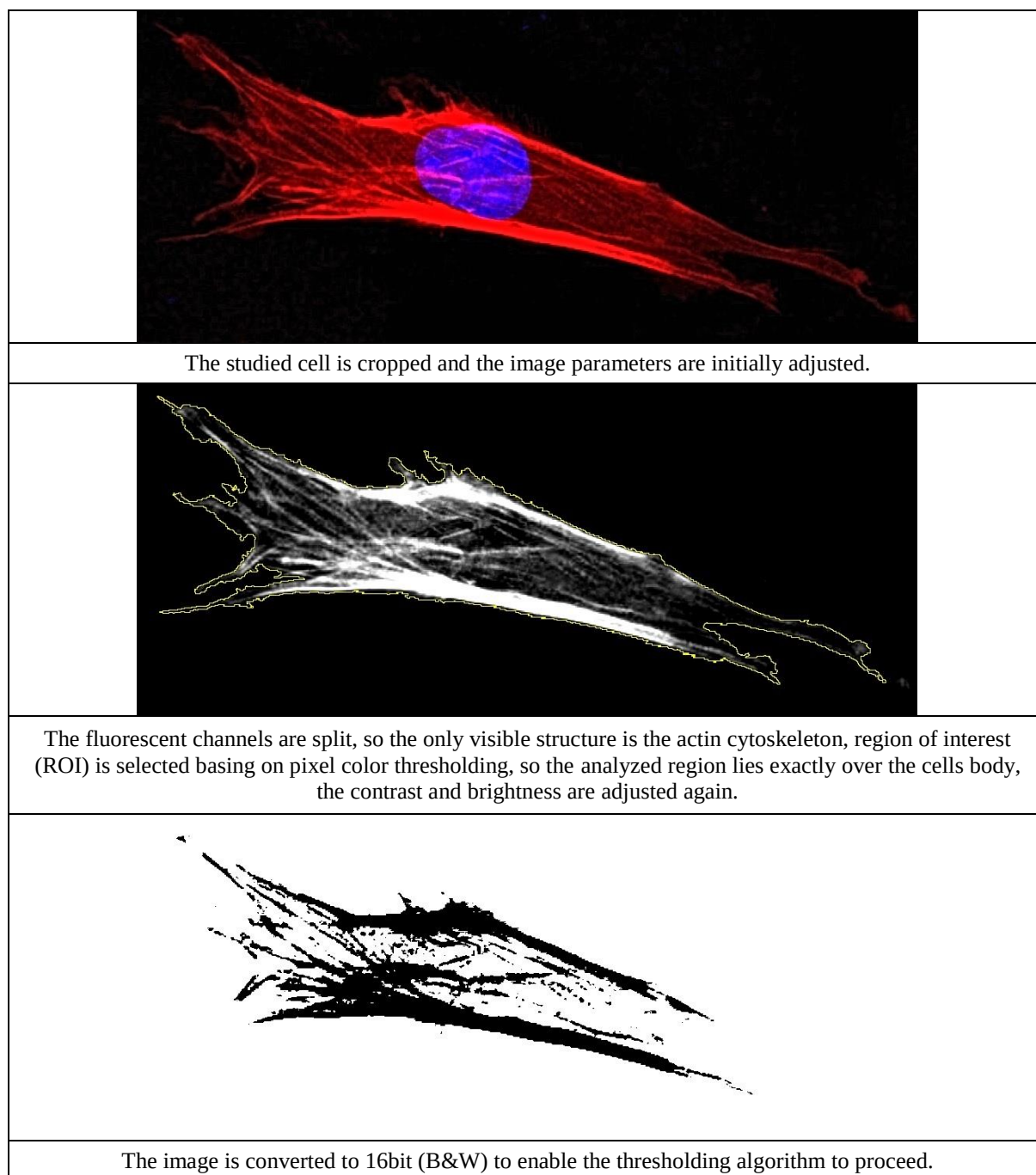


Fig. 90 Quantative analysis of actin cytoskeleton for the entire cell

Similar procedure was conducted for different cell regions. A region $10\mu\text{m} \times 10\mu\text{m}$ was selected on each microscopic image, enlarged, processed and analyzed. To study the distribution of actin filaments throughout the cell, a region of identical dimensions was selected for each one near the edge, in the middle of the cell body, and over the nucleus. The process of selecting region location on a cell and further image analysis is shown below.

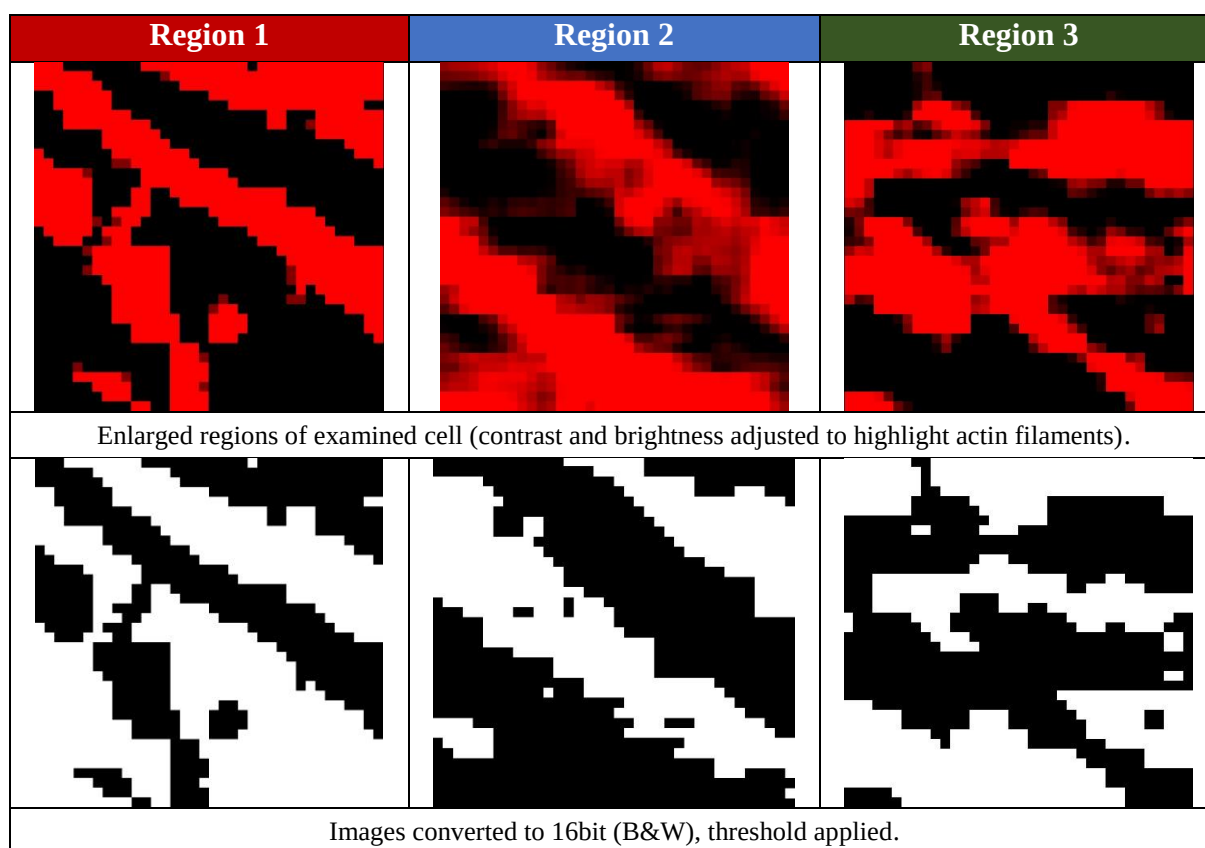
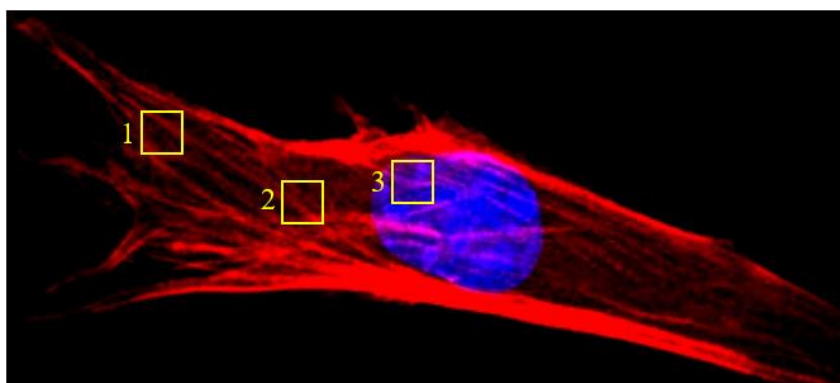


Fig. 91 Illustration of the subsequent stages of the actin cytoskeleton quantitative analysis basing on its spatial organization in the cell.

The results obtained for the studied cell groups are presented below, the percentage value of area occupied by actin fibers in “whole cell” approach is compared in Table 30 :

Substrate	Glass	Polylactic acid			Polyhydroxyoctanoate		
Solvent Cell no.		Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate
1	48.45	33.45	34.41	33.78	38.96	39.92	39.65
2	44.63	35.77	36.76	34.14	39.56	38.62	33.62
3	55.32	39.67	38.98	38.09	40.25	37.12	39.82
4	48.96	45.16	40.09	29.65	33.85	36.22	42.12
5	42.36	33.15	33.14	33.65	36.98	33.54	44.52
6	56.98	39.03	39.14	39.01	33.12	30.98	36.98
7	60.23	36.18	33.01	44.44	31.03	36.62	34.56
8	42.32	33.09	35.16	41.09	33.85	40.12	39.22
9	52.69	31.12	33.45	29.06	39.65	29.85	38.65
10	41.09	39.02	40.06	34.15	40.58	29.15	37.25
11	61.02	40.01	41.21	33.23	42.59	36.95	32.65
12	55.63	44.15	29.01	38.89	33.26	37.15	39.85
13	52.02	35.62	35.22	37.06	31.06	39.25	34.75
14	44.23	34.02	37.89	32.15	29.65	40.33	39.12
15	40.15	40.01	33.19	30.09	31.97	46.23	35.42
16	44.98	44.16	30.09	31.13	36.15	42.59	39.26
17	47.63	35.19	28.93	33.86	39.96	33.11	36.62
18	49.85	38.01	31.09	34.76	40.02	28.35	37.85
19	50.06	37.76	32.15	35.98	44.16	26.52	39.05
20	52.32	39.38	31.78	40.09	29.82	33.95	37.36
AVERAGE	49.55	37.70	34.74	35.22	36.32	35.83	37.92
SD	6.23	3.93	3.82	4.09	4.49	5.11	2.86

Table 30 Percentage area occupied by actin filaments in relation to the entire cell.

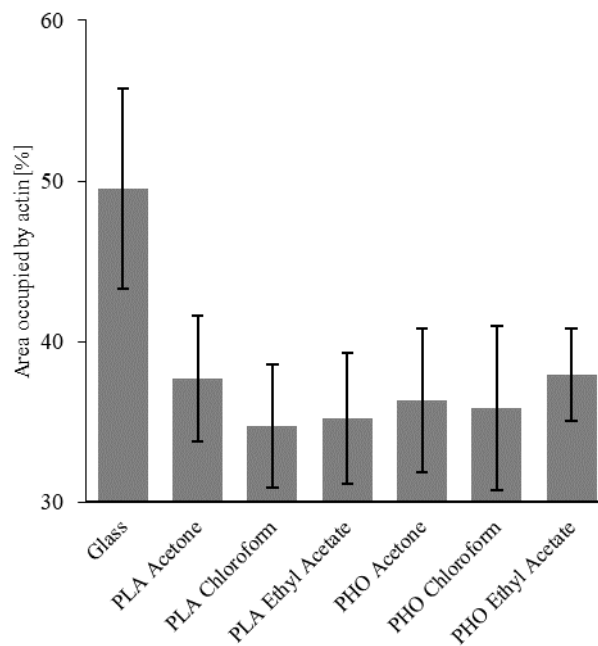


Fig. 92 Comparison of the area occupied by actin in the whole cell with division into different types of substrates.

<i>Substrate</i>		<i>Glass</i>		
Cell no.	<i>Region</i>	1 - lamellipodium	2 - cell body	3 - nucleus
1		40.74	61.47	51.91
2		43.56	66.72	55.12
3		39.54	59.21	49.76
4		44.76	45.12	44.12
5		55.23	56.23	51.09
6		41.72	71.09	61.01
7		44.11	62.31	59.12
8		42.09	67.83	43.56
9		45.74	60.09	50.92
10		40.07	61.21	52.31
11		39.54	66.19	55.02
12		42.12	59.13	49.08
13		52.32	57.94	46.12
14		55.13	60.01	50.19
15		42.33	59.88	51.42
16		44.64	55.32	52.39
17		51.09	50.87	54.02
18		52.45	60.12	55.13
19		43.23	66.28	53.09
20		34.51	60.04	54.14
AVERAGE		44.75	60.35	51.98
SD		5.65	5.89	4.34

Table 31 Collective data containing area [%] occupied by actin cytoskeleton in different regions for MEF 3T3 cells grown on glass substrate.

Substrate		Polylactic acid							
Solvent	Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate
Region Cell no.	1 - lamellipodium			2 - cell body			3 - nucleus		
1	29.54	25.51	29.02	31.28	34.12	35.56	46.23	46.51	47.12
2	30.88	29.98	33.12	32.42	37.98	35.09	44.23	45.18	44.14
3	22.92	31.09	35.23	33.76	33.12	38.92	45.12	50.02	43.09
4	35.12	34.62	29.76	39.76	30.09	40.23	34.91	55.12	50.09
5	40.03	33.13	41.09	23.12	32.12	29.01	49.09	34.13	45.13
6	29.71	39.61	34.13	41.09	33.61	33.01	51.13	45.09	45.24
7	28.12	24.12	33.19	28.98	37.13	42.19	44.02	50.02	49.09
8	38.12	29.81	21.09	36.77	40.05	23.12	45.13	46.09	47.11
9	35.25	31.09	27.15	32.09	41.31	29.98	44.85	49.19	39.02
10	33.19	25.13	29.09	41.09	40.02	35.16	42.02	50.09	43.09
11	30.09	27.82	29.23	32.19	39.95	37.09	44.78	51.11	45.98
12	21.03	26.55	30.09	29.01	35.61	38.24	50.09	45.19	51.02
13	29.34	32.41	33.65	27.12	33.09	33.09	43.12	39.13	52.42
14	28.93	40.08	40.09	32.76	30.03	31.39	32.43	44.16	59.01
15	27.12	38.09	32.91	36.89	29.15	40.23	44.61	42.38	45.12
16	33.11	26.78	20.04	35.13	35.12	36.09	40.02	44.82	45.19
17	30.19	30.07	32.13	33.88	35.59	35.19	38.95	41.09	38.13
18	34.07	32.16	29.09	35.09	36.63	33.52	50.12	44.18	37.92
19	28.01	33.08	22.13	39.01	35.12	40.01	39.97	47.09	42.09
20	25.34	29.82	28.03	33.12	39.02	39.82	44.23	48.36	45.03
AVERAGE	30.51	31.05	30.51	33.73	35.44	35.35	43.75	45.95	45.75
SD	4.72	4.57	5.46	4.65	3.59	4.64	4.80	4.65	5.02

Table 32 Quantative data describing area occupied by actin fibers for MEF cells grown on PLA.

Substrate	Polyhydroxyoctanoate								
Solvent	Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate
Region Cell no.	1 - lamellipodium			2 - cell body			3 - nucleus		
1	37.52	39.25	39.12	30.16	36.12	30.21	49.84	50.02	55.13
2	34.13	33.65	39.03	29.12	30.05	33.48	52.02	55.32	50.96
3	21.09	37.02	37.12	33.54	29.22	40.06	49.52	49.32	49.52
4	39.12	40.02	33.62	39.05	22.06	29.33	47.06	49.12	41.12
5	37.15	36.29	35.01	44.12	40.03	31.06	55.32	56.02	55.21
6	40.09	29.23	40.12	29.21	39.06	33.65	49.25	55.23	52.09
7	34.12	33.05	29.95	29.25	33.11	39.02	47.32	51.21	50.01
8	33.12	30.52	31.29	30.25	39.01	33.82	44.02	39.25	49.85
9	35.34	39.23	33.54	36.28	36.52	39.05	49.65	50.52	44.12
10	39.09	36.02	36.21	39.01	37.82	29.93	55.32	47.28	43.21
11	38.13	40.02	39.15	40.03	34.19	26.65	57.02	46.62	53.08
12	40.09	40.02	29.98	28.92	36.05	34.59	45.03	49.12	50.09
13	29.67	33.21	36.98	41.05	31.03	36.16	43.01	50.12	52.16
14	39.45	32.02	32.16	50.01	30.02	37.45	47.98	55.16	50.87
15	36.29	33.16	39.92	29.64	33.94	39.25	49.95	47.92	48.71
16	37.02	38.12	42.23	33.25	40.31	33.06	52.02	46.25	44.95
17	34.12	39.54	26.12	30.09	39.21	23.12	55.14	44.33	49.65
18	32.61	40.06	49.85	37.25	34.02	32.57	51.65	42.09	50.32
19	24.02	40.01	39.05	30.19	29.12	33.48	44.25	49.32	51.26
20	40.09	29.85	36.32	33.08	22.13	34.63	47.82	49.02	55.12
AVERAGE	35.11	36.01	36.34	34.68	33.65	33.53	49.66	49.16	49.87
SD	5.19	3.84	5.24	5.97	5.39	4.38	4.03	4.34	3.90

Table 33 Percentage area occupied by actin cytoskeleton in MEF 3T3 cells cultured on PHO based substrates.

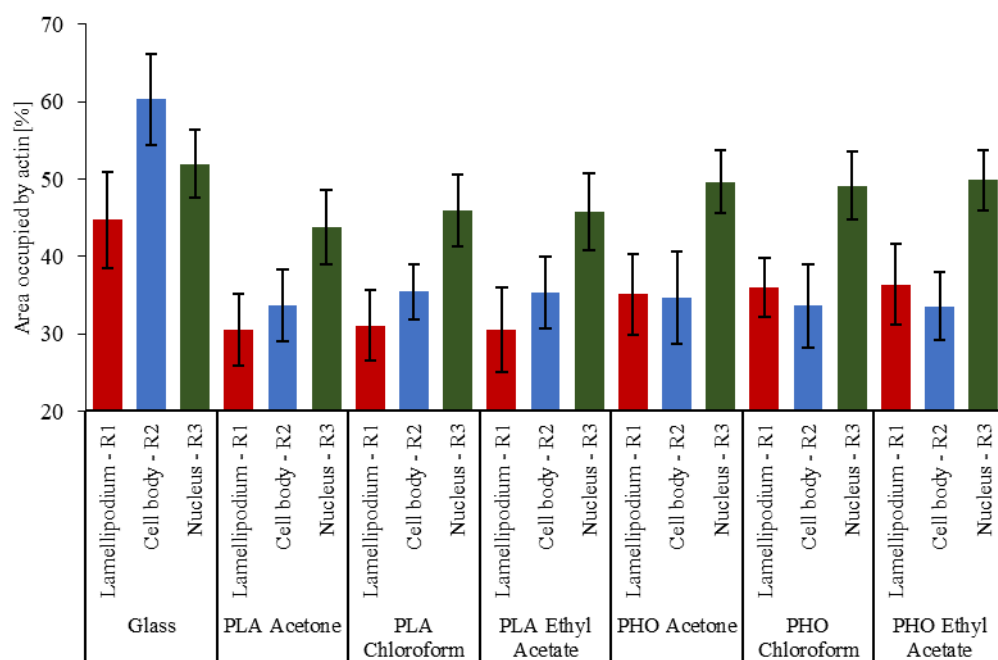


Fig. 93 Comparison of area occupied by actin divided into individual cell regions and substrates.

The obtained data indicates that both in “regional” and global approach cells grown on glass substratum own actin cytoskeleton which occupies more area. This trend is clearly visible in lamellipodium where cells cultured on both types of biopolymer exhibit thin but densely spaced actin bundles while in those grown on glass the filaments are thicker but rarely distributed (Fig. 83). Actin filaments in cells grown on both PLA and PHO often create a complex network with many bifurcations (Fig. 85, Fig. 88). In case of cells cultured on glass substrate a thick actin bundles running along the entire cell are present. There were no significant differences between the cell groups grown on media casted from different solvents. Both microscopic pictures themselves and the subsequent quantitative analysis indicate that the actin cytoskeleton architecture differs for cells grown on glass and on biopolymers. In regional analysis, the actin cytoskeleton for cells grown on PHO takes up slightly more space than analogically on PLA, but the differences are within the limits of the standard deviation for the results group, so they cannot be treated as statistically significant (Fig. 93). In the case of whole cell analysis, this differentiation disappears, however the dominance of cytoskeleton area for cells grown on glass remains prominent (Fig. 92). The considerable deviations in the results obtained from the analysis (Table 33) result from the fact that the studied cytoskeletal structures as well as the cells themselves are dynamic objects and their behavior and morphology depend on a number of different factors that are not included in the study^{206–209}. During the experiments, however, efforts were made to minimize the influence of peripheral factors through identical culturing conditions, passage and staining protocols, or microscopic observation parameters itself.

c. Microtubules

Microtubular cytoskeleton was observed in confocal technique with two fluorescent channels active. Acquisition parameters used for actin cytoskeleton morphology analysis are presented below:

Parameter	Value	
<i>Image size (px)</i>	1024 x 1024	
<i>Objective</i>	Plan-Apochromat 40x/1.4 Oil DIC M27	
<i>Number of fluorescence channels</i>	2	
	<i>Channel 1</i>	<i>Channel 2</i>
<i>Laser wavelength[nm]</i>	405	488
<i>Excitation wavelength [nm]</i>	405	488
<i>Emission wavelength [nm]</i>	455	563
<i>Detection range [nm]</i>	410-500	495-630

Cells grown on glass substrate:

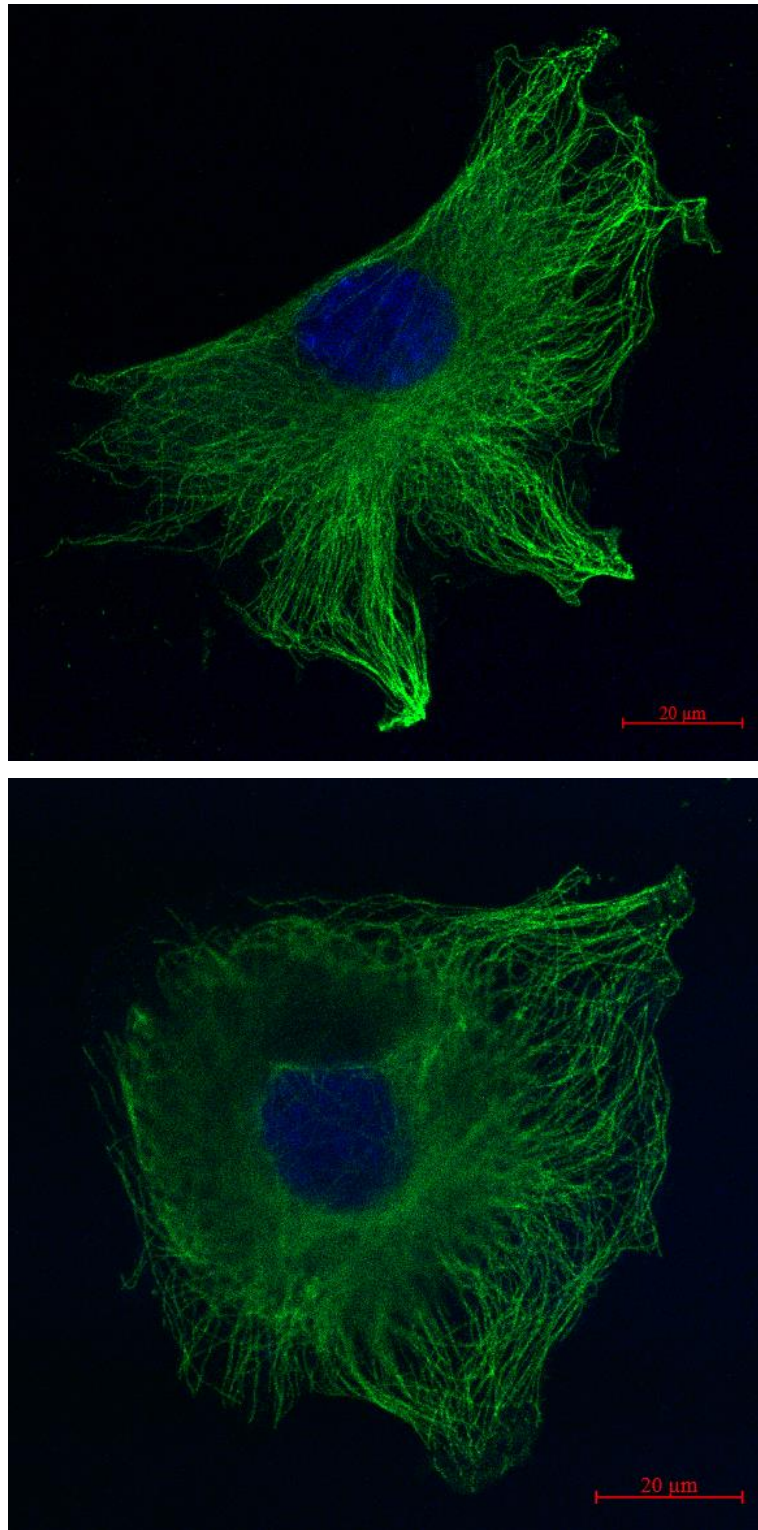
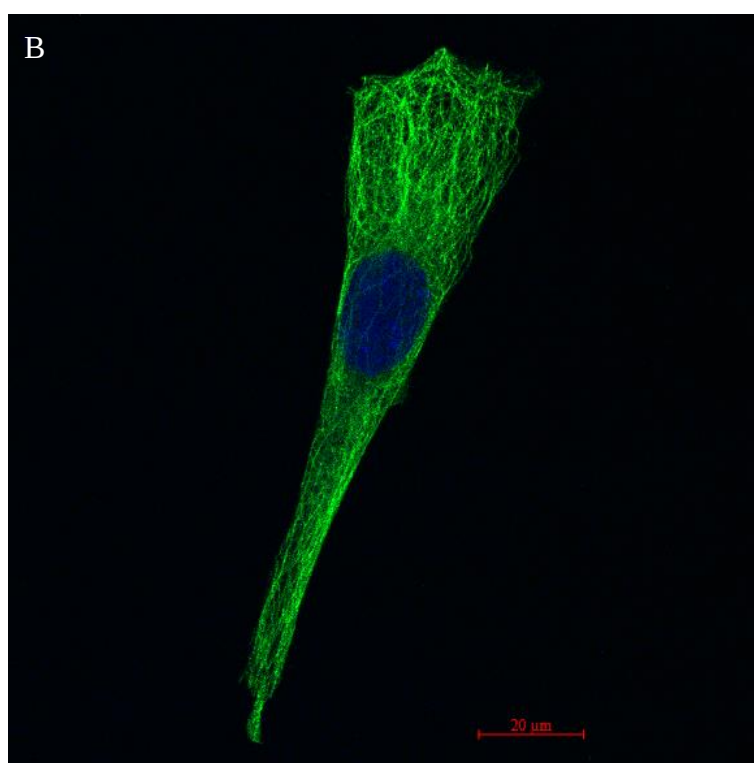
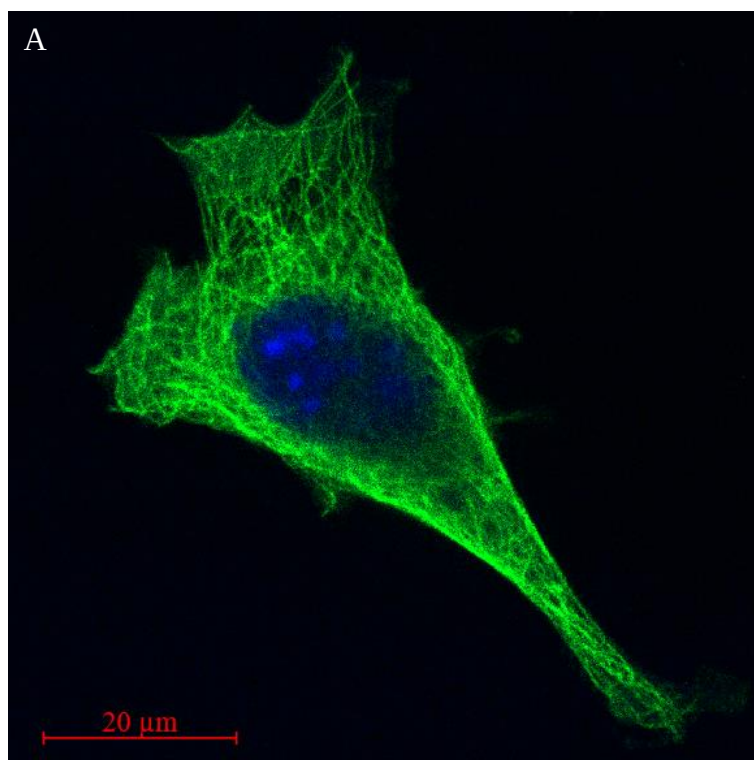


Fig. 94 Microtubular cytoskeleton in MEF 3T3 cells grown on glass substrate.

Cells grown on PLA substrates:



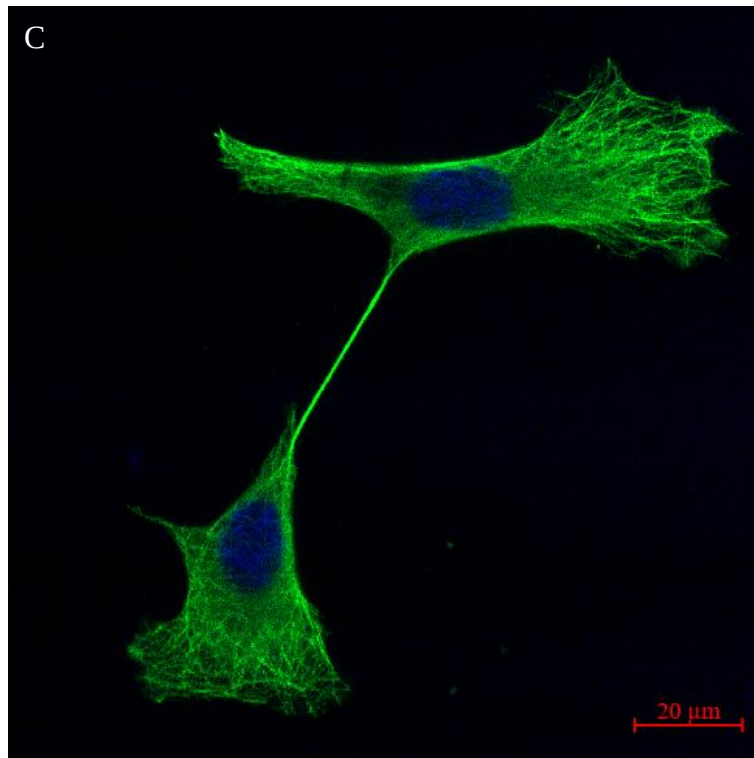
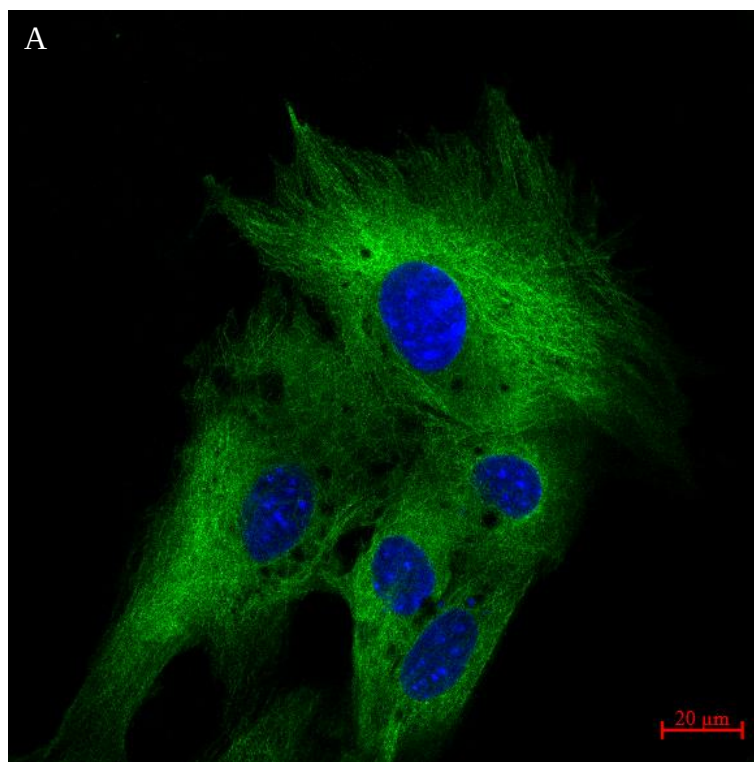


Fig. 95 Fluorescent images of microtubular fibers in cells grown on PLA substrates casted from acetone (A), chloroform (B) and ethyl acetate (C).

Cells grown on PHO substrates:



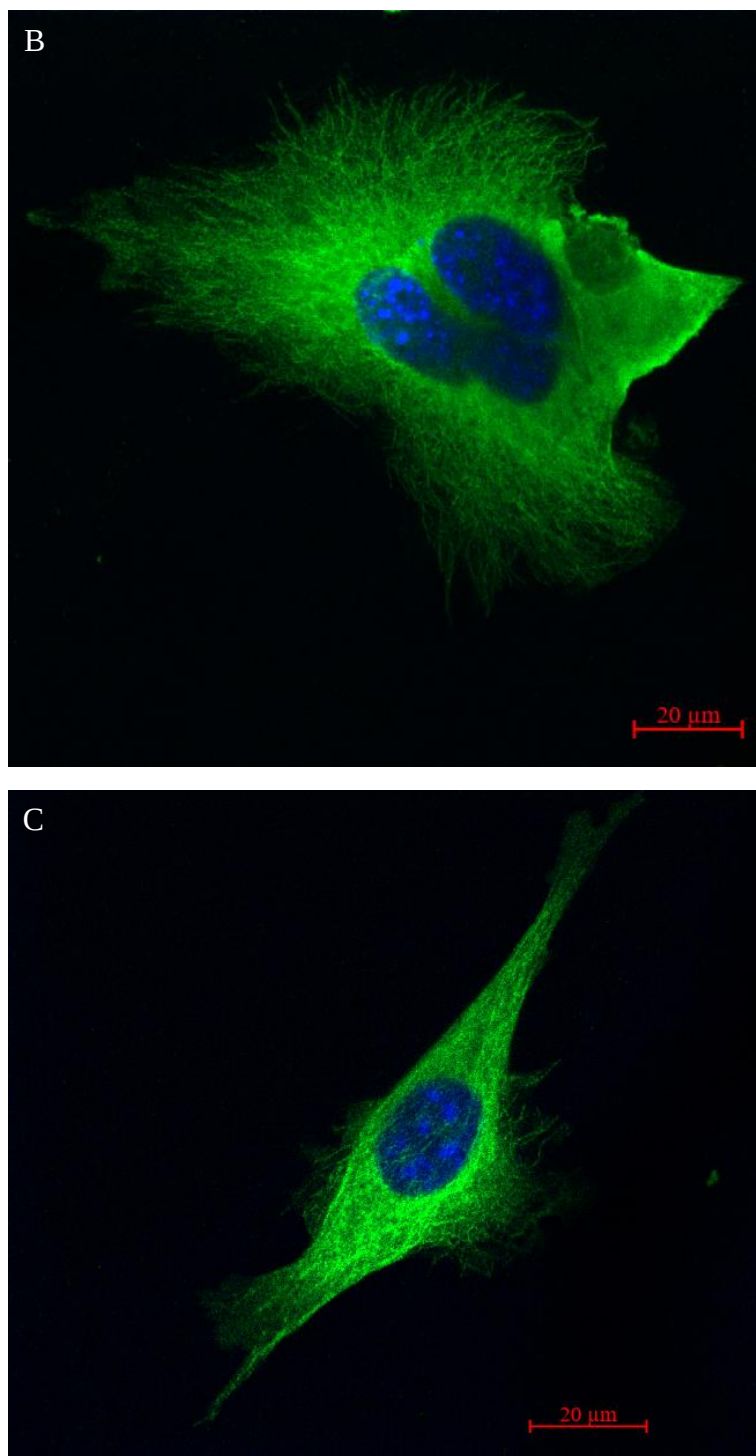


Fig. 96 Microtubular cytoskeleton in MEF 3T3 cells grown on PHO substrate casted from acetone (A), chloroform (B) and ethyl acetate (C)

Microtubular cytoskeleton analysis

Similar to the actin cytoskeleton, quantitative analysis was also carried out for microtubules. In this case, in “regional” approach only two regions were analyzed (lamellipodium and middle of the cell body), because over the nucleus the microtubule network is practically non-existent or very rarely arranged. For the analysis, 15 cells images were used for each of the examined substrate and solvent. The image analysis method is analogous to that used for actin and is described on Fig. 98 taking into account the spatial distribution of microtubules in the cell (ROI selection) and Fig. 97 when the entire cell area was analyzed. The results obtained during the analysis are summarized in tables as well as shown in the graphs to allow direct comparison of the substrates impact on the appearance and distribution of actin fibers in cell.

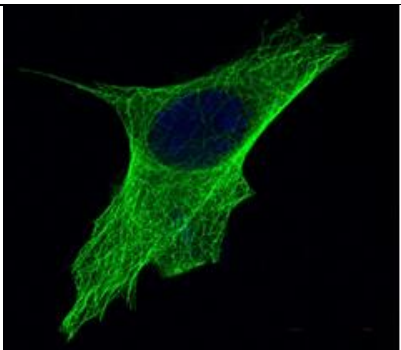
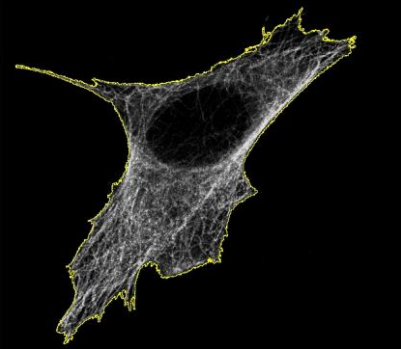
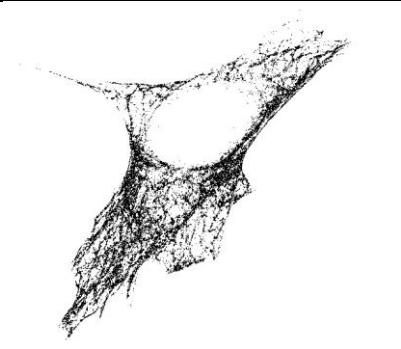
		
The studied cell is cropped and the image parameters are initially adjusted.		
		
The fluorescent channels are split, so the only visible structure are the microtubules, region of interest (ROI) is selected basing on pixel color thresholding, so the analyzed region lies exactly over the cells body, the contrast and brightness are adjusted again.		
		
The image is converted to 16bit (B&W) to enable the thresholding algorithm to proceed.		

Fig. 97 Subsequent stages of the microtubules analysis in the whole cell approach.

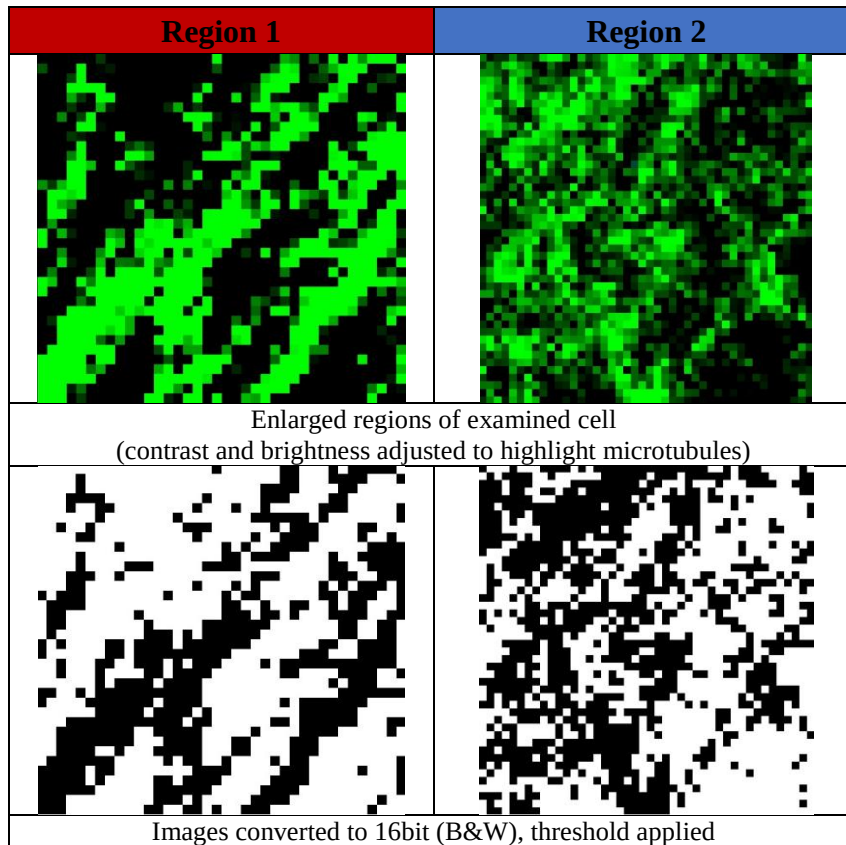
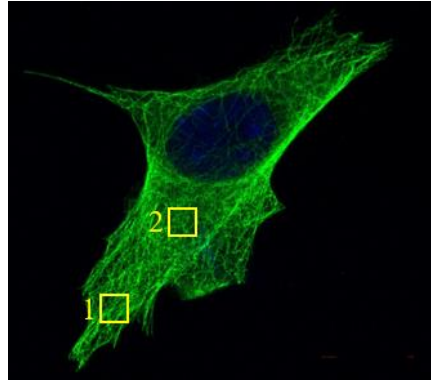


Fig. 98 Microtubular cytoskeleton quantitative analysis method basing on different regions in the cell.

The results obtained for the studied cell groups are presented below, the percentage value of area occupied by actin fibers in “whole cell” approach is compared in Table 34.

Substrate	Glass	Polylactic acid (PLA)			Polyhydroxyoctanoate (PHO)		
Solvent Cell no.		Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate
1	40.36	42.32	46.67	42.25	55.12	51.13	50.01
2	44.52	48.87	44.23	40.43	54.09	54.34	55.62
3	42.23	44.23	44.15	45.52	50.03	59.14	44.16
4	40.63	49.98	51.09	44.09	51.76	50.98	56.23
5	39.05	51.12	39.83	41.12	44.53	44.76	50.17
6	46.25	42.09	50.09	39.08	52.15	42.14	51.93
7	44.02	39.01	49.16	55.62	46.89	50.98	50.19
8	40.06	37.15	49.42	50.09	49.92	55.14	55.16
9	41.12	55.23	47.81	40.45	56.09	50.09	43.09
10	49.32	51.56	51.01	39.29	43.89	51.63	49.96
11	51.12	40.02	49.21	44.13	59.08	54.34	48.85
12	36.64	55.89	42.14	45.62	50.17	55.19	50.27
13	52.18	40.98	40.04	49.32	52.34	52.02	52.23
14	43.12	44.52	40.56	48.42	50.19	43.16	55.19
15	44.16	43.31	43.32	46.74	45.14	51.87	43.78
AVERAGE	43.65	45.75	45.92	44.81	50.76	51.13	50.46
<i>SD</i>	4.48	5.93	4.10	4.67	4.37	4.67	4.24

Table 34 Quantative data indicating percentage of area covered by microtubules in relation to the entire cell body.

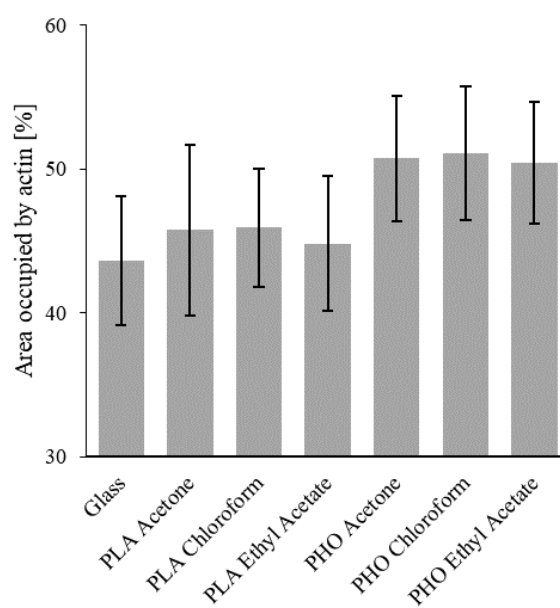


Fig. 99 Direct comparison of the actin filament network area in the whole cell with division into different types of substrates.

Substrate	Glass	
Region Cell no.	1 - lamellipodium	2 - cell body
1	38.24	46.39
2	38.28	45.11
3	40.09	50.07
4	37.15	59.13
5	33.87	43.66
6	42.12	45.13
7	39.95	44.09
8	31.09	52.15
9	33.76	55.67
10	37.15	42.01
11	39.09	40.96
12	40.02	41.62
13	41.12	43.62
14	38.19	50.02
15	42.33	49.88
AVERAGE	38.16	47.30
<i>SD</i>	3.19	5.37

Table 35 Microtubular network density summary among cells grown on glass substrate.

Substrate	Polylactic acid (PLA)					
Solvent	Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate
Region Cell no.	1 - lamellipodium			2 - cell body		
1	42.35	47.52	47.52	41.56	49.62	50.16
2	40.16	48.09	48.09	44.26	45.32	45.89
3	39.98	42.16	42.16	49.85	44.09	48.02
4	39.23	44.13	44.13	52.16	49.62	50.67
5	43.25	35.16	35.16	55.95	44.51	54.16
6	41.58	33.12	33.12	59.82	55.23	49.07
7	43.25	50.09	50.09	55.63	55.87	47.78
8	47.06	47.16	47.16	52.09	50.09	52.13
9	43.19	42.37	42.37	49.95	44.16	40.09
10	44.36	42.18	42.18	42.36	48.23	39.98
11	45.21	33.78	33.78	41.03	49.03	37.19
12	39.85	39.93	39.93	39.25	47.19	44.23
13	42.39	37.09	37.09	55.22	50.02	44.04
14	51.56	35.62	35.62	56.09	51.12	51.19
15	39.05	43.04	43.04	51.36	55.03	55.09
AVERAGE	42.83	41.43	41.43	49.77	49.28	47.31
<i>SD</i>	3.34	5.50	5.50	6.53	3.91	5.34

Table 36 Percentage area occupied by microtubular cytoskeleton in MEF 3T3 cells cultured on PLA based substrates.

Substrate	Polyhydroxyoctanoate (PHO)					
Solvent	Acetone	Chloroform	Ethyl Acetate	Acetone	Chloroform	Ethyl Acetate
Region Cell no.	1 - lamellipodium			2 - cell body		
1	55.15	49.32	47.21	52.39	55.32	56.36
2	56.13	48.25	45.62	56.26	59.21	49.65
3	44.09	52.14	55.23	51.39	56.31	55.12
4	48.14	55.63	50.18	47.82	49.23	54.85
5	52.09	52.03	49.85	59.22	52.39	48.63
6	50.46	44.79	47.63	49.32	51.14	56.13
7	43.19	47.93	44.12	51.06	40.79	52.32
8	48.56	49.63	43.69	58.23	50.32	37.23
9	49.03	46.25	52.03	59.03	56.63	54.11
10	59.09	49.32	50.06	46.13	50.32	49.35
11	55.18	51.23	49.93	44.95	54.19	55.54
12	44.13	55.63	47.09	48.09	49.21	49.65
13	49.15	44.11	52.13	50.19	52.36	50.06
14	51.97	40.06	46.23	54.31	59.31	52.33
15	54.02	49.69	49.06	56.29	46.36	50.23
AVERAGE	50.69	49.07	48.67	52.31	52.21	51.44
SD	4.74	4.17	3.16	4.70	4.93	4.79

Table 37 Quantative data describing area occupied microtubules in MEF cells grown on PHO.

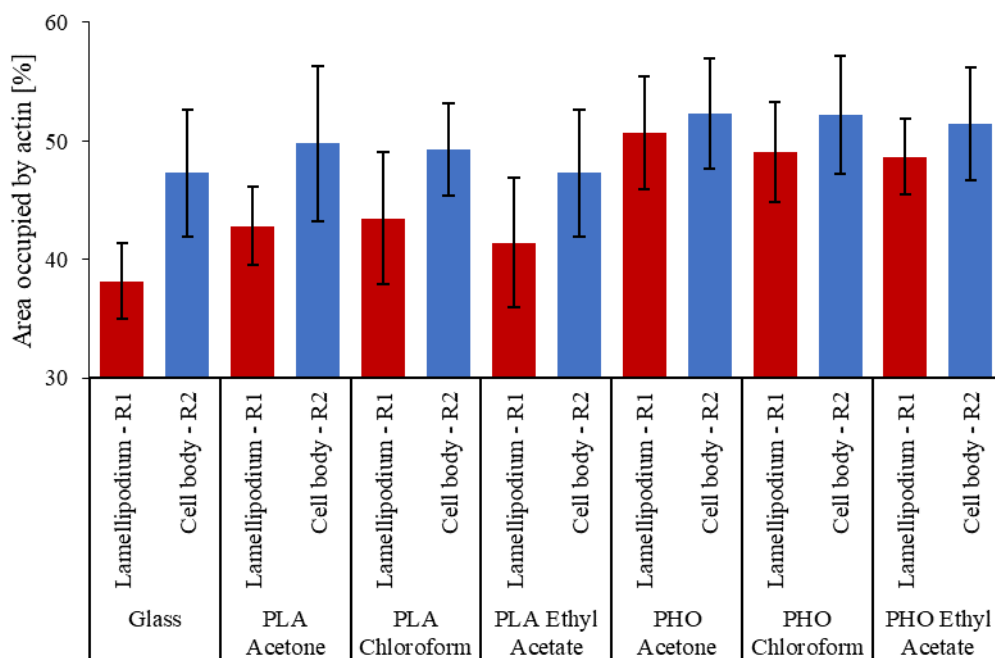


Fig. 100 Comparison of area occupied by microtubular network divided into individual cell regions and different substrates.

In microtubular cytoskeleton analysis the differentiation between certain substrates is visible already in the microscopic pictures. In case of glass and PLA substrates a thick and clearly separated microtubular bundles are visible (Fig. 94, Fig. 95). In case of substrates made out of polyhydroxyoctanoate the structure of the microtubules takes the form of a mesh of very thin, densely arranged fibers (Fig. 96). These observations are confirmed by the results from the quantitative analysis both in the regional (Fig. 100) and for whole cell approach (Fig. 99). The density of microtubules is the smallest on the edge of the cell and increases with the proximity of nucleus. The results for both regions for glass and PLA substrate are similar, they are clearly higher for PHO, where a dense network of thin filaments takes up more space. The results vary even more when the whole cell analysis is performed. Relatively large standard deviations in result groups (Table 37) arise from the fact that the observed structures are located in living cells, dynamic objects whose morphology and behavior cannot be described by means of a single parameter^{210–213}.

8. Practical importance and economical aims of the research



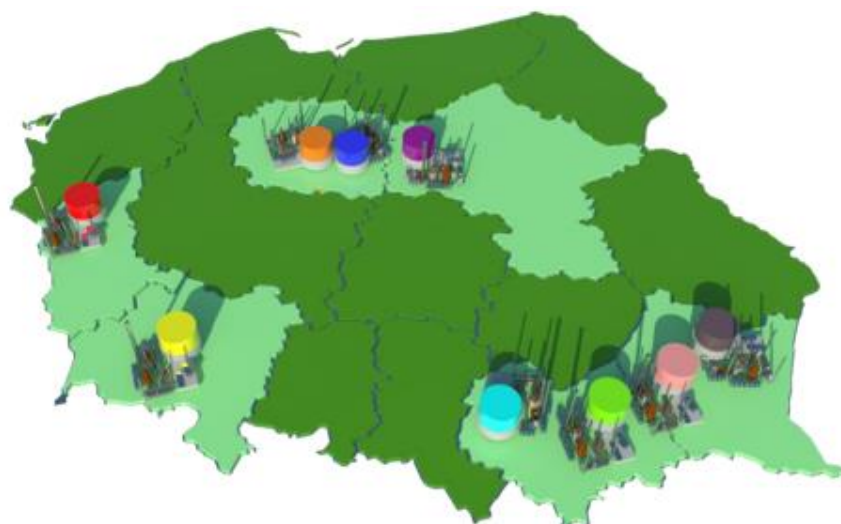
In this chapter several potential applications of PHA based polymers in the circular economy are discussed. An economic analysis of the entire biopolymer group is performed. A list of industries in which PHA may have the highest chance to be applied in the future is also depicted. A circular economy model for biopolymers production, consumption and utilization is presented.

8.1. Global petroleum-based polymers market

The global traditional plastics market is projected to reach USD 586.24 Billion by 2021, at a CAGR of 6.84%. By volume, the market is projected to reach 332.4 Million tons by 2021, at a CAGR of 4.68%. Polyethylene (PE) is the largest type segment of the global plastics market, followed by the polypropylene (PP) segment. The high demand for PE is mainly due to its effective and substantial usage in consumer products, especially in packaging materials²¹⁴. In 2016, the plastics sales markets flexible packaging, construction products, and rigid packaging dominated: These three segments account for about 65% of the total demand for plastics. The sales markets with the highest growth rates of more than 3% per year are, however, the segments automotive and electrical and electronics²¹⁵.

8.2. Polish plastics market

The consumption of plastics in Poland in 2018 is estimated at approx. 3.5 million tons, which means an increase of 9%. compared to 2017²¹⁶. This is a very high score when compared with the European market, where this increase is estimated at 3.5%, and the demand for 51.7 million tons. The quantities of plastics consumed for processing in Poland constitute approx. 6.8 percent. average European demand. Poland remains sixth in terms of demand for plastics in Europe after Germany, Italy, France, Spain and Great Britain²¹⁷. The plastics industry in Poland is represented by around 8,000 companies employing nearly 160,000 employees. According to *PlasticsEurope Polska* among largest plastics processing companies in Poland (Fig. 101), more than two-thirds are companies with Polish capital, which implement 56%. turnover of this group of companies. Foreign capital comes from 20 countries, and the most investors come from Germany, France, Austria and Italy²¹⁶.



Polymer type	Company name and location	
<i>PE, PP</i>	Basel Orlen Polyolefins - Płock	
<i>PVC</i>	Anwil - Włocławek	
<i>PS, synthetic rubber</i>	Synthos - Oświęcim	
<i>PA6, POM</i>	Grupa Azoty - Tarnów	
<i>PUR, PU</i>	PCC Rokita – Brzeg Dolny	
<i>Epoxy and Polyester resins</i>	Ciech Sarzyna – Nowa Sarzyna	
<i>Phenol and Polyester resins</i>	LERG - Pustków	
<i>PET</i>	Indorama - Włocławek	
<i>PA6</i>	Solvay Engineering Plastics Poland – Gorzów Wielkopolski	

Fig. 101 The main plastic production plants in Poland.

In Poland, all primary plastic types are manufactured: polyolefins (HDPE, LDPE, PP), polyvinyl chloride (PVC), polystyrene (PS, EPS) and polyethylene terephthalate (PET). The construction plastics like PA6 and POM are also produced, as well as polyesters, epoxy and

phenolic resins or polyurethane systems. All leading world producers of plastics are also present on the Polish market (Fig. 101).

8.3. Biopolymers market

The biomaterials market is projected to reach USD 149.17 Billion by 2021 from USD 70.90 Billion in 2016, at a CAGR of 16.0%. The growth can be attributed to increased funds granted by governments worldwide, the growing implantable devices market and technological advancements. This trend is related to high growth in geriatric population coupled with growing incidence of cardiovascular diseases²¹⁸. The major companies in the global biomaterials market are Royal DSM (Netherlands), BASF SE (Germany), Corbion N.V. (Netherlands), Covestro (Germany), Invibio Ltd. (U.K.), Carpenter Technology Corporation (U.S.), Evonik Industries AG (Germany), Berkeley Advanced Biomaterials, Inc. (U.S.), CAM Bioceramics BV (Netherlands), and Celanese Corporation (U.S.). Most of the biopolymer market is dominated by Asian based producers, however, companies operating in Europe are responsible for over 25% of global production (Fig. 10)⁴⁴. Bioplastics are a growing, innovative industry that can play a key role in the transformation to a bio-based circular economy²¹⁹. The upward trend can be clearly seen on graph presenting expected production capacities of bioplastics in years 2015-2021 (Fig. 102).

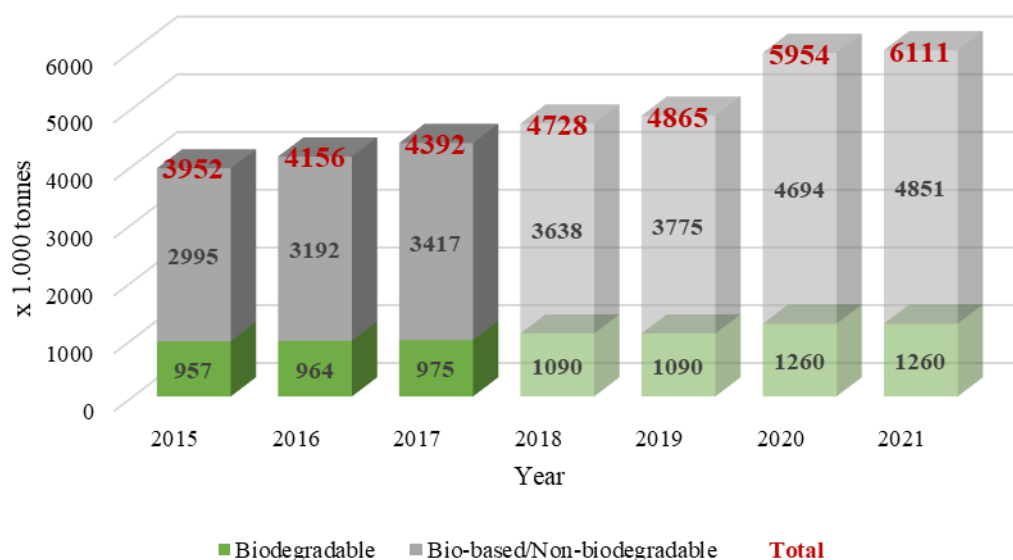


Fig. 102 Global production capacities of bioplastics²¹⁹, for 2018-2021 forecasted values (European Bioplastics, novo-Institute, 2016)

8.4. Commercialization potential

Aging society and extending life expectancy build demand for better healthcare. This entails more and more advanced medical procedures which in turn forces innovation in development of new medicines and biomedical products. All these factors drive the demand for production of materials used in medical coatings and tissue engineering. Economic studies point to a clear increase in the value of the commodity market of materials used in biomedical engineering.

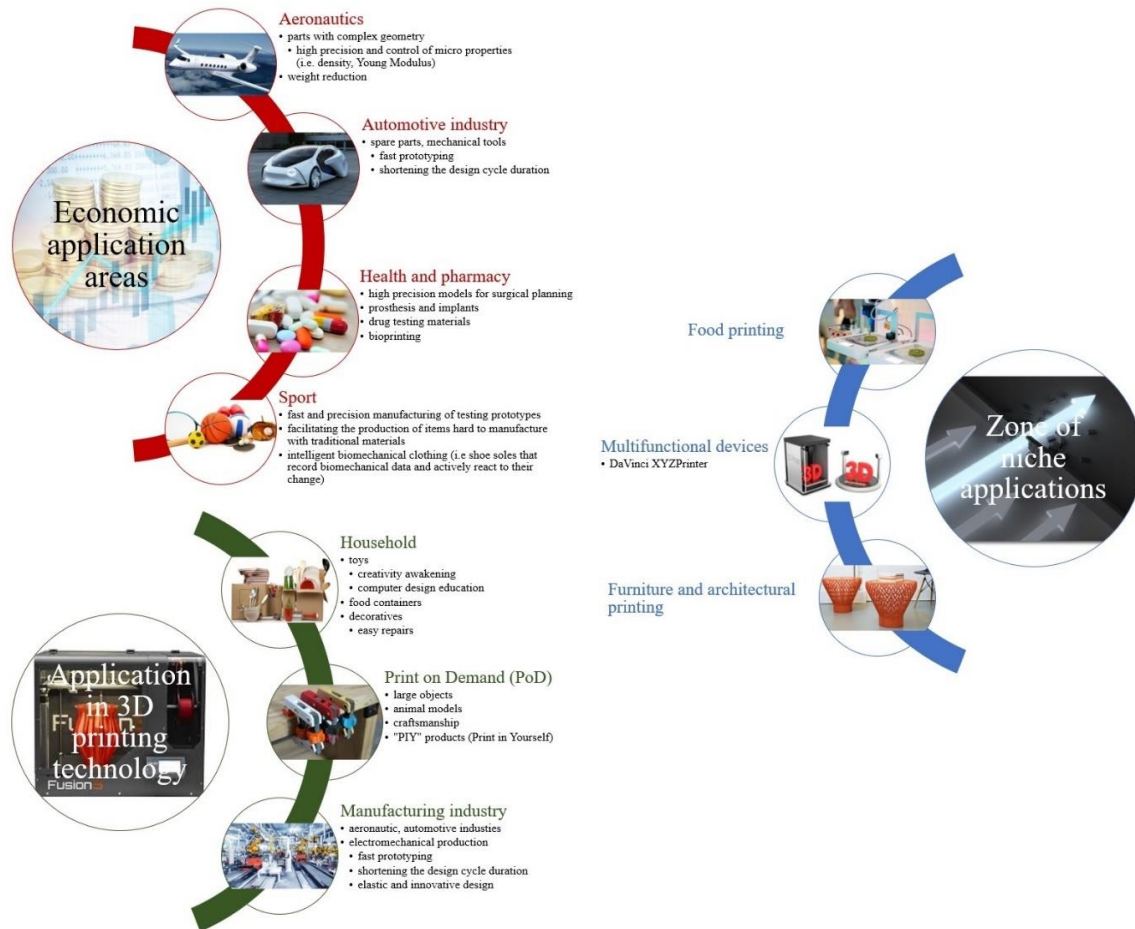


Fig. 103 Areas of PHAs applications.

8.5. Biopolymers in circular economy

Circular economy is defined as “restorative and regenerative by design, which aims to keep products, components and materials at their highest utility and value at all times, distinguishing between technical and biological cycles”²²⁰. Bio-based polyhydroxyalkanoates fit in perfectly with this innovative economic concept by opposing to the traditional linear economy and favoring a more circular product management model. In the case of traditional plastics derived from non-renewable resources, such as hydrocarbons, the life cycle of a product can be illustrated by a scheme: production ► usage ► recycling ► disposal. Bioplastics are characterized by a very similar scheme; however, both differ significantly in their timescales. Traditional plastics undergo lengthy degradation processes lasting thousands of years, whereas biopolymers degrade within months to years. Therefore fossil-based products generate huge CO₂ footprint, while polymers from renewable resources minimize this footprint by capturing CO₂ during their lifetime (Fig. 104). Human existence as we know is increasingly under threat from the pressure placed on biosphere by population growth and rising production and consumption rates²²¹. Thus, diversion of traditional economy models towards these based on bio-based products offer promising basis for the production of sustainable chemicals and materials. It is important to grow a bio-based economy, displacing the unsustainable petroleum-based energy and chemical production, while managing natural resources in order to reduce waste²²².

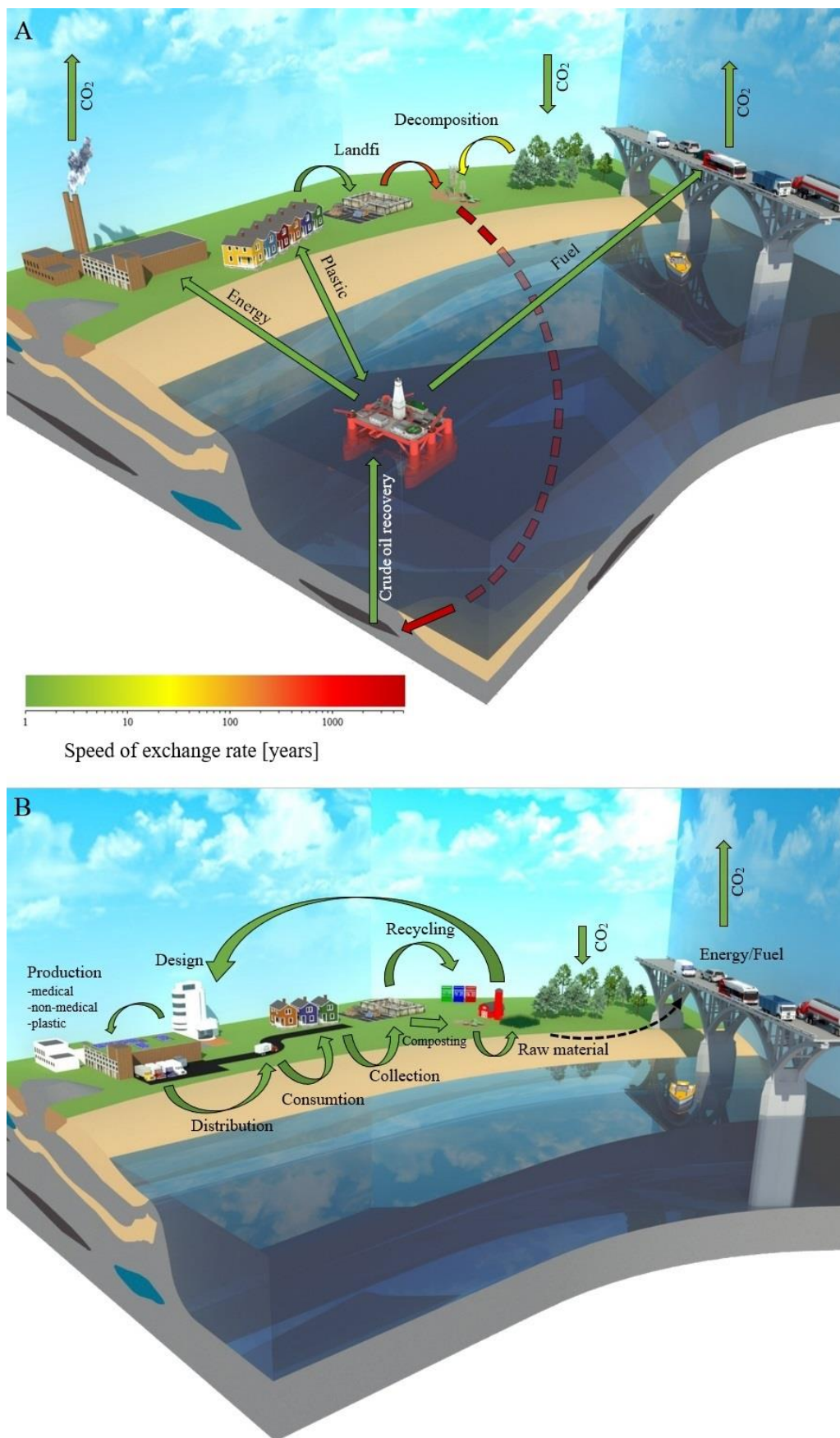


Fig. 104 The lifecycle of traditional plastics (A) versus biodegradable polyhydroxyalkanoates plastics (B).

8.6. Practical applicability in medicine and biomedical sciences

Thanks to their natural origin and good biocompatibility with mammalian cells polyhydroxyalkanoates may not only become an alternative to traditional petroleum-based plastics but they can easily find applications in niche areas where currently applied materials do not fulfill their role in full range. Such a region of material sciences where biodegradable and biocompatible materials are desired are widely understood biomedical applications. An applied medicinal product has to be fully biocompatible with the patient's body to prevent inducing an inflammatory reaction or any other undesirable body response. In certain specific applications it is also required that a medical product interacting with the body gradually degrades while releasing the drug directly at the place of insertion. Polyhydroxyalkanoates are perfectly suitable for this kind of application because they are biodegradable, moreover the biodegradation rate can be modified for individual applications, and thanks to their chemical structure polymers are easy to be physically blended or chemically modified with i.e. inflammatory drugs or antibiotics. Due to the thermoplastic nature of the polymers, the production of medical products is possible with application of techniques not available for traditional plastics or other materials like electrospinning or high-resolution 3D printing. The use of completely new manufacturing techniques characterized by high precision of production may result in the development of new, less invasive, faster and more effective methods of treatment.

8.7. Future perspectives of PHAs

The observed trend of continuous consumption of traditional plastics followed by a steady growth in PHAs production will ultimately lead to gradual displacement of fossil-based polymers. Unremitting development of new genetically engineered strains in parallel with bettering of the existing wild type microorganisms employed for PHA production, while constantly improving fermentation protocols, will allow in near future for the manufacturing processes to become more competitive with traditional production of polymers^{223,224,225}. Several technologies have also been developed to harvest effluents rich in PHB from wastewater plants in order to produce large quantities of cheap PHB material²²⁵. Similarly field studies of genetically modified plants already have shown a perspective for coproduction of PHA polymers in parallel to harvesting other essential agricultural crops^{226,227}. All of this development in PHA manufacturing area will lead to novel niches of their applications.

Similarly, as for other biopolymers (i.e. PLA) it is foreseen that virgin PHAs or PHA-based materials will find uses in areas such as production of consumables materials, agriculture, medicine, energy and many more. There are already magnitudes of basic science studies and development projects being undertaken supported by designated grant proposal calls in order to deliver new technologies and new products to markets in the near future.

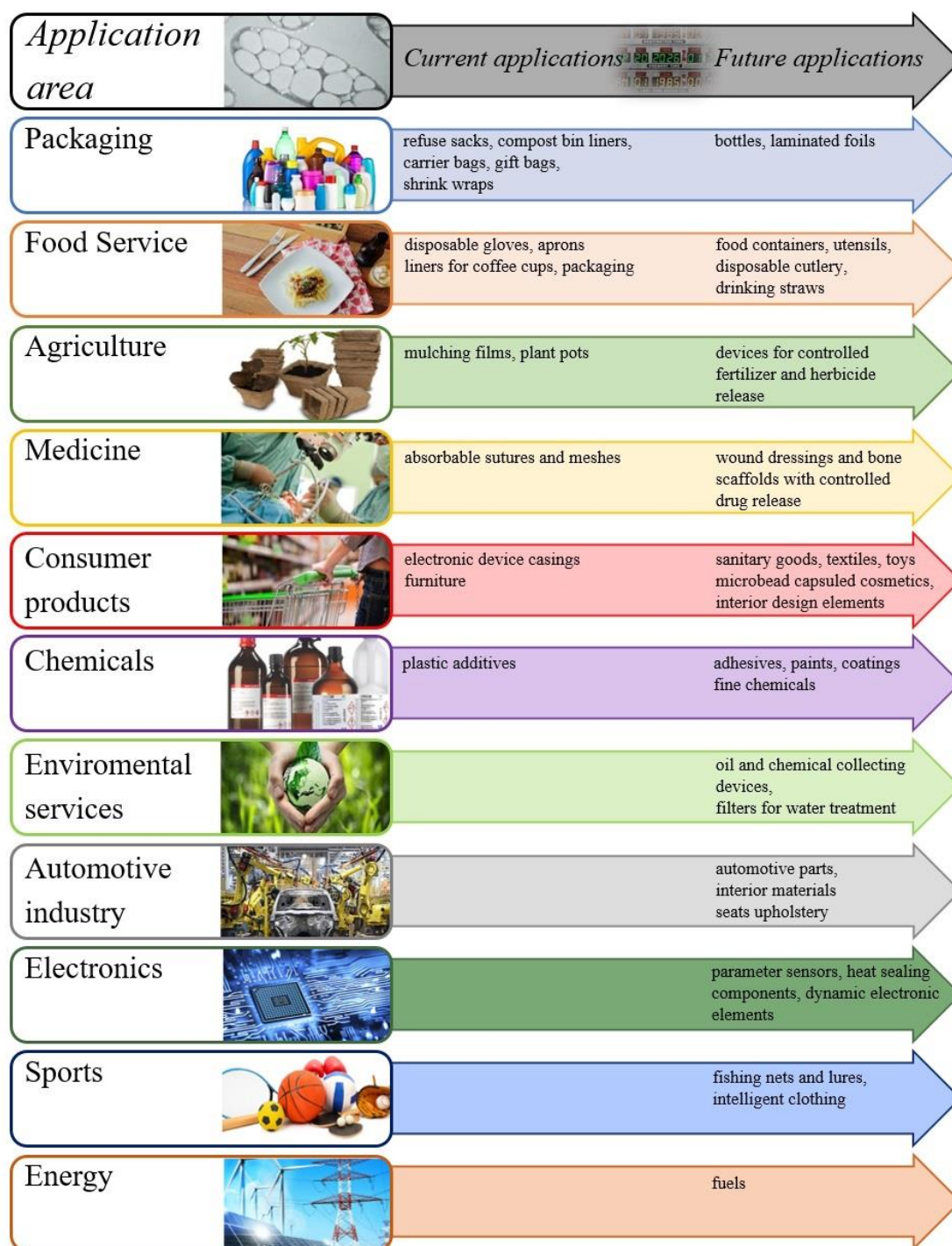


Fig. 105 Current and future applications of PHA polymers

9. Discussion



In the face of dwindling resources of fossil fuels and the negative impact of traditional plastics on the environment, we must look for alternative materials that can soon disown and replace popular in households and industry polymers whose production is based on crude oil and which are not biodegradable. The level of environmental pollution causes that individual governments to consider a legal ban on the use of plastics in selected areas of application. Plastic disposable items such as plates, cutlery, straws and cotton buds, which in total account for over 70% of marine waste is to be banned in the EU from 2021, according to a resolution adopted on 10th October by the EP environmental committee²²⁸. However, apart from the aspect of nature conservation, new biomaterials also create an opportunity for medical sciences. They can replace the materials used so far as well as extend the areas of therapy thanks to their unique properties. Polyhydroxyalkanoates, due to their biocompatibility and possibility of modification, both through chemical reactions of compound attachment and physical blend formation, seem to be ideal candidates for these particular applications. PHAs create a group of biopolymers that offer a wide range of physical properties^{77,229} which makes them a universal material for the production of biomedical products. One of the representatives of this group is polyhydroxyoctanoate, a polymer belonging to the medium chain length (mcl) PHAs group. With physical properties similar to traditional polymers available on the market, chemical structure allowing simple chemical modification and even change of hydrophobic properties by surface modification, it seems to be an ideal material for further research and development of bacterial biopolymer technologies. It is produced by bacteria, therefore it does not cause depletion of fossil fuel resources and does not leave a negative carbon footprint in the environment. Further research on polyhydroxyoctane can bring measurable profits to both the natural environment and the progress of medicine, often hindered by the lack of appropriate technologies or material constraints. Extensive investigation and understanding of the physical, chemical and biological properties of the material is an indispensable step for inserting a biopolymer for widespread use in general applications as well as in medicine and biomedical sciences.

The aim of the experimental work carried out in this thesis was to examine the physical, chemical and biological properties of polyhydroxyoctanoate in order to identify its possible applications in medicine and biomedical sciences. A multilayered study of a polyhydroxyalkanoate polymer was conducted and its properties were compared with glass and polylactic acid. Investigations of mechanical properties of the polymer were made by nanoindentation method, results were consistent with these obtained with AFM technique as well as with values provided by literature in previous reports^{1,115,178}. The obtained results indicate that the solvent from which polymer is casted has no significant effect on the final

value of both the Young's modulus and the hardness, but it is clearly visible that the biopolymer needs sufficient time after preliminary drying to achieve its final mechanical properties. Young's modulus values in the range of 33-41 MPa indicate that PHO can map the mechanical properties of popular polymers such as polyethylene (PE) or polystyrene (PS). The material hardness in range between 3 and 4.5 MPa indicates that it can find applications where elastomeric properties are desired. The combination of the Young modulus value characteristic for popular polymers with the hardness characteristic of elastomers enables application of PHO in areas where use of composite materials was required so far like elastic wound dressings, cartilage implants and coatings or vein and arterial prostheses. As part of biopolymer's mechanical properties studies, a scratch test was performed to delimitate the friction coefficient value. The obtained value of 0.2 indicates similar slip properties of PHO biopolymer for materials such as polypropylene (PP), polyethylene (PE) and polyvinyl chloride (PVC). Obtained value of the friction coefficient combined with the lower fragility of the material than i.e. PHB or PLA^{14,12} while maintaining the high value of the Young modulus, may suggest the use of medical implants covered with PHO in joint endoprotheses. They not only are subjected to considerable loads (hip joint, knee joint) but also their movement in relation to contact with bones and cartilage has to be as smooth as possible and the material should not be subject to excessive abrasion.

In order to make the material suitable for medical applications, it is necessary to determine its effect on living organisms. As a model cell line, mouse embryonic fibroblasts (MEF 3T3) were chosen because this cell line responds and participates in such processes as healing wounds or epidermis regeneration. Fibroblasts are the most common cells of connective tissue in animals. The first stage of cell biopolymer testing was to conduct cytotoxicity tests, the result of which was the most important in the further research planning and the potential suitability of the biopolymer for medical applications. Tests were carried out involving the simultaneous staining of living cells with fluorescein diacetate (FDA) and dead with propidium iodide (PI). The cytotoxicity results indicate that the vitality rate of MEF cells is comparable to that grown on glass substrates, whereas in the case of PHO, for the first 24 hours after the preliminary drying process, the presence of organic solvents is high enough to affect the cells survivability. The dry polymer (from hour 24h onwards), but not fully matured, is completely biocompatible with fibroblast cells and has no toxic effect on them. High cell viability indicates that PHO can be used in medical products that come into contact with cells of the living body.

A positive result of material's biocompatibility test with mammalian cells allowed to carry out subsequent stages of cellular research. Due to the fact that the cells show morphological

sensitivity to their immediate surroundings^{55,59,148}, it was decided to check whether the presence of the biopolymer significantly influences changes in the morphology of the cells. Due to the fact that the determined biopolymers Young modulus differs significantly from glass substrate (1GPa) in order to eliminate the influence of this factor for the morphology comparison^{14,150,230}, polyacrylamide substrates with the elasticity values of 8, 16 and 40kPa were used. The obtained results from the confocal microscope indicate that the tendency of increasing cell height together with the decreasing Young's modulus of the substrate is preserved, however on the PHO cells are relatively high and have spherical nucleus, they maintain good contact with the substrate and numerous filopodia can be noticed. It can be concluded that the biopolymer does not exert additional influence on morphological changes in cells, and additionally cells strongly adhere to the substrate. At the later stages of research with advanced fluorescence and confocal microscopy appliance, the morphology of cytoskeleton components such as actin fibers and microtubules and the possible biopolymer influence on their appearance were examined. In the case of these studies, a quantitative analysis was applied to check what percentage of the cell surface or its fragments is occupied by cytoskeleton components. In the case of the actin cytoskeleton basing on images captured with the confocal microscope, it is observed that on the glass substrate there are thick bundles of actin fibers most often running along the whole cell, whereas on the PHO substrate the actin fibers take on a more subtle form, the thin fibers do not cluster into branches but have numerous ramifications. The quantitative analysis revealed that on glass substrates there is a significant difference between fibers density between lamellipodium part and the cell body in favor of the latter. In cells cultured on PHO substrate there is no significant difference between the lamellipodium and cell body regions, however, the fiber density is clearly higher in the area above the nucleus of the cell. In the analysis of the entire cell, it can be seen that in the case of cells grown on glass, the actin cytoskeleton occupies much more surface area than those cultivated on polyhydroxyoctane. Thus, it can be noticed that the presence of PHO in the medium affects the morphology of the actin cytoskeleton, it becomes more complex and the fibers thinner. During microtubular cytoskeleton analysis already fluorescence images revealed distinct changes between the structure of the fibers in cells cultured on glass and PHO. In the case of fibroblasts grown on glass substrates, microtubules form spaced thick bundles and in the case of polyhydroxyoctanoate, this structure takes the form of a dense network of thin fibers that populate throughout the entire cell volume. Quantitative analysis confirms these observations, in the case of a regional approach, it can be seen that in the case of PHO the larger area is occupied by microtubules. The whole cell approach shows that this effect actually takes place and the differences become even more

pronounced. It can be seen that the PHO substrate affects the microtubule morphology, the fibers do not form thick bundles, but proteins form a dense mesh over the entire cell area. Cellular studies have confirmed that although the polymer is biocompatible, it does affect both the morphology of the whole cell and the components of the cytoskeleton.

In the last part of the thesis an economic analysis of biopolymers market has been performed. The main factor driving the world biopolymer market are the environmental protection and necessity of fossil fuel consumption reduction. The European and global biopolymer market is growing from year to year, which is stimulated by the gradual institutional limitations in the admission of traditional plastics in selected applications²²⁸. Thanks to their physical properties like the Young's modulus value or the fact that they are thermoplastic materials, biopolymers from the group of polyhydroxyalkanoates are suitable for the production with use of modern methods such as 3D printing. The use of biopolymers in industry and medicine can contribute not only to nature protection and reduction of CO₂ emissions but they can contribute to faster development in industries and biomedical sciences where until now the material from which the object was manufactured constituted a limitation in the development of certain technology or prevented the effective implementation of medical therapy. The biopolymer market in Poland is still undeveloped and most of the production taking place in the country focuses on the production of traditional plastics based on crude oil²¹⁷. This fact means that research on biopolymers and their subsequent introduction onto the Polish market are important and have a future. Research shows that the amount of plastic garbage lying in garbage dumps in Poland is well above the European average, which causes problems on a national scale. The combination of thorough scientific research on the properties of polyhydroxyalkanoates with a broad analysis of the global and European market can be a great step to expand potential applications and popularize the topic of bioplastics in society.

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Supplementary materials



Supplementary materials are available online. Below corresponding web addresses for each section are supplied:

13. Alternative PHAs accumulation pathways

<http://www.biophysics.fais.uj.edu.pl/documents/7122345/142105785/Alternative+PHAs+accumulation+pathways.pdf/>

14. Nanoindentation - different indenter types

<http://www.biophysics.fais.uj.edu.pl/documents/7122345/142105785/Nanoindentation+-+different+indenter+types.pdf>

15. Methods not described in thesis main text

<http://www.biophysics.fais.uj.edu.pl/documents/7122345/142105785/Methods+not+described+in+thesis+main+text.pdf>

16. Experimental data not included in thesis main body

<http://www.biophysics.fais.uj.edu.pl/documents/7122345/142105785/Experimental+data+not+included+in+thesis+main+body.pdf>

17. Physicochemical analysis of PHO polymer¹

<http://www.biophysics.fais.uj.edu.pl/documents/7122345/142105785/Physicochemical+analysis+of+PHO+polymer.pdf/>