4.2. Cellulose Nanocrystals Based Hydrogels

Hydrogels are polymeric materials, chemically or physically crosslinked, characterized by a three-dimensional (3D) and elastic network capable to swell or de-swell when immersed in aqueous solutions. In particular, chemically crosslinked hydrogels are prepared either through water-soluble polymer crosslinking or by converting hydrophobic into hydrophilic polymers, which in turn are then crosslinked to form a network [70]. Lately, a particular class of polymer hydrogels has gained more interest in the scientific community; the so-called smart or stimuli-responsive hydrogels, which are able to change their size and shape in response to an external stimulus, such as temperature, pH, ionic force, pressure, electric and magnetic field. The change in solubility or the degree of swelling are due to a fine balance among competing interactions such as electrostatic forces and hydrophobic dehydration [71]. Moreover, some systems have been developed to combine two or more stimuli-responsive mechanisms into one polymer system. Recently, dual stimuli-responsive or ternary stimuli-responsive polymer hydrogel microspheres were prepared and applied in various fields, especially in controlled release drug delivery systems [72]. Hydrogels were the first biomaterials to be rationally designed for use in humans. They are biocompatible, their aqueous environment can protect cells and fragile drugs (such as peptides, proteins, etc.), they present good transport of nutrients to cells and metabolic products from the cells, they may be easily modified with cell adhesion ligands, or they can be injected in vivo [73]. All these properties make it an interesting candidate for biomedical and pharmaceutical applications [74]. The main areas in which hydrogels are used as biomaterials are in contact lenses, synthetic wound coverings, drug-delivery systems, permselective membranes, and in organ and tissue replacements, such as skin, tendon, cartilage, heart valve stents and bone [75]. However, because of the random nature of the crosslinking reactions produced by a large number of organic crosslinker polymer, hydrogels exhibit poor mechanical properties, which strongly limit their use in structural applications. For such a reason, different nanofillers, such as silicates [76], ceramics [77], metals [78], magnetic particles [79] and graphene [80] have been introduced into the hydrogel matrices, thus obtaining the corresponding nanocomposites. The incorporation of nanoparticles into the hydrogel 3D matrix, producing a class of materials known as nanocomposite hydrogels (NHPs), has been a widely investigated strategy to improve some existing physical properties or to provide them with new physical or chemical features. Considering the excellent dispersion of cellulose nanocrystals in water, they are obvious candidates to prepare NHPs, having many advantages compared to other polymeric or metal nanoparticles [54]. Cellulose-based hydrogels represent an important material class in biomedical fields due to their biocompatibility and biodegradability. Several water-soluble cellulose derivatives can be used

as mono-components of multi-component systems and they can be used also as reinforcement phases in some other biodegradable polymers to form hydrogel networks with specific properties in terms of swelling capability and sensitivity to external stimuli [81]. However, the current trend in the design of cellulose-based hydrogels is associated with the use of non-toxic crosslinking agents or crosslinking chemical treatments, to further improve the safety of both the final product and the manufacturing process [11], and few recent studies have investigated the incorporation of CNC with particular surface modifications in the preparation of NPHs, thus acting as both filler and cross-linker to reinforce hydrogel systems. Zhou et al. [82] demonstrated that the CNC acted as reinforcing agents, and also as multifunctional cross-linkers, accelerating the formation of hydrogels and increasing their effective cross-linking density. Moreover, chemically cross-linked gelatin/CNC hydrogels were prepared using oxidized CNC as cross-linkers [83]. The authors proved that the dialdehyde groups of oxidized CNC reacted with the free amine groups of gelatin to cross-link the hydrogel framework and the final properties of these materials were dependent on the amounts of CNC aldehyde groups.

Recently, Sanna et al. [71] reported on the synthesis and characterization of thermoresponsive poly(N-vinylcaprolactam), PNVCL, nanocomposite hydrogels containing nanocrystalline cellulose produced by a frontal polymerization technique, which is a convenient, easy and low-energy-consuming method of macromolecular synthesis. The authors proved that the presence of CNC resulted in a significant increase of the mechanical properties even at very low CNC concentrations, as confirmed by rheological tests indicating that the nanocellulose has a great potential to reinforce PNVCL polymer hydrogels. Finally, Yang et al. arguably reported the first injectable hydrogels reinforced both physically and covalently with CNC, based on a carboxymethyl cellulose (CMC)/dextran system [84]. Their approach was based on coextruding aldehyde functionalized CNC with dihydrazide-modified CMC and aldehyde-modified dextran solutions through a double-barrel syringe. The produced NPHs and their components revealed no evident cytotoxicity to NIH 3T3 fibroblast cells. The authors hypothesize that these CNC reinforced injectable polysaccharide hydrogels are of potential interest for TE applications where longer term dimensional stability and enhanced mechanical strength are desirable [84].

5. Concluding Remarks

Over the past few years, a growing interest has emerged in applying cellulose structures, and in particular cellulose nanocrystals, as biomaterials for the development of advanced functional bionanocomposites which could find a wide range of potential applications in TE. This Chapter has attempted to provide a general overview of the potential of CNC in the design of these functional

nanomaterials, through various examples involving different approaches and processes. Recent studies demonstrated that different types of nanomaterials presenting desired properties and functions could be produced from CNC. Research on nanocomposite films, membranes and hydrogels with potential application in TE strategies was summarized.

The use of lignocellulosic materials in drug-delivery devises has been studied for several years while the use of cellulose derivatives as excipients in drug formulations is already a standard in the pharmaceutical industry. In the future, the appearance of commercial formulations that are able to control the release rate and timeframe of different drugs, is expected. However, it is necessary to attract the industry to this growing market opportunity capable of creating sustainable technological improvements in bionanocomposites.

The positive results obtained by the research activity concerning increased performance of the bionanocomposites, will contribute to sustain these market developments although it is important to validate the most sustainable supplies of lignocellulosic materials. In addition, it is also relevant to create mechanisms to contribute to the certification of these nanostructured biomaterials according to common methods and standards.

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Chitin Nanofibrils-Chitosan Composite Films: Characterization and Properties

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Abstract: In this research, an attempt to prepare a prototype of fully biodegradable nanocomposite films for food packaging was made by using commercial chitosan (CS) and chitin nanofibrils (CN). If food products could be packaged in these innovative films instead of paper (cellulosic) ones, the speed of deforesting in Europe will be diminished, thus improving the ecological situation and climate. In the paper, properties of the CS/CN films are described. The prototype films have been prepared from aqueous viscous CS/CN slurries by casting technique. The effect of many operating variables on morphology and properties of the nanocomposite CS/CN films such as sorption of water vapors, oxygen permeability, contact angle, mechanical stability and thermostability is analyzed and discussed. The examined variables are CN content, type of plasticizers (polyglycerols) and their content, type of metal ions such as Mg^{+2}, Ca^{+2}, Ba^{+2} and type of various additives (gelatin, lignin and monolignols) or surface modifiers (polylactides, polyglycerol polyricinoleate). Special attention was paid to the study of the effect of the storage time of the slurries on their aging and mechanical characteristics of CS/CN films.

1. Introduction

This research solves several particular problems of the global European project Bioeconomy, designed to become the basis of a new lifestyle for future generations. The goal of Bioeconomy is to create a more sustainable future, where all natural resources will be used in the most rational and efficient way for existence of a "zero waste" society. As a concrete example of Bioeconomy in action, the utilization of waste from the fishing industry can be considered. The production of chitin nanofibrils (CN) and chitosan (CS) from exoskeletons of crustaceans, which are accumulated in huge quantities in fisheries daily, improves not only the ecological situation but also creates additional sources of renewable feedstock. New products based on CS and its derivatives and based on CN have been already applied in medicine, dermatology, cosmetics, and material science.

Nature is the most skillful designer of myriad nanocomposites. Crustaceans' exoskeletons (the polysaccharide-protein-mineral nanocomposites) are typical examples [1]. The integuments of crustaceans contain the linear water-insoluble homopolymer of β-(1→4)-linked *N*-acetyl-D-glucosamine (GlcNAc) in the form of nanofibrils composed mainly from α-chitin with antiparallel orientation of GlcNAc chains. Each chitin nanofibril, about 3 nm in diameter and 300 nm long [1], is formed by a bundle of 20–30 α-chitin chains interconnected with multiple hydrogen bonds and hydrophobic interactions. CN, as is well known, are nanocrystals [2–4], which reinforce the non-crystalline proteins in carapaces just as the steel reinforcement strengthens concrete panels. The surface of CN serves for proteins enveloping them perhaps as a template. Micro- and nano-crystals of calcium carbonate and phosphate are distributed chiefly between protein molecules and probably affect their mobility and spatial arrangement [1], finally enhancing their rigidity.

In material science, mainly chitosan (deacetylated chitin) has been of interest since its discovery in 1811 by Henri Braconnot. Owing to solubility in acidified water at pH 4–4.5, a lot of remarkable properties of chitosan, including its excellent film-forming ability, have been discovered and recently exploited in many applications [5]. In contrast to rigid CN, CS chains bearing protonated amine groups in glucosamine (GlcN) rings are repelled and the chains acquire flexibility. However, the hydrophobic parts of CS chains are less flexible due to intra-chain hydrogen bonds between the atom HO3 of one sugar unit and the O5 atom of the next monosaccharide in the same chain that reduces the conformational variability. The chains with block-distribution of GlcNAc rings form the micelle-like aggregates which are interconnected by almost fully deacetylated CS chains stretched by electrostatic repulsion. The molecular dynamic simulations have shown that in such aggregates, the density of chain packaging is higher than that of aggregated chains with uniformly distributed acetylated rings. These phenomena are enhanced by increasing the acetylation degree (DA) affecting the viscosity of CS solutions [6]. After evaporation of plasticizing water molecules, the dissociation of acid molecules decreases, the protonation of CS chains is suppressed, and their repulsion becomes so negligible that they come together forming homogeneous and transparent CS films. The dried flexible CS films are stable so long as they are not in contact with water, which can promote their swelling and even complete dissolution. The non-protonated CS films are stable in water at neutral pH. They are obtained when counterions and free acid molecules, which remained inside after drying the slurries, are removed from the dried films. This is usually achieved by dipping the dried CS films in the alkaline solution followed by rinsing with water and drying. For preparing chitosan films stable in aqueous solutions in a wide pH range, the CS chains must be crosslinked with covalent bonds using bifunctional agents (e.g., glutaric

dialdehyde, genipin and, etc.). Both procedures make the practical realization for production of CS films at large scale difficult.

An alternative simple method based on "physical crosslinking" of CS chains with chitin nanofibrils is described in this paper. It has been developed for preparing biodegradable water-insoluble-at-neutral-pH nanocomposite CS/CN films for use in food packaging.

2. Experimental Methods

2.1. Solid State ^{13}C CP/MAS NMR

1D solid-state NMR spectra were measured using a Bruker Avance 500 NMR spectrometer. Magic angle spinning (MAS) frequency of the sample was 10 kHz. In all cases the dried samples were placed into the ZrO_2 rotors and stored under silica-gel to prevent rehydration. Amplitude-modulated cross-polarization (CP) with duration 1 ms was used to obtain ^{13}C CP/MAS NMR spectra with 5 s recycle delay. The ^{13}C scale was calibrated with glycine as external standard (176.03 ppm—low-field carbonyl signal).

2.2. Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR spectra were recorded using a spectrophotometer Perkin-Elmer Paragon 1000PC and Attenuated total reflection technique Specac MKII Golden Gate Single Reflection ATR system with a diamond crystal; the incidence angle was 45°. The 16 scan-spectra with 4 cm^{-1}-resolution were recorded in the range of wavenumbers: 4400–450 cm^{-1} and evaluated using Spectrum 2.00 software. The samples were directly applied without modification to the diamond crystal and measured.

2.3. Size Exclusion Chromatography (SEC)

The commercial chitosan (Giusto Faravelli SpA, Italy) (DA = 22%) was analyzed by SEC with dual light scattering-concentration detection: a light-scattering photometer (DAWN DSP-F, Wyatt Technology Corp.) measuring at 18 angles of observation and a differential refractometer (Shodex RI 71) were the detectors. The mobile phase was the acetate buffer, 0.15 M ammonium acetate/0.2 M acetic acid buffer (pH 4.5) and chitosan solutions (1–5 $mg \cdot mL^{-1}$) were filtered through 0.2-μm pore size disposable syringe filters (Watrex) before injection. Two columns PL Aquagel OH-MIXED B was the separation system.

For studied chitosan samples, molecular weight was also determined by measuring the intrinsic viscosity [η]. The viscosity-average molecular weight M_η was calculated using the Mark-Houwink equation with the constants for chitosan in the acetate buffer experimentally obtained by Yomota et al. [7]. In the range of molecular

weights $1.15 \times 10^3 < M < 1.59 \times 10^6$, the constants K and a equal to $0.199/\mathrm{mL \cdot g^{-1}}$ and 0.59, respectively, were used in calculations. For measuring the intrinsic viscosity, the classic Ubbelohde viscometer modified for foaming solutions with the capillary diameter of 0.42 mm was used [8].

2.4. Rheological Measurements

The rheological experiments were performed at room temperature, using a rheometer Physica MCR 501 (Anton Paar GmbH, Austria), equipped with an anti-slipping parallel-plate geometry. Viscoelastic properties in oscillatory shear flow were measured in the linear viscoelasticity region. The start-up tests at small shear frequencies were performed to investigate the dependence of solutions viscoelasticity on time. The measurements were started after 3 s pre-shearing at the shear rate $0.03\mathrm{s^{-1}}$, to ensure the same shear history for the measured samples. The homogeneous slurries with CS/CN proportion 70/30 wt. % containing 25 wt. % of a plasticizer from the total content of CS and CN in the slurry were tested.

2.5. Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM)

SEM was used to examine the surface structures of CS/CN films and their fractures. The observation was performed on field-emission gun scanning electron microscope Quanta 200 FEG (SEM; produced by FEI, Czech Republic) in the secondary electron mode at accelerating voltages of 5 kV. The samples were sputtered with platinum (~5 nm; Vacuum sputter coater SCD 050, Balzers) to avoid charging and sample damage due to electron beam.

TEM observations of CN dispersions were performed on a Tecnai G2 Spirit Twin 120kV (FEI, Czech Republic). The solution of bromophenol blue (0.1 wt. %) was used as a color marker to increase the contrast of the chitin nanofibrils. A drop (2 μL) of the solutions was put onto a copper TEM grid (300 mesh) coated with a thin carbon film transparent for electrons. The excess of solution was sucked out by touching the bottom of the grid with filtering paper. This fast removal of the solution was performed after 1 min to minimize oversaturation during the drying process. The sample was left to dry completely at ambient temperature and then observed with a TEM microscope using bright field imaging.

2.6. Atomic Force Microscopy (AFM) of CS/CN Films in the Dried State

For characterizing the film surface at the micrometer and nanometer scale, atomic force microscopy (AFM) was applied for obtaining information about the surface topography and about the film properties, such as the surface heterogeneity. An Icon Dimension instrument, Bruker equipped with the SSS-NCL probe, Super Sharp Silicon^{TM} - SPM-Sensor (NanoSensors^{TM} Switzerland at the spring constant 35 $\mathrm{Nm^{-1}}$ with resonant frequency $\approx$ 170 kHz) was used. Measurements

were performed under ambient conditions using the tapping mode AFM technique. The scans covered the sizes from 0.35 × 0.35 to 30 × 30 μm^2. The upper and bottom surfaces of the samples were also analyzed.

2.7. AFM of CS/CN Films in the Swollen State

AFM characterization of CS/CN films was performed on Atomic Force Microscope Dimension ICON (Bruker). All images were acquired as topographical scans in Peak Force Tapping mode in water using silicon nitride tip ScanAsyst-Fluid (Bruker) with typical spring constant k = 0.7 N/m and scan rates within the range of 0.7–0.9 Hz. Prior to testing the CS supports CN adsorbed on their surface, they were double rinsed with fresh Milli-Q water to remove an excess of no adsorbed CN.

2.8. Sorption of Water Vapors

Water vapor sorption isotherms were determined by a gravimetric method using the sorption balance IGA-003 (Hiden Isochema, UK). In the used isothermal static procedure, the source of water vapors was liquid water added to the IGA reservoir. The film sample was loaded into the microbalance, weighed, and evacuated until constant weight was reached. After determining the mass of the dry sample, it was equilibrated at the isothermal temperature (25 °C). During the measurements, the pressure of water vapors was increased step by step to achieve the constant values equal to 5, 10, 15, 20, 25 or 30 mbar. At each pressure value, the saturated state of water vapors was reached. Values of each relative pressure at 25 °C were calculated using the software program, which also restricted the maximum pressure below condensation of water vapors. The amount of adsorbed water vapors (S_W) was evaluated as a percentage from the mass of a dry film.

2.9. Contact Angle Measurements

The hydrophobicity of CS/CN films was evaluated by the values of contact angles measured using an optical device OCA20 system (DataPhysics, Germany). The measurements were performed using the sessile drop method in the static mode. The samples have been prepared as follows: film disks (1.8 cm in diameter) were placed on a glass microscope slide and their borders were fixed in four points using double-sided Scotch tape (1 mm in diameter). Before measuring, all film samples were cleaned with compressed air. Both surfaces of the studied films were analyzed at least twice if difference between the measured values was high. Three to five 30 µL-drops of water were applied to each film disk at the rate of 5 µL/s. Values of the contact angles were calculated based on the Young-Laplace fitting.

2.10. Thermogravimetric Analysis (TGA)

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) were used to characterize the thermal behavior of CN and CS/CN films. TGA measurements were performed on Perkin Elmer Thermogravimetric Analyzer Pyris 1 in air atmosphere to determine among others also the thermo-oxidative stability of the studied samples. The temperature interval of measurements was usually 30–800 °C, with gradual temperature rise of 10 °C/min and air flow 50 mL/min on samples of about 10 mg.

2.11. Measuring the Mechanical Characteristics of CS/CN Films

Tensile tests were carried out at ambient temperature using an Instron 5800 apparatus at a crosshead speed of 1 mm/min. At least eight specimens were tested for each sample. The stress-at-break, σ_b (variation coefficient < 2%), elongation at break, ε_b (variation coefficient < 5%), and Young's modulus, *E* (variation coefficient < 6%), were evaluated. Test specimens according to ISO with a length of working part 10 mm and width 2 mm were cut from cast films with thickness < 0.1 mm. The samples were conditioned in a hermetic box at 43% RH and 22 °C for 5 days before testing.

2.12. X-ray Diffraction Studies

Small Angle X-ray Scattering (SAXS) experiments were performed using a 3-pinhole camera (Molecular Metrology SAXS System now Rigaku). A X-ray beam produced by the microfocus X-ray tube (Bede Microsource) operating at 45 kV and 0.66 mA (30 W) was monochromized and focused by multilayer spherical optics (Osmic Confocal Max-Flux). SAXS patterns were measured using a multi-wire, gas-filled area detector with an active area diameter of 20 cm (Gabriel design). The probed q region was from 0.049 to 10.3 nm^{-1}, $q = (4\pi/\lambda) \sin(\vartheta)$, where $\lambda = 0.154$ nm is the wavelength and 2ϑ is the scattering angle. Data were always merged from two overlapping measurements in high- and low-resolution regions. Calibration was performed using Silver Behenate powder. Measurements took up to 2 h, depending on the quality and intensity of diffraction from sample.

3. Results and Discussion

3.1. Chitin Nanofibrils

The aqueous dispersions of CN (Figure 1a,b) resemble milky fluids. Their color may be different from slightly yellowish to brownish due to some admixture of carateno-protein components remaining bound to CN surface.

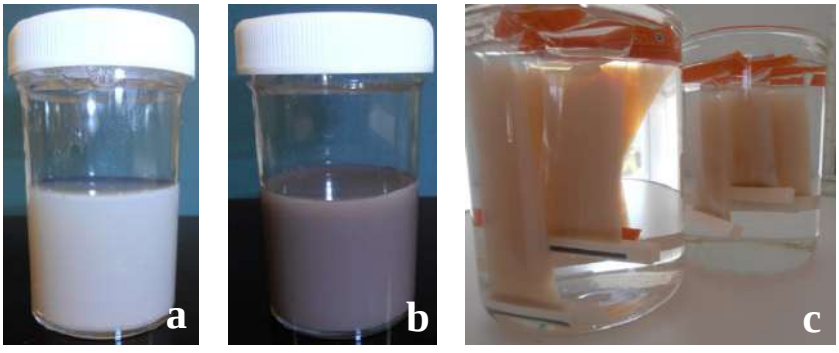

Figure 1. (**a**,**b**) Aqueous commercial dispersions of chitin nanofibrils (MAVI Sud Srl, Italy), (**c**) Clarification of commercial CN dispersions by dialysis against distilled water through 3.5-kDa SpectraPor dialysis cellulosic film. The precipitate and supernatant of CN were separated by centrifugation at 5000 rpm for 30 min.

The commercial CN dispersions (MAVI Sud Srl, Italy) contain sodium benzoate (0.5 wt. %) protecting them against microbial growth. As usual, the chitin nanofibrils do not precipitate at storage for at least a half a year but at their clarification by dialysis against distilled water (Figure 1c), a part of CN has been precipitated due to changing in the charge state of the CN surface after removal of the surface-adsorbed molecules of sodium benzoate. Initial pH of CN dispersions is shifted from acidic values (1.8–2) to more neutral (about 5.2–5.5), which are more favorable for aggregation of CN facilitating their precipitation.

The characterization of chitin nanofibrils in the swollen state was performed using AFM in the peak force tapping mode in water. In both precipitate (Figure 2a,c) and supernatant (Figure 2b,d), the CN had a spindle-like shape. They were slightly longer and thinner in the precipitate (Figure 2a,c) than in the supernatant (Figure 2b,d). The shape of CN in the swollen state was quite similar to that in the dried state (Figure 3a,b).

In AFM (Figure 2), SEM (Figure 3a) and TEM (Figure 3b) the average length of both swollen and dried CN did not exceed about 400–500 nm but their average diameter was about twice as small (about 35–40 nm) as that of the swollen CN (75–80 nm). The diameter of a single CN was considerably less (about 3 nm) [1]. It seems that the method of production of CN [4] has allowed the isolation of the chitin nanoparticles consisting of a higher quantity of bundles of nanofibrils.

We already know that the dimensions of CN have been affected by temperature and some salts such as $CuSO_4$ and $Cu(CH_3COO)_2$. The effect of copper salts on the structure of CN has been enhanced with time and depends on the type of added salt. The copper sulfate molecules were able to fix on the CN surface (Figure 3b) without their destruction. When the CN supernatant was heated at 60 °C for 30 min

in the presence of copper acetate, the formation of bundles of single chitin chains was observed (Figure 3c).

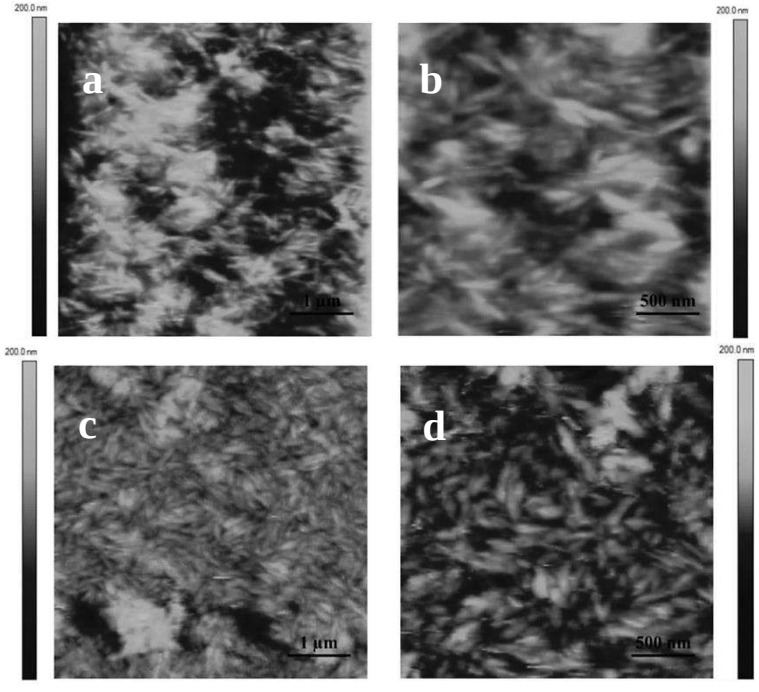

Figure 2. AFM images of CN in the swollen state in precipitate (**a**,**b**) and in supernatant (**c**,**d**). The scan size: 5 × 5 µm (**a**,**c**) and 2.5 × 2.5 µm (**b**,**d**). The CN were previously adsorbed on the surface of CS films during a day.

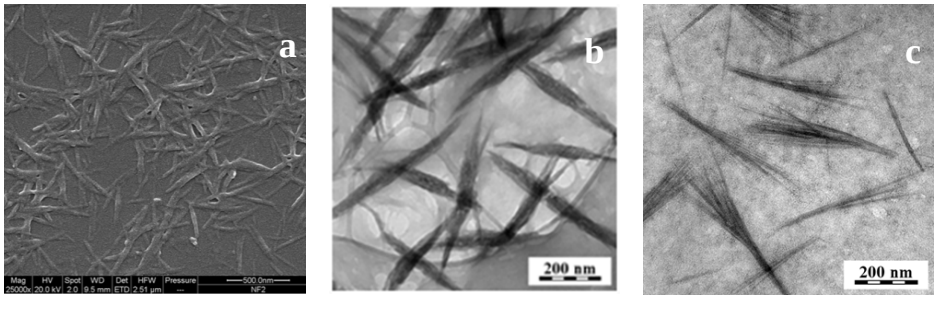

Figure 3. (**a**) SEM and (**b**,**c**) TEM images of dried CN: (**a**) initial (reprinted from [9]); (**b**) with surface-adsorbed $CuSO_4$, and (**c**) heated at 60 °C for 30 min in the presence of Cu $(CH_3COO)_2$.

This is the clear evidence that some protein molecules enveloping the chitin chains are tightly fixed on the surface of CN. In the absence of metal salts, there were no essential changes of CN dimensions even during heating of CN supernatant free of metal salts at 60 °C for 1.5 h. Although the CN became thinner and somewhat shorter (Figure 4c,d vs. Figure 4a,b), their structure was not destroyed, and the reinforcing ability did not change.

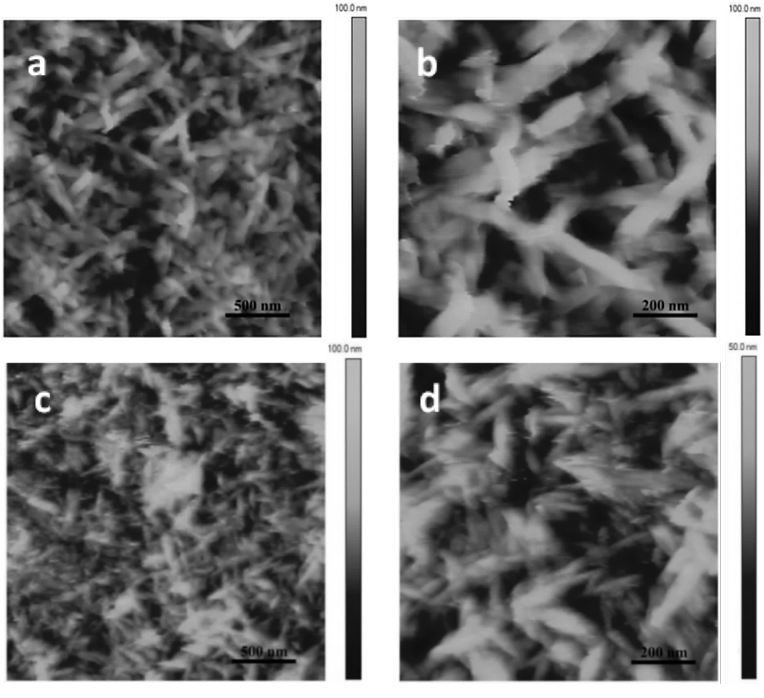

Figure 4. AFM topography images of CN in water: (**a**,**b**) initial supernatant, (**c**,**d**) after its heating at 60 °C for 1.5 h. The scan size: (**a**,**c**)—2.5 × 2.5 μm, (**b**,**d**)—1.0 × 1.0 μm. The CN were previously adsorbed on the surface of CS films during a day.

The crystallinity of CN [4] for all the samples has been tested by SAXS. The character of the X-ray diffraction pattern (Figure 5) is typical for a well-ordered crystalline phase.

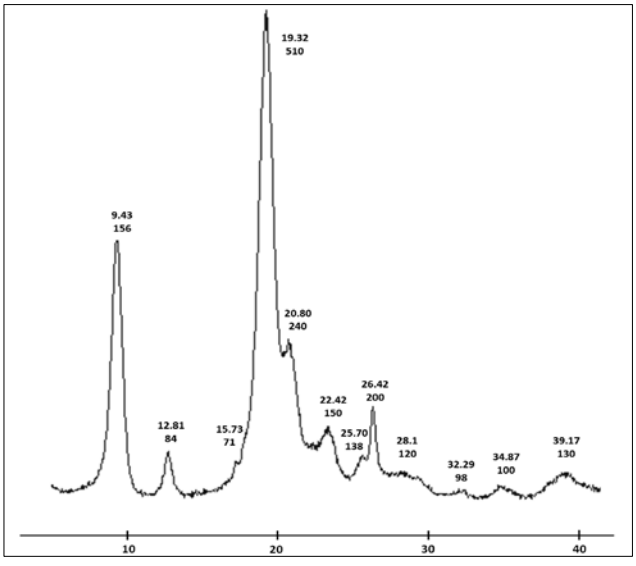

Figure 5. The X-ray diffraction pattern of α-chitin nanofibrils. The upper and bottom digital labels (e.g., 9.43 and 156) at diffraction peaks denote the respective diffraction angle (in 2θ degrees for $CuK\alpha_{12}$) and X-ray intensity (in arbitrary scale), respectively.

Broadening of peaks corresponds to crystal imperfections due to different lengths of polysaccharide chains forming CN and due to small crystalline domains of nanofibrils. Positions of peaks uniquely identify the crystalline phase of α-chitin. This agrees (Table 1) with other observations [10].

Table 1. Characteristic peaks of the α-chitin nanofibrils in the X-ray diffraction pattern.

Diffraction Angles (in 2θ Degrees) at the Main Peaks in X-ray Spectrum of α-Chitin							**Reference**
1	2	3	4	5	6	7	
9.4	12.8	19.3	20.8	22.4	26.4	32.3	Figure 5
9.2	12.6	19.2	20.6	23.2	26.2	32.2	[10]

The measured diffraction pattern of dried α-chitin (Figure 5) corresponds to the well-ordered crystalline polymer. It remains unchanged, i.e., the crystalline quality and average size of nanofibrils are identical no matter if the diffraction angle (in 2θ degrees) has been recorded on aqueous CN dispersion or dried CN flakes, on

a CN monolith or CS/CN plasticized or non-plasticized films. However, heating the aqueous CN dispersion in the presence of nitrates of such metals as Cu^{+2}, Be^{+3} and Ag^{+} almost completely destroyed the crystallinity of CN.

3.2. Chitosan

As a rule, four production steps (demineralization, deproteinization, discoloration and deacetylation) are common for all manufacturers of chitosan from carapaces of crustaceans [9,11]. Nevertheless, the quality of various commercial chitosans can differ essentially because of the difference in the type of crustacean carapaces and in processing conditions (temperature, reaction time, concentration of alkali, pretreatment of the chitin and its concentration, particle size, concentration of dissolved oxygen and intensity of stirring). The variation of these parameters during the processing of chitin affects the properties of CS such as molecular weight and polydispersity of molecular distribution, the DA and solubility, content of mineral salts, and admixtures.

Usually, the targeted use of any packaging film determines the choice of raw materials, the quality of which must be sufficient for realization of this goal. One of the possible applications of completely biodegradable CS/CN films is, initially, one-off packaging for sandwiches, sliced cheese, or sausages, and, perhaps, packaging of food products with a short shelf life. Undoubtedly, the cost of such packaging film should be minimized because its price is included in the cost of packed products.

In our study, the cheap commercial CS of technical grade (Giusto Faravelli SpA, Italy) with molecular weight (M_W) 1425 $\pm$ 35 kDa determined by SEC and DA 21% was mainly used for preparation of CS/CN films. In some rheological experiments, the high-quality CS with M_W 425 kDa and DA 11% (HMC^{+} GmbH, Germany) was also tested.

Preliminary investigations have shown that the yellowish CS powder of the technical grade with sizes of particles from 0.1 mm up to 6 mm was not completely soluble at ambient temperature in distilled water acidified with acetic acid to pH = 4. After filtering the CS solution, the undissolved particles were retained on the surface of a non-woven polyester Histar filter (Figure 6a).

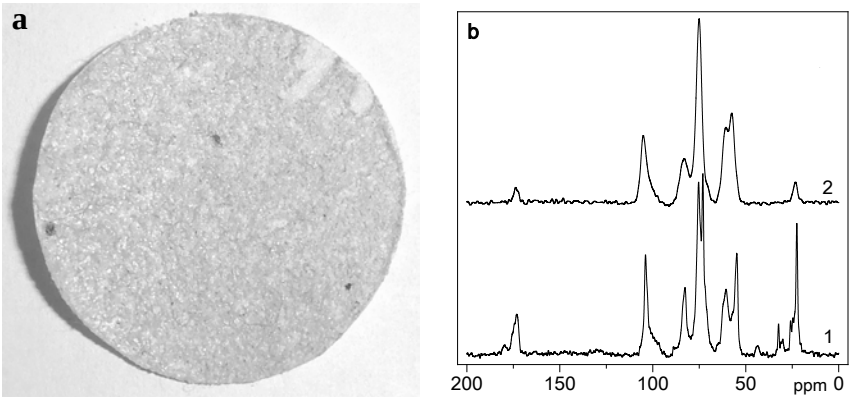

Figure 6. (**a**) Insoluble (at pH 4.0) CS particles retained on the non-woven Histar filter; (**b**) 13C CP/MAS NMR spectra of insoluble and soluble CS (curves 1 and 2, respectively) at pH 4.3.

As was determined by the solid state 13C CP/MAS NMR (Figure 6b), the insoluble particles with the DA value equal to 63% could be identified as chitin, since it is generally accepted that if the DA of the product is above 50 percent, it is characterized as chitin [12].

When prepared from raw chitosan solution, the composite CS/CN films contained additional microparticles (CM). In the SEM images (Figure 7a–c), these films looked unattractive from a commercial point of view because of both high roughness of their surfaces and morphological heterogeneity. Moreover, the films containing chitin microparticles together with CN were less mechanically stable (Table 2).

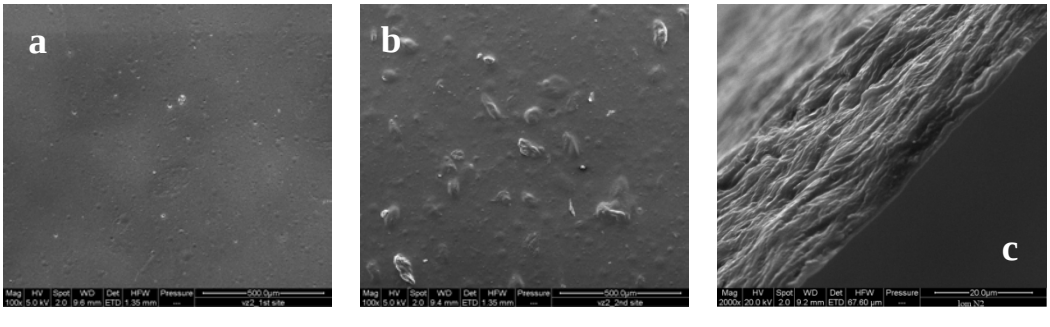

Figure 7. SEM images of (CS/CM)/glycerol film with the formulation: (40/60)/30 wt. %. (**a**,**b**) upper and bottom surfaces, respectively, (**c**) fracture of a film.

Table 2. Mechanical characteristics of the chitosan films containing CN or CM. Y—the Young's modulus; σ—the maximum tensile stress; ε—the strain at break of the film.

Film	Formulation	Y, MPa	σ, MPa	ε, %
(CS/CN)/glycerol	(40/60)/30 wt. %	3093 ± 780	61.6 ± 4.4	9.3 ± 2.0
(CS/CM)/glycerol		211 ± 35	21.6 ± 1.8	19.5 ± 2.5

Therefore, to standardize the preparation process of the CS/CN films, the commercial CS powder was sieved through a set of standard sieves and two fractions with particles' dimensions less than 80 μm and (80–140 μm) were used.

Both CS and composite CS/CN films prepared from the sieved CS powder looked rather homogeneous. Nevertheless, they still contained some microparticles undissolved at pH 4.3 at the ambient temperature. To dissolve them completely, the CS solution had to be heated at least for an hour at 60 °C.

3.3. Rheological Behavior of CS/CN Slurries

The CS/CN slurry consisting of two phases—liquid (aqueous chitosan solution) and solid (chitin nanofibrils)—behaved as a structural viscoelastic liquid, the rheological properties of which depend on multiple interactions between the components [13].

In both aqueous CS solution and CN dispersion, there are both repulsive and attractive forces. In CS solution at pH 4–4.5, repulsion of protonated GlcN rings in some parts of CS chains dominates over attraction of GlcNAc rings in other parts of chains that prohibit the fast aggregation of CS molecules. The CN dispersions tend not to settle also for very long time, due to steric hindrance and repulsion of protonated GlcN rings existing on the surface of nanofibrils. Both systems, if they stand undisturbed during some time, are stabilized through the networks of multiple hydrogen bonds and hydrophobic interactions combining all constituents in each system. In CS/CN slurry, new interactions between CS chains and CN become dominant because of the high surface potential at the liquid/solid interface [14]. All these reasons are valid for freshly prepared CS solutions and slurries if their testing was carried out for two days but no longer, since the longer-term storage has caused irreversible changes of their rheological properties.

The rheological behavior of pure CS solution, CS/CN slurries without and with added plasticizers (glycerol, polyglycerol-3 or PEG-600) or saturated solutions of calcium, magnesium or barium hydroxides was investigated in detail [15]. These investigations were the basis for optimization of the composition and concentration of CS/CN slurries that, in turn, has led to improvement of the mechanical properties of composite films.

It was found that in all tested systems, the self-assembly process has ended by formation of *reversible thixotropic gels*, the characteristic feature of which was the loss of its internal microstructure in shear and the subsequent recovery in rest. Moreover, the CN appeared to be a strong gelling agent accelerating the self-assembly of slurries. In contrast, the addition of plasticizers retarded the formation of gel. Thus, e.g., for pure CS solution, the gel point has not been achieved even after 3.5 h-rest, whereas 28 min and 80 min were needed for gel formation of non-plasticized and PEG-600-plasticized CS/CN slurries, respectively [15]. The gel point of the slurries containing ions of the alkali-earth metals depended on the type of introduced metal ion. Ions of Ca^{+2} had the highest effect on the rate of gelation: in two glycerol-plasticized CS/CN slurries with and without Ca^{+2} ions, the gel has been formed after 12 min and 22min in rest, respectively. Ions of Mg^{+2} had delayed the formation of the plasticized gel up to 49 min. The effect of added Ba^{+2} on gelling the glycerol-plasticized CS/CN slurry was considerably stronger. The same pre-shearing conditions were insufficient for breaking the internal microstructure of the gel when saturated solution of barium hydroxide instead of calcium or magnesium hydroxides were added into CS/CN slurries (Figure 8).

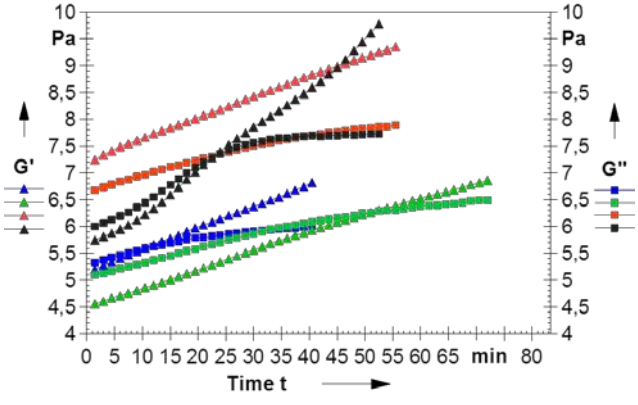

Figure 8. A self-organization process in the chitosan slurries—the storage G′ and loss G″ dynamic moduli are plotted as functions of time: the slurry CS/CN/glycerol without metal ions (▲, ■), with Ca^{+2} ions (▲, ■), with Mg^{+2} ions (▲, ■), with Ba^{+2} ions (▲, ■).

In contrast to the loss modulus G″ being a measure of the energy dissipated in the slurry under the applied shearing deformation, the storage modulus G′ characterizes the elastic deformation, at which the energy stored in a slurry under shear is then expended to return its parameters to the initial state when the shearing force is removed. For slurries containing transition metal ions, the values of the

storage modulus G′ increase in the range: CS-Mg^{+2} < CS-Ca^{+2} < CS < CS-Ba^{+2} that can be interpreted as an increase in springiness of the slurries.

The found thixotropic behavior of the slurry is a key factor at casting the CS/CN slurry on a template [15]. It characterizes the ability of the slurry to be easily applied to a surface during casting owing to breaking down of the microstructure of gel and rebuilding its viscosity in rest so that the coating does not drip and does not spread over the surface [13].

Among the most important rheological aspects describing the quality of coating is considered the *leveling*, which refers to the ability of slurry to flow laterally and diminish differences in thickness of adjacent areas of the coating, thus improving the smoothness, uniformity, and mechanical properties of CS/CN films.

The time lag for leveling of the cast slurry, after which its viscosity is restored, depends on viscosity of the slurry: the higher the viscosity, the slower leveling of coating. The time lag leveling was about 20 min for the slurries with the CS/CN proportion (70/30) wt. % plasticized with glycerol (30 wt. %), which have been used in preparation of CS/CN films.

An equally important parameter describing the rheological behavior of the slurry is the *yield stress* indicating the maximum value of the shear stress, at which the disruption of internal structure of the thixotropic CS/CN slurry will happen.

It was shown that some efforts (pre-shearing) must be applied to CS/CN slurries for disruption of their internal microstructures to force them to flow. The value of critical stress (*yield stress*) was about twice as high for the non-plasticized CS/CN slurry (6.8 Pa) than for plasticized ones with glycerol (3.9 Pa) or PEG-600 (3.1 Pa) if commercial CS (HMC$^+$ GmbH, Germany) with low-molecular-weight (Mw 374 kDa) and low DA (11%) was used (Figure 9).

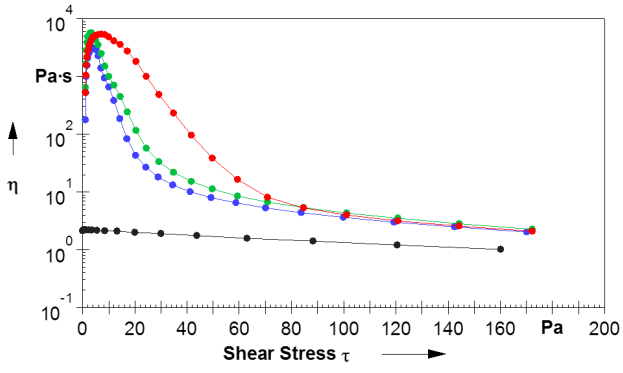

Figure 9. Steady shear viscosity vs. shear stress of the pure HMC$^+$ chitosan solution (●) and the slurries: CS/CN (●), CS/CN/PEG (●), CS/CN/glycerol (●). The (CS/CN)/plasticizer proportion was (65/35)/30 wt. %.

Values of the yield stress of non-plasticized CS/CN slurry have increased about three times (up to 18.2 Pa) if the CS (Giusto Faravelli SpA, Italy) with higher molecular weight (1425 kDa) and DA (20%) was used. For CS/CN slurries plasticized with glycerol and polyglycerol-3, the yield stress was less and equal to 14.5 Pa independently on the plasticizer type (Figure 10).

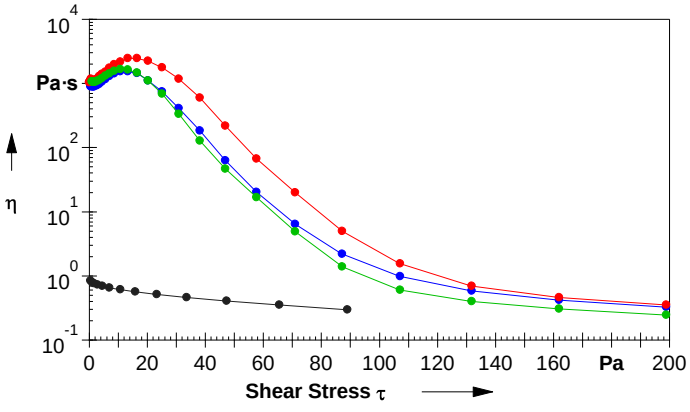

Figure 10. Steady shear viscosity as a function of the shear stress of the CS (Giusto Faravelli SpA, Italy) solution (●) and the slurries: CS/CN (●), CS/CN/glycerol (●), CS/CN/polyglycerol-3 (●). The (CS/CN)/plasticizer proportion was (70/30)/25 wt. %.

In both slurries, the CN dispersion (MAVI Sud Srl, Italy) with DA = 95% and pH = 5.12 has been mixed with CS solution. The slurries behaved as "fully" elastic fluids below the yield stress that had been manifested in their ability to absorb the shear energy as solids, which remain stationary, until the yield stress was not achieved. Above the yield stress, the internal microstructure has broken, and the slurries started to flow.

From a practical point of view, the gentle joggle or pulsed sonication of the CS/CN slurries could be effective for preventing the formation of their internal microstructure. It is reasonable to consider this recommendation for obtaining homogeneous coatings in the large-scale production of films using casting on the templates.

In this study, the homogeneous composite CS/CN films have been prepared from the slurry containing 70 and 30 wt. % of CS and CN, respectively, and 30 wt. % glycerol of total amount of CS and CN. The rheological parameters of this slurry (Figure 10, green curve) were determined as follows: the yield stress equal to about 15 Pa corresponded to the maximum steady shear viscosity about 1×10^3 Pa s.

Rheological testing provided useful information about rheological changes in time of the CS/CN slurries that allowed determination of the limit of their storage. The viscoelastic characteristics of the slurries had steadily getting worse. After their storage at 6 °C for 19 weeks, the slurries started to behave similarly to Newtonian fluids, the characteristic feature of which is the independence of their viscosity on the shear conditions (Figure 11).

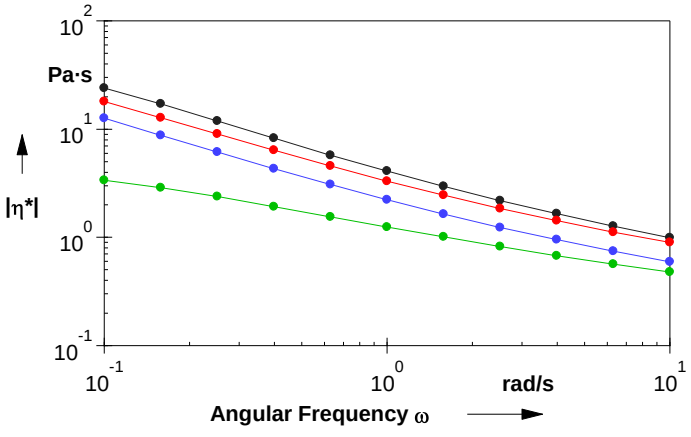

Figure 11. The absolute value of complex viscosity as a function of angular frequency of the slurry CS/CN/glycerol (70/30)/25 wt. % measured after various storage times: 1 day (●), 2 weeks (●), 6 weeks (●) and 19 weeks (●).

The absence of the rheological features typical for the physical network inside the slurries has indicated the clear evidence that CS chains had degraded into short fragments unable to form the extended microstructure of a thixotropic gel.

3.4. Composite CS/CN Films

The composite CS/CN films have been prepared by casting technique [16]. The used preparation procedure consisted of four steps (Figure 12).

Bearing in mind the effect of aging of CS/CN slurries on their rheology, its effect on the mechanical stability of the films has been also checked. It was found (Table 3) that all mechanical characteristics of the films worsened in strength the more significantly the longer the slurries were stored. Therefore, the slurries were always used as quickly as they were prepared.

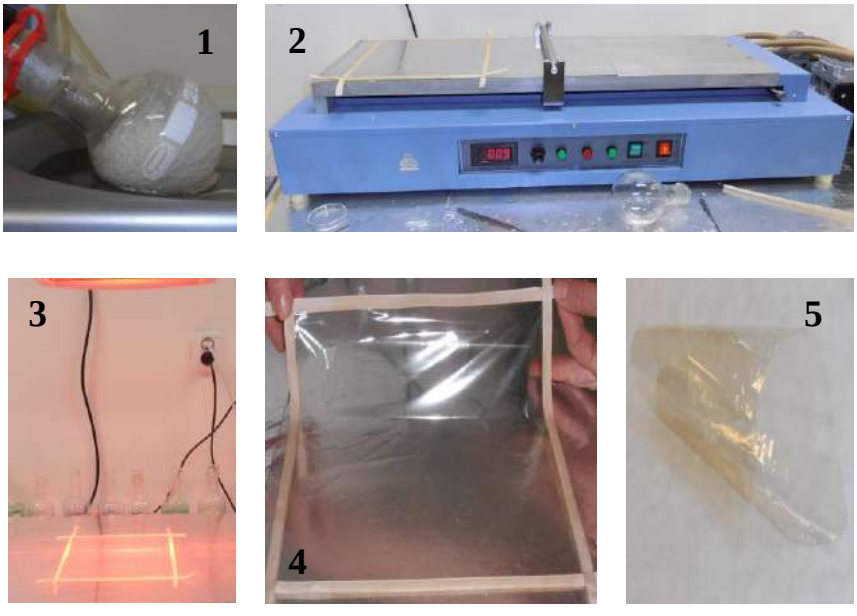

Figure 12. Visualization of the preparation steps of composite CS/CN films by casting technique. (**1**) deairing and concentrating the slurry using a rotary vacuum evaporator; (**2**) the slurry cast on a Dura Lar (Grafix Co., USA) support placed on a coater MSK AFA L800 (MTI Corp., USA) using a Doctor Blade; (**3**) drying the cast slurry by its heating at 50–55 °C using a source of the infrared light; (**4**) taking off the dried film from a support; (**5**) a died composite CS/CN film.

Table 3. Effect of the storage of a slurry on mechanical properties of (CS/CN)/glycerol films with the formulation: (70/30)/30 wt. %. I—used as prepared, pH = 4.25; II, pH = 4.43 and III, pH = 4.35—storage in fridge for 2 weeks and 1 month, respectively; The slurry II and III contained Ca^{+2} ions. The slurries II and III were additionally concentrated by vacuum evaporation prior to their casting on the supports.

Parameter	I	II	III
Y, MPa	2784 ± 582	460 ± 224	201 ± 44
σ, MPa	57.6 ± 5.9	37.8 ± 5.7	31.2 ± 4.9
ε, %	10.6 ± 2.4	25.2 ± 4.6	28.2 ± 3.2

It should be noted that vacuum evaporation used for mixing the aqueous solutions of the components, their deairing and concentrating allowed the preparation of very homogeneous slurries with desirable viscosity, which was close to the gel point but did not achieve it. This procedure took about a half of hour.

Evaporation of an excess of water from the slurry (about 25% from its weight) was carried out in a water bath with temperature at 60 °C.

The right choice of a support, on which the slurry is cast, becomes very important, if not the main factor in preparing the films of large dimensions. To avoid any defect in the dried film during its removal from the support, it is extremely important to use such a support, which will ensure easy removal of the dried film from its surface.

Some researchers were successful in preparation of experimental CS-based films having small dimensions when poly(vinyl chloride) sheets [17], polystyrene [18] or polypropylene plates [19], metallic [20] or non-stick trays [21], acrylic [22] or even Teflon [23] plates were used. Sometimes, the CS solutions were cast onto plastic [24] or glass [25] Petri dishes.

In our study, the CS/CN slurries have exhibited good adhesion to poly(vinyl terephthalate) films (Dura Lar, Grafix. Co., USA) and to stainless steel plates (Figure 13) and, at the same time, have ensured rather easy removal of the dried films from their surface by applying minimal efforts.

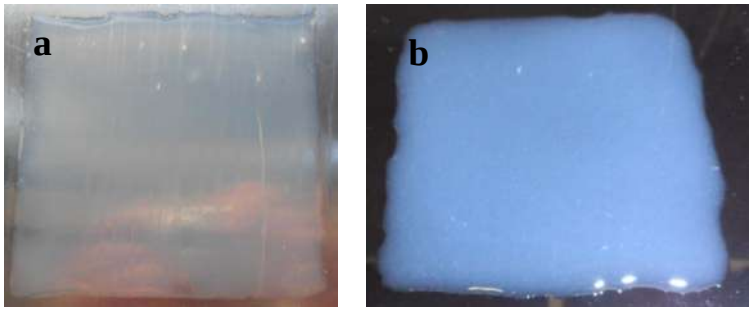

Figure 13. An optical view of the (CS/CN)/glycerol slurry at the proportion of components: (70/30)/30 wt. % cast on: (**a**) Stainless steel plate, (**b**) Dura Lar film.

The cast slurries on these supports formed uniform layers with thickness of about 0.75 mm. The thickness of dried CS/CN films plasticized with glycerol was equal to 46 ± 16 μm. For determination of the optimal formulation of the CS/CN slurry, the content of CN was increased from 3 wt. % to 40 wt. % (from the CS content). Chitin nanofibers introduced in CS solution promoted creating new spatial arrangement of chitosan chains in the dry CS/CN films, which differed considerably from the original one existing in the chitosan itself after its drying (Figure 14a–d). In aqueous acidified solution, the protonated CS chains preferred to interact with functional groups on the surface of rigid chitin nanofibrils than with each other. During the fixing on the surface of chitin nanofibers, the CS chains are packed more densely than in solution, acquiring a more energetically favorable spatial

arrangement. The reinforcing effect of CN on CS phase was ensured by a high surface potential of chitin nanofibrils with a highly developed surface (180 m^2 per 1 g of dried CN) [4]. The compatibility of CS with CN was excellent owing to similarity of their chemical structures differing only in the quantitative proportion of GlcN and GlcNAc rings and their distribution in polysaccharide chains. From the structural point of view, the CS/CN films can be considered to be composites consisting of one polymer. That is why there was no phase separation after increasing the content of CN in CS solution up to 80 wt. %. This newly formed interconnected spatial structure differed from the previous one in CS by the apparent new heterogeneity at the nano and micro level.

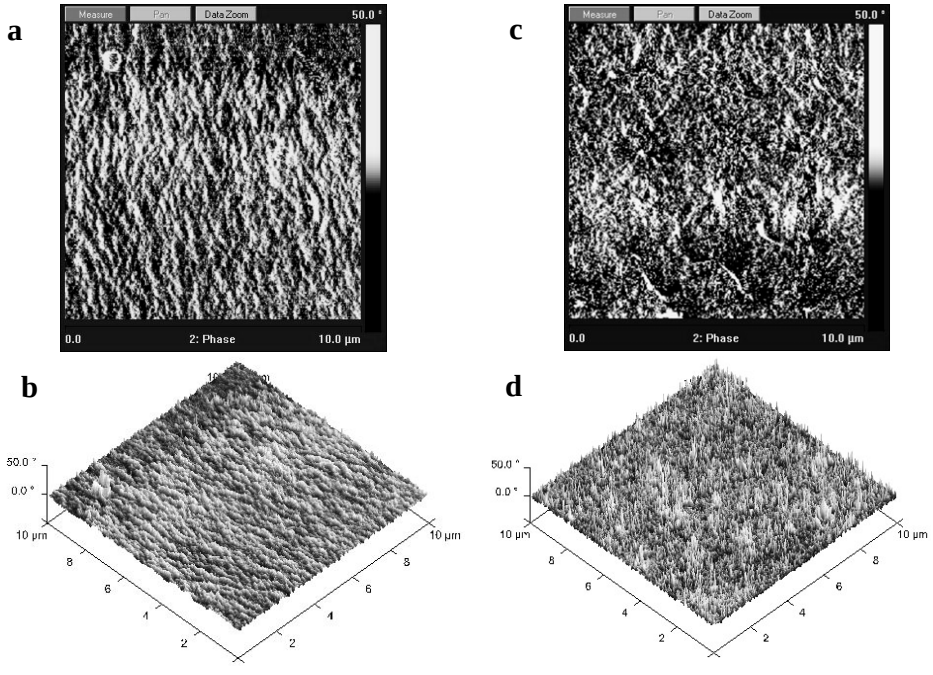

Figure 14. AFM phase images of the dry non-plasticized films: (**a**,**b**) CS; (**c**,**d**) (CS/CN) film at the proportion of components equal to (60/40) wt. %.

The increase in CN content in CS phase increased the stiffness of CS/CN films since the elastic deformation of the films has required applying stronger tensile stress at increase in CN content to a definite value (Figure 15). The ultimate tensile stress increased with CN content and achieved its maximum value for the CS/CN film with 10 wt. % of CN in CS solution. After further increase in CN content up to 40 wt. %, values of the maximum tensile stress decreased gradually. The effect of CN content on elongation of CS/CN films was the most perceptible. The value of

the strain at break of a film with 40 wt. % of CN was about half of that value of a chitosan film free of CN.

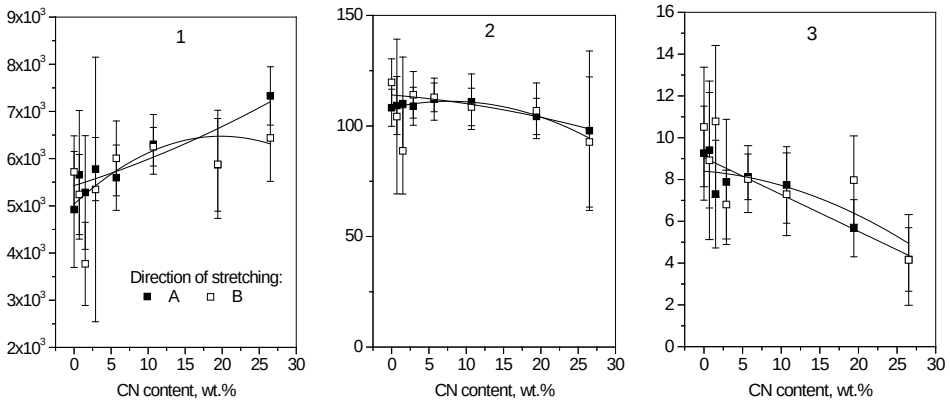

Figure 15. Effect of CN content in CS phase on the mechanical properties of non-plasticized films measured in two mutually perpendicular directions A and B of the applied tension. (1) Young's modulus; MPa, (2) Maximum stress, MPa; (3) Strain at break, %.

When the CN content exceeded 10 wt. %, the saturation limit of CS phase with CN was reached. An excess of CN was pushed out from CS phase and formed a surface-adjacent layer enriched with chitin nanofibrils (Figure 16).

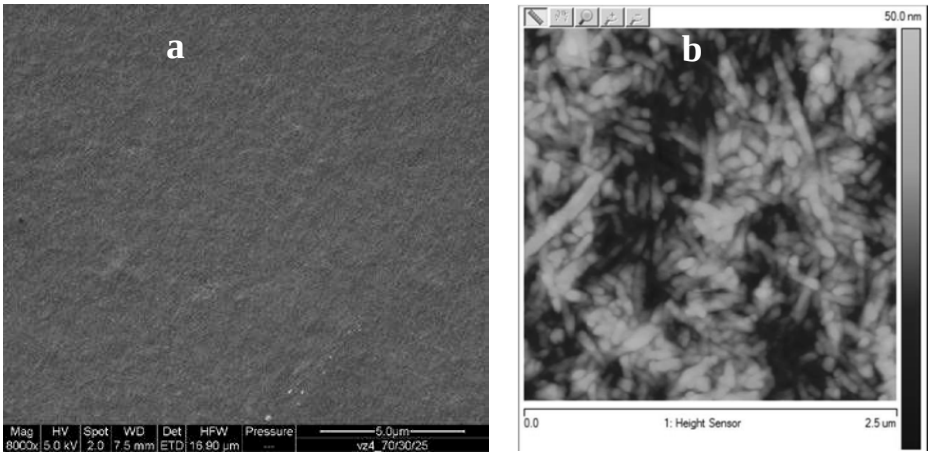

Figure 16. Images of the upper surface of a (CS/CN)/glycerol film at the proportion of components (70/30)/25 wt. % in the dry and swollen state in water. (**a**) SEM; (**b**) AFM images.

The bonds between chitosan chains and the surface groupings of chitin nanofibrils were so stable that the CS/CN films did not dissolve in water despite some excess of free molecules of acetic acid, which have remained inside the films after their drying. After dipping in water, the dimensions of CS/CN films reinforced with 30 wt. % of CN have increased only a little despite the presence of plasticizing glycerol molecules weakening interactions between the components in the films (Figure 17). However, an increase in the stiffness of the CS/CN films, especially those with high CN content, resulted in a decrease in their elasticity. Such film was more brittle.

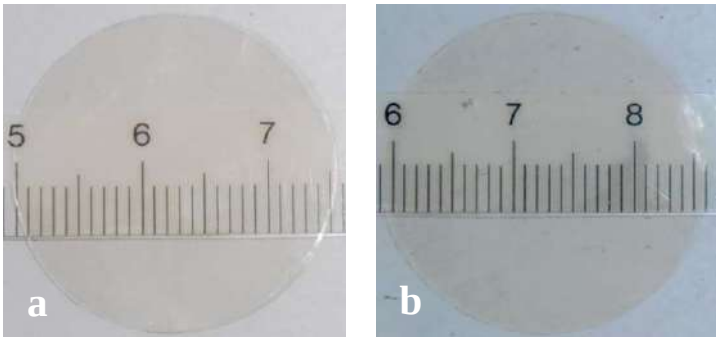

Figure 17. Dimensions of a (CS/CN)/glycerol film at the proportion of components (70/30)/25 wt. %. (**a**) dry film; (**b**) swollen film.

This problem is usually overcame by using plasticizers [26]. Small molecules of plasticizers penetrated between chitosan chains and weakened the bonds between them (Table 4). The comparison of the effect of the tested plasticizers on mechanical characteristics has shown that the elasticity of the CS/CN films increased considerably at the presence of a plasticizer and depended on both its quantity and its type.

For a film with the CS/CN proportion (85/15) wt. %, values of the elastic modulus decreased with increase in the plasticizer content in a great extent. Simultaneously, values of the ultimate tensile stress decreased for this film by about half but the values of its strain at break increased by about three-fold. It seems that it is not reasonable to increase the content of plasticizers higher than 20–30 wt. % because of dramatic worsening of all mechanical characteristics of CS/CN films.

The temperature of drying the CS/CN films was the next important parameter affecting their morphology (Figure 18) and mechanical characteristics (Table 5). The internal heterogeneity of CS/CN films became more pronounced when a cast slurry was dried using the infrared irradiation (Figure 18c,d) for half an hour instead of its drying at the ambient temperature (Figure 18a,b) for 12 h. The differences in

morphology of the dried films were observed on the micro level only. No noticeable differences in the appearance of the films were observed.

Table 4. Effect of type and content of a plasticizer on mechanical characteristics of CS/CN films with the formulation (85/15 wt. %). Support: Dura Lar film.

Plasticizer, wt. %		Y, MPa	σ, MPa	ε, %
no	0	5720 ± 350	107.5 ± 7	6.7 ± 2.4
Glycerol	20	1390 ± 600	43.0 ± 5	20.4 ± 4
_"_	30	300 ± 50	35.4 ± 4	27.6 ± 4
_"_	40	50 ± 12	17.0 ± 6	36.0 ± 4.5
pG-2	20	1835 ± 177	48.6 ± 8	25.4 ± 4.7
_"_	30	700 ± 65	36.7 ± 5	32.5 ± 5.7
_"_	40	98 ± 33	25.6 ± 5.8	44.3 ± 7.4
pG-3	20	1390 ± 100	33.5 ± 6	26.7 ± 8
_"_	30	379 ± 47	25.0 ± 5	35.0 ± 6
_"_	40	65 ± 8	24.4 ± 4	50.0 ± 4
pG-4	20	2320 ± 110	41.4 ± 4.9	13.7 ± 5
_"_	30	1380 ± 50	30.0 ± 4.5	17.0 ± 5
_"_	40	370 ± 57	28.6 ± 6	31.6 ± 7

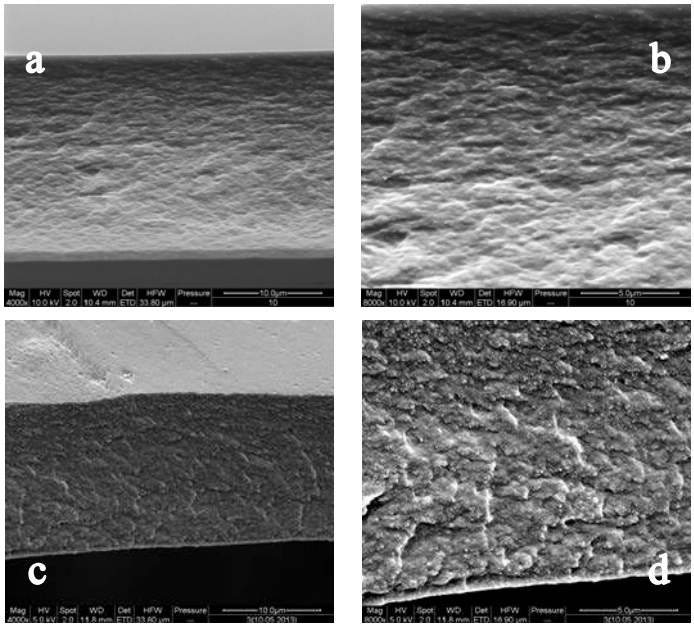

Figure 18. SEM images of fractures of (CS/CN)/glycerol films with the formulation (85/15)/30 wt. % dried at: (**a**,**b**) ambient temperature and (**c**,**d**) IR irradiation at 55 °C.

Table 5. Effect of the temperature of drying on the mechanical properties of (CS/CN)/glycerol films with the formulation (85/15)/30 wt. %.

Parameter	22–25 °C	50–55 °C
Y, MPa	1462 ± 306	2495 ± 380
σ, MPa	25.0 ± 3.6	45.0 ± 4.5
ε, %	12.6 ± 3.0	12.0 ± 3.0

Under exposition of the tested polysaccharides' films to UV light or microwave irradiation for 8 days and 30 min, respectively, the oxidative reactions were triggered inside the films. The fact of their existence was detected by analysis of UV spectra of the irradiated films (Figure 19).

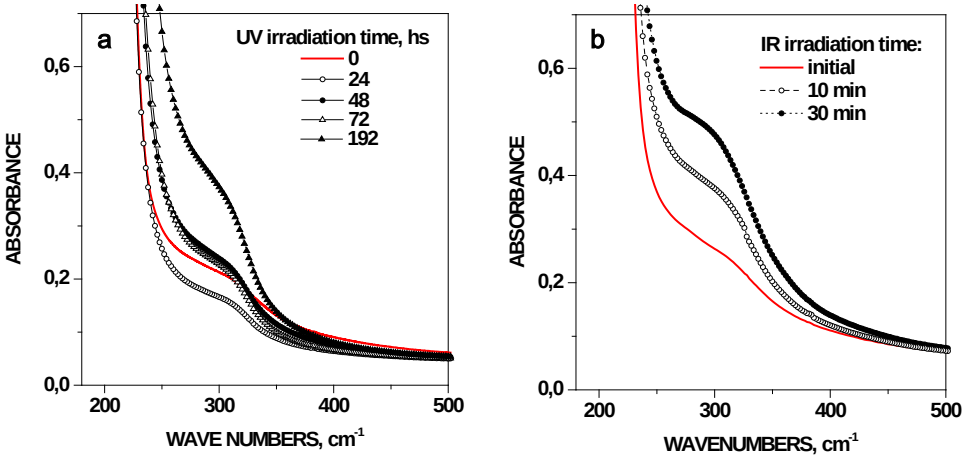

Figure 19. UV spectra of (CS/CN)/glycerol films after: (**a**) UV irradiation (0.5 N/m^2) for 192 hs and (**b**) microwave irradiation up to 30 min with the 1000 W-magneton power. The formulation of the slurries: (**a**) (40/60)/30 wt. %, (**b**) (20/80)/30 wt. %.

It is reasonable to suggest that drying the films using IR irradiation was also accompanied by accelerating the oxidative reactions in CS/CN films resulting in changes in packaging of CS chains that is manifested in morphological changes of the films (Figure 20c,d).

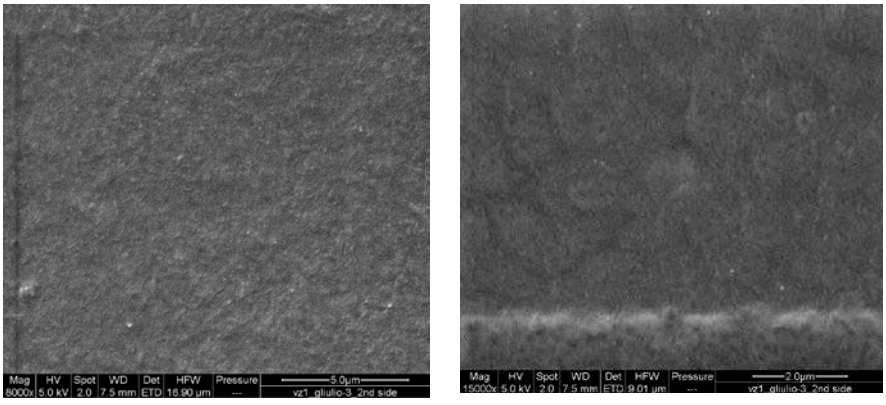

Figure 20. SEM images of the bottom surface of (CS/CN)/glycerol film with the formulation (70/30)/25 wt. % obtained by casting the slurry on a stainless steel plate.

Investigations of the degradation of CS/CN films at the elevated temperatures by thermogravimetric analysis (Table 6) has revealed that CN consisting of the densely packed chitin chains required higher temperatures than raw CS powder or reprecipitated CS. It should be noted that a sample of reprecipitated CS has lost 40% of its mass within a very narrow temperature interval with its maximum at 306 °C. This value was about 12 °C higher than the temperature maximum on a TGA curve of the raw CS. It means that to improve the thermostability of raw CS, the reprecipitation could be used to remove low-molecular-weight oligosaccharides and increase the fraction of high-molecular-weight CS. The decomposition of chitin nanofibrils occurred at temperature approximately 50 °C higher than that of CS.

It is interesting that two maximums on TGA curves of CS/CN films have been observed. The decomposition temperature of the films rose to higher values (for the first and second decomposition steps from about 304 to 311 °C and from 361 to 371 °C, respectively) with the increase in CN content from 15 wt. % to 35 wt. %. The loss of the mass of a sample decreased from 30% to 23% for the first degradation step. Simultaneously, at the second degradation step, the loss of the mass of a sample increased from about 20% to 26%. For CS/CN films having the same formulation (85/15)/30 wt. % but containing polyglycerols of different type, the higher temperature was required for decomposition of the film plasticized with polyglycerol-4 having longer molecules. For CS/CN films plasticized with polyglycerol-3, the decomposition temperature rose with an increase in the content of plasticizer at both steps of their degradation. The detailed description of TGA and DSC analysis of the composite films based on CS and CN will be published elsewhere.

Table 6. TGA analysis of CS, CN, and composite CS/CN films. T1, T2, T3 – temperature, °C. ΔW1, ΔW2, ΔW3 – loss of a sample's weight, wt. %.

Sample	Composition	T1	ΔW1	T2	ΔW2	T3	ΔW3
CS raw		294.4	47	–	–	–	–
CS *		306.0	40	–	–	–	–
CN		358.2	66	–	–	–	–
CS/CN	85/15	303.7	30	360.9	19.5	–	–
–"–"–"–	75/25	306.5	28	371.0	22.0	–	–
–"–"–"–	65/35	310.9	23	370.9	25.5	–	–
(CS/CN)/pG-2	(85/15)/30	261.5	22.5	315.4	23.5	384.5	18.0
(CS/CN)/pG-3	–"–"–"–	303.4	40.0	374.6	19.5	–	–
(CS/CN)/pG-4	–"–"–"–	304.4	41.0	385.2	21.0	–	–
(CS/CN)/pG-3	(85/15)/20	314.1	37	384.8	18.0	–	–
–"–"–"–	(85/15)/30	312.0	41.0	384.6	17.5	–	–
–"–"–"–	(85/15)/40	304.9	55.5	401.1	17.5	–	–
–"–"–"–	(85/15)/50	305.7	63.0	400.0	12.0	–	–

Analyzing the SEM images in Figure 20, everyone can clearly see that the (CS/CN)/glycerol films have denser upper and bottom layers and moreover, the film surface facing a support seems to be considerably denser than that contacting with air.

It was observed (Table 7) that not only the type of the surface (upper or bottom) of CS/CN film but also the chemical nature of a support on which it was formed remarkably affected the values of contact angles of both film surfaces. As a rule, the values of contact angles of the bottom surfaces were higher than those of the upper ones exposed to air. In contrast to the transparent glycerol-plasticized CS/CN films formed on the Dura Lar supports from poly(vinyl terephthalate), those formed on the stainless steel plates looked matte because of higher surface roughness of the latter support (Figure 20).

Table 7. Contact angles of the surfaces of glycerol- or pG-3-plasticized CS/CN films.

Support / Surface	DL	SS	DL	SS	DL	SS
	Non-plasticized		30% Glycerol		30% pG-3	
Upper	102.2 ± 4.4	105.6 ± 2.7	105.2 ± 5.1	106.5 ± 3.5	103.2 ± 4.1	104.7 ± 1.8
Bottom	107.1 ± 3.8	125.2 ± 4.8	107.0 ± 3.8	115.4 ± 2.6	106.5 ± 3.9	109.7 ± 0.6

The chemical nature of a support, on which the slurry was cast, influenced also the composition of chemical groupings on the formed film surface. The X-ray photoelectron spectroscopy analysis of both surfaces of glycerol-plasticized CS/CN films, which differed in formulations (Table 8), has revealed that the hydrophilic groupings (–C–O–, –C–O–C– and –N–C=O–) were predominantly located on the

upper surface of the films. In contrast, the bottom surfaces formed on the glass plates silanized with ethyltrimetoxysilane had more hydrophobic groupings (–C–C– and –C–H–).

Table 8. Binding energy of chemical groupings in the spectra of C 1 s electrons.

Binding Energy, eV	(CS/CN)/Glycerol Proportion, wt. %				Chemical Groupings
	(80/20)/20		(60/40)40		
	Upper	Bottom	Upper	Bottom	
285.0	29	37	28	33	–C–C–; –C–H–
285.6	9	10	10	10	$–C–NH_2$
286.6	52	46	50	47	–C–O–
288.2	10	7	12	10	–C–O–C–; –N–C=O–

The surface wettability of CS/CN films with water (Figure 21) depended on both chemical groupings on the surface of films and on the density of packaging of their structural elements.

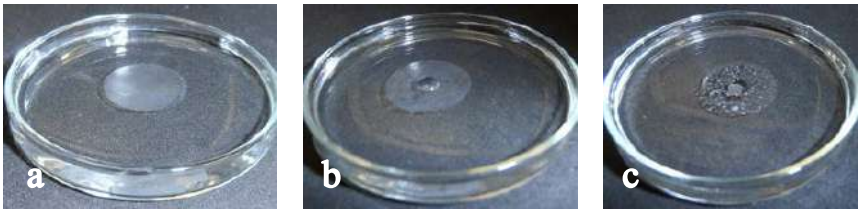

Figure 21. Wettability of a (CS/CN)/glycerol film with the formulation (75/25)/30 wt. %: (**a**) just after placing on the water surface; (**b**,**c**) after 5-min- or 2-h contact with water, respectively.

The suction of water inside such films occurred when the content of an admixture of low-molecular-weight chitooligosaccharides together with some excess of the acetic acid molecules were high and these components have transferred into water creating the channels inside the films dipped into water. This phenomenon was often observed when CS solution was heated at 60 °C for one hour to dissolve small CS microparticles for obtaining homogeneous slurry or when the slurry has been stored in the fridge for two days.

It should be noted that despite good wettability, these films did not dissolve in water and did not change their size during at least 10 days of contact with water.

Both high permeability and adsorption of water (Table 9) have been also detected for the CS/CN films containing the ions of alkali earth metals such as Ca^{+2}, Mg^{+2} or Ba^{+2}. Freshly prepared saturated solutions of hydroxides of these metals free of

carbon dioxide were added to CS/CN slurries with the aim of neutralizing an excess of the acetic acid used for dissolving of CS at preparation of the slurries.

Table 9. Adsorption of water vapors with CS/CN films.

Film	Components	Support	Composition	S_W, wt. %		$\frac{S_{W2}}{S_{W1}}$
			wt. %	Air-Dried [1]	Swollen [2]	
1	(CS/CN)/PEG-600	DL	(70/30)/25	7.6	25.2	3.3
2	(CS/CN)/glycerol	–"–"–	(75/25)/30	5.3	24.8	4.7
3	(CS/CN)/pG-3	–"–"–	(85/15)/30	7.4	20.8	2.8
4	–"–"–"–	–"–"–	(75/25)/30	6.8	33.5	4.8
5	–"–"–"–	–"–"–	(65/35)/30	5.5	32.5	6.1
6	(CS/CN)/glycerol, Ca^{+2}	DL	(70/30)/25	45.2	38.1	0.8
7	–"–"–"–	SS	–"–"–	43.9	35.7	0.8
8	(CS/CN)/glycerol	DL	(70/30)/10	8.3	22.3	2.7
9	–"–"–"–	SS	–"–"–	6.8	24.0	3.5
10	(CS/CN)/glycerol, PGPR	DL	(70/30)/30	7.2	20.0	2.8
11	–"–"–"–	SS	–"–"–	6.0	25.1	4.2
12	(CS/CN)/glycerol, PLA	DL	(70/30)/30	10.3	22.2	2.2
13	–"–"–"–	SS	–"–"–	8.4	21.8	2.6
KFC	cellulose			5.4	13.0	2.4

Comparison of the air-dried CS/CN films has shown that the water content was always higher in the films prepared using Dura Lar supports than in those obtained using the stainless-steel plates. The content of water in CS/CN films plasticized with polyglycerol-3 decreased with the increase in CN content from 15 to 35 wt. %. The CS/CN films with the surface-adsorbed polyglycerol polyricinoleate or polylactide had lower water content if the stainless-steel plates instead of Dura Lar ones have been used as the support for casting the slurry. The glycerol-plasticized CS/CN films containing calcium ions retained the highest amount of water in the air-dried state. Most of this water was bound with metal ions in their hydration shells. At equilibrium with water vapors at 25 mbar, the saturation state was not achieved for these films and they contained only 80% of water from its content in the air-dried films. In the sorption experiments, the sorption of water proceeded slowly by the films and took several hours until equilibrium with water vapors was achieved at each partial pressure. Being equilibrated with water vapors at 25 mbar, the swollen CS/CN films contained from 3 to 5 times more water than the air-dried ones. In the moistened state, the CS/CN films prepared by casting the slurry on the stainless-steel plates contained more water than those obtained by using Dura Lar supports. This difference can be explained by the structural differences of the films.

These are the structural features of the films that determine the dependence of the change in the moisture vapor transmission rate through a film on its equilibrium state at each change of the partial pressure of water vapors (Table 10).

Table 10. Moisture vapor transmission rate for the (CS/CN)/glycerol film with the formulation (70/30)/30 wt. %. The film contained 0.234 mmol of Ca^{+2} ions.

Δp, mbar	0–0.45	0.45–5.2	5.2–10.4	10.4–15.4	15.4–20.3	20.3–25.3
MVTR, g/(m^2 × 24 h)	4.1	31.6	65.1	111.5	186.1	180.1
ΔMVTR/Δp	9.1	6.1	6.3	7.2	9.2	7.1

At low water vapor pressures, the rate of MVTP change (ΔMVTR/Δp value) was high since water molecules have adsorbed onto the outer surfaces and on the surface of the available pores of a dry film free from any adsorbed water at zero pressure of water vapors in the apparatus chamber at the beginning of the sorption process.

When the partial water vapor pressures achieved 15.4 mbar the second increase in the rate of MVTR changing occurred due to the changes of packaging the CS chains and the increase in the distance between them in the moistened film.

The difference in distance between CS chains in the dry and swollen films influenced their permeability for gases (Table 11). The permeability of hydrogen, oxygen and nitrogen through a swollen glycerol-plasticized CS/CN film was about triple higher than that of the dry film. The molecules of carbon dioxide penetrated through the swollen film about five times faster compared with the dry film. Comparison of the kinetic diameters and molecular mass of gases with the rate of their transfer through a CS/CN film allows us to conclude that the decisive factors defining the film permeability (P) for gases are their diffusivity (D) and solubility (S) controlled by numerous interactions of the diffusing molecules inside the CS/CN films.

Table 11. Permeability of a (CS/CN)/glycerol film with the formulation: (70/30)/25 wt. % for gases.

Film	P	H2	O2	N2	CO2	CH4
Dry	barrer	0.04	0.012	0.005	0.142	0.01
Swollen		0.137	0.038	0.013	0.761	–
Kinetic diameter	Å	2.89	3.46	3.64	3.30	3.8
Molecular mass	D	2	32	28	44	18

Analysis of the transfer of oxygen through the composite films with the same content of CS and CN but containing various plasticizers has elucidated the correlation between the structure of the composite films and their permeability for gases.

The most permeable for oxygen were the films plasticized with PEG-600 (Table 12). The permeability and the diffusion coefficient of oxygen in these films were about three and five orders, respectively, higher than those of the films plasticized

with glycerol. In contrast, the solubility of oxygen in the former films was about three orders lower than that in the latter ones. High diffusivity together with low solubility of oxygen in the PEG-plasticized films ensured their high permeability. The observed differences in the transport performance of the films were determined by the difference in their spatial structures, on which the different plasticizers were affected in different ways.

Table 12. Oxygen transport through dry plasticized CS/CN films with the formulation: (70/30)/30 wt.% * 1 barrer = 1×10^{-10} cm^3 (STP) $\times$ cm /($cm^2 \times s \times$ cm Hg) [27,28].

Film	P		D	S
	10^{18} mol/(m×Pa×S)	barrer*	10^{15} m^2/s	10^6 mol/(m^3×Pa)
(CS/CN)/PEG-600	172	0.512	1.21×10^6	0.14
(CS/CN)/glycerol	0.18	0.00054	2.25	80.7
(CS/CN)/pG-2	1.28	0.0038	14.4	88.7
(CS/CN)/pG-3	1.21	0.0036	25.9	46.8
(CS/CN)/pG-4	0.13	0.00037	2.43	51.7

For decreasing wettability and permeability of CS/CN films for water vapors, the hydrophobization of their surfaces was carried out.

This procedure was performed by dipping the films into dioxane solution of polylactide (PLA) with molecular mass of 7.7 kDa or polyglycerol polyricinoleate (PGPR) for half an hour. Both surfaces of the plasticized CS/CN film treated in such a way have acquired their water-repellent properties (Figure 22) and simultaneously their mechanical characteristics were considerably improved.

The effect of surface modifiers on the mechanical characteristics of (CS/CN) films containing various low- and high-molecular-weight substances are summarized in Tables 13–16. From the results (Table 13), we can get an idea of the effect of glycerol, gelatin, and CN on the magnitudes of the elastic modulus (Y), the ultimate stress (σ) and the strain at break (ε) of the CS-based films. In contrast to glycerol being the disintegrating agent for the CS phase, gelatin molecules have combined the CS chains promoting their tighter packaging but the introduction of glycerol in the CS/gelatin slurry-2 caused another disintegration of the formed intermolecular bonds.

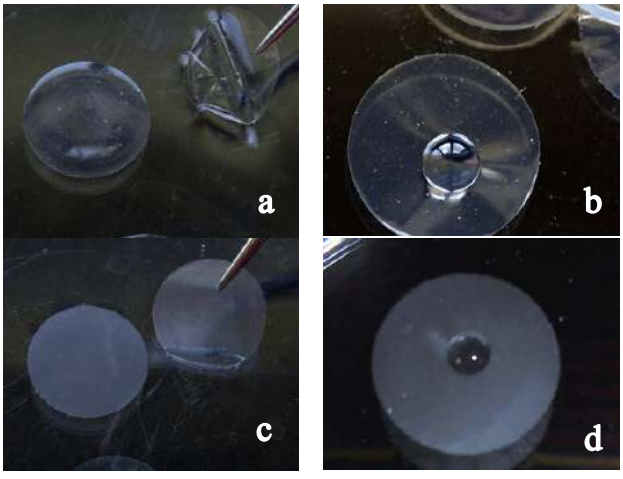

Figure 22. Photos of PLA-modified (CS/CN)/glycerol film with the formulation: (70/30)/30 wt. %. The slurry was cast on: (**a**,**b**) Dura Lar support; (**c**,**d**) stainless steel plate.

Table 13. Mechanical characteristics of CS-based films with different formulations.

Slurry	Components	Formulation	Y, MPa	σ, MPa	ε, %
1	CS/glycerol	70/30	1442 ± 55	34.0 ± 3.5	18.8 ± 2.9
2	CS/gelatin	90/10	3702 ± 129	69.9 ± 3.3	4.9 ± 2.2
3	(CS/gelatin)/glycerol	(90/10)/30	1714 ± 79	35.5 ± 4.2	16.7 ± 4.4
4	[(CS, gelatin)/CN]/glycerol	[(90/10)/25]/15	3000 ± 164	55.3 ± 5.0	14.3 ± 2.5
5	–"–"–"–"–"–	[(90/10)/25]/25	1263 ± 544	36.9 ± 6.1	16.2 ± 4.4
6	–"–"–"–"–"–	[(90/10)/30]/30	325 ± 48	33.3 ± 7.1	18.7 ± 3.9

Table 14. Mechanical characteristics of (CS/CN/glycerol films with the formulation: (70/30)/30 wt. % with 0.234 mmol $Ca(OH)_2$ and 0.1 mmol $Ca_3(PO_4)_2$ or with 10 wt. % gelatin from CS amount.

Additive / Parameter	Gelatin			$Ca(OH)_2$, $Ca_3(PO_4)_2$		
	None	PLA	PGPR	None	PLA	PGPR
Y, MPa	634 ± 95	3993 ± 447	4032 ± 264	176 ± 42	670 ± 246	1363 ± 868
σ, MPa	36.1 ± 4.3	61.1 ± 8.6	58.8 ± 1.9	20.3 ± 5.8	53.8 ± 11.2	42.9 ± 6.7
ε, %	20.3 ± 2.2	11.8 ± 2.9	12.2 ± 1.4	19.7 ± 5.8	20.6 ± 5.2	15.4 ± 2.7
θ, °	93.2 ± 1.3	95.1 ± 3.1	99.3 ± 3.2	94.1 ± 0.1	96.8 ± 2.4	100.9 ± 2.4
S_W, %	23.8	ND	ND	ND	37.2	ND

Chitin nanofibers, which are powerful reinforcing agents, have leveled the disintegrating effect of the plasticizer at its small amount in slurry 4 (Table 13). However, all mechanical characteristics of the composite films have dramatically worsened with increasing content of glycerol in slurries 5 and 6.

The mechanical characteristics of the composite films obtained from slurry 6 were practically restored after their modification with polylactide (PLA) or polyglycerol polyricinoleate (PGPR) (Table 14).

Table 15. Mechanical characteristics of (CS/CN)/glycerol films with the formulation: (70/30)/30 wt. % with 5 wt. % monolignols or 1 wt. % nanolignin from CS amount. Surface modifier: PLA—polylactide, 7.7 kDa or PGPR—polyglycerol polyricinoleate.

Additive / Parameter	Monolignols			Nanolignin		
	None	PLA	PGPR	None	PLA	PGPR
Y, MPa	293 ± 66	3503 ± 457	3236 ± 472	455 ± 56	3883 ± 405	4027 ± 400
σ, MPa	26.3 ± 3.6	57.1 ± 9.8	45.9 ± 9.8	24.1 ± 3.7	51.4 ± 4.9	50.9 ± 4.9
ε, %	20.8 ± 3.2	13.1 ± 2.7	14.7 ± 1.6	14.6 ± 2.4	10.9 ± 2.2	12.9 ± 2.0
θ, °	96.0 ± 1.7	96.5 ± 1.2	104.9 ± 3.2			
S_W, %	Not determined		21.5			

Table 16. Mechanical characteristics of (CS/CN)/glycerol film with the formulation: (70/30)/30 wt. % with 0.178 mmol Mg^{+2}, 0.234 mmol Ca^{+2} or 0.321 mmol Ba^{+2} ions.

Modifier / Parameter	PGPR			PLA		
	Mg^{+2}	Ca^{+2}	Ba^{+2}	Mg^{+2}	Ca^{+2}	Ba^{+2}
Y, MPa	3987 ± 134	2807 ± 186	2535 ± 506	3940 ± 200	3597 ± 271	3034 ± 262
σ, MPa	67.2 ± 7.2	55.1 ± 5.6	49.6 ± 9.8	65.1 ± 4.7	62.9 ± 5.2	55.9 ± 5.6
ε, %	9.9 ± 2.4	13.0 ± 2.5	15.6 ± 2.4	9.5 ± 2.4	11.8 ± 1.5	13.7 ± 2.5

The mechanical properties of CS/CN films prepared from slurry 6 (Table 13) have been improved considerably after modification of the films with PGPR or PLA. The values of the elastic modulus increased six-fold compared with unmodified film. The values of the ultimate tensile stress increased about twice while simultaneously decreasing the strain at break of both modified films.

Strengthening of the films containing mineral substances has also occurred but the values of their elastic modulus and strain at break changed considerably less after modification with PLA and PGPR. In both cases, the increase in the values of contact angles was observed, especially for films modified with PGPR. It should be noted that the adsorption of PLA and PGPR within the plasticized CS/CN films was probably accompanied by the extraction of the molecules of both acetic acid and glycerol from the films into dioxane.

Practically the same changes in mechanical characteristics of CS/CN films containing monolignols and nanolignin occurred after their modification with PLA or PGPR (Table 15).

It is interesting that the order of strengthening of CS/CN films containing ions of the alkali earth metals coincided with the order of increasing their ionic radii equal

to 66 pm, 99 pm and 134 pm for Mg^{+2}, Ca^{+2} and Ba^{+2}, respectively. The highest and lowest values of the elastic modulus and the ultimate tensile stress have been observed for PGPR- and PLA-modified CS/CN films containing Mg^{+2} and Ba^{+2} ions. The most strengthened films with Mg^{+2} ions have the lowest values of strain at break (Table 16).

Commercial wrapping paper from cellulose used for food packaging and PLA- or PGPR-modified plasticized CS/CN films named CHITOPACK have the comparable values of elastic modulus and ultimate tensile stress (Table 17). However, elasticity of CHITOPACK films are about 3–4 times higher than that of commercial paper such as KFC paper widely used in fast food restaurants for packaging of sandwiches.

Table 17. Comparison of the mechanical characteristics of the commercial films for food packaging with PLA- or PGPR-modified (CS/CN)/pG-3 films with the formulation: (70/30)/25 wt. %.

Film	Composition	Surface Modifier	Y, MPa	σ, MPa	ε, %
1	CHITOPACK	PGPR	4792 ± 496	81.2 ± 7.5	9.7 ± 2.8
2	–"–"–"–"–	PLA	4891 ± 594	74.7 ± 5.1	7.7 ± 2.2
3	Packaging paper	unknown	4489 ± 136	44.8 ± 5.7	2.1 ± 0.7
4	KFC paper	unknown	4891 ± 211	71.8 ± 6.8	2.3 ± 0.3
5	DOMOPACK	none	196 ± 23	15.6 ± 1.8	623.0 ± 111

4. Conclusions

Summarizing the results of the research, it should be concluded that the native linear polysaccharide polymer extracted from crustaceans' carapaces in the form of chitin nanofibrils (CN) has proved to be a potent "physical crosslinker" forming numerous stable noncovalent bonds with chitosan chains by such a way that the CS/CN films do not dissolve in contact with water for a long time without any additional crosslinking.

It was found that there was a limit of saturation of CS phase with CN at about 10 wt. %-content from CS amount in slurry. At higher CN content, the unbound CN were concentrated in the surface-adjacent layer with higher density of packaging components because some chitosan chains present between chitin nanofibrils.

The CS/CN slurries have good compatibility with various low- and high-molecular-weight substances such as ions of the alkali earth metals (Mg, Ca, Ba) and monolignols, nanolignin and gelatin. These substances exhibited some reinforcing effects on the CS chain community. In contrast, the plasticizers disintegrated the interactions between CS and CN in the films. Therefore, their content should be limited by 20–30 wt. %.

The surface modification of CS/CN films by sorption immobilization of PLA or PGPR exhibited great effect on improvement of both mechanical stability and hydrophobicity of CS/CN films. This finding opens the wide avenue for optimizing characteristics of the films in the manner similar to that used by nature in the creation of a polysaccharide-mineral nanocomposite having a hydrophobic layer consisting of hydrocarbon'' molecules on the surface of crustaceans' carapaces.

Chitosan is a very sensitive and malleable polymer, the properties of which are strongly influenced by such factors as temperature, conditions and duration of storage, exposure to light, infrared, ultrasonic or microwave irradiation, intensity of stirring and acidity of its solutions, the microheterogeneity and chemical nature of a support used for casting the CS/CN slurries, the conditions and temperature of their drying, and subsequent hydrophobization of the obtained films. It should be emphasized, that in addition to factors mentioned above, it is extremely important to control the quality of raw materials in order to produce of the CHITOPACK packaging films with reproducible characteristics.

The formed CS/CN composite films seem to be promising candidates for production of completely biodegradable films comparable in their mechanical stability with commercial wrapping paper that is used nowadays for one-off packaging of some food products. Indisputably, the innovative CHITOPACK films will gain an extremely widespread in the future, especially, in the Floating Cities [29] owing to easy biodegradability of the films' components and their bio-compatibility with the Environment.

Author Contributions: Preparation of chitin nanofibrils/chitosan composite films, analysis of the results and coordination of experimental work (Galina Tishchenko); coordination of the research and preparation of chitin nanofibrils (Pierfrancesco Morganti and Marco Stoller); measuring and analysis of mechanical properties of the composite films (Ivan Kelnar and Ludmila Kaprálková); investigation of rheological properties of chitin nanofibrils/chitosan slurries (Jana Mikešová); thermogravimetric analysis and differential scanning calorimetry of the composite films (Jana Kovářová); investigation of the effect of temperature and copper ions on crystallinity of chitin nanofibrils (Jindřich Hašek and Radomír Kužel); determination of molecular weight of chitosans by size-exclusion chromatography (Miloš Netopilík); SEM and TEM of the surfaces and fractures of composite films (Ewa Pavlová); AFM analysis of the morphology of chitin nanofibrils and surfaces of the composite films in the swollen state in water (Eliška Chanová) and in the dry state (Milena Špirková); measuring the sorption of water vapors, gas permeability and contact angles of composite films (Libuše Brožová); FTIR spectroscopy of chitosan nanofibrils, chitosans and composite films (Michal Pekárek); the solid state 13C CP/MAS NMR of chitosan nanofibrils, chitosans and composite films (Libor Kobera); synthesis of polylactides (Dana Kubies); surface modification of the composite films by PLA and PGPR (Zdenka Sedláková).

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Baby Diapers Past and Present: A Critical Review

Pietro Febo and Alessandro Gagliardini

Abstract: Disposable baby diapers are a popular consumer product, the use of which contributes nearly 77 million tons of solid waste to landfills, with a degradation period of at least 500 years! Moreover, their use continues to increase worldwide. More than 200,000 trees are lost each year to their manufacture in the US market alone, with a consumption of 3.4 billion gallons of fuel oil, which contributes to the production of greenhouse gas emissions, furthering the environmental strain due to the Earth's climate changes. It is therefore necessary to change the way these products are consumed and to find more eco-compatible solutions, by using biodegradable polymeric plastics and/or re-usable cloth diapers. This chapter reports the historical evolution of baby diapers, its global market, the connected environmental problems, and the efforts that are being made in order to create bioactive and biodegradable baby diapers.

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1. Introduction

The continuous increase of the world's population and the high level of human development has created a negative ecological footprint (i.d. 1.2 kilograms per person per day), with an annual cost in natural capital degradation estimated at US$4.7 trillion/year [1,2]. Thus, the current global waste levels are approximately 1.3 billion tons/year and are expected to increase to around 2.2 billion/year by 2025, i.e., from 1.2 to 1.42 kg per person per day in the next 15 years.

Managing waste properly is, therefore, essential not only for building sustainable and livable cities and developing countries, but also for reducing greenhouse emissions and natural environmental disasters, the latter of which have seen their frequency increase year by year. For this purpose, it is interesting to underline that the majority of waste is produced by food production, distribution and consumption, estimated to be around 0.5/1 billion tons/year and by disposable baby diapers that reached a worldwide figure of 3.5 million tons/year. Thus, it is necessary to create a sustainable chain of food and to produce baby diapers made of biodegradable and natural polymers in order to achieve zero waste, driving industrial changes towards a new techno-economic system, the so-called bio-economy.

Technological advances, in fact, are set out to replace finite resources and conventional industrial processes with procedures and components that are biologically derived. These innovative bio-based processes, expected to be more

sustainable because of their use of renewable resources and their decreased levels of CO_2 emissions, should ensure economic growth as well as contributing to the achievement of environmental and climate goals.

In conclusion, promoting a more inclusive and sustainable industrialization by using natural biopolymer obtained from waste materials, will foster innovation and provide access to more affordable economic growth through the development of new markets and employment potential. New ways of producing biodegradable and reusable baby diapers could be part of these innovative processes.

2. The Diaper's Story

From time immemorial, there has been a need to protect babies due to their physiological needs; infants were supposed to be wrapped in swaddling bands in many societies and since antiquity. The swaddling bands were made of strips of linen or wool that were wrapped tightly around each limb and then crosswise around the body (Figure 1).

Figure 1. Different types of baby diapers used in different periods [3].

After their use, the diapers were seldom washed. They were usually just hung by the fireplace or outside to dry and then used again.

Between the end of the 19th and the beginning of the 20th century, infants in Europe and North America started wearing what could be considered the prototype of the modern diaper. A square or rectangle of linen, cotton flannel, or stockinet was folded into a rectangular shape and held in place with safety pins. Such new diapers were originally made with white cotton or linen fabric (or similar), which are absorbent natural materials (Figure 2).

Figure 2. Linen fabric and similar natural materials [3].

It seems that the first mass-produced cloth diapers were introduced by Maria Allen in 1887 in the United States [4–7].

At the beginning of the 20th century, many mothers had concerns related to the negative effects caused by diapers such as rashes or redness of the skin (Figure 3).

Figure 3. Diaper rash (diaper dermatitis) [8,9].

Diapers were also associated with the presence of bacteria, viruses and fungi. Concerned mothers understood the need to eradicate, or at least be able to control them. The mothers began using boiled water in order to reduce the common rash problem. The identified process consisted in putting used diapers in a big pot of

boiled water; however, this required great amounts of energy and time. Skin rash was a serious problem in those days.

It is presently still not yet clear who can be credited as the real inventor of the disposable diaper. Current knowledge establishes that the first disposable diaper concept was most probably made by using unbleached craped cellulose tissue just after the second world war in Europe and specifically in Paulistrom, Sweden.

A few years later in the United States, a Westport housewife named Marion Donovan invented the Boater, a waterproof covering for cloth diapers (Figure 4) [10].

Figure 4. The Donovan Boater [11].

Her first model of the disposable diaper was made of shower curtain plastic into which a conventional cloth diaper was inserted. She obtained four granted patents for the designs, including the use of plastic snaps that replaced the traditional and dangerous safety pins.

Following this new innovative design, the first disposable diaper made with non-woven fabric was created in 1949 in the USA. In the same year, a British mother developed a two-piece disposable diaper. Following this, disposable diapers with a rectangular one piece diaper were invented, followed by the launch of the roll diaper (Figure 5).

Figure 5. The Roll Diaper [12]. For a timeline of major diaper development, see reference [13].

Following these years, the disposable diaper was still considered a luxury item and used only for special occasions such as vacation trips and the like.

The first truly disposable diapers were made using a very simple rectangular design. The absorbent core structure was made of several layers of tissue paper, and using a plastic film with no tape on the outside, which came with the product.

In 1957, Mölnlycke entered the market with a product made of paper pulp encapsulated in tissue and surrounded by a knitted net. The sanitary napkin, on the other hand, experienced a rapid growth in the European and North American markets. It was not until the end of this decade when Vic Mills, who worked for the Procter and Gamble company, invented "Pampers", as he was looking for better products to use for his baby grandson. The diaper was, however, not launched on the market until 1961 [14].

From the 1960s onwards, the disposable diaper evolved quickly as the industry was able to gauge mothers' needs. Tissue was replaced with pulp a decade after the first disposable sanitary napkins arrived on the market. In 1966, Pampers launched

a new C-fold design and by 1969 they initiated a third sizing option. A typical commercial diaper machine ran at speeds of 150 diapers per minute.

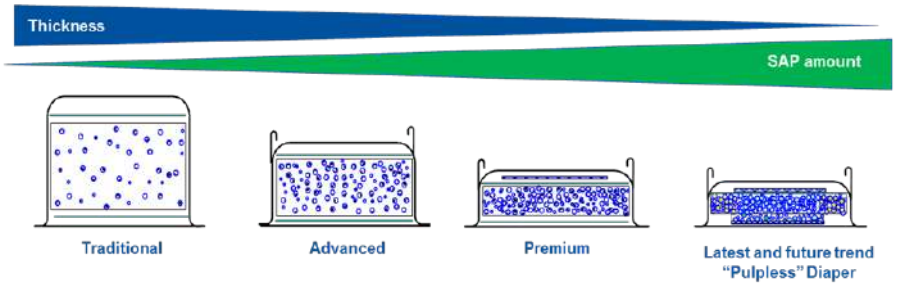
The 1970s proved to be the literal baby boom for the disposable diaper industry in developed countries and even in some other, less developed areas of the world. Competition between Procter and Gamble and Kimberly Clark to gain control of the world diaper market resulted in quick diaper design improvements and lower prices for the consumer. In 1976, Kimberly Clark introduced its shaped Huggies diapers. Lateral elastomeric was used at the end of the decade by most producers in an attempt to improve the fit.

Then, in 1982, Unicharm introduced its concept of SAP (super-absorbent) [15] in Japan, following its use in sanitary napkins.

As of 1985 and to this day, there are huge and continuous developments in the diaper's structure and composition. Today's market allows for several companies producing different shapes and product designs. As reported in Table 1, the ratio of cellulose to pulp/SAP has significantly changed over the years in combination with the reduced thickness of the product.

Table 1. Development in the structure of baby diapers over the years.

~ 1980	1990/1995	2000/2012	Present / Future



3. Technical Requirements and Performance

In today's global market, there are several types and structures of disposable diaper products designed to satisfy the needs of all babies in all countries (Figure 6).

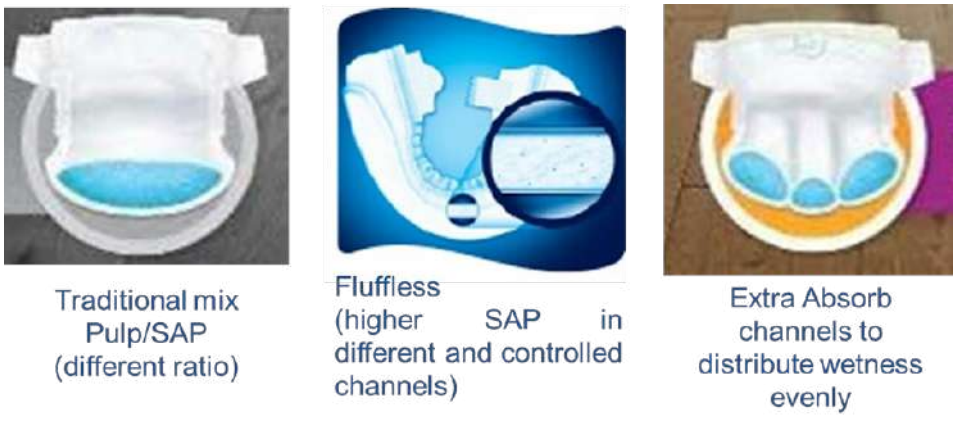


Figure 6. Different types of baby diapers used currently and globally.

There are numerous different factors related to, among others, country, culture, religion and baby age that can affect the type of diaper. However, there are some needs that are universal:

- a meaningful ratio between product cost and quality; and
- no side-effects such as skin dryness or rashes.

These differences incite the different diaper manufacturers to constantly research and improve on their current designs in order to reflect customer needs.

In addition to the dryness performance of diapers, all companies are paying attention to developing and delivering a new generation of diaper products, paying particular attention to including minor details such as the softness, gentleness, sustainability and naturalness.

Figure 7. Safety and security of baby diapers according to publicity claims.

As reported in Figure 7, the safety and security of baby diapers are actually based on eliminating the use of some components considered to cause allergies and sensitivity problems. However, the real issue at hand is the necessity to make these products 100% bio-degradable, and to ensure that they do not use fossil materials for the sake of later generations. Thus, future baby diapers should be obtained from agricultural waste and the industrial by-products necessary to produce the natural bio-polymers that are indispensable to making disposable diaper components.

In this way, we could eliminate the many environmental problems linked to diapers and produce skin-friendly and environmentally-friendly products, thus safeguarding the ecosystem and biodiversity of our planet.

4. Environmental Problems Linked to Diapers

An infant requires up to 7000 diaper changes before leaving diapers behind. This typically requires 300–600 cloth diapers from birth to potty training, adding roughly 14 kg of cotton to landfills. In addition, the use of cloth diapers also entails other costs, such as greater water and energy use: about 76,000 liters of water are needed to launder diapers for one infant. The 450 billion disposable diapers used each year contribute to nearly 77 million tons of solid waste to landfills, and a disposable diaper takes at least 500 years to degrade [16], (Figure 8).

Figure 8. Disposable baby diapers in a landfill [17].

Efforts are being made to reduce these impacts. In 2000, the Mexican company Absormex created a disposable bioactive diaper that degraded 200 percent faster than ordinary disposables. The technology is based on a catalyst additive added to the plastic to enhance biodegradation [18,19]. However even if this diaper has been demonstrated to pass the ASTM D883-99 test, there are still many doubts about the times required for degradation under particular conditions, e.g., in anaerobic conditions.

Another approach currently under investigation is diaper recycling. The idea behind this is to separate a diaper's components (essentially plastic and organic compostable matter) in a process that neutralizes the potential biological hazard, e.g., by sterilizing the used diaper [20–23].

Recently, biodegradable and compostable bio-based resins such as Ecovio [20] and Mater-bi [21] have been commercialized. These resins could be a suitable substitute for the polyolefins and polyesters currently used in diapers.

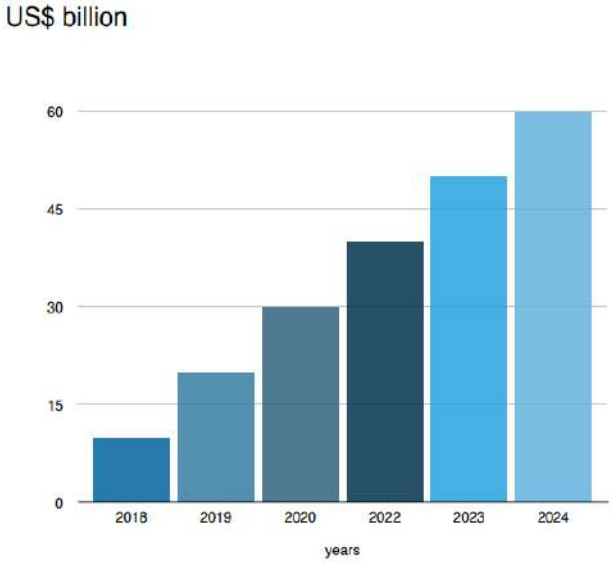
5. What About the Global Baby Diaper Market?

Different market analyses [22,23] have projected that the global market for baby diapers should reach around US$60 billion by 2024 (Table 2), driven by the growing number of young mothers in the workforce. Further predictions indicate a decline in diaper prices as a result of the mass commoditization of the product, its increasing use to maintain hygiene and prevent rashes on babies' skin, the launch of smaller pack diapers, and a growing preference for diaper pants over open diapers.

Additionally, in developing countries, increasing birth rates, rapid urbanization and continuously improving economic conditions have fueled the growth of the baby diaper industry. Moreover, this market is also benefiting from growing awareness of the convenience offered by diapers in markets like India, South Africa, Mexico, Venezuela and Turkey, due to the ubiquity of parenting websites, baby blogs and social media. Thus, while economies such as the USA, the EU and Japan are characterized by maturing conditions due to declining birth rates, Asia Pacific represents the largest and fastest growing market worldwide. Furthermore, Europe was the largest regional producer market in 2013, followed by Asia Pacific, which is expected to have the fastest growth of 8.3% during the forecast period. Still, disposable diapers have exhibited the largest market share in North America owing to environmental regulations and the adoption of eco-friendly diapers in the American market.

As a result of the abovementioned factors, biodegradable diapers are expected to reach their highest growth rate during the forecast period [24].

Table 2. Baby diaper global market forecasts (2018–2024).



6. Conclusive Remarks

The demand for baby diapers depends highly on two factors [24]: fertility rates and the penetration of the diaper market across geographic areas. As an example, in Africa the fertility rate is high, but the market penetration rate is low, hence the sale of diapers in the region is also low.

In North America and Europe, the penetration of the diaper market is high, but the fertility rate has steadily decreased. However, the worldwide sensibility leaning toward more environmentally friendly practices is continually growing; therefore, we expect that the future market will see a higher growth of biodegradable and eco-friendly baby diapers.

Thus, it is necessary to increase research studies to recover and produce innovative bio-composites made by natural polymers produced through the use of waste materials and sustainable industrial processes.

This is our dream, together with all the scientists involved in the EU research project PolyBioSkin.

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Production of Electrospun Nonwoven Materials as a Blending of Chitin Nanofibrils and Other Natural Polymers

Angelo Chianese and Paola Del Ciotto

Abstract: Electrospinning is an electrostatic fiber fabrication technique that has evinced much interest and attention in recent years due to its versatility and potential for applications in diverse fields. The sub-micron range spun fibers produced by this process offer various advantages, like high surface area to volume ratio, tunable porosity, and the ability to manipulate nanofiber composition in order to get a set of desired properties and function. This chapter deals with a preliminary investigation on the production of nonwoven materials obtained by the electrospinning of a blending of nanochitin fibrils and lignin, by using polyethylene oxide as the solvent. The adopted blend was carefully prepared as sol-gel material at a suitable temperature, mixing conditions, and time of ageing. The blends were characterized by the measurements of viscosity and electroconductivity. Many factors may influence the quality of the electrospun product, among them the main ones include the following: the applied voltage between the two electrodes, the distance between the tip and the collector, and the rotation of the collector. In this work, these operating parameters have been investigated using a preliminary factorial analysis experimental campaign. For this purpose, a pilot scale electrospinning machine (model Nanospider NS LAB 500 supplied by Elmarco) was used. The characterization of the produced electrospun nanofibers was performed by using the Field Emission Scanning Electron Microscope Auriga Zeiss. This instrument provides images of the nanofibers with an accuracy of less than 10 nm and allows the determination of the chemical composition by using the microanalysis device EDS 123 Mn-KeV supplied by Bruker.

1. Introduction

In recent years, a large number of research groups have carried out works focused on the development and production of new and improved wound dressings by synthesizing and modifying biocompatible materials [1,2].

In particular, efforts are devoted to the use of biologically derived materials such as chitin and its derivatives, which are capable of accelerating healing processes at the molecular, cellular, and systemic levels. Chitin is a readily available and inexpensive biological material obtained from invertebrate skeletons

as well as the cell wall of fungi. It is a linear 1, 4-linked polymer composed of N-acetyl-D-glucosamine residues.

For its chain rigidity, chitin's dissolution in many solvents is a hard task, and for this reason, it is usually used as nanofibril, which are highly crystalline and rigid, thus allowing for the improvement of mechanical performance of composites. Based on infrared spectroscopy and x-ray diffraction data, chitin can be found in one of three crystalline forms: α-chitin, β-chitin, and γ-chitin. The molecules in orthorhombic α-chitin are arranged very tightly in an anti-parallel fashion. In this work, α-chitin, mainly present in shells of crabs, lobsters, and shrimps, was considered. High crystalline chitin is often called chitin nanocrystals, or chitin nanofibrils (CTN).

Chitin and its derivative, chitosan, are biocompatible, biodegradable, nontoxic, antimicrobial, and hydrating agents, and in general act as nanofillers in the reinforcement of both natural and synthetic composites [3]. Due to these properties, they show good biocompatibility and positive effects on wound healing. Previous studies have shown that chitin-based dressings can accelerate the repair of different tissues, thus facilitating the contraction of wounds and regulating the secretion of inflammatory mediators such as interleukin 8, prostaglandin E, interleukin 1 β, and others [4]. The effectiveness of three chitin nanofibril-based preparations, a spray (Chit-A), a gel (Chit-B), and a gauze (Chit-C), in healing cutaneous lesions was assessed macroscopically and by light microscopy immunohistochemistry [5]. These evaluations were compared to the results obtained using a laser co-treatment.

Ja.li Ji et al. [6] have underlined that chitin nanofibrils are an emerging novel filler. They have reinforcing effects on synthetic and natural fibers, and thus they can give rise to efficient scaffolds for tissue engineering. A number of techniques have been developed to fabricate nanofibrous tissue with unique properties. Among these techniques, electrospinning technology has become the most popular for the fabrication of tissue engineering scaffolds in recent years because it is a simple, rapid, efficient, and inexpensive method for producing nanofibers by applying a high voltage to electrically charged liquid [7,8]. Recently, Naseri et al. [9] successfully produced electrospun chitosan-based nanocomposite mats reinforced with chitin nanocrystals for wound dressing. For spinning solutions, chitosan and PEO were blended in a 1:1 ratio and used in a matrix, while CTN was used as reinforcement in order to produce final solutions of 3 wt % polymer in 50% aqueous acetic acid solvent.

The electrospinning process involves the application of a high voltage between a syringe filled with a polymer solution and a collector mounted at a fixed distance from the needle/syringe setup.

An electrical charge builds up on the surface of the solution that is attracted to the collector. The large potential difference overcomes the surface tension of

the fluid droplet at the tip of the needle. Under specific conditions of voltage, flow rate, and distance, a jet of fluid is ejected from the needle and subjected to whipping and splaying instabilities due to stresses of electrostatic origin [10]. The solvent evaporates over the jet path, and polymer nanofibers are formed on the collector. Various factors affect the electrospinning process such as solution properties, process parameters (flow rate, voltage, electrode distance), and ambient conditions. Hence, different requirements should be met in order to have an efficient process [11,12].

In this work, chitin-based nanofibers are prepared by a sol-gel material produced by mixing an aqueous solution of chitin with polyethylene oxide (PEOX). The effect of the operating variables of the electrospinning machine was investigated in order to optimize the quality of the electrospun textile.

2. Experimental

2.1. Materials

The materials used in this study include: Chitin, purchased from Primex (Siglufjordur, Iceland), PEOX (polyethylene oxide), purchased from Amerchol (Dow Italia, Italy), Chitin nanofibrils (CN) and CN-Lignin complex purchased from MAVI sud S.r.l. (Aprilia, Italy).

The sol-gel mixture prepared for the electrospinning tests was obtained mixing the CN-Lignin complex (30.1% w/w) with deionized water (up to 100% w/w) at temperature of 15 °C for few minutes. Then PEOX (7% w/w) was added to the solution, under stirring until completely dissolved. This last step took 24 h to obtain a homogeneous gel without agglomerations. The properties of the sol-gel materials are as follows:

- pH: 10.52
- Viscosity: 8.4 P
- Conductivity: 7.8 mS

2.2. Electrospinning

The electrospinning process was performed by using the pilot scale machine Elmarco Nanospider NS LAB 500 based on the nozzle-less technology (S. Petrik, M. Maly, "Production Nozzle-Less Electrospinning Nanofiber Technology", Elmarco s.r.o.). The proof of concept of this technique is that a rotating drum is dipped into a bath of the liquid solution. The thin layer of solution is carried out on the drum surface and exposed to a high voltage electric field. If the voltage exceeds the critical value a number of electrospinning jets are generated. The jets are distributed over the electrode surface with periodicity. This is one of the main

advantages of nozzle-less electrospinning, i.e., the number and location of the jets is set up naturally in their optimal positions.

The setting parameters of the machine were:

Voltage: 45–75 kV
Collecting electrode (CE): cylinder
Spinning electrode (SE): cylinder
Distance SE/CE: 10–16 cm
CE rotation: 2–8 rpm
Substrate material: Spunbond, 30 gsm, polypropylene 100% with antistatic treatment

The photo of the electrospinning set-up is reported in Figure 1.

Figure 1. The used electrospinning setup.

2.3. Rheological Measurements

Dynamic rheological properties of the sol-gel material were determined at 25 °C using the rotational rheometer supplied by Brookfield model HA DV-E 230. The applied operating conditions were: spindle code 05, speed of rotation 12 rpm, time 1 min

2.4. Fiber Diameter Characterization

The surface morphology of electrospun nanofibers was characterized by a field emission electron microscope–FESEM Auriga Zeiss, including microanalysis EDS 123 Mn-Kα eV(Bruker) and EBL –7 nm resolution (Raith). Samples cut from the electrospun material mounted on aluminum stubs were coated by an ultrathin layer of platinum for better conductivity during imaging. The samples were observed at

magnifications between 100 and 40,000 times their original sizes to visually evaluate the electrospinnability and existence of beads.

Fiber diameters were also determined using Image-J image processing software. For each electrospun material, at least 100 fibers were considered from three different images to calculate the average diameter.

2.5. Factorial Experimental Plan

The main aim of the experimental work has been to determine the best set of the operating parameters of the electrospinning machine in order to optimize the quality of the produced fibers. The process parameters which could be chosen were: the distance between the electrodes, the voltage range applied along each run, and the rotational velocity of the cylindrical electrode. On the basis of a preliminary explorative work it was ascertained that the better operating range of these parameters are those reported in Table 1.

Table 1. The operating conditions of the electrospinning machine.

Electrodes Distance (cm)	Voltage (kV)	Rotational Velocity (rpm)
10	45–60	2
16	45–70/75 (MAX)	8

In order to minimize the experimental runs it was decided to carry out an experimental campaign based on the 2^3 factorial design. In fact, this technique can reduce the number of experiments to be performed by studying multiple factors simultaneously. Additionally, it can be used to find both main effects (from each independent factor) and interaction effects (when both factors must be used to explain the outcome).

Additionally, it can be used to find both main effects (from each independent factor) and interaction effects (when both factors must be used to explain the outcome). The operating conditions of the 8 runs of the experimental campaign are represented in Table 2.

Table 2. The operating conditions of the 8 experimental runs.

Run	Variables Level	Electrodes Distance (cm)	Voltage (kV)	Rotational Velocity (rpm)
(1)	- - -	10	45–60	2
a	+ - -	16	45–60	2
b	- + -	10	45-MAX	2
ab	+ + -	16	45-MAX	2
c	- - +	10	45–60	8
ac	+ - +	16	45–60	8
bc	- + +	10	45-MAX	8
abc	+ + +	16	45-MAX	8

All the operating variables but those ones indicated in Table 1 were maintained constant in all the runs. The investigation was focused on only one effect, i.e., the average diameter of the fibers. In Table 3 the obtained values of this variable, together with the relevant statistical parameters, are reported for all the experimental runs.

Table 3. Average diameters of the electrospun fibers obtained in the factorial campaign.

Run	(1)	a	b	ab	c	ac	bc	abc
Diameter, nm	193	146	184	148	163	144	167	138
Variance	5484	1898	2338	2822	2655	2509	3523	1171
E%	13	10	9	12	10	12	12	8

The average diameter was determined on the basis of more than 100 measurements. The obtained results show significant changes of the fibers diameter for the different operating parameters set, within the overall measured range 138–198 nm. The smaller diameter values are those obtained by adopting the upper value of the electrode distance. In order to evaluate in quantitative way the effect of each operating variable the results of the factorial experiment design were analyzed by means of the ANOVA method. This method is based on the comparison of variance of results corresponding to a single effect or a combination of effects with the variance of the experimental error. In this work, it was assumed that factor interactions are negligible. Under this assumption, the average estimate of high order interactions is considered an estimation of experimental error.

In Table 4 the variance in correspondence of each single effect, in background color, and the zero-variance corresponding to the interactions effect is reported.

Table 4. The variance of single effects and zero variance.

Effect	Variance	Zero Variance	Fisher Factor
A	2145		40.5
B	10		0.19
C	435		8.2
AB	0.71		
AC	151	53	
BC	4		
ABC	55		

Finally, the F-test was made by comparing the ratio between the variance of each single effect and the zero variance with the value of the F distribution at 95% of significance.

In the examined case this value of F distribution is equal to 7.71. By comparing the F-test with the F-distribution it is possible to state that the fiber diameter is strongly affected by the electrode distance, is not affected at all by the voltage and is only slightly affected by the rotational speed of the spinning electrode. The fibers obtained at the best conditions, that is that one of the run abc (upper value of all the three operating variables) is reported in Figure 2.

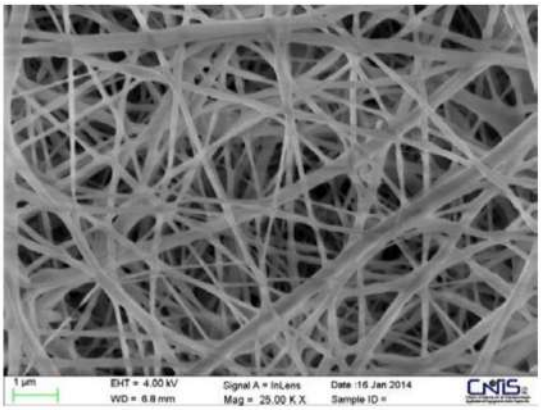

Figure 2. Image of the electrospun nonwoven tissue produced in run abc.

The fibers are elongated and quite regular without evidence of beads. It was not the case for some other runs performed at different operating conditions. For instance, for run b, carried out at lower values of the distance between pin and collector and of the voltage a quite bad tissue was obtained, as shown in Figure 3.

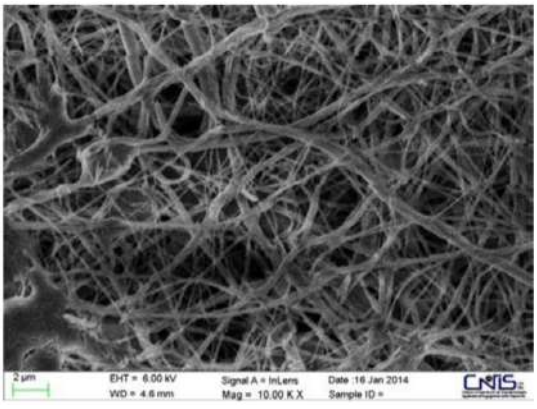

Figure 3. Image of the electron spun nonwoven tissue in run b.

The importance of the distance between tip and collector in order to minimize the beads and to obtain more regular fibers was noticed by other authors [13], in particular it was observed that beads are generated with too small and too large distance and a minimum distance is required to obtain uniform fibers. The applied voltage had no effect on the performances of the electrospinning. This is in agreement with the research work of Reneker and Chun [14], who has showed that there is not much effect of electric field on the fiber diameter with electrospinning of polyethylene oxide. As far as the effect of rotational speed is concerned it is well known that its increase resulted in more uniform and thinner fibers, potentially due to the higher stretching level imposed on them. In our particular case, the increase of the rotational speed from 2 to 8 rpm induces an improving of the electrospinnability of the tissue, even if it is not remarkable.

Obviously, the characteristics of the sol-gel material used for the electrospinning plays an important role. The used material was produced at a relatively low temperature, around 15 °C. If the sol-gel material is produced at a higher temperature, around 20 °C, its viscosity is lowered down to 7 P and the electrospun fibers, produced at the best operating conditions above reported, exhibit a larger size, i.e., of 191 nm. size. It has been found that with very low viscosity there is no continuous fiber formation and with very high viscosity there is difficulty in the ejection of jets from the polymer solution, thus there is a requirement of optimal viscosity for electrospinning. Fong et al. [15] have studied polyethylene oxide (PEOX) to study nanofiber formation at different viscosities and found that a range of viscosity between 1 and 20 poise is suitable for production of uniform nanofibers by electrospinning. The values of viscosity of the sol-gel material produced in this work in presence of PEO were well inside of the suitable range viscosity outlined by Fong et al. [15] and thus it's a confirmation of their results.

3. Conclusions

In this work, the influences of the operating conditions of a rotating electrospinning machine on the characteristics of a non-woven chitin-based tissue has been investigated. In order to minimize the experimental efforts, a factorial campaign of experiments have been designed and performed. The effects of the electrode distance, the voltage range and the rotational speed of the spinning electrode were considered. By means of preliminary experiments, the variables' operating ranges to be adopted were identified. The experimentation showed that the most important operating variable is, in our particular case, the distance between the electrodes, as its increase gave rise to a reduction of the fiber diameter down to less than 150 nm. A second significant effect was exhibited by the collected electrode rotational speed. By operating at the best set of operating variables, a very good electrospinnability of the tissue was obtained with parallel elongated fibers, but the size ratio of the produced fibers was quite high. Future work should be done to find out the best operating conditions and to improve the non-woven tissue, by an investigation within the domain of variables identified by this study.

Conflicts of Interest: The authors declare no conflict of interest.

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PART IV
Applications

Chitin-Hyaluronan Block Copolymeric Nanoparticles for Innovative Cosmeceuticals

Hong-Duo Chen, Li Yuan Hong, and Pierfrancesco Morganti

Abstract: Chitin (C8H13O5N)n is a low-cost available unbranched polysaccharide widely distributed in nature as supporting structure of the cell wall of fungi and exoskeleton of arthropod and insects. Chitin nanofibrils are easily metabolized by the body's endogenous enzymes and thus used in cosmetic dermatology and biotextiles. Hyaluronan is anionic, nonsulfated glycosaminoglycan which, as major component of the Extracellular Matrix (ECM) plays several biological roles, showing expensive age-related changes. A successful model of an innovative block-polymer nanoparticle (BPN) based on phosphatidylcholine, hyaluronan, and chitin nanofibrils entrapping amino acids, vitamins, and melatonin has been formulated. Both the in vitro and in vivo results obtained demonstrate the efficacy of the injected block-polymer nanoparticles in reducing skin wrinkling and ameliorating the signs of aging. Chitin nanofibrils, protecting both corneocytes and intracorneal lamellae, help to maintain cutaneous homeostasis, neutralize the activity of radicals and trap them in their structure, regularizing the correct cell turnover. The BPN seems to be useful for improving the activity of permanent fillers, rendering it useful as an anti-aging remedy for the plastic surgery armamentarium.

Raymond Reed and Albert Kligman brought about the concept of "cosmeceutical" more than 50 years ago [1,2]. According to these authors, *cosmeceuticals* are topical Cosmetic-Pharmaceutical hybrids intended to enhance the health and beauty of skin, being based on the use of biologically active ingredients with medicinal or drug-like benefits.

At this purpose, many substances, either chemically synthesized or extracted from plants or animals, are used as functional ingredients. Nowadays, many cosmetic products with biologically active ingredients have been developed and marketed, though there are discrepancies in relation to their regulations and approvals by the government [3–5]. However, a number of topical cosmeceutical treatments for conditions such as photoaging, hyperpigmentation, wrinkles, and hair damage have come into widespread use.

In the cosmeceutical arena, nanotechnology has played an important role. Using new techniques to manipulate matter at an atomic or molecular level, they have been at the root of numerous innovations, opening up new perspectives for the future of the cosmeceutical industry.

Nanotechnology-based cosmeceuticals, in fact, offer the advantage of diversity in products, increase the bioavailability of active ingredients, ameliorating the aesthetic appeal of cosmeceutical products with prolonged effects [6–8].

There is a great potential in the marine bioprocess industry to convert and utilize most marine resources and marine food by-products as valuable cosmeceutical ingredients. Current available potential cosmeceuticals from marine resources include: seaweed extract, phlorotannins, polysaccharides, carotenoid pigments, fucosterol, microalgae extract, collagen, bioactive peptides, chitooligosaccharide derivatives, enzymes, sea mud, sea water, and minerals. Potential health benefits of marine-derived cosmetic active ingredients on human skin include anti-aging, antioxidant, anti-wrinkling, anti-whitening, cytoprotective, anti-tyrosinase, anti-acne, anti-inflammatory, and UV photo protective effects [9].

Chitin ($C_8H_{13}O_5N)_n$ is a long-chain glucose-derived of a N-acetylglucosamine widely distributed in nature. It is a modified polysaccharide which, containing nitrogen, is distributed in the cell wall of fungi and exoskeketon of arthropods and insects. This polymer available at low cost, has been shown to be bio- and eco-compatible, with a very low level of toxicity. At present, the world offshore disposal of this natural waste material is estimated to be around 250 billion tons per year.

Chitin is an underutilized resource and has the potential to supply a wide range of useful products if suitably recycled, thus contributing to sustainable growth and a greener economy [10,11]. In addition, it is a good inducer of defense mechanisms in plants, being a fertilizer that can improve overall crop yields. Chemically-modified chitin forms edible films as an additive to thicken and stabilize foods and pharmaceuticals. It also acts as a binder in dyes, fabrics, adhesives, a reproducible form of biodegradable plastic, and as a promising substrate for engineering human tissues. Chitin's flexibility and strength make it favorable as surgical thread. Its biodegradibility means it wears away with time as the wound heals and it might have some unusual properties that accelerate the healing of wounds in humans [12].

Recently, it has become possible to industrially produce pure chitin crystals, named "chitin nanofibrils" (CN) for their needle-like shape and nanostructured average size (240 × 5 × 7 nm) [11] (Figure 1).

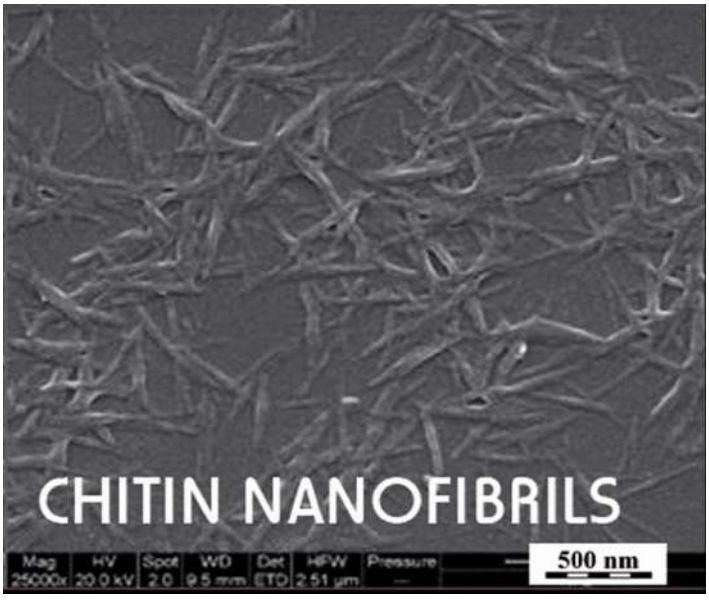

Figure 1. Chitin Nanofibrils at SEM.

Having a backbone like hyaluronic acid, chitin nanofibrils are easily metabolized by the body's endogenous enzymes and thus used in cosmetic dermatology and biotextiles. Moreover, because it occurs naturally and is considered as a safe raw material, it is safe to use. Chitin nanofibrils were recognized as a strong "gelling agent" [13].

As the nanofibril has an average size one-quarter that of a bacterium, 1 g of the product covers a surface area of 400 m^2. Many studies have shown that chitin nanofibrils can activate the proliferation of keratinocytes as well as fibroblasts, regulating not only collagen synthesis but also cytokine secretion and macrophage activity [11,13–15].

Chitosan is a natural biopolymer derived from the deacetylation of chitin and known to have various biological activities such as antifungal, antitumor, and antibacterial activity. It has been applied in various fields including wastewater treatment, agriculture, fabric and textiles, cosmetics, nutritional enhancement, and food processing.

Nanocomposite biomaterials based on chitosan and chitin are widely investigated for their antimicrobial activity, biocompatibility, and biodegradability [11,13]. P. Morganti's group has obtained interesting results showing how chitin nanofibrils can not only ameliorate the appearance of photoaged skin but also promote wound healing by reducing hypertrophic scar formation [6,15–20] (Figure 2).

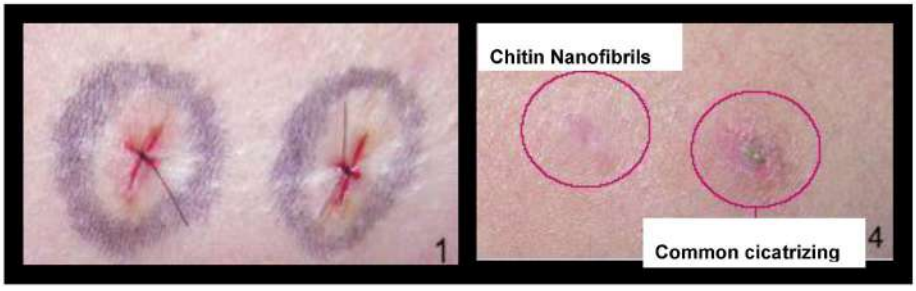

Figure 2. Cicatrizing activity of a gel based on chitin nanofibrils and chitosan (courtesy of P. Mezzana, MD [20]).

In vitro studies have shown how chitin nanofibrils can increase the reproduction of fibroblasts with a subsequent increase in collagen synthesis and in adenosine triphosphate production. In an in vivo double-blind study, skin hydration and superficial skin lipids were improved, with a simultaneous reduction in lipid peroxides and transepidermal water loss (TEWL) [21–23].

Hyaluronan is an anionic, non sulfated glycosaminoglycan distributed widely throughout connective, epithelial, and neural tissues. Hyaluronan is a polymer of disaccharides, composed of D-glucuronic acid and D-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds. Polymers of hyaluronan can range in size from 5000 to 20,000,000 Da in vivo [24,25].

Hyaluronan is found in many tissues of the body, such as skin, cartilage, and the vitreous humour. Therefore, it is well-suited to biomedical applications targeting these tissues. Native hyaluronan has a relatively short half-life, so various manufacturing techniques have been deployed to extend the length of the chain and stabilize the molecule for its use in medical applications [6,11,22,26].

The first hyaluronan biomedical product was developed in the 1970s and is approved for use in eye surgery. Hyaluronan has been used in attempts to treat osteoarthritis of the knee by injecting it into the joint [27–29].

Dry skin can be treated with a prescription skin lotion containing sodium hyaluronate as its active ingredient. In some cancers, hyaluronan levels correlate well with malignancy and poor prognosis. Hyaluronan is often used as a tumor marker for prostate and breast cancer and may also be used to monitor the progression of the disease [29]. Moreover, it may also be used postoperatively to induce tissue healing, notably after cataract surgery [30], as well as in the synthesis of biological scaffolds for wound-healing applications [31].

Hyaluronan is a common ingredient in skin-care products, so that hyaluronic acid fillers have been injected using a classic sharp hypodermic needle, cutting through nerves and vessels, with increasing risks of causing pain and bruising.

Nanotechnology is not only essential for marketing-oriented chemical companies, but is also a tool for developing science-based solutions for innovative

therapeutics and cosmetics, enhancing well-being and addressing anti-aging issues. Nanomaterials and nanobiotechnology have the potential to radically change the way cosmetics and drugs deliver their benefits.

Specifically, nanoparticles are being developed to encapsulate a wide range of ingredients beneficial to the skin. To obtain nanoparticles, two principles approaches are used: (a) the bottom-up method in which nanoparticles are assembled from the molecular dimension; (b) the top-down approach that reduces larger particles through the use of physicochemical methods. In cosmetics, the top-down approach is more commonly used to produce different kinds of structures. Examples of such structures include nanosomes, cubosomes, niosomes, and liposomes [6,32].

A successful model has been formulated by P. Morganti's group: an innovative block-polymer nanoparticles (BPN) based on phosphatidylcholine, hyaluronan, and chitin nanofibrils entrapping amino acids, vitamins, and melatonin.

The specific formulation for each milliliter contained: hyaluronan salt 1 mg; phosphatidylcholine 3 mg; creatine 0.1 mg; caffeine 0.1 mg; ascorbyl tetraisopalmitate 0.5 mg; vitamin E 10 mg; chitin nanofibrils 1 mg; melatonin 0.1 mg; glucosamine 0.1 mg; glycine 0.1 mg; arginine 0.1 mg; sodium phosphate dibasic 2 mg; potassium di hydrogen phosphate 0.2 mg; sodium chloride 9 mg; sterile water for injection to 1 mL.

The injection was based on the mesotherapy technique, using 1 mL solution and a 30 g needle positioned at 45 to the skin surface. The injection rate was at all times less than 0.3 mL/min. A firm massage, with the index finger inside the mouth and the thumb outside, was then used to remove any unevenness. The 1mL quantity is sufficient to treat the entire face [5,6].

Both the in vitro and the in vivo results obtained demonstrate the efficacy of the injected block-polymer nanoparticles in reducing skin wrinkling and ameliorating the signs of aging. Subjects were satisfied with the general aspect of their skin, which appeared softer and more hydrated during the first month of treatment.

In line with their self-evaluation, the appearance of fine wrinkling was notably reduced and the consequent skin softness and firmness enhanced during the entire treatment period. The general amelioration remained during the regression period and 30 days after the interruption of the treatment (Figure 3).

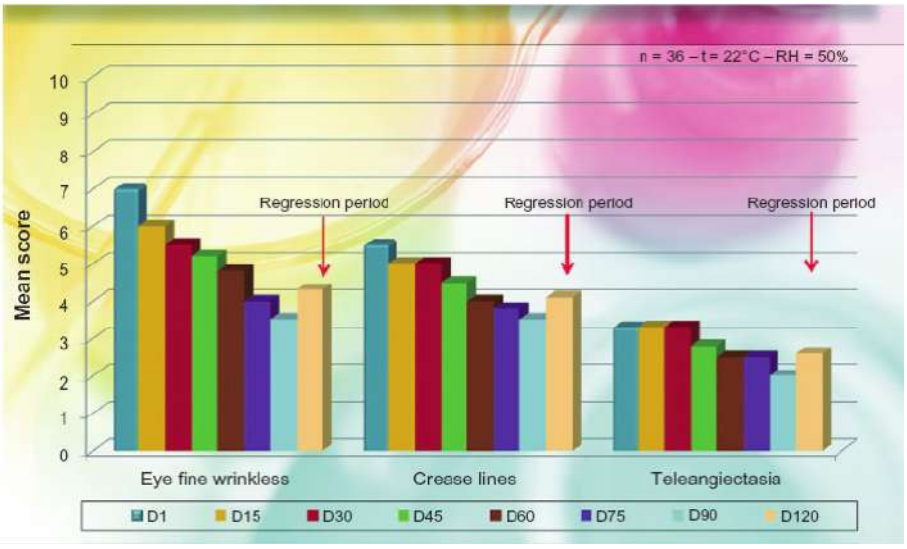

Figure 3. Dermatological mean evaluation on signs of photoaging after injective treatment with phosphatidylcholine-hyaluronic acid-chitin nanofibrils encapsulating active compounds (BPN). Note: All P values are highly significant as to baseline ($p < 0.005$). Abbreviations: BPN, block-polymer nanoparticles; RH, relative humidity.

In this formulation, the high content in linoleic acid of the phosphatidylcholine used allowed the active BPN to quickly reestablish the skin-barrier function. Thus, while the phosphatidylcholine fatty acids of the BPN composition contribute to balancing the disturbed composition and organization of lipids at the level of epidermal keratinocytes and consequently of corneocyte lamellae, the high level of linoleic acid should contribute to reintegrating the reduced level of ceramide 1, structural and stabilizing component of the stratum corneum [5,6,33].

Chitin nanofibrils, protecting both corneocytes and intracorneal lamellae, help to maintain cutaneous homeostasis, neutralize the activity of free radicals and trap them in their structure, and regularize the correct cell turnover [24,34–37]. All these activities are modulated and increased by the chitin nanofibrils/hyaluronan (CN-HA) encapsulation methodology made using the gelation method [38,39] (Figure 4).

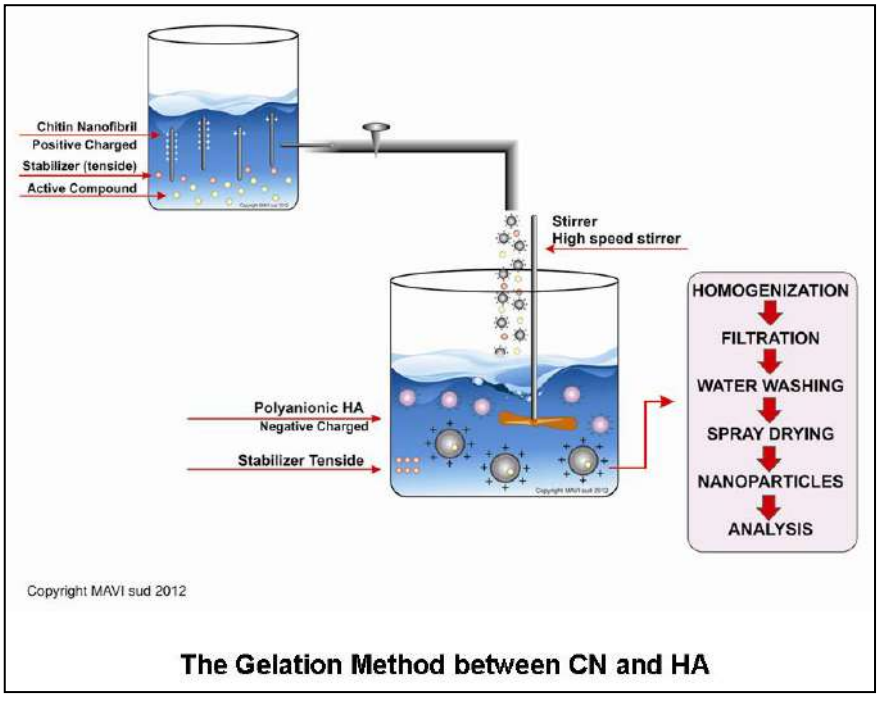

Figure 4. Gelation method for producing Chitin Nanofibril-Yaluronic acid (CN-HA) block polymeric nanoparticles.

In summary, the BPN encapsulating the active ingredient used seems to be useful in improving the activity of permanent fillers, rendering it useful as an antiaging remedy for the plastic surgery armamentarium.

In conclusion, this innovative biostimulating medical device should be used for wrinkle treatment and rejuvenating looks, as well as an adjuvant in soft-tissue augmentation and stretch-mark corrections [6,40].

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Conflicts of Interest: We declare that Hong-Duo Chen and Li Yuan Hong have no conflict of interests. Pierfrancesco Morganti works as Head of the R&D Centre of Nanoscience, Mavi Sud, s.r.l, Italy.

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Chitin Nanocomposite Scaffolds for Advanced Medications

Xue-Gang Xu, Xing-Hua Gao, Hong-Duo Chen and Pierfancesco Morganti

Abstract: The rapid development of nanotechnology, especially in the biological material sciences, has stimulated the demand for new hybrid materials and biocomposites for wide range of applications. Chitin, the second most abundant polysaccharide after cellulose, is a naturally occurring biopolymer found in yeast, fungi and exoskeletal structures of numerous invertebrates. Chitin meets the several desirable properties of biomaterial, which includes mechanical strength, chemical and thermal stability, biocompatibility, etc. Both chitin and its deacetylated product chitosan are considered of great economic values because of their versatile biological activities which made them proper to various applications in personal care products, pharmaceutical, medicine, food, agriculture and environmental sectors. This chapter gives an overview of the extensive research and recent developments on chitin/chitosan based nanocomposite scaffolds for application as advanced medicines. Several kinds of nanocomposite scaffolds have been applicable or under investigations, such as chitin-chitosan-gelatin scaffolds, pectin-chitin/$CaCO_3$ nanocomposite scaffold, et al. Here we highlight the most recent research on different aspects of chitin based nanocomposite scaffolds, including their preparations, properties and applications, especially the applications of chitin nanocomposite scaffolds in tissue engineering, stem cell technologies, and vaccine preparations.

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1. Introduction

There has been rapid development of nanotechnology recently, especially in the biological material sciences, which stimulates the demand for new hybrid materials and biocomposites of specific and defined properties for a wide range of applications. Nanomaterials exhibit novel characteristics compared to bulk materials, such as high mechanical strength, high surface-area-to-volume ratio, and extremely miniaturized size [1].

The interest in chitin as a raw material began in the 1920s owing to market pressure for low cost fibers, which promoted research into artificial silks. In particularly, chitin's biocompatibility and wound-healing properties made it attractive for different biomedical applications including tissue engineering scaffolds, wound dressing and sutures, and biocompatible devices. Chitin is the second most abundant polysaccharide after cellulose. It is a natural biopolymer found in fungi, yeast and the exoskeletal

structures of numerous invertebrates including insects, sponges, worms and aquatic arthropods [2].

The industrial processing of shellfish such as crab, shrimp and krill for human food generates a huge amount of waste and it is said that 50–60% of the total weight is chitin. Annually, worldwide production is about 1.44 million metric tons dry weight [3]. These abundant and renewable marine wastes can be commercially exploited for the extraction of chitin [3]. However, traditional chitin extraction processes employ harsh chemicals and produce other waste-water or solvent. Advances in the biological extraction of this natural polymer, which reduces energy cost and waste water—producing valuable by-products—will certainly have high economic and environmental impacts [3]. Thus, the bio-extraction of chitin from crustacean shell waste has been increasingly studied in order to develop eco-friendly, cleaner, and economical processes.

Due to their molecular and supramolecular structures characterized for their intrinsic antimicrobial and wound healing properties, chitin and derived compounds (chitosan and chito-oligosaccharides) have been identified as suitable bio platforms to make specialized polymers functionalized for the advanced medicine.

What is chitin? As a derivative of glucose, it is a long-chain natural occurring polymer of *N*-acetyl-D-glucosamine with a molecular length varying from 5000 to 8000 *N*-acetyl-D-glucosamine units found in crabs, to up to only 100 units contained in yeast.

Chitosan, a chitin-derived compound, is usually produced by chitin deacetylation with concentrated alkali solutions at elevated temperatures. This process of deacetylation, which does not proceed to completion, implies that chitosan, obtained from commercial sources, is in reality chitin with a low degree of acetylation. When the number of the units is higher than 50%, the polymer is termed chitin, when less than 50% it is termed chitosan.

In conclusion, chitin and chitosan may be considered as two points of a continuum material that share the same basic structure differing in their acetylation degree. As to the function of its cell structural support and defense against environmental aggressions, chitin may be compared to human keratin, which supports skin, hair and nails [4].

It has therefore proven useful for several medical and industrial purposes from tissue engineering to making advanced medications for wounded and burned skin [5], to producing smart and innovative colored dressings, imitating the iridescent colors used by birds and butterflies in nature. Birds' plumage and butterfly wing scales, in fact, are often organized into stacks of nano-sticks or nano-layers made of chitin nano-crystals, which produce various iridescent colors thin-film interference (Figure 1) [6,7].

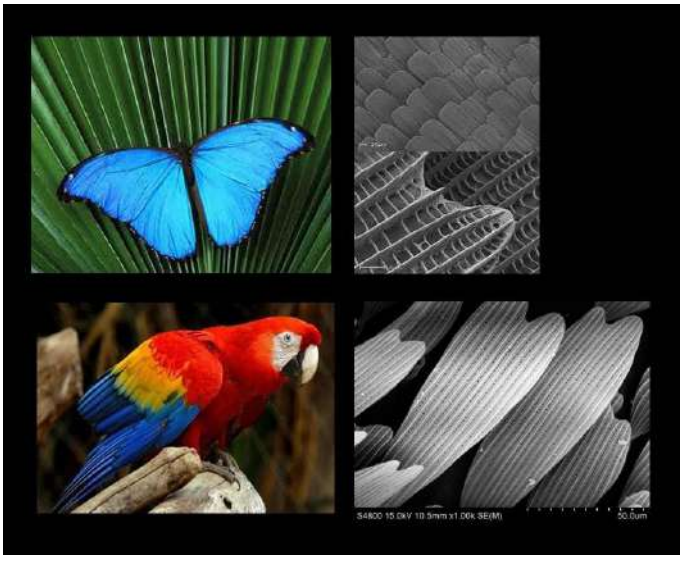

Figure 1. The different colors and iridescences are due to chitin nanocrystals. Source: Morganti 2012 [6].

Chitin is insoluble in aqueous solution and relatively difficult to process. To improve its processability, many different approaches have been studied for the successful employment of this polymer in the areas of advanced medicine including stem cells, tissue engineering etc.

Chitosan displays interesting physicochemical properties, differing in its properties and organized structure. In a solid state, relatively rigid crystals are formed due to its regularly arranged hydroxyl and amino groups, while in solution the hydrogen bondings drive the formation of microfibrils, depending on the chitosan concentration. Furthermore, chitosan is easy to develop into various designs, i.e., films, sponges, scaffolds and hydrogels, which result in the ability to make various kinds of tissue-engineering materials and wound dressings [7]. Due to their unique structural, physico-chemical and functional properties, both chitin and chitosan are good candidates for the preparation of scaffolds and dressing materials for tissue regeneration [8].

In recent years, considerable attention has been attracted to ameliorating the functionality of these biopolymers for improving their properties by increasing their solubility or introducing selective active functions or making blends with other intrinsically bioactive polymers [9].

This chapter tries to give an overview of the recently reported chitin and chitosan nanocomposite scaffolds, emphasizing their characteristics and applications in tissue engineering and other applications.

2. Characteristics of Potential Chitin/Chitosan Nanocomposite Scaffolds

2.1. Nanohydroxyapatite/Gelatin/Carboxymethyl Chitin Composite Scaffold

Sagar et al. developed a novel 3D scaffold from the unique combination of nanohydroxyapatite/gelatin/carboxymethyl chitin (*n*-HA/gel/CMC) for bone tissue engineering by using the solvent-casting method combined with vapor-phase cross-linking and freeze-drying [10]. An optimized (composition and processing parameters) ratio of *n*-HA:gel:CMC (1:2:1) exhibited an ideal porous structure with regular interconnected pores (75–250 μm) and a higher mechanical strength.

Their results suggested that the divalent (Ca^{2+}), carboxyl (COO^{-}), amino (NH_4^{+}), and phosphate (PO_4^{3-}) groups created favorable ionic interactions which facilitated structural stability and integrity of the composite scaffold. Further, hemocompatibility and biocompatibility with MG-63 osteoblast cells indicated that the structural and dimensional stability of a composite scaffold provided an optimal mechanosensory environment for the enhancement of cell adhesion, proliferation, and network formation [10].

2.2. Pectin-Chitin/$CaCO_3$ Nanocomposite Scaffold

Kumar et al. developed a nanocomposite scaffold using a mixture of chitin, pectin and nano $CaCO_3$ through the technique of lyophilisation, with specific biomedical applications for tissue engineering and drug delivery [2].

The developed composite scaffold showed controlled swelling and degradation, compared to the control scaffold. Moreover, cells attached onto the scaffolds started to proliferate after 48 h of incubation and demonstrated negligible toxicity towards cells. Drug delivery through the scaffold was confirmed using a bisphosphonate called Fosamax. The results suggested that the developed composite scaffold possessed the essential requisites for their applications in the fields of drug delivery and tissue engineering.

2.3. α-Chitin Hydrogel/Nano Hydroxyapatite Composite Scaffold

In another paper, Kumar et al. synthesized α-chitin hydrogel/nano hydroxyapatite (*n*HAp) composite scaffold using a freeze-drying approach [11] with nHAp and α-chitin hydrogel. Hydroxyapatite [(HAp), $Ca_{10}(PO_4)_6(OH)_2$] is a major inorganic ceramic material and an essential component of bone. nHAp is used for various applications like dental filling material, bone tissue engineering, etc. Because it offers a high surface area to volume ratio, a small concentration is sufficient to enhance

its bioactivity and osseointegration. Hap nanoparticles were mixed with α-chitin hydrogel at concentrations of 0.5% and 1% (*w*/*w*), stirred for 30 min, frozen at −20 °C and lyophilized to get a microporous nanocomposite scaffold [11]. The prepared composite scaffolds were characterized using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Thermogravimetric analysis and differential thermal analysis (TG-DTA) and scanning electron microscopy (SEM). Porosity, swelling ability, protein adsorption, in vitro biodegradation and biomineralization of the scaffolds were evaluated. These supports showed interconnected micro-pores, sufficient swelling ratio of 15–20, good protein adsorption, controlled degradation and wonder biomineralization ability.

2.4. Chitin-Chitosan/Nano ZrO_2 Composite Scaffolds

According to de Moraes et al. [11], innovative biomaterials can provide a promising new direction for the treatment of bone defects, stimulating a proper repair process with no damage to the adjacent tissues [12]. Collagen, in fact, is one of the most used biomaterials due to its biocompatibility and bioactivity [13]. Thus, Soares et al. [14], evaluating the odontogenic potential of human dental pulp cells in contact with a porous system of chitosan-collagen mineralized with calcium-aluminate, concluded that this scaffold seems to be an interesting candidate for in vivo applications to exposed pulp tissue.

Owing to its good mechanical strength and biocompatibility, zirconia is also considered to be one of the most used materials after titanium over a period of about 20 years, especially in dentistry. Cultured osteoblasts proliferate and differentiate in zirconia with no adverse reaction. Jayakumar R et al. tried incorporation of nano ZrO_2 onto the chitin-chitosan scaffold to enhance osteogenesis [15]. They fabricated a nanocomposite scaffold using a lyophilisation technique with chitin-chitosan and nano ZrO_2. The prepared nanocomposite scaffolds were characterized by FTIR, SEM, XRD and TGA. The swelling, degradation, cell attachment, cell viability and biomineralization of the composite scaffolds were also detected. The results showed better swelling and controlled degradation compared to the control scaffold [15]. Cytocompatibility studies proved the non-toxic nature of chitin-chitosan/nano ZrO_2 scaffolds against MG-63, L-929 and hMSCs [15]. Additionally, cell attachment studies showed the nanocomposite scaffold significantly increased the cell attachment when compared to control scaffolds. All these results suggested that the developed nancomposite scaffolds possess the prerequisites for tissue engineering scaffolds and could be used for bone tissue engineering and other bio-engineering purposes of human interest.

3. Chitosan/Gelatin/nSiO$_2$ Composite Scaffold

Kavya et al. [16] fabricated a 3D nanocomposite scaffold of chitosan, gelatin and nano-silica by lyophilisation to produce a better candidate for bone tissue engineering compared to pure chitosan and chitosan/gelatin scaffolds.

To prepare composite scaffolds with better biological compatibility and hydrophilicity, they added gelatin to enhance the performance of chitosan. Unique biocomposites of amorphous silica, chitin, and crystalline aragonite have been discovered in marine sponges. Silica is believed to be essential in skeletal development so that critical amounts of silicon ions are found to up-regulate genes like collagen type-, BSP, osteocalcin and osteopontin in osteoblasts.

To this purpose, these authors developed a 3D composite scaffold of chitosan/chondroitin sulfate/nSiO$_2$ to bring out the combined properties of chitosan, gelatine and nSiO$_2$ to facilitate bone regeneration. Porosity, swelling, density, mechanical integrity, degradation, biomineralization and protein adsorption studies, favored it in comparison to the conventional chitosan and chitosan/gelatin scaffolds. In vitro cyto-compatability, cell attachment-proliferation and ALP activity studies by using MG-63 cells, advocated its remarkable performance. These cumulative results indicate the chitosan/gelatin/nSiO$_2$ nanocomposite scaffold as a suitable candidate for bone tissue engineering.

Chitosan-Graphene Oxide Network Structure Scaffold

Graphene—a single layer of sp2 bonded carbon atoms in a two-dimensional hexagonal lattice—has attracted considerable attention as a potential biomaterial because of its physic-chemical properties such as a large surface area, high hydrophilicity and dispersibility. Chitosan-graphene network structure scaffolds were synthesized by covalent linkage of the carboxyl groups of grapheme oxide with the amine groups of chitosan [10].

The covalent incorporation of graphene oxidant into a CS network favorably modulated the biological response of osteoblasts, such that cell attachment and growth were significantly enhanced. Thus, related to a combination of a number of physic-chemical factors, including a large surface area, nanoscale roughness, the presence of pendant groups, a hydrophilic nature and high water retention ability, this network is believed to be a promising material for tissue engineering applications in regenerative medicine (Figure 2) [17,18].

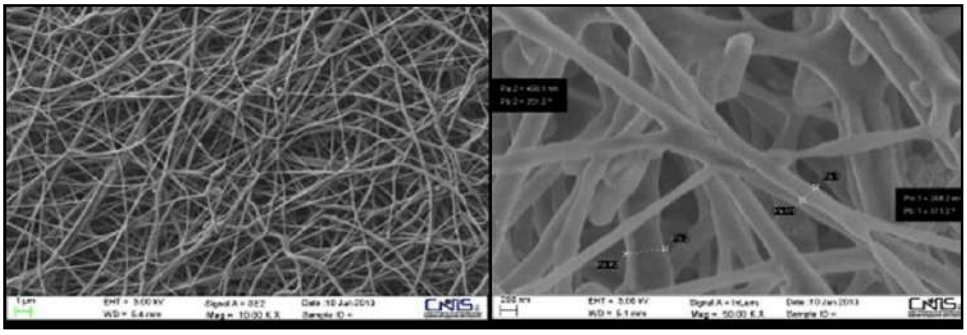

Figure 2. Engineered non-woven tissue made by chitin nanofibrils with scanning electron microscopy (SEM). Source: Morganti et al. [19].

4. Applications of Nanocomposite Scaffolds for Advanced Medications

Tissue Engineering

Extensive research is in progress to develop biosmart materials in the field of tissue engineering, being considered of prime importance for biomedical and microbiological applications.

Chitin, chitosan and its derivatives have received much attention because of their biocompatibility and other advantages. Chitin composite scaffolds have sufficient mechanical strength. And due to their viscoelasticity in wet conditions, they can be cut, deformed, and fitted according to bone defects. This allows us to use them in cases of bone cysts and large defects with smaller openings.

These scaffolds can spontaneously grow by consuming the calcium and phosphate ions from the surrounding fluid which render them osteoinductive structures, analogous to living bone.

Besides being a matrix of a composite material, chitin has also been confirmed to promote osteogenesis in mesenchymal stem cells, accelerate wound healing, enhance cell migration, and form granulation tissue with angiogenesis [19–22]. These effects might be mediated by the production of cytokines and growth factors by fibroblasts that come into contact with the chitin material. For tissue engineering applications, the ideal situation would be gradual but complete scaffold degradation concomitant to tissue remodeling, while foreign material might elicit an adverse host tissue response in the long term, so the degradability of polymeric scaffolds should be of primary concern.

Chitin is susceptible to lysozyme and chitotriosidases, which are ubiquitously present in humans. In this regard, it is interesting to underline that this natural polymer has to be considered as a pathogen-associated molecular pattern (PAMP) regulating the macrophage function and inflammation, depending on its size [23].

Moreover, it has been shown that chitin nanofibrils (240 × 7 × 5 nm) stimulate the defensive production of defensine-2, contextually reducing the production of metallo-proteinases and inflammation [24,25]. This is another advantage for chitin in the application of tissue engineering. Combinations of chitin/chitosan with other polymers have been fabricated to nanocomposite scaffolds for tissue engineering as we mentioned above. Recent work established that a chitin-hydroxyapatite composite, loaded with mesenchymal stem cell-induced osteoblasts, was able to support bone regeneration when implanted into bone defects in a rabbit femur [26].

Composite scaffolds of chitin with nanohydroxyapatite and nanotitania, obtained by dispersing the particles in a chitin hydrogel cast from a $CaCl_2$/methanol solvent system and freeze-drying of the mixture, supported apatite deposition and adhesion of a variety of cell lines [11,15]. Composite scaffolds, prepared with *n*-HA/gel/CMC, have sufficient mechanical strength and, along with their viscoelasticity in wet conditions, they can be cut, deformed, and fitted according to bone defects which allows them to be used in cases of bone cysts and large defects with smaller openings [10].

Another composite scaffold, based on α-chitin hydrogel/nano hydroxyapatite composite scaffold, showed that its viability in the presence of scaffold leachables and *n*HAp did not affect its cyto-compatibility. It was also found that the scaffolds were cytocompatible and cells were well attached and distributed throughout these structures [11]. Moreover, scaffolds made by chitin nanofibrils developed antibacterial and anti-inflammatory activity and increased the production of defensine-2, speeding up the skin repairing process in wounded and burned skin [17,24–27].

All these results suggested that these scaffolds can be used for bone and wound tissue engineering also because of their effectiveness and safeness. However, biopolymers offer a highly effective flexibility to design porous matrices by chitosan, chitin, collagen, alginate and other natural compounds. Moreover, when at the nanodimension, they have to be considered the best candidates for drug delivery applications due to their controllable pore sizes, high surface area with favorable properties.

5. Stem Cell Technologies

Because of the fast development in technologies, these years, stem cell had bright prospects in regenerative medicine and organ transplantations. Chitin and other biomaterials can help to realize the vast potential of stem cells in regenerative medicine by: (1) playing a role in providing substrates that support stem cell self-renewal, while maintaining stem cell pluri- or multipotency; (2) favoring the provision of a matrix permissive to stem cell differentiation; (3) representing a matrix of composite material as physical support of cells to regenerate organs; and (4) guiding their differentiation

when appropriate signals are provided [28]. To this purpose, the advantages of a chitin matrix in supporting stem cell proliferation and differentiation has extended its applications in vivo, where chitin has been shown to be effective as a carrier material for mesenchymal stem cells in the treatment of large physical defects.

Mesenchymal stem cells seeded or encapsulated in water-soluble chitin-alginate fibrous scaffolds have been differentiated into chondrogenic and osteogenic lineages by immersion in the respective differentiation media [29]. Nanocomposite scaffolds of chitin or chitosan would definitely have more broad application prospects.

6. Vaccine Preparation

Vaccination is one of the major keys to maintaining a good public health and wellbeing status of society. It induces specific adaptive immune responses and memory responses against infections, tumors, etc. [30]. Unfortunately, not all vaccines are as effective, often showing low efficiency of antibody production with a weak host T cell response and T cell memory, which require repeated booster injections to obtain longer host memory immune responses.

To solve this problem, adjuvants [31] are often used to augment the effects of a vaccine by stimulating the immune system to respond to the vaccine more vigorously, thus providing increased immunity to a particular disease. There are many adjuvants, such as aluminium salts, virosomes, etc. Aluminium—the only adjuvant approved by the Food and Drug Administration (FDA) for clinical use—stimulates B cell response for antibody production but is not very effective at inducing host T cell responses and does not work well with all antigens.

Chitin nanocomposite scaffolds are a biodegradable polymer, which not only has immune adjuvant effects on its own [23,24] but is also able to release incorporated cytokines and antigens in a controlled manner, therebby synergistically boosting adjuvant effects. Chitin also accelerates macrophage migration and fibroblast proliferation with a particular role in vascularisation [32].

For clinical applications, chitin/chitosan is frequently cited to possess low immunogenicity as an advantage, and at the same time, the ability to act as an immunoadjuvant. Glycolic acid-g-chitosan-gold nanoflower nanocomposite scaffolds, for example, were confirmed to be used for the sustained delivery of drug [33]. This property would also help chitin/chitosan nanocomposite scaffolds to be used in vaccine preparation as an adjuvant.

7. Conclusions

Recent progress in chitin and chitosan nano-composite scaffolds highlights great potential usage in wound-healing and tissue engineering due to their unique structural, functional, physical and chemical properties. Another area of particular interest is also gene-therapy, referring to methods aimed at influencing gene

expression in living organisms through the delivery of integrating exogenous DNA or RNA to treat or prevent disease [34]. For these reasons, many innovative approaches are emerging in recent years with the use of novel materials and technologies. However, biomedical modifications of these biopolymers and their preparation in different designs have been reported extensively, but until now with limited commercial impact. Further studies on the clinical applications of these chitin and chitosan nanocomposite scaffolds would be the most important issue in this field.

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Flexible Food Packaging Using Polymers from Biomass

Maria-Beatrice Coltelli, Vito Gigante, Patrizia Cinelli and Andrea Lazzeri

Abstract: The use of polymers from biomass in the production of flexible packaging is an important challenge to give an answer to both the reduction of oil-derived materials and the increase of waste production. Oil-derived materials are now employed in recyclable packaging, but, although the management of waste recycling is improving, it cannot allow the complete mechanical recycling of the plastic fraction. It would be important to optimize the system by replacing packaging difficult to be recyclable, such as multi-layer flexible packaging mainly based on the use of polyolefines, with alternative packaging consisting of biodegradable materials, thus managing its disposal by considering composting. In the present paper, a classification of polymeric materials available from biomass for flexible packaging is reported. Moreover, the biodegradable and renewable materials investigated or yet employed in this sector are described. In addition, some insights on the actual production of flexible packaging is given, to describe also what technical specification the polymers from biomass should have to replace commodities. Finally, the perspectives in the field of flexible packaging for polymers from biomass are discussed.

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1. Polymeric Materials from Biomass

As material scientists, we often need to study and select the best material for a specific application. In many cases, especially for applications requiring the combination of lightness and resistance, polymeric materials are quite interesting, as well as blends or composites obtained by using them blended with other polymers or with inorganic or organic fillers.

In fact, polymers, with respect to other materials, show a higher versatility due to the possibility of modulating their macromolecular structure by controlling the industrial synthesis in order to achieve specific final properties.

The structural materials selected by nature in plants and animals are based on polymers too. The main structural polymer for plants is cellulose, a polyether, which is the polymer of D-glucose having each repeating unit linked to the following one by a β(1-4)-linkage. The main structural polymers for animals are proteins, characterized by a polyamide structure.

In general, polymers can be natural, artificial or synthetic (Table 1). An example of a natural polymer is cellulose, contained in paper or cotton fabric; among

natural polymers are also the polymers produced by microorganisms, such as poly(hydroxyalcanoates) (PHAs). An example of an artificial polymer, obtained by chemical modification of natural polymers, is cellulose acetate, a plastic material, or cellulose xanthate, a fiber for textile applications, or nitro-cellulose, which can be an explosive, but with a low degree of nitration is employed in varnishes and enamels in the cosmetic sector [1].

The natural and artificial polymers show the advantage of being renewable; that is, they are obtained not from petrochemical resources but from natural sources.

Examples of synthetic polymers are given, in the packaging sector, by the so-called commodities, consisting in polyethylene (PE), polypropylene (PP), poly(ethylene terephthalate) (PET) or polystyrene (PS).

Currently biosynthetic polymers are distinguished from synthetic polymers because they were industrially produced by traditional polymerization methods, but starting from monomers available from natural sources [2]. Hence they are renewable. Poly(lactic acid) (PLA) is an example of biosynthetic polymer. The advantage of biosynthetic polymers with respect to natural and artificial polymers is the possibility of controlling the primary structure of the polymer and consequently its processability and its properties.

Table 1. Classification of polymers with respect to renewability and biodegradability.

Class	Renewability	Biodegradability
natural	YES	YES [a]
artificial	YES [b]	POSSIBLE
synthetic	NO	NO [c]
bio-synthetic	YES	YES/NO

[a] although some evaluations about it were made [3], natural rubber cannot be considered biodegradable; [b] the renewability of the chemicals used for modification should be considered as well; [c] some petrochemical-based polymer are biodegradable.

From the point of view of biodegradability [4,5] usually natural polymers are biodegradable where biodegradability is the capability of resulting in the complete oxidation in the environment giving CH_4, CO_2 and other simple compounds. This is typical of all polymers, but for synthetic polymers very long times—centuries—are required (Figure 1). Artificial polymers can be biodegradable, but usually it depends much on the degree of chemical modification reached. For instance, cellulose acetate, that can have an acetylation degree between 0 and 3, is reported to be biodegradable with an acetylation degree below 2.5 [6]. Biosynthetic polymers are usually both bio-based and biodegradable, such as PLA, but also polymers defined as synthetic, such as PE, traditionally obtained by petrochemicals, can now be produced from natural sources. These polymers are not biodegradable.

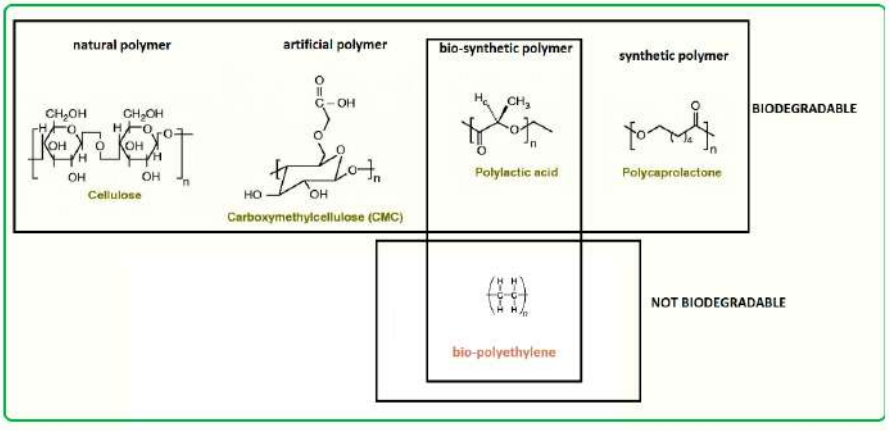

Figure 1. Scheme related to bioplastics with examples of specific polymers.

In addition, in bioplastics also the polymers currently not yet synthesized by biotechnology but from not biobased monomers, such as poly(butylene succinate) (PBS), polycaprolactone (PCL) and poly(butylene adipate-co-terephthalate) (PBAT) et cetera, are included, because they are biodegradable. The bioplastics family thus regroups polymers coming from renewable and/or biodegradable sources [7].

In the field of packaging, the most interesting options for polymers in applications depend much on the end-life option [8,9]. While in durable applications biodegradability is not an interesting property, in the food-packaging sector it could be so, especially if the perspective of composting is selected for the waste packaging.

More important than biodegradability is compostability. The latter is related to the capability of a material to effectively be disintegrated because of microorganism activity in the environment in standard conditions. Biodegradable and compostable products should be certified according to EN 13432/14995 standards [10,11], defining procedures for testing the effective compostability, granting the correct behavior of the material in the composting plants.

The other possible option for packaging management is recycling, which requires the possibility of separating the different polymers, avoiding contamination as well as good durability of the plastic material, especially in re-processing conditions. Thus, it is an option suitable for mono-material packages.

2. Commercially Available Polymeric Materials from Biomass

In considering bioplastics that can be employed in flexible packaging preparation, polymers produced on an industrial scale and yet available in the market and validated in processes commonly used in plastic industry have to be taken mainly into account.

Two different types of basic formulations were developed on a mature technological level: starch-based materials and poly(lactic acid) (PLA)-based materials.

Starch is a natural polymer, consisting of amylose and amylopectin, with the former being linear and water soluble, and the latter branched and water insoluble. The processing of starch was possible some decades ago by controlling the plasticizing effect of water [12], culminating in the thermoplastic processing of starch at approximately its natural water content (about 15%) at a temperature of about 100 °C. Amorphous thermoplastic starch (TPS)-based polymers were thus obtained. An important characteristic of thermoplastic starch formation is the thermal and mechanical (shear-based) destructuring of the starch granules to form a homogeneous melt, the formation being accompanied by swelling. In particular, the gelatinization of starch occurs [13] thanks to the processing, in which the starch granules become swollen and destructured and lose amylose by diffusion. This process, having a typical temperature dependent on the water content, results in the destruction of amylopectin crystallites and molecular order in the granules. The gelatinization represents an undesired state. Hence the range of temperature for processing is superiorly limited by the gelatization point. From a rheological point of view TPSs show the possibility of being processed only in a restricted screw speed range, but in that range the behavior is shear thinning, similar to the one of low density polyethylene, with apparent viscosity decreasing with the increase of screw speed [14]. Destructured starch behaves as a thermoplastic polymer and can be processed as a traditional plastic; when alone, however, its sensitivity to humidity makes it unsuitable for most of the applications. The main use of destructured starch alone is in soluble compostable foams such as loose-fillers, and other expanded items as a replacement for polystyrene.

The attaining of processable starch-based formulations suitable for flexible film production was possible by blending starch with thermoplastic hydrophilic synthetic polymers such as poly(caprolactone) [15] and poly(ethylene-vinyl alcohol) [16]. Usually plasticizers such as glycerol [17] or polyethylene glycols [12] are used for optimizing TPS processing. Cassava, also known as manioc or yucca, is a plant producing tuberous roots, typical of South America. Ezehoa et al. [18] reported the preparation of cassava starch-based formulations employed for preparing films by blown film extrusion. The films were obtained by adding poly(vinyl alcohol) (PVA), which is the matrix in the system. Ali et al. [19] also reported the preparation of films for packaging containing starch, but these films consisted mainly of polyethylene. However, in general it is not possible to employ starch for packaging films without blending it with plasticizers and polymers. The granules available on the market and widely employed for soft packaging, especially for pouches, were developed by considering this approach. NOVAMONT, which is the main producer of MATER-BI material, also follows this approach, but using renewable

and biodegradable self-produced additives. These intermediates are produced by vegetable oils and are defined polymeric complexing agents [20]. In fact, they interact with the starch, incorporating it. Hence the processing and mechanical properties were easily modulated and the starch is protected by environmental humidity by the barrier properties of the host polymers/additives.

Another promising material for packaging application, because of its cost, which is now not much higher than the one of PS (at about 2 €/kg in Europe [21]), is poly(lactic acid) (PLA), which is renewable and biodegradable. Because of its rigidity it is not suitable alone for flexible packaging applications. However, commercial granules suitable for flexible packaging applications are available. In fact, in the last decade, many studies were carried out about PLA blends with other biodegradable polyesters or plasticizers. Plasticized and nano-filled films were prepared by flat die extrusion by Scatto et al. [22]. The rheological measurements carried out by capillary viscometer suggested that the processability of plasticized and nano-filled PLA is suitable for the industrial production of cast films. Plasticization reduces the glass transition temperature of the plastic material, making it ductile at room temperature. A good miscibility of the plasticizer is important, to avoid demixing and loss in transparency of films. Moreover, the plasticizer should not migrate out of the film. The production of film based on PLA, biodegradable plasticizers and nano clays was investigated in the running EC project DIBBIOPACK "Development of injection and extrusion blow molded biodegradable and multifunctional packages by nanotechnologies" [23].

Blending with other commercial polymers is also another possibility for increasing PLA flexibility. Blends of PLA and poly(butylene adipate-co-terephthalate) (PBAT) show a good flexibility and ductility in a proper composition range [24,25]. The addition of a plasticizer to PLA/PBAT blends can also provide a further alternative for properties modulation [26].

The use of chain extenders [27] or peroxides [28] can be also important for modulating rheological properties introducing an increase in melt viscosity, some branching and hence a shear thinning behavior.

Blends of PLA and PBS were also studied [29,30] for application in flexible film preparation, thanks to the good compatibility between these polyesters.

Many companies are developing new PLA-based formulations of granules suitable for flexible packaging applications and many different granules can be found in the market. The formulations are often very complex, as they contain fillers, such as calcium carbonate or talc, or nucleating agents, to control crystallization of PLA during processing by increasing resistance without compromising the transparency of films. Our research unit has recently developed novel copolymers consisting of PLA, organic plasticizers with epoxy functions and biodegradable elastomeric polyesters, which allows the production of transparent PLA-based

films, maintaining compostability [24,31] and activities for exploiting this know-how producing commercial granules are ongoing.

3. Production of Flexible Packaging by Using Biobased Polymers

The production of flexible packaging is based on the preparation of films. Plastic films and multilayer systems can be manufactured using different converting processes such as blown film extrusion, flat die extrusion, extrusion coating, extrusion laminating and co-extrusion. These processes have advantages and disadvantages depending on the material type in use, the width and thickness of film and the required film properties. The use of starch or biopolyester in such processes introduced some modification with respect to the use of polyolefins (high density poly(ethylene)—HDPE and low density poly(ethylene)—LDPE) and PET. Biopolyesters showed a processability similar to traditional plastics. The control of humidity of biopolyesters is significant for limiting the increase of melt fluidity due to chain scission, hence industrial plants must be equipped with proper drying sections. In this equipment usually biopolyester pellets are heated in presence of a dried air flow, with a low dew point, maintaining them stirred [32] to avoid undesired agglomeration, before extrusion.

One of the most commonly employed methods for preparing plastic film is blown film extrusion [30,33]. The produced film is tubular, hence this process is usually employed for the production of pouches, industrial bags or packaging films for shrink wrapping. The necessary industrial equipment consists of an extruder equipped with an annular die. The blown film process involves the biaxial stretching of annular extrudate to make a suitable bubble according to the product requirements. During this film-blowing process, the molten polymer from the annular die is pulling upward applying the take-up force; air is introduced at the bottom of the die to inflate the bubble and an air ring is used to cool the extrudate. The nip rolls are used to provide the axial tension needed to pull and flatten the film into the winder (Figure 2). The speed of the nip rolls and the air pressure inside the bubble are adjusted to maintain the process and product requirements. At a certain height from the die exit, molten polymer is solidified due to the effect of cooling followed by crystallization, and a freeze line height (FLH) can be observed. Above this point the bubble diameter is assumed to be constant.

Bubble size is maintained by controlling the air through a hole in the die face. Addition of air inside the bubble will expand it to a larger diameter and vice versa. This inflation process will stretch the bubble in the circumferential direction (CD). The ratio of this expanded bubble diameter and the die diameter is defined as blow-up ratio (BUR). To pull the extrudate in the upward direction, an axial force is applied by means of nip rollers and hence another stretching in the axial or machine direction (MD) occurs. Draw-down ratio (DDR), which is another important process variable

is defined as the ratio of the linear speed of the film at the nip rolls and the average melt velocity at the die exit. Melt rheological properties are important in the blown film extrusion to determine the processability, shape and stability of the film bubble and the onset of surface roughness.

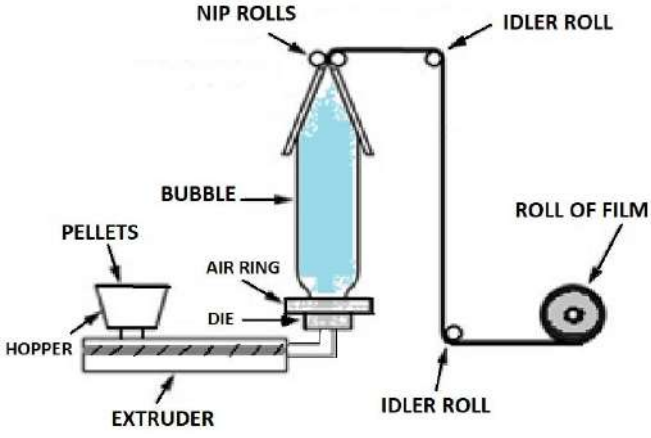

Figure 2. Schematic diagram of the blown film process.

Usually the polymer employed in this process for film production is low-density polyethylene (LDPE) or linear low-density polyethylene (LLDPE). The degree of branching and the length of the branching are particularly important for the success of the process. Generally, a high short chain branching of LDPE influences much the capability of crystallizing of the polymer, much influencing the mechanical and optical properties of the film. A polymer having a more irregular structure is thus more transparent. Long chain branching (LCB) is usually necessary because, considering the rheological behavior, it is correlated to shear thinning (viscosity decreasing by increasing shear rate in a stationary flow) and tension thickening (viscosity increasing by increasing the extensional flow) [34].

PLA-based materials show generally a melt fluidity too high to be processed in these plants. Usually chain extenders are used to improve the viscosity of the melt and the melt strength to allow the production of films, and commercial ones are available on the market [35]. Lazzeri et al. developed a patent to produce transparent PLA-based blends containing an epoxidized molecule acting both as plasticizer, compatibilizer and fluidity regulator [24], thus allowing a good control of processability and final properties. With respect to traditional polyolefins, the tearing strength of these blends should be improved. Recently it was evidenced that by using the epoxidized molecule in combination with a nucleating agent it was possible to improve the tearing strength of the blends [36] reaching values comparable to those of PP.

Flat die extrusion consists of an extrusion through a linear die of adjustable thickness (die gap) usually between 3 and 1.4 mm. This technology allows the production of polymeric sheets and films (with thicknesses ranging from 50 microns to a millimeter) and consists of the extrusion of the molten polymer through a die of rectangular geometry. The geometry generally used for the flat die distribution channel is the coat hanger die. The material comes out from the die in the form of a molten plate that is immediately in contact with a thermostatic roller to allow cooling and solidification. Due to the motion of the roller, the film undergoes elongation with a consequent reduction in thickness. The film then passes through a second roller and to a measuring, cutting and winding station.

The operating parameters to be controlled during flat die extrusion are the extrusion and windup rolls temperatures, distance between die and the first roll and the draw ratio (ratio between windup roll speed and polymer speed at the die exit).

All of these parameters have a big influence on the final product characteristics (morphologically and mechanically). (Figure 3).

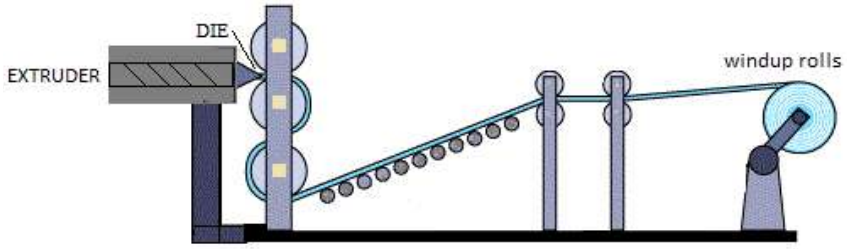

Figure 3. Schematic diagram of the flat die extrusion.

In flat die extrusion, as well as in blown extrusion, the necessity of increasing the melt viscosity of PLA-based blends is often an issue, but usually melt fluidity values higher than those adopted in the blown extrusion are suitable for this processing technique.

Films produced through this technique (Figure 3) can be used in flexible packaging and they can be used also in multilayer systems. Diaper top-sheets are generally made by using polyolefins with a specific patterning of holes on the surface allowing the controlled passage of liquids through the film. Currently the ongoing POLYBIOSKIN European project [37] is active in developing a diaper top-sheet based on biobased formulations to replace traditional ones. The final objective is developing diapers completely compostable and with improved compatibility with skin. Hence PLA-based granules are selected and processed by flat die extrusion to obtain flexible films (Figures 3 and 4).

Figure 4. Flat die extrusion of PLA (**left**) and flat die extruded films based on PLA/PBAT blends (**right**).

Extrusion coating consists of the extrusion of a film of plastic onto a preformed film made of plastic, cellulose or aluminum, hence it is a technique suitable for multilayer packages. The current process of extrusion coating involves extruding resin from a slot at temperatures up to 300 °C directly onto the moving substrate. The control of velocity at which the substrate runs allows to control the coating thickness. High extensional flows are encountered at non-isothermal conditions, especially in industrial conditions requiring higher and higher output speeds. Currently it is very difficult for biopolyester-based materials to have the suitable processing properties in terms of fluidity and melts strength typical of polyolefins to perform extrusion coating in industrial plant currently used for producing PE/aluminum bilayer typical of the most employed multilayer packages such as TETRAPACK [38].

Coating of plastic films with water-based solution containing whey was studied in the framework of the WHEYLAYER European project [39]. The project results showed that the layer made of whey, coming from the waste cheese industry, a protein in between other polymeric films (for instance PET and PE films), allowed separation of different materials in a water bath containing enzymatic detergents [40]. Hence a multilayer package allowing the full recovery and recycling of all the polymeric components was developed in the framework of this project. Moreover, the whey layer shows interesting barrier properties towards oxygen.

Another technique for producing multilayer packaging is extrusion laminating. It is similar to the extrusion coating process except that the extruded melt polymer acts as the adhesive to a second film of material (Figure 5). Specific scientific papers about this technique could not be found. In any case, in this sector, the development of biodegradable glues is an interesting topic of current research [41].

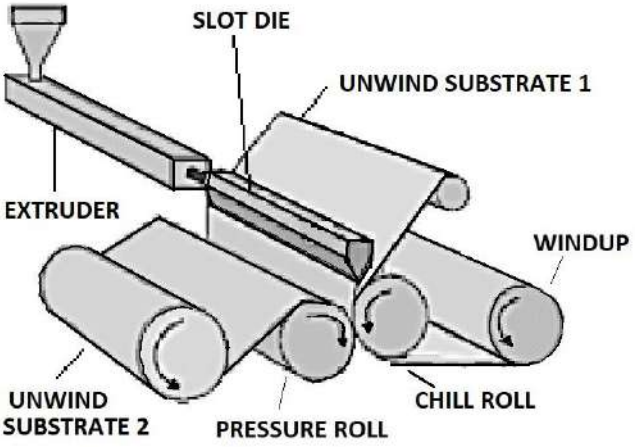

Figure 5. Schematic diagram of extrusion laminating.

Co-extrusion is a similar process to extrusion coating but with two or more extruders coupled to a single die head in which the individually extruded melts are brought together and finally extruded as a multi-layer film. In this case, the viscosity differences between the different layers are of critical importance to avoid distortions of the produced multilayer system. Regarding this process applied to bioplastics, only a patent reported the co-extrusion of PLA with poly(ethylene-co-vinyl acetate) (EVA), a not biodegradable polymer, to obtain multilayer films [42]. Certainly the study of processes allowing to obtain completely biodegradable systems would be useful.

In general, for the processing of biobased polymers, structural and rheological properties of extruded polymers are fundamental for optimizing industrial processes. In general, as stated above, usually for extrusion coating, less viscous polymer melts are required than for blown film extrusion, but a polymer having proper shear thinning and tension thickening behavior must be selected to avoid instabilities and defects thus avoiding the breakage of the film, leading to the undesired interruption of the industrial production [43].

Regarding the processing of starch-based materials, the method based on blown film extrusion is quite common. In fact, the commercial starch-based materials are used for producing pouches and shopping bags. The use of these bags to replace polyolefin ones is supported by EU regulation [44]. Regarding the processing of PLA, films are produced by flat die extrusion [37] or blown film extrusion [36].

4. Innovative Plastic Formulations for Flexible Packaging

Innovative biopolymer-based materials are developed by academies and companies all over the world. Many studies are focused on improving the

properties of starch or PLA-based formulations, but also in fostering the use of other biopolymers by making easier their preparation and finding new applications or also by trying to develop new materials employing natural abundant polymers such as polysaccharides (cellulose or chitin) or proteins. In both cases the studies are focused onto waste coming from agricultural or food industries as starting materials.

Innovative materials based on starch are currently developed by preparing blends with other biodegradable polymers to improve processability and mechanical properties. Recently Hwang et al. [45] prepared PLA/starch blends by melt blending by using maleic anhydride in the presence of a peroxide in order to obtain in one processing step both functionalization of PLA and reactive compatibilization between starch and PLA. Although some improvements in compatibility were observed, the tensile properties were very similar to the one of pure PLA. Reasonably, the combination of increased compatibility with biopolyesters and a better knowledge about the effect of plasticizers coming from renewable sources, as more and more of them will be obtained by new biorefineries [46], will lead towards the formulation of materials with improved properties.

PLA-based materials for flexible packaging applications will be improved by deepening the knowledge about nano-composite preparation and properties and selecting suitable and performing plasticizers. PLA nano-composites are promising because the presence of nano-fillers can improve some functional properties (barrier properties, thermo-mechanical properties, anti-microbial properties, etc.) without affecting the density of material, because generally only a few percent of nano-fillers is needed to improve physical-chemical and mechanical properties. These properties are in fact very important for flexible packaging applications. The addition to PLA of layered nano-filler was widely studied [47] as these kinds of fillers affect barrier properties and so are very interesting in the attempt to develop packaging films. The barrier properties of PLA to O_2, CO_2 and water vapor are reported by Auras et al. [48] and summarized on Table 2. It can be noticed that in general the permeability properties are slightly lower than the ones of PET, with the exception of water vapor permeability, where the PLA is slightly better.

Table 2. Barrier properties of PET and PLA on the basis of Auras paper [48].

	CO_2 Permeability	**Oxygen Permeability**	**Water Vapor Permeability Coefficient (WVPC)**
PLA	1.99 [a]–2.77 [b] $\times 10^{-14}$ g·m/(m^2·s·Pa) at 0% RH from 25 °C	1.39 [a] –1.21 [b] $\times 10^{-15}$ g·m/(m^2·s·Pa) at 25 °C and 70% RH	1.61 [a]–1.65 [b] $\times 10^{-11}$ g·m/(m^2·s·Pa) at 30 °C in the 40% to 90% RH range
PET	1.73 $\times 10^{-15}$ g·m/(m^2·s·Pa) at 0% RH from 25 °C	1.88 $\times 10^{-16}$ g·m/(m^2·s·Pa) measured at 25 °C and 70% RH	1.1 $\times 10^{-12}$ g·m/(m^2·s·Pa) at 25 °C in the 40% to 90% RH range

[a] PLA 4040-D commercial product; [b] PLA 4030-D commercial product.

On the basis of these data, PET and PLA are both hydrophobic films that absorb very low amounts of water and show similar barrier property behavior. Hence the replacement of PET in flexible packaging applications would be possible.

As stated above, many researchers tried to improve the barrier properties of PLA by preparing nano-composites. The dispersion of phyllosilicates into polymers or blends at the nanometer scale [49] allowed improvement of the properties of the polymer matrix such as thermal stability. PLA nano-composites that contain phyllosilicates are considered superior for providing improved gas barrier properties, due mainly to the strong effect of confinement as the result of a high surface:volume ratio (i.e., reducing chain mobility and permeability [49]), as well as to the enhancement of tortuosity [50] of the path required for small molecules to permeate through a polymer film due to the presence of silicate lamellae.

New synthetic and processing routes were investigated by Castiello et al. for the production of PLA-based nano-composites containing a modified clay [51]. The direct synthesis of PLA by ring opening polymerization in the clays was compared in terms of structure and property features of nano-composites with a method based on melt processing in a laboratory batch mixer adding clay or modified clay to PLA. Although the in situ polymerization could be promising, the latter method showed evident advantages in terms of time and cost. Successively, the processing was successfully scaled up in a semi-industrial extruder in order to prepare extruded films for flexible packaging [22,23]. However, since the addition of plasticizer was necessary for attaining the suitable flexibility, the barrier property improvements due to the clay addition was counterbalanced by its reduction due to plasticization, making the material in the amorphous state at room temperature and so more suitable for gases and vapors diffusion.

Nano-cellulose, which represents the crystalline part of cellulose fibers, is also very interesting as a filler to be dispersed in PLA in an attempt to improve the barrier properties of PLA to oxygen, as evidenced by several authors [52,53].

Chitin is another interesting source, as it is very abundant because it represents a waste of the food industry being present in the exoskeletons of crabs, krills, lobsters and shrimps [54]. From chitin it is possible to obtain on an industrial scale chitin nano-fibrils [55] up to now employed in the sector of cosmesis and biotextiles [56]. The addition of chitin nano-fibrils to PLA is very innovative and plasticized, and nano-filled PLA showed composition-dependent tensile properties [57] suitable for flexible packaging. As an antimicrobial and antifungal behavior was reported for chitin [58] and chitin nano-fibrils [59], the possibility of exploiting this approach for producing nano-reinforced antimicrobial films is quite interesting. This represented one of the objectives of the European project N-CHITOPACK [60]. One of the aims of the project was to develop a biodegradable nanostructured film containing chitin nano-fibrils. The observed transparency of the films was in agreement with

a nano-scaled dispersion of chitin nano-fibrils inside the material (Figure 6). This result was achieved by studying a suitable method for adding chitin nano-fibrils to polymers during extrusion avoiding nano-fibril agglomeration. These films were biodegradable, as evidenced by Cinelli et al. [61].

Figure 6. Commercial compostable biobased films (**left**) based on BioComp® BF 3051; films (**Right**) obtained with the same material but with addition of chitin nano-fibrils.

In the field of polyesters, polyhydroxyalcanoates (PHAs) should also be considered [62], as they are produced by microorganisms. Since the biotechnology for obtaining them is not yet at a mature level, the price of these polymers is quite high (4–5 €/kg [63]). Several researchers are targeting optimization of production and extraction process and the use of waste material-based substrates for the growth of microorganism producing PHAs, as developed in the EC project Oli-PHA "A novel and efficient method for the production of PHA polymer-based packaging from olive oil waste water" under the scientific coordination of our research unit [64]. Their processability changes as a function of the molecular weight and also primary structure of the PHAs. However, in general these materials are brittle and very viscous when melted. Hence the use of a proper plasticizer is fundamental for processing. The necessity of controlling the migration of plasticizer from PHAs to avoid stickiness of the material and loss of ductility was evidenced by Farris et al. [65].

The other important class of biomaterials is represented by proteins, widely available as by-products or waste of the agricultural and horticultural industries and the industry of food or leather.

Hence different kinds of proteins are also studied as potential plastic materials for producing films. As a result, proteins from plants (wheat gluten, soy, sunflower, and corn) and animals (gelatin, keratin, casein, and whey) were employed in plastic formulation [66–68].

However many studies have been carried out using casting and compression molding techniques. In fact, the processing of proteins is still difficult to control with a conventional extrusion machine, as evidenced by Verbeek et al. [69].

In particular the employment of flat die extrusion and calendering is quite difficult. In this case, a successful production of protein-based laminates was reported by blending with non-biodegradable polymers such as poly(ethylene-co-vinyl acetate) (EVA) [70]. Moreover, new fully biodegradable composites based on whey/PBS blends were developed in the framework of the BIOBOARD European project [38,70] but it was possible to prepare films with a higher thickness than conventional films used in packaging. On the other hand, to exploit the barrier properties of whey, the application of layer coating made of protein onto a polymeric seemed easier, as evidenced by Cinelli et al. [36]. If techniques based on casting from water solutions are considered, also chitosan, the polymer obtained industrially by the deacetilation of chitin, can be a very interesting material for the production of coatings [71,72].

Chitosan is a biodegradable polymer that may be used to elaborate edible films or coatings to enhance shelf life of foods. Its water vapor permeability is about 20 times higher with respect to PS, so it is too high with respect to conventional packaging. However, it was demonstrated to have an anti-microbial activity that can be interesting for the production of biopolymer-based biodegradable packaging materials with additional bioactive functions [60].

The necessity of improving the chitosan barrier properties was evidenced by Morreno-Osorio et al. [73], who added to chitosan a natural compound acting as cross-linker, thus improving both mechanical and barrier properties. More frequently nano-fillers were used to improve chitosan barrier properties. Azeredo et al. [74] demonstrated that cellulose nano-fibers (CNF) can improve the mechanical and water vapor barrier properties of chitosan films. A nano-composite film with 15% CNF and plasticized with 18% glycerol was comparable to some synthetic polymers in terms of strength and stiffness, but with poorer elongation and water vapor barrier, indicating that they can be used for applications that do not require high flexibility and/or water vapor barrier. The more important advantage of such films when compared to synthetic polymer films is their environmentally friendly properties.

However up to now, despite of the production of cast films based on chitosan being successfully set-up [75], there are not yet suitable continuous machines for preparing chitosan-based films. In addition, the packaging of wet food cannot be done, as the material can dissolve in water and has a too much high water permeability. However anti-microbial properties are reported for chitosan films and biotextiles [76]. Hence the preparation of multilayer systems including one layer of chitosan should be very interesting.

5. Multi-Layer Systems: The Most Environmental Friendly Application in Food Packaging

The production of multilayer packaging films is very important in the field of packaging because it offers the opportunity of protecting the content of packaging from the interaction with different gases. As an example, the case of traditional multilayer sheets based on poly(ethylene terephthalate) (PET) and poly(ethylene) (PE) can be reported.

The recycling of these multilayer packages is not easy because the different polymeric, cellulosic or metal-based materials must be separated. Natural polymers are much used in flexible packaging, since cellulose is employed in paper-based packaging. Usually this kind of packaging consists of two layers for dried food, as the paper does not have the suitable barrier properties to water (but it has good barrier properties towards oxygen). Hence, the paper is present in a multilayer system in which also a polyethylene layer, granting the suitable water barrier properties, is present. For non-dried food, creams or liquids, also an aluminum foil layer is present to grant a total barrier effect for the packaging. For cellulose-based materials the option of recycling is possible also by considering multilayer systems. The recycling of cellulose is actually carried out in paper plants, by repulping the grinded recovered packaging material. A residual fraction (about the 30% by weight of the material) consists of aluminum and polyethylene, which can be employed in the injection-molding sector. The recycling process should be optimized, for example by separating the aluminum from the PE or replacing the PE with a biodegradable polymer and recovering Al after composting. These possible options for multilayer paper-based packaging are the object of the BIOBOARD European project [38] research activities. In the perspective of having a composting option for future packaging, the possible future scenario can be the one described in Figure 7. The monolayer plastic-based, the multilayer plastic-based and the multilayer paper-based flexible packaging systems need to be modified by replacing the layer with compostable ones. Overall, the packaging must grant the same properties in terms of content protection as the synthetic polymer-based options.

Extensive research has been devoted to the set-up of new synthetic routes for compostable polymer synthesis [77,78], and to the recovery process of materials from biomass in order to make them available for employment in materials production [79–81]. Many papers have been devoted to the blending of currently available polymers with processing aids in order to allow them to have the same properties of commodities [82,83] and others (less numerous) have been trying to find out new industrial technologies suitable for biomass derived materials [84]. All this research work can contribute to the aim of replacing flexible packaging with compostable and renewable ones in the future, but it should be stressed that the last point is fundamental. In fact, often the new materials cannot be processed

exactly as commodities. Given that the plastic industrial sector mainly consists of numerous and small enterprises, plastics converters requiring new biobased plastics that have the same characteristics and processability of conventional plastic-based counterparts, as the conversion of industrial lines to new ones, could require huge investments for them.

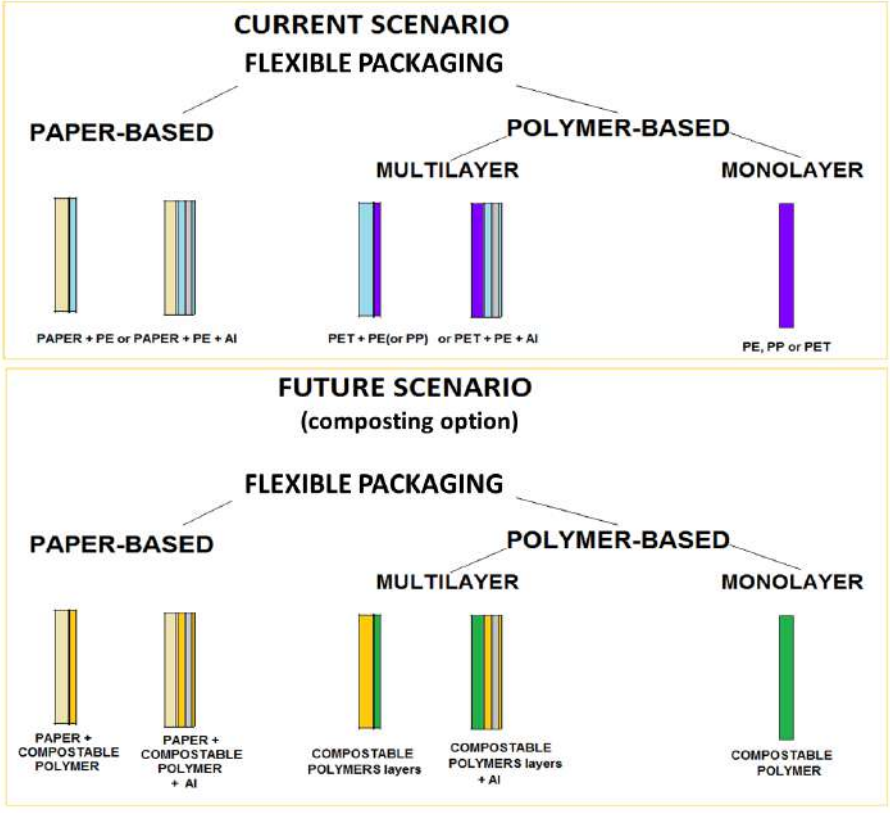

Figure 7. Polymeric materials currently employed in packaging and future perspectives in the hypothesis of composting option for flexible packaging.

The preparation of nano-composites can be effective in reducing gas permeability, hence a system in which the different layers are nanostructured can allow an optimized set of properties for a specific packaging.

Multilayer systems should be prepared preferentially by co-extrusion or lamination. Hence the material employed for making the different layers must be processable by these techniques. It is fundamental for controlling the structure of the polymers and especially their rheological behavior. However recently some researchers published a work about a new technique for preparing a peculiar

multilayer system by using a wet method [85]. A PLA film was immersed in a aqueous solution (Figure 8) of chitosan and successively in a aqueous suspension of sodium montmorillonite (MMT).

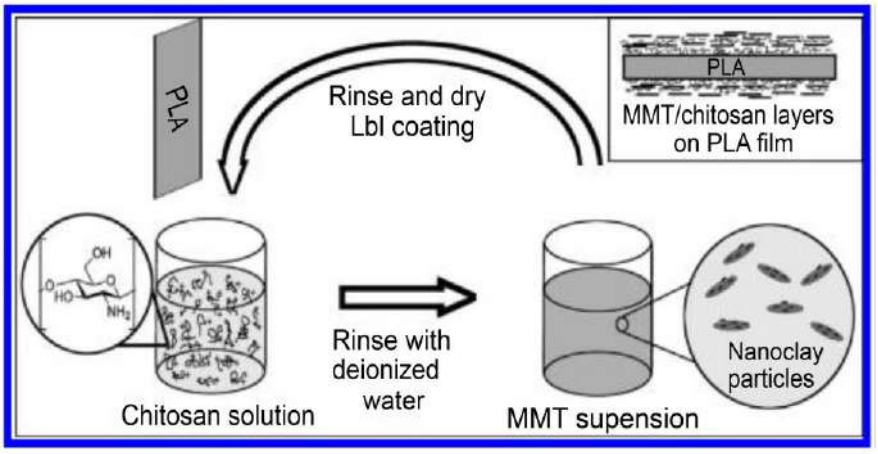

Figure 8. Illustration about LBL technique for depositing a chitosan/montmorillonite nano-structured coating on the surface of a PLA film. Reprinted with permission from [80], Copyright 2012, American Chemical Society.

By making 40 successive immersion steps, 40 alternate layers of chitosan (having a positive charge) and MMT platelets (having a negative charge) were deposited on the PLA films, thus producing a nanostructured coating. The peculiar technique was called LBL (layer by layer). Successively Laufer et al. [86] employed again the technique for preparing multilayer nano-coatings made with three food contact-approved components (chitosan (CH), poly(acrylic acid) (PAA), and montmorillonite (MMT) clay). They deposited the layers onto PET and polylactic acid (PLA) substrates. At 38 °C and 90% relative humidity (RH), the oxygen transmission rate (OTR) of 500 μm PLA was reduced from 50 to 4.6 cc/(m^2 day atm), which is lower than 179 μm PET film under the same conditions. This good gas barrier is believed to be due to the nano-brick wall structure present in this thin film, where clay platelets act as bricks held together by polymeric mortar. These assembled thin films are also very transparent, which combined with ambient processing and the use of renewable and food contact approved ingredients, makes this a promising foil replacement technology. This technology is quite interesting even if it is not easy to apply it on an industrial scale.

The coating of polymeric films made with biodegradable polymers with a film made with chitosan or another hydro-suspendable biopolymer-based formulation can be obtained by continuously spraying a film at the end of a flat die extrusion

plant. In this way a bilayer system can be obtained continuously without introducing enormous changes in the plant.

These papers evidence that the preparation of a multilayer containing biopolyesters and chitosan, also with the presence of nano-filler, is a quite interesting scientific and technological challenge.

The preparation of multilayer packaging containing paper layers and biobased polymers can be very interesting for producing fully biodegradable soft packaging with optimal barrier properties. Coltelli et al. reviewed the use of proteins from many natural sources in paper and paperboard coatings, making an extensive comparison in mechanical and barrier properties between proteins from several sources and having different primary structures [87].

In multilayer systems the formulation of biodegradable glues for lamination or biodegradable hot melt for extrusion coating is an interesting topic for current research [41]. For binding, protein or starch-based adhesives can be used, whereas for hot melt glues, PLA or PCL can be used. Sodergard et al. [88,89] studied blends of lactic acid-based hot melt adhesives with oxidized potato starch and poly(ethylene glycol). Thanks to this approach the authors increased the disintegration rate of the glue, making it suitable for the production of compostable multilayer packaging.

6. Challenges and Perspectives

The use of biobased plastics in flexible packaging is possible by employing different products. The main commercial products are based on starch or PLA. In both cases biopolymers, plasticizers, chain extenders and fillers are added to the material to modulate their properties. Some commercial products are available on the market, but research is in progress to better understand the correlation between formulation (new biobased additives are continuously introduced in the market especially thanks to the ongoing developments of biorefineries [46]), processability and final properties of such bioplastic materials.

In the field of starch-based materials, systematic research aimed at developing a knowledge about the integrated effect of biopolyesters, plasticizers and fillers in the starch-based material could be useful. PLA-based nanostructured composites, containing nano-fillers (such as phillosilicates or cellulose or chitin nano-fibrils) can be promising to improve the barrier properties of biodegradable films without modifying their optical properties (transparency).

A bioplastic material for flexible packaging applications should have rheological and mechanical behavior similar to polyolefins. In particular, these conditions are difficult to respect for materials based on natural polymers, such as polysaccharides or proteins. In this case, the application to flexible packaging requires further efforts. In the case of PLA the melt viscosity is low, and usually for developing the blown film extrusion or flat die extrusion a chain extension approach, also leading to branching,

can be followed. On the other hand, in the case of proteins, their tendency to give extensive crosslinking by heating during processing can be counterbalanced by the addition of plasticizers or reactive molecules, which results in the decrease in melt viscosity.

In addition, the preparation of multilayer systems by employing biopolymer sheets can be a promising technique for flexible packaging. The replacement of current multilayers packaging with fully biodegradable ones, by keeping into account the scheme of Figure 7, will require a good adhesion between the different polymers and paper sheets. In the case of starch-based materials the good adhesion is granted by the high chemical affinity between starch and cellulose. In some cases the necessity of improving the adhesion by the use of proper biodegradable glues, by the application of surficial treatments (mechanical, plasma or corona) or by peculiar thermal and mechanical treatments to films could be useful to better design multilayer packages taking into account their end-life management.

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Naturally-Made Hard Containers for Food Packaging: Actual and Future Perspectives

Patrizia Cinelli, Maria-Beatrice Coltelli and Andrea Lazzeri

Abstract: The huge amount of plastic used in packaging application and the related problems of disposal of the packaging after its use have gained research and industrial interest in both bio-based polymers that are not biodegradable but produced by renewable resources and bio-based compostable polymers, to be used for production of packaging. The production of bio-polymers is more costly than that of conventional fossil fuel-based polymers. Moreover, the range of applications of polymers derived from natural sources was limited due to difficulties in processing natural materials, moisture sensitivity, incompatibility at the interface between natural fillers and polymeric matrices, possible toxicity related to natural material degradation, poor mechanical properties, etc. Mass transfer properties are very important for packaging application, and at present, bio-based packaging generally lacks in maintaining barrier properties compared to traditional petro-derived polymers. The combination of traditional polymers and biodegradable ones in multilayer systems allowed obtaining a good balance of mass transfer properties, but the presence of the non-biodegradable layer negatively affects, and in some cases compromises, the composting of the final packaging. Recent advances in technology are reducing the cost of manufacturing bio-based plastics and are producing materials with an expanded range of properties that has made them suitable for low cost and high demanding applications such as packaging. Chemical modifications of the bio-based polymers, as well as blending with biodegradable additives or polymers are the preferred solutions to improve and control the properties of these materials. In this work, several materials derived from biomasses such as polyesters (poly lactic acid, poly hydroxyl alkanoates), polysaccharides (starch), vegetal and animal proteins, etc., which can be used in hard packaging applications are reviewed. Modification and processing of bio-based products with additives, polymers and natural fibers (cellulose, wood fibers, etc.) are discussed as well. The suitability of these materials for the industrial processing required for the production of hard food packaging is reported critically in order to evidence the challenges and perspectives for polymers derived from renewable resources to be applied in this sector.

1. Introduction

Plastic materials applied in packaging can match almost all requirements, perfectly meeting customers' demands, offering important advantages such as low weight, protection of the packaged good (and consequent reduction of food waste), suitable mass transfer properties and low cost. Hard packaging comprises containers, bottles, canisters, jars, cups, buckets, trays, clamshells, blisters and the like.

In 2012 in the European Union, the post-consumer plastic waste ending up in the waste upstream was 25.2 million tons; 62% of this waste was recovered through energy recovery and recycling processes, while 38% still was sent to landfill [1].

The recent European Commission (EC) regulation promotes the bio-recycling of plastic waste through composting or anaerobic digestion, as well as the utilization of renewable resources for the production of plastics. Most recently, the development of environmental-friendly bio-based and or biodegradable polymeric materials from renewable sources has attracted extensive interest. This is due to the waste accumulation, but also to the limitation of crude oil or gas supply. Crude oil is the starting point of any conventional plastic, and it will only last for a few more decades, since it is used mainly to serve the energy demand of the population on a worldwide scale. An early conversion to renewable sources is thus important for the plastics industry.

Although only 4% of the global oil consumption is used to produce plastics (and a further 4% is used to produce the energy for the production of plastics), the new technologies required to process renewable sources thus preparing for the "post-oil era" require a sufficient time to be developed. About 46 million tons of plastics were used in the European Union in 2012 [1], about 40% for packaging production, and ca. 12 million tons of this plastic were used for rigid packaging.

In a recent fact sheet [2], European Bioplastic reports that the bioplastic producers are engaged to life cycle thinking and further improving their products, increasing the yield of their processes, thus producing more with less and ensuring sustainable resource supply to decrease the impact on the environment. Various life cycle analyses are in agreement with the better performance of bio-based rigid packaging, in particular in the impact categories of global warming potential (often referred to as a carbon footprint) and in the category of the consumption of fossil resources.

Some bio-based plastics are commercialized or are announced to be ready for the market such as polylactic acid (PLA), polybutylene succinate (PBS), polyhydroxy alkanoates (PHA), polyethylene furanoate (PEF) and also bio-based commodity plastics such as polyethylene (PE), polyethylene terephthalate (PET) or, in the very near future, polypropylene (PP). These polymers can be used for the production of rigid packaging.

The traditional market of rigid containers for food and beverages consists essentially of two different categories: the first one, usually defined as mono-material packaging, is based on unique plastic materials; the second is based on two or more materials (multi-material).

In the first category, the most used polymers are poly(ethylene terephthalate) (PET), high density poly(ethylene) (HDPE), poly(propylene) (PP) and polystyrene (PS). PET and HDPE are employed for the production of bottles, as they can be processed by blow molding, whereas PP and PS are much employed in the production of rigid food trays or jar as they can be extruded in sheets and thermoformed. At room temperature, all plastic materials are rigid, but HDPE and PP are above their glass transition temperature. Hence, the rigidity is provided by the presence of the crystalline phase domains, embedded in the main amorphous matrix. As many molecular motions are possible above the glass transition, this peculiar morphology can explain the performance impact properties of these polymers.

Multilayer rigid packaging can consist of thermoplastic polymers or of different materials, such as a multilayer system consisting of plastic, board and aluminum, with board representing the most abundant material. The former is processed usually by thermoforming starting from extruded sheets, whereas the latter is processed by properly creasing and sealing the board-based multilayer sheet.

On the market, rigid packaging based on bio-based and biodegradable polymers is already present, and some examples are reported in Figure 1.

Figure 1. Examples of rigid packaging produced with bio-based polymers.

In all cases, as the market of products is huge, the containers, which are designed purposely for preserving the specific properties of food or beverages, can consist of many different polymeric materials. The polymers cited above are the most employed, because of their wide-scale production and low price. However, many other polymers with specific properties can be addressed to allow the achievement of the desired requirement, and some examples are reported in Table 1.

Table 1. Main application of bio-based polymers in hard packaging.

Hard Packaging	Materials					
	PLA	**PHA**	**Starch**	**PBS**	**BioPET**	**BioPE**
Bottles	x	-	-	-	x	x
Trays	x	-	x	-	x	x
Containers	x	x	x	x	x	x
Caps	x	x	x	x	-	x
Blisters	x	-	x	x	x	-
Foamed packaging	x	-	x	x	-	-
Cutlery	x	-	x	x	-	x

PLA = poly lactic acid, PHA = poly hydroxyl alkanoate, PE = poly ethylene, PET = poly ethylene terephthalate. x = used and present on the market, - = not commonly used.

The applications of these packaging have still a huge potential to increase, and at present, most of the rigid packaging from renewable resources is represented by cardboard and its laminates. This is due to difficulties in processing of bio-based biodegradable polymers for the production of hard packaging and the cost being higher than that of petro-derived polymers.

Thus, an overview of processing technologies for the production of hard packaging is reported below with particular focus on issues related to processing of bio-based-biodegradable polymers.

2. Processing for Production of Hard Packaging

2.1. Injection Molding

The production of plastic rigid containers is carried out by different processes. Among those, injection molding is a processing method applied for polymeric thermoplastic materials. In this process, the polymer is melted in a heated barrel and injected in a mold to produce pieces with a defined shape. The automation of the process allows for series production. This method can be employed for example for producing caps, thick jars for cosmetics, cutlery and coffee capsules. The process consists of three main phases: the injection, in which the partial filling of the mold with the molten polymer is achieved; the holding pressure and plastification, in which

the mold is completely filled by the molten polymer and kept in the mold at a defined pressure and temperature; and the ejection, in which the solid piece is extracted from the mold (Figure 2).

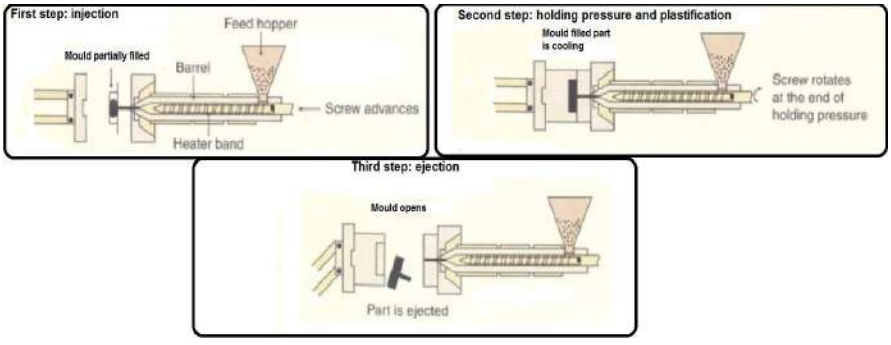


Figure 2. Three main steps of the injection molding process.

The selection and control of the temperature is fundamental in the first step of the process, since, when processing polymers with this technique, a very low viscosity at a high shear rate is required in order to grant a rapid and perfect filling of the mold. The knowledge and control of the rheological behavior of the molten polymer is thus very important to allow a good processing. In particular, melt flow index (MFI) determination at the same temperature of injection molding can be useful, and generally, thermoplastic polymers having MFI above about 10 g/min are suitable for injection molding. In some cases, the dimensions of the filling channel in the mold should be well dimensioned as a function of the melt rheology of the thermoplastic material employed.

In the second step, the temperature of the mold, the holding pressure and the holding time are the parameters that are important to be controlled. Some polymers can crystallize during the holding step; hence, the temperature of the mold and the holding time are quite important to allow the material to reach the desired crystalline morphology, as the amount and distribution of crystals in the material influence its final properties. This is particularly the case for bio-polyesters such as PLA and PHAs.

A specific selection of parameters is necessary also for the further steps. In fact, the ejection step should be done when the piece is solid and resistant enough, in order to avoid the breakage of the piece inside the open mold. The thermal properties such as the glass transition temperature of the material and/or its crystallization kinetics can greatly influence this step. Hence, the determination of the heat deflection temperature (HDT) usually allows one to know the maximum temperature of the mold suitable to allow the ejection without deformation of the prepared item.

This point is critical as the deformation or breakage of the item would require interrupting the processing cycle, thus wasting time, material and energy, but at the same time, in the least time possible, to avoid the waste of time and energy as well.

2.2. Injection Stretch Blow Molding Process

The injection stretch blow molding process, which is the most advanced method for producing bottles, consists of the preliminary injection molding of a pre-form, having the shape of a test tube with a threaded neck. Then, this piece is transferred to a mold where it is blown with an air jet in order to obtain a container having the shape of the mold. The container wall is thus bi-oriented, that is stretched both in the direction of air-flow and in the radial direction. By using this method, bottles of many different shapes and dimensions can be obtained. Transparent bottles available for water or fizzy beverages are made of PET, whereas the opaque ones, used for milk or liquid detergents, are made of HDPE. PLA is also suitable for this process, and most recently, bottles produced from PLA have been proposed; one example on the market is reported in Figure 3 [3–5].

Figure 3. Plastic bottle based on PLA.

2.3. Thermoforming

Arrays, plastic cups, blisters and jars are produced by another important processing method, thermoforming. Whereas in the injection molding or injection stretch blow molding, granules are fed into the equipment, in this case, the polymeric material must be fed in sheets having a thickness in the range of 50–300 microns for packaging production. The process can be applied also to thicker sheets, but in this case, the applications are in the automotive or electric and electronic fields, for the production of bodies or shells. Hence, a preliminary flat die extrusion step

is necessary for producing suitable sheets for thermoforming. In the packaging field, PS or PP is usually employed, but positive results and interesting products were produced also with PLA and PLA filled with natural fibers. In Figure 4 are reported examples of rigid packaging trays and egg containers produced by thermoforming of sheets based on PLA and wood fibers, produced by the authors in the activity of the EC project FORBIOPLAST "Forest Resource Sustainability through Bio-Based-Composite Development". GA 212239 [6].

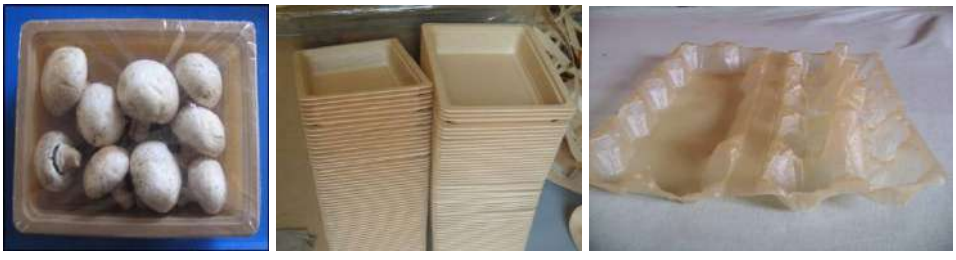


Figure 4. Trays and egg containers based on PLA and wood fibers.

The method consists of heating the material above its glass transition temperature, but below its melting point, thus obtaining a softened sheet usually by using infrared heaters. Then, a mold is inserted (or a vacuum is applied), which gives the softened sheet the desired shape (Figure 5).

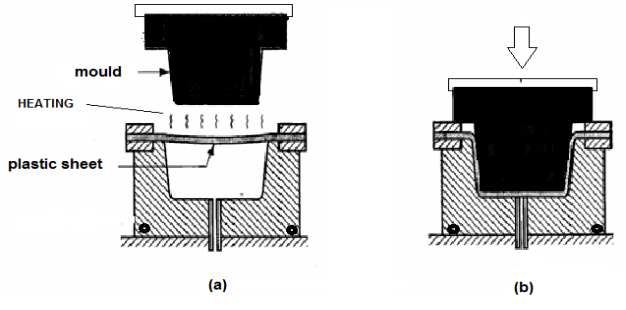


Figure 5. Thermoforming process.

2.4. Multi-Material Packaging

The methods listed up to now are suitable for packages consisting of one single material. However, usually, fresh food requires packaging with enhanced barrier properties, and for these reasons, packaging consisting of two or more layers is necessary. An interesting example is the packaging consisting of one layer of PET and one layer of poly(ethylene) (PE). These bi-layer sheets can be obtained by lamination

of PE and PET or also by co-extrusion, usually employing compatibilizers consisting of ethylene copolymers, such as poly(ethylene-co-vinyl acetate) (EVA) in between the two layers to enhance adhesion and barrier properties. The multi-layer sheets can be thus thermoformed to obtain arrays or blisters, especially employed to pack fresh foods in supermarkets because of their structural, barrier and optical properties (high transparency).

The production of rigid packaging for liquids can be made also by using board-based materials. The usual rigid multilayer system consists of different layers (Figure 6) of different thicknesses, with a layer of PE in contact with the food. In dependence of the perishability of the liquid, a total barrier layer of aluminum may be necessary. This is the case of rigid containers for milk. However, also in the most complex multilayer sheet, the content of cellulose is at least 70% by weight, as the board is the main structural material of the packaging.

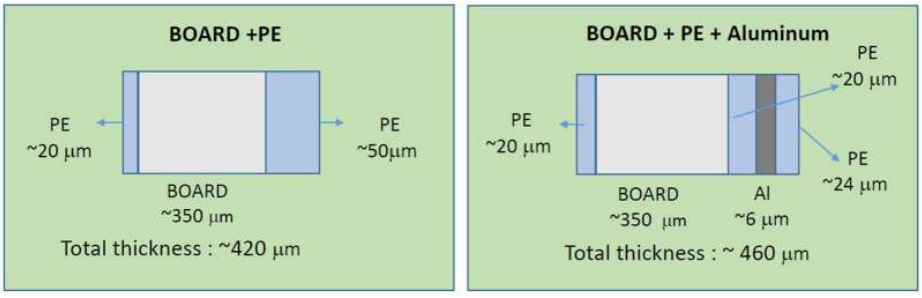


Figure 6. Structure of typical board-based multilayer packaging.

The sheets can be obtained by extrusion coating of PE on Al foil and co-extrusion of PE and board. To obtain the final packaging, the sheets must be properly creased and folded (Figure 7) [7].

Usually, the multilayer sheet is produced by worldwide producers, then adapted to the final users by the conversion plant, where also the printing is realized, whereas the folding is usually carried out in the packaging plant.

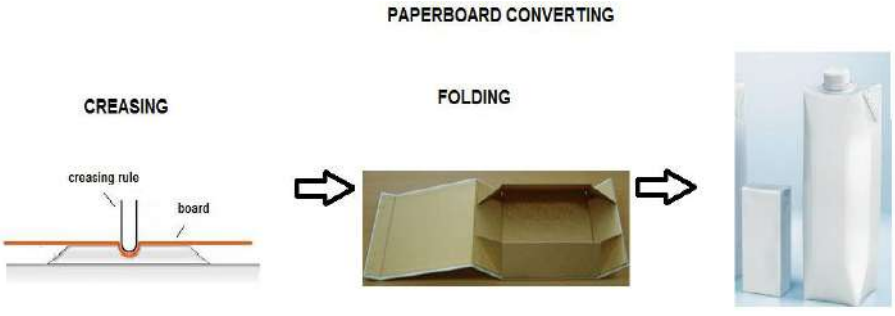


Figure 7. Scheme of paperboard-based packaging conversion.

In Table 2, a summary of the different processes used for the different types of rigid packaging is reported.

Table 2. Examples of processes used for the production of rigid packaging.

Rigid Packages or Products	Process	Polymers
Caps, coffee capsules, jars, etc.	Injection molding	HDPE, PP, PLA; PHA
Bottles	Injection stretch blow molding	PET, HDPE, PLA
Arrays, cups, jar, trays, blisters, etc.	Thermoforming	PS, PP, PET, PET/PE, PLA
Bricks for beverages or cups, etc.	Extrusion coating/laminating, creasing, folding	Cellulose/PE(/Al)

3. Adapting Biobased Polymers to Process for Rigid Packaging

3.1. Poly Lactic Acid

PLA is an aliphatic, thermoplastic polyester derived from renewable resources such as starch and appears as one of the best sustainable alternatives to petrochemical-derived products for applications in packaging [8].

PLA has been found to have good stiffness and strength, and it can be processed with conventional plastic processing machinery (extrusion, extrusion blow molding, injection molding, vacuum forming) and is being used in several applications for hard packaging such as boxes, jars, trays, water and milk bottles.

Products made from PLA degrade in the environment, fragmenting into small molecules, and can be compostable, as, depending on their thickness, they can fully disappear in less than 30 days in ideal conditions [9,10].

Lim et al. [11] explained that the processing of PLA requires a preliminary drying step in order to reduce the detrimental effect of water on the PLA properties [12]. Thus, during the processing in the melt, water can result in the chain scission of PLA macromolecules because of hydrolysis. The hydrolysis is due to the nucleophilic attack of the water molecules on the carbonyl ester groups of PLA. All the authors that processed PLA for producing blends [13,14] adopted this strategy or took into account

the effect of hydrolysis on the final properties of their material. Lim [11] evidenced that commercial-grade PLA pellets are usually crystallized. This permits drying at higher temperatures to reduce the required drying time. In fact, if amorphous pellets are available, they must be dried below the Tg (~60 °C) to avoid pellets sticking together. In this case, drying under vacuum is suggested.

Industrial desiccators are available to control the water content before processing. Drying of PLA is usually attained using a closed-loop dual-bed regenerative desiccant-type dryer, where the dew point of the drying air should be at least −40 °C or lower.

The extrusion of PLA is the processing step allowing granules to be obtained. To ensure the melting of all the crystalline phases and to achieve an optimized melt viscosity for processing, the heater set point is usually set at 200–210 °C. Commercial-grade PLA can be processed in a conventional extruder equipped with a general purpose screw of an l/d ratio in the range of 24–30. Screws for extruding PET, which are low-shear to grant mild mixing (to minimize chain scission and acetaldehyde generation), are also suitable for processing PLA [10]. The compression ratio (the ratio of the flight depth in the feed section to the flight depth in the metering section) for PLA extrusion is in the range of 2–3 [15].

In injection molding, the cycle time is an important parameter, which is often minimized to maximize the production throughput. The filling, holding and cooling events that occur during injection molding have an important implication on the shrinkage of the injection molded articles, which must be controlled to avoid undesired piece deformation. In general, injection molded PLA pieces are relatively brittle, and this brittleness is attributed to the rapid physical aging of the polymer, as the ambient temperature is only about 25 °C below the glass transition temperature. Cai et al. [16] showed that endothermic enthalpy relaxation measured in the glass transition region by DSC increased with increasing aging time. Moreover, it was observed that when molded specimens were aged at room temperature for 3–8 h, they became very brittle [17]. This occurrence was attributed to the reduction of the free volume of the polymer due to rapid relaxation to the equilibrium amorphous state.

Increasing the crystallinity of the polymer can reduce the aging effect [18]. Several authors investigated and are still investigating the effect of nucleating agents on PLA [19–21] in order to make compatible effective crystallization, mechanical stability during time and short injection molding cycles enabling series production. In fact, the formed crystallites act like physical crosslinks to retard the polymer chain mobility. In the framework of the DIBBIOPACK European project *"Development of Injection and Extrusion Blow Moulded Biodegradable and Multifunctional Packages by Nanotechnologies"* GA 280676 [22], some nucleating agents were studied in combination with a plasticizer, to control the evolution of crystallinity in injection

molded specimens [23]. To reduce the brittleness, the plasticization [24–26] and the blending with biodegradable elastomeric polymers [27,28] was also investigated and successfully applied, in some cases following reactive blending [29] or reactive extrusion [30,31] approaches.

Interestingly, by considering the injection stretch blow molding process, it was observed that the molecular orientation induced by this process limits the effect of aging by stabilizing the polymer free volume [17]. Aging is also reduced by the crystallites produced during strain-induced crystallization acting as physical crosslinks to stabilize the amorphous fraction, thus reducing the brittleness. Similarly to PET, PLA exhibits strain-hardening when stretched to high strain. This is desirable for blow molding of pre-forms to minimize wall thickness variation. As strain-hardening occurs only when the PLA is highly stretched, the pre-form must be designed as a function of mold dimension such that optimal stretch ratios are achieved during blow molding. It was noticed that the crystallinity after stretching decreases by decreasing the stereo isomeric fraction of the polymer [22]. Thus, the optimal stretch ratios depend on the grade of employed PLA.

The thermoforming of PLA can be generally made with aluminum molds in the range of 80–110 °C. Molds, trim tools and ovens designed for thermoforming PET and PS can be used for forming PLA containers. On the other hand, molds for thermoforming of PP cannot be used likewise for PLA, since PP shrinks significantly more than PLA during cooling [11].

Many processing methods are thus available to obtain rigid packages from PLA, especially if its properties are modulated by using proper additives, such as other polymers, fillers or low molecular weight additives such as stabilizers and chain extenders.

3.2. Poly Hydroxy Alkanoate

PHAs are gaining attention among biodegradable polymers to be used for packaging production due to their characteristic properties (such as high biodegradability in different environments such as soil and marine water, not just in composting plants) coupled with high barrier properties to oxygen and moisture and relatively high thermal stability [32–35]. These properties differentiate PHAs from PLA and are particularly interesting because of addressing the issue of plastic waste accumulation in the oceans.

The brittleness of polyhydroxy Butyrate (PHB) and polyhydroxy butyrate-co-valerate (PHB/V) is due to the secondary crystallization of the amorphous phase that occurs during storage at room temperature; in fact, the glass transition temperature (Tg) of PHB is close to room temperature. The crystallization kinetics of PHB usually starts from homogeneous nuclei, unless specific nucleating agents are added, since this polymer is free of heterogeneities. In fact, PHB does

not contain catalyst residues or other impurities that can act as heterogeneous nuclei [36–40]. To achieve high elongation at break and a higher flexibility for modified/formulated PHB, the glass transition temperature must reach a lower value than the testing temperature. By adding plasticizers, the molecular mobility is improved and the glass transition temperature is lowered, as well as the melting temperature. For processing, it must be considered also that PHB thermally decomposes at temperatures just above its melting point. Short exposure of PHB to temperatures near 180 °C could induce severe degradation. The main reaction consists of a random chain scission that involves a cis-elimination reaction of β-CH and a six-member ring transition, which results in a rapid decrease in molecular weight accompanied by production of the degraded products of olefinic and carboxylic acid compounds, such as crotonic acid and various oligomers [41–44].

Several blends among PHB and other biodegradable polymers and several types of plasticizers have been investigated [45,46]. Materials of relatively low cost, biodegradable and possibly produced by renewable resources are preferred as plasticizers. Examples are: oxypropylated glycerin (or laprol), glycerol, glycerol triacetate, 4-nonylphenol, 4,40-dihydroxydiphenylmethane, acetyl tributyl citrate, salicylic ester, acetylsalicylic acid ester, soybean oil, epoxidized soybean oil, dibutyl phthalate, triethyl citrate, dioctyl phthalate, dioctyl sebacate, acetyl tributyl citrate, di-2-ethylhexylphthalate, tri(ethylene glycol)-bis(2-ethylhexanoate), triacetin, fatty alcohols with or without glycerol fatty esters, polyethylene glycol (PEG), as well as low molecular weight polyhydroxybutyrate since PHAs with a medium chain length are elastomers with a low melting point and a relatively lower degree of crystallinity [47,48]. In our research unit, we have recently investigated the performances of different degrees of polyethylene glycol (PEG) as plasticizers for PHB-based blends compared to tributyl citrate and the effect of the kinetic of crystallization by the use of these plasticizers [49]. PEG400 and tributyl citrate are very efficient plasticizers for PHB, and PEG400 resulted in being an efficient lubricating agent for the production of composite based on PHB and wood fibers, allowing better processing of the viscous melt containing up to 30% by weight of wood fibers [50]. Blends based on either PHB or PHA and PEG400 have been used for the production of hard packaging jars intended for cosmetic applications, in the research activity of the EC project Oli-PHA [51], as reported in Figure 8.

Although plasticization was the object of many studies, it must be noticed that the improvement in mechanical properties is often limited. For instance, for PHBV, Martino et al. [52], in the framework of the ECOBIOCAP project "Ecoefficient Biodegradable Composite Advanced Packaging" GA No. 265669 [53], obtained an improvement of the elongation at break of 6% starting from 2% from the pure PHBV. Hence, the improvement in properties of PHA polymers is still an open issue in current bioplastics research.

Figure 8. Cosmetic jars produced with PHB.

3.3. Starch

Polysaccharides such as starch are considered for the production of rigid packaging due to relatively low cost and high degradability, in compost, soil and marine water. Thus, as previously addressed, the long-term impact of plastic waste in the marine environment is a primary issue since birds, mammals and fish may become entangled in plastic films or ingest plastic particles [54].

Non-durable plastic hard packaging includes plates, bowls and cups, as well as peanuts used for packaging fragile materials. Polystyrene (PS) is the plastic mostly used in plastic food service items (79%), and a low recycling rate is reported for the packaging waste made of PS also due to difficulties in collection and cleaning of the packaging after use [55]. Polymers such as PS are especially persistent and difficult to degrade in the environment, and for foamed items, their light weight promotes the dispersion in the environment by wind or storm drains [56]. Native starch is not a thermoplastic material, and it thermally degrades before its glass transition temperature (Tg) when its melting temperature (230–240 °C) is reached [57,58].

However, as evidenced in the previous section, when starch is heated in the presence of plasticizers such as water or polyols, the semi-crystalline structure of the starch granule is disrupted. Then, the glass transition temperature decreases below the thermal degradation temperature, and the starch actually behaves as a thermoplastic, it is then denominated thermoplastic starch (TPS) [56]. In extrusion processing, the TPS melts at much lower moisture content (10–20%) than that used for conventional cooking methods [59,60].

Several companies have introduced starch-based products on the market, Novamont (Novara, Italy), Cereplast (Seymour, IN, USA), BASF (Suffolk, VA, USA), Biotec GmbH (Gütersloh, Germany), Plantic (Altona, VIC, Australia) and Biolice

(Ennezat, France), among others. Some images of starch-based hard packaging (peanuts and clams shell) are reported in Figure 9.

Figure 9. Image of hard packaging made by starch.

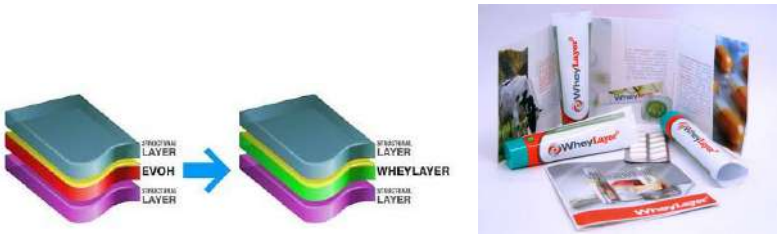
An alternative procedure to produce foamed starch-based packaging is based on a baking technology that was firstly developed for the food industry and was successively adapted for making starch-based foam food service products with a process similar to that used to produce waffles [61–64]. A starch dough is first prepared containing gelatinized or pre-gelatinized starch, native starch, water, fiber, fillers and other additives and then mixed for about 10 min. A predetermined amount of aqueous starch dough is placed into a preheated (150–200 °C) mold cavity. The dough rapidly heats, and the starch component is gelatinized, forming a melt that fills the mold cavity. A skin forms on the upper and lower surfaces where the dough contacts the mold surfaces. The steam formed during the process acts as a blowing agent and allows the formation of a foam structure in the core region of the product. Steam is then allowed to vent from the mold, and within about 45–60 s, the product dries and solidifies into the desired shape. The starch-based products are very similar to PS foam and are marketed by Biopack (Graz, Austria), Apack-IBEK Verpackungshandel GmbH (Markt Erlbach, Germany), Earthshell (Santa Barbara, CA, USA) and Biosphere Industries (Carpinteria, CA, USA).

Packages based on starch are sensitive to water; hence, some researchers studied the possible cross-linking of starch to decrease its solubility in water [65,66] with the aim of using starch for application as paper coating.

3.4. Proteins

Proteins (casein, collagen, gelatine, corn, soy, wheat, etc.), can be obtained from a variety of agricultural commodities and/or wastes and food products. Proteins can be processed by casting or by melt extrusion in the presence of plasticizers or other polymers [67–79]. The mechanical and barrier properties offered by protein-based films are generally superior to those offered by polysaccharide-based

films due to, and contrary to polysaccharides, which are mainly homopolymers, the specific structure (based on 20 different monomers), which confers functional properties and high intermolecular binding. High molecular weight proteins are insoluble, or only partially soluble, in water and, thus, present themselves as very interesting film-forming molecules for the formation of water-resistant films, particularly after crosslinking [80]. As proteins are hydrophilic, they adhere very well to polar surfaces such as paper, thus acting as barriers to oxygen and carbon dioxide. Materials with protein coatings are expected to show good barrier properties offering an alternative to nonrenewable polymers such as ethylene vinyl alcohol (EVOH) or silica-based coating. Achievements in this application were gained by the EC project WHEYLAYER "Whey Protein-Coated Plastic Films to Replace Expensive Polymers and Increase Recyclability" and the following demo action WHEYLAYER2 "Barrier Properties for Sustainable Packaging" [81]. An image of the WHEYLAYER approach and of the products are reported in Figure 10.



(**a**) (**b**)

Figure 10. (**a**) WHEYLAYER approach and products; (**b**) EVOH, ethylene vinyl alcohol.

In the WHEYLAYER-based packaging, a whey-based coating is applied on a plastic film substrate, such as PET, and the coated film is used for the production of multi-layer packaging.

It is possible to achieve a separation of the different layers of the packaging by removing the whey protein layer by washing with water containing a protease enzymatic detergent [82,83]. This procedure promotes the recyclability of the material that constitutes the packaging. When WHEYLAYER is applied on a compostable substrate such as PLA, it is possible to produce a completely biodegradable multi-layer material with improved barrier properties [84].

Furthermore, in rigid paperboard packages, the main innovation can be the introduction of a new bio-based layer that can replace, at least partially, polyethylene, thus increasing the bio-content of the packaging and allowing a better management of the package end life.

More research activity is running on this very promising approach of the application of protein in packaging. The most recent project BIOBOARD [85] aims

at developing a food waste-based protein-based film by flat die extrusion. In this project, both whey protein from the dairy industry and potato pulp from the starch industry are used [86,87].

The project LEGUVAL"Valorisation of Legumes Co-Products and by-Products for Package Application and Energy Production from Biomass" GA No. 15241 [88] aims at the use and valorization of co-products and by-products of processed grain legumes thanks to the extraction of their proteinaceous fraction, which can be used as a raw material in packaging.

4. Conclusions

This overview of processing technologies for the production of hard packaging from bio-based polymers and the strategies for maximum valorization of bio-based polymer in this application outlines the potentiality and the growing attention not just of researcher, but also of producers and consumers towards bio-based materials. The renewable origin, as well as the biodegradability and compostibility of several products based on bio-based and biodegradable polymers represent a benefit for the environment and human life.

The research will continue to improve the properties of packaging based on these materials, as well as optimize their processing, with also the aim to lower the cost of bio-based products, which at present represents the main limit to the wide spreading of their applications. With the growing awareness of the need for environment and resource preservation, the market for bio-based products applied in single use applications is forced to increase. Thus, we expect more and more achievements and innovative bio-based products suitable for use in the production of hard packaging.

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Conflicts of Interest: The authors declare no conflict of interest.

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PART V

Biological Activity, Safety & Patent Application & Legal Considerations

Biological Activity of Innovative Polymeric Nanoparticles and Non–Woven Tissue

Giovanna Donnarumma, Brunella Perfetto, Adone Baroni, Iole Paoletti, Maria Antonietta Tufano, Paola Del Ciotto and Pierfrancesco Morganti

Abstract: In the last few years many studies have focused their attention on different potential biomedical applications for biocompatibile polymers, especially nanopolymers, in shape of nanotubes, nanofibers, and nanoparticles. Polymer nanocomposites arising from different chemistries and constructions include aliphatic polyester such as polylactide (PLA) and poly (DL-lactic-cd-glycolic acid) (PLGA), poly (ε-caprolactone) (PCL), poly(*p*-dioxanone) (PPDO), poly(butylenes succinate) (PBS), poly (hydrdroxyalkanoate), and natural biopolymers, such as starch, cellulose, chitin, chitosan, lignin, and protein. In medicine, they contribute to applications in surgery, dentistry, and pharmachology for scaffolds for tissue regeneration, tissue engineering, drug delivery devices, and gene transfection. Bionanopolymers can be used in many other fields including food, cosmetics and agriculture. The research efforts are focused on the study of new polymeric nano-constructs that exploit the body's natural biological response and are environment-friendly.

1. Introduction

For thousands of years humans made tools and devices from naturally available compounds, and later artificial compounds were created to make them.

The interdisciplinary field of biotechnology and particularly nanobiotechnology, which combines biology, chemistry, engineering, nanopharmacology, and nanomedicine, is revolutionizing the development of drug delivery systems and tissue engineering, such as tissue engineered for vascular grafts and wound healing. Research in this area has provided unlimited potential to improve human health [1–3]. A large variety of materials can be used and are classified as synthetic, natural, or hybrid. The synthetic materials can be further classified in degradable and non-degradable materials. The synthetics include polymers, such as (poly(ethyleneglycol), *N*-(2-hydroxypropyl) and methacrylamide co-polymers, Natural polymers can be classified as those obtained from natural sources such as animal, microbial, and vegetable sources. They are usually natural proteins or polysaccharides, such as chitin, chitosan, (dextran (α-1,6 polyglucose), dextrin (α-1,4 polyglucose), lignin, collagen, gelatin, and hyaluronic acid. Interestingly, in the interaction of nanopolymers with the human immune system, is that chitin and hyaluronic acid show the same backbone (Figures 1 and 2).

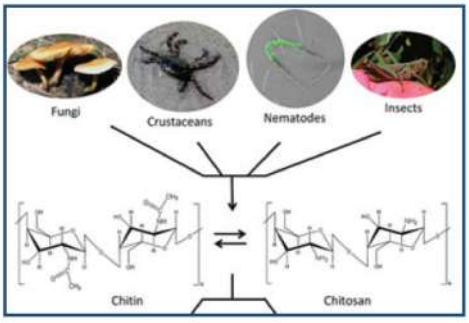

Figure 1. Chemical structures of Chitin and Chitosan from natural sources such as crustacean exoskeleton and fungi wall. Adapted from Morganti, P. et al. [4].

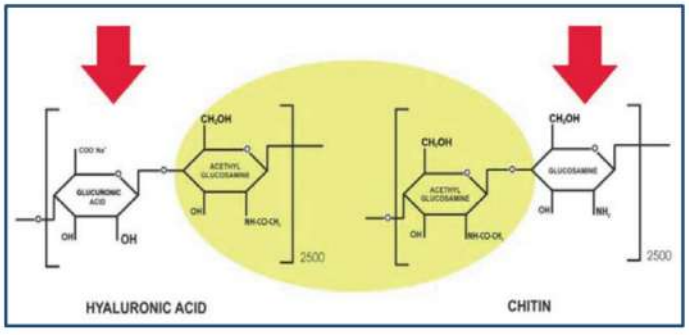

Figure 2. Chitin and hydrauronic acid structures. Adapted from Morganti, P. et al. [4].

Others include linear polyamidoamines and pseudosynthetic polymers the man-made poly (amino acids) poly(L-lysine), poly(L-glutei acid), poly(malic acid), and poly(aspartamides).

Natural polymers have the disadvantage of high biodegradability, while synthetic polymers can be synthesized and modified in a controlled manner to produce constant and homogenous physical and chemical properties and stability. However, the latter are biologically inert and do not offer the therapeutic advantages of natural polymers. Ongoing research is focused on the development of hybrid biodegradable materials for specific applications using new resorbable biomaterials and applying computational and combinatorial approaches to develop biomimetic polymer structures with unique chemistries, thus increasing diversity [4–8]. Tsao and coworkers [9] used a polyelectrolyte complex consisting of chitosan and (-poly (glutamic acid) (-PGA) as a wound dressing material. This complex combination offered good mechanical properties, suitable moisture content, and favorable removal without the damage of regenerated tissue. To form a suitable wound dressing Kim and coworkers [10]

used chitosan with poloxamer. A semi-interpenetrating polymer network provided enhanced compatibility and mechanical strength. Novel wound dressing applications have been devised by novel bioprocesses and advances in organic chemistry, thus enabling the development of enhanced smart polymers as candidates for specialized wound dressings that elicit favorable biological, physical, and chemical responses. Hydrocolloid dressings, the most widely used dressings, are obtained from colloidal materials that are gel-forming agents combined with other materials such as gelatin, elastomers, carboxymethylcellulose, pectin and adhesives. These agents can be bonded together to produce a thin film, sheet, or foam with the properties of hydrocolloids, thus forming a gel on the wound surface to promote moist wound healing Recent advances have been made in wound healing and dermal substitution. The main component of every wound is the connective tissue matrix, thus there is an overall consensus that in order to heal wounds effectively, it is necessary to ensure the effective substitution of the main component. Wound dressings are an essential part of wound management and care in order to enhance the natural wound healing process. The development of new intelligent dressings is under way, and these promise to play an active role in promoting healing of both acute and chronic wounds [11].

Other important nano-biopolymer applications are drug delivery devices and tissue engineering. Innovative delivery strategies based on nano and micro-particulate systems are currently being investigated for pulmonary delivery to improve drug transport to its target [12], and to develop a compatible small-diameter engineered tissue as a scaffold for vascular graft. This requires a compliant polymer scaffold to which endothelial cells can adhere, form an anti-thrombogenic luminal surface, exhibit vasoactive properties, and improve patency, and within which smooth muscle cells can migrate, deposit functional vascular ECM, and become contractile [13]. When choosing a material for surgical or pharmacological devices, it must meet specific requirements, and of utmost importance is the biocompatibility and biodegradability. The aim of this chapter is to provide an overview of the biological activity of innovative nanoparticles and non–woven tissue.

2. Bionanotechnology

Recent improvements have been made in the technique to produce biopolymers in the form of nanosized fibers [13], biodegradable polymeric nanospheres, nanorods, and nanotubes. Several important characteristics can be seen by reducing the diameters of polymer fiber materials from micrometers to submicron or nanometers, such as a very large surface area to volume ratio; the ratio of nanofiber in comparison with that of a microfiber may be as much as 10^3 greater. This also provides greater mechanical support in the functionalities, such as tensile strength and stiffness, and improved flexibility when compared to any other known form of the same material [14,15].

Electrospinning, a spinning technique, is a widely used technology for electrostatic fiber formation, which utilizes electrical forces to produce polymer fibers with diameters ranging from 2 nm to several micrometers using polymer solutions of both natural and synthetic polymers. This is a unique approach to produce fine fibers from polymer solutions or melts, which have a larger surface area and a thinner diameter (from nanometer to micrometer) than those obtained from conventional spinning processes. There are two standard electrospinning setups, vertical and horizontal [14,16]. With the expansion of this technology, more sophisticated systems that can fabricate more complex nanofibrous structures in a more controlled and efficient manner have been developed. Electrospinning is conducted at room temperature under atmospheric conditions. An electrospinning system has three components: a high voltage power supply, a spinneret (e.g., a pipette tip), and a collecting plate (usually a metal screen, plate, or rotating mandrel). It utilizes a high voltage source to inject a charge of a certain polarity into a polymer solution or melt, which is then accelerated towards a collector of opposite polarity. Most polymers are dissolved in solvents before electrospinning, and when they completely dissolve, they form a polymer solution. The polymer fluid is then inserted into the capillary tube for electrospinning. In the electrospinning process, a polymer solution held by its surface tension at the end of a capillary tube is subjected to an electric field, thus inducing an electric charge on the liquid surface. When the electric field applied reaches a critical value, the repulsive electrical forces overcome the surface tension forces. A charged jet of the solution is then ejected from the tip of the Taylor cone and an unstable and rapid whipping of the jet occurs in the space between the capillary tip and collector, which leads to the evaporation of the solvent, leaving a polymer behind. The jet is only stable at the tip of the spinneret and after that instability starts [17–19] (Figures 3 and 4). A wide range of polymers that are able to form fine nanofibers within the submicron range suitable for varied applications are used in electrospinning. Electrospun nanofibers have been produced from natural polymers, synthetic polymers, or a blend of both, including nucleic acids, proteins, and even polysaccharides. More than 200 polymers have been electrospun successfully from natural polymers and characterized according to their applications. When used in biomedical applications, naturally occurring polymers normally exhibit good biocompatibility and low immunogenicity when compared to synthetic polymers. An advantage of natural polymers for electrospinning is their capacity to bind to cells since they carry specific protein sequences, such as RGD, arginine/glycine/aspartic acid sequences [17]. Nanoparticles can be defined as particulate matter having at least one dimension that is less than 100nm and an exceptionally high surface to volume ratio, which contributes to their unusual properties and behavior. Furthermore, because of their high surface area, the surface structure also differs from that of the core. Specific and suitable functional groups

can be attached to the surface of a nanoparticle to allow it to reach its target and interact with the biological system [11].

Polymeric nanoparticles (NP) are a promising resource for drug delivery. These carriers must be designed for a specific purpose since the material used affects the distribution inside of the body and the uptake into certain cells. The NP size affects the ability to penetrate barriers in the body. It is important to select an appropriate approach for the specific drug (hydrophobic vs. hydrophilic) and delivery route. Drug incorporation by adsorption or entrapment can alter the size and physicochemical properties that determine the NP interactions [20,21].

Different methods are used for gelatin NP preparations: the desolvation method and the water-in-oil emulsification method. The protocols for chitosan NPs include the ionotropic gelation method and another method that includes complex coacervation, emulsion evaporation, nanoprecipitation, and radical polymerization [22]. For synthetic polymer preparations, such as PLGA nanoparticles, which are made from a copolymer of polylactic acid and polyglycolic acid, the salting out method or nanoprecipitation, also known as solvent diffusion or solvent displacement, is used. The emulsification methods include emulsion diffusion, a top-down technique that starts by dissolving the pre-formed polymer in an organic solvent that is partially miscible with water (e.g., ethylacetate, dichloromethane, or acetone/methanol) and emulsion evaporation, another top-down method used for the formation of PLGA NPs. The emulsion dispersion polymerization method is used to prepare polyalkyl(cyano)acrylate (PCA) nanoparticles [23].

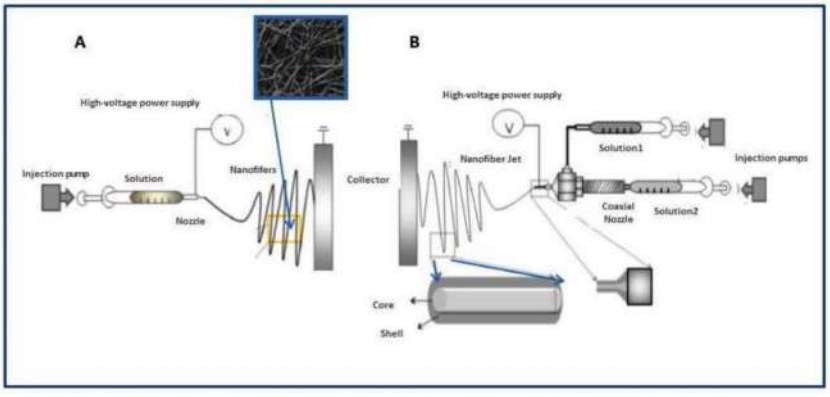

Figure 3. Schematic diagram of electrospinning setup consisting of injection pump/s, power supply, nozzle and conducting collector. A: One type of solution. B: Two type of solution and a core-shell nozzle design used to encapsulate drugs within the nanofiber. Adapted from Shin, S.H. et al. [24].

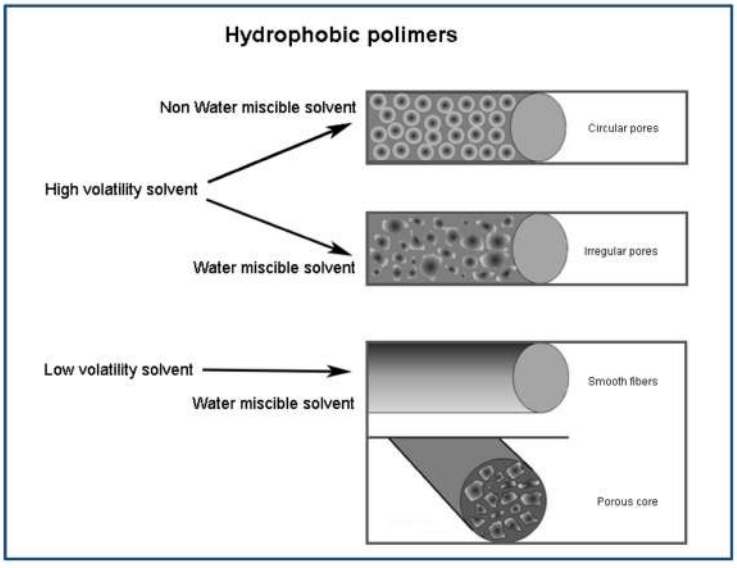

Figure 4. Fibers for hydrophobic polymers, electrospun at high percent RH, obtained with different solvent, show different morphology. Adapted from Nezarati, R.M. et al. [19].

3. Biological Activity

Like any biomaterial, a functional polymeric system aimed at serving for a limited period of time before degradation and elimination from the body must first fulfill strict criteria related to biocompatibility and biofunctionality. Therapeutic devices made from biomaterials, especially polymers, must permit in vivo: no or acceptable toxicity, no immune reaction, no carcinogenic mutation and no thrombus formation to meet the strict criteria of biocompatibility. Regarding biofunctionality, these devices must offer adequate mechanical, chemical, thermal, physical, and biological properties, and must be sterile and easy to handle [23]. Increasing attention to the environment has brought about an in-depth study of the biological activity of natural polymers or artificial polymers that respect the characteristics of biodegradability and biocompatibility, such as PLA or PLGA. Besides the application of chitin as a starting material for the synthesis of chitosan and chito-oligosaccharides, chitin itself has been a center of many therapeutic applications, and is thought to be a promising biomaterial for tissue engineering and stem-cell technologies. In 2013, Bae and coworkers [25] demonstrated that the oral administration of chitin (α and β forms) is beneficial in preventing food allergies; the oral administration of chitin was accomplished by milling it to particle size less than 20 m and mixing it with the feed. Their results showed that the α-form reduced the serum levels of peanut-specific IgE and both the forms decreased the levels of

interleukin IL-5 and IL-10, and increased the levels of IL-12. Dietary supplementation of chitin was shown to exert positive immunomodulatory effects; the antibacterial activity of chitin, prepared from shrimp-shell waste, was reported by Benhabiles et al. [26]. Chitosan, a natural nontoxic biopolymer that is produced by the deacetylation of chitin, has been noted for its application as a film-forming agent in cosmetics, a dye binder for textiles, a strengthening additive in paper, and a hypolipidic material in diets. It has been used extensively as a biomaterial owing to its immune-stimulatory activities, anticoagulant properties, antibacterial and antifungal action, and its action as a promoter of wound healing in surgery. The antimicrobial activity of chitosan has been demonstrated against many bacteria, filamentous fungi, and yeasts. Chitosan has a wide spectrum of activity and high killing rate against Gram-positive and Gram-negative bacteria but a lower toxicity toward mammalian cells. Due to the presence of hydroxyl, amine, and acetylated amine groups, chitosan, low molecular weight chitosan, and chito-oligosaccharides (COS) interact with various cell receptors that trigger a cascade of interconnected reactions in organisms, which results in anti-inflammatory, anticancerogenic, antidiabetic, antimicrobial, anti-HIV-1, antioxidant, antiangiogenic, neuroprotective, and immunostimulative effects [27]. Both in the form of nanoparticles or nanofibers these biopolymers play an important role in helping the human body in its outstanding ability to self-repair.

3.1. Nanoparticles

Nanoparticles can be defined as ultradispersed solid supramolecular structures with a submicrometer size ranging from 10 to 1000 μm, used especially to produce drug delivery devices (Figure 5). The nanoparticle matrix with the drugs (dissolved, entrapped, encapsulated, or attached) acts as a reservoir for particulate systems and plays an important role as a drug delivery device, e.g., in oncology [28]. Nanoparticles fabricated from polysaccharides, proteins and biocompatible/biodegradable polymers, such as polyethylene glycol (PEG), poly(γ-benzyl l-glutamate) (PBLG), poly(D,L-lactide), poly(lactic acid), poly(D,L-glycolide), poly(lactide-co-glycolide), polycyanoacrylate, chitosan, gelatin, and sodium alginate are called PNPs [27]. The various materials used in NP preparation show different drug release kinetics, which can be achieved through desorption of surface-bound drugs, diffusion through the NP polymer wall, or erosion of the NP matrix. Drugs can be released with a burst mechanism or in a sustained manner. Synthetic polymers can be more easily engineered to produce a sustained release than natural polymers. In addition to the choice of polymer, the loading method has a strong impact on the release rate. However, the choice of the loading method depends on the drug, as certain drugs are more effective when released in a large burst, but controlled release is better for long-term effects; the size of the particle is equally important. The drug encapsulated will have an impact on the nanoparticle size, but it is difficult to predict which

method of encapsulation will impact the size. This parameter is crucial to determine which organs the particle may reach and whether or not it will be opsonized by macrophages. Once NPs are in the circulation they can pass, according to their size, through fenestrations in the endothelial barrier. Table 1 shows the materials commonly used for NP preparation.

Chitosan has been the impetus for the development of safe and effective drug delivery devices. Its primary hydroxyl and amine groups that are located on the backbone permit chemical modifications to control its physical properties. The interaction of the hydrophobic moiety with a chitosan molecule determines an amphiphile that can form self-assembled nanoparticles that are able to encapsulate a quantity of drugs and deliver them to a specific site of action. Chemical attachment of the drug to the chitosan throughout the functional linker may produce useful pro-drugs, exhibiting the appropriate biological activity at the target site [29].

Synthetic polymers, when compared to natural polymers, are generally more homogenous in composition and have a higher purity, thus making the preparation of NPs more reproducible. However, it should be noted that not all of the synthetic polymers are suitable for drug delivery as they need to be biodegradable and exert low cytotoxicity. NPs made from synthetic polymers are polyesters, which include poly(lactic acid), poly(glycolic acid), their co-polymer: poly(lactide-co-glycolic acid) (PLGA), as well as polyalkyl(cyano)acrylates. These are not new and have been extensively used in clinical settings, e.g., PLA has been used for surgical sutures and implants, while PCAs have been studied for their use in sealing wounds. Knowledge of the degradation kinetics of such polymers allows for the preparation of better formulations for a more controlled release. Because of the large amount of materials used for NP preparations (Table 1) and the extensive field of application as drug delivery devices, it is not possible to provide a comprehensive overview. We have limited our intervention to some recent studies that investigated the use and biological activity of different NPs.

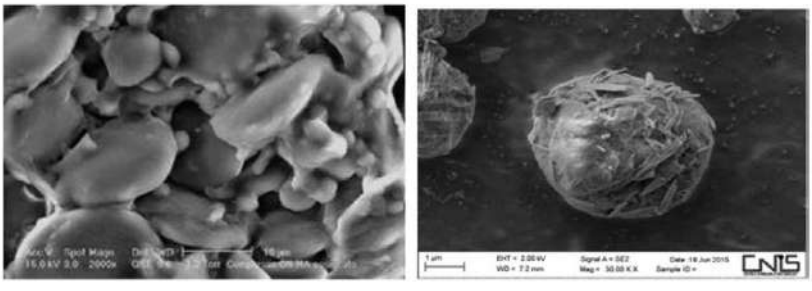

Figure 5. Micro/nanoparticles of the complex chitin nanoparticle-nanolignin. From Morganti, P. et al. [4].

Table 1. List of organic materials used to formulate nanoparticle drug carriers.

Polymeric Based Nanoparticles	Non-Polymeric Based Nanoparticles
Natural sources	
Gelatin Chitosan Alginate Nano-crystalline cellulose	Carbon-based carriers Liposomes Solid lipid nanoparticles
Synthetic sources	
Poly(lactic-co-glycolic)acid Poly-n-(cyanoacrylate) Polycaprolactone	

Lai [22], Dinavard [30], Nitta [31], and coworkers offer a good overview of the preparation and ability of polymeric nanoparticles as a drug-delivery system for gene deliver, anticancer drug delivery, and antibiotics delivery. Recently, in the treatment of pulmonary infections, the use of antibiotics for inhalation has gained increasing attention, particularly for cystic fibrosis (CF) patients. Aerosolized antibiotics offer an interesting way to deliver high drug concentrations directly to the site of infection, which reduces the toxicity and enhances the therapeutic potential of the antimicrobial agents against resistant microorganisms.

In 2009, Tahara et al. [32] demonstrated in vitro that chitosan-modified PLGA NSs (CS-PLGA NSs) are preferentially taken up by human lung adenocarcinoma cells (A549). Cellular uptake of PLGA NS was confirmed using fluorescence spectrophotometry and was visualized in A549 cells with confocal laser scanning microscopy (CLSM). The cytotoxicity of non-and CS-PLGA NS systems were compared in vitro using a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium (MTS) assay; CS-PLGA NSs did not show cytotoxicity to A549 cells. The cellular uptake of non- and CS-PLGA NSs is a time-, temperature-, and concentration-dependent saturable event mediated by clathrin-coated pit endocytic pathways, and that of PLGA NSs is related to a particle diameter. CS modification through electrostatic interactions between the CS adsorbed to the NS surface and the negatively charged cell membrane improved the cellular uptake of PLGA NSs. PLGA NS and CS-PLGA NS (200-nm) were internalized by A549 cells through adsorptive endocytosis started by nonspecific interactions between NS and cell membranes, triggered partially by a clathrin-mediated process. NSs have been evaluated for the delivery of different types of therapeutic agents, such as DNA, peptides, and proteins. Moreover, NSs protect the encapsulated drugs from enzymatic degradation and therefore provide a sustained release. In conclusion,

CS-PLGA NSs are better drug carriers because of a high cellular uptake due to their strong interaction with the cells and a low cytotoxicity.

Ungaro and coworkers [33] designed and developed a pulmonary delivery system for antibiotics based on spray-dried lactose/PLGA nano-embedded microparticles (NEM), engineered at the nanosize and microsize levels. To test the use of NEM, Tobramycin (Tb), the first choice antibiotic in lung infections for CF treatment, has been chosen. The results showed that to improve the size and modulate the surface properties of Tb-loaded PLGA NPs, poly(vinyl alcohol) (PVA), and chitosan are essential, and the use of alginate (Alg) allows an efficient Tb entrapment within NPs and its release up to one month. The optimized formulations of NP spray-drying with lactose offered NEM with particular flow and aerosolization properties without altering the NP features. The Tb-loaded PLGA NPs showed good in vitro antimicrobial activity against *P. aeruginosa* planktonic cells. In addition, the bio-distribution studies showed that PVA-modified Alg/PLGA NPs reached the deep lung, while CS-modified NPs remained in the upper airways lining lung epithelial surfaces. In conclusion, the composition of PLGA NP plays a crucial role in determining the technological features of NPs, and when NPs are processed in the form of NEMs, their in vitro/in-vivo deposition pattern is also modified

Recently Piras et al. [34] evaluated as an antimicrobial protein drug model, chitosan in a new formulation of nanoparticles loaded with lysozyme (LZ). LZ-loaded nanoparticles (LZ-NPs) of 150 nm diameter were prepared by inotropic gelation. They demonstrated that, these nanoparticles preserved the antibacterial activity of the loaded enzyme, which was slowly released over three weeks in vitro and remained active against *Staphylococcus epidermidis*, up to five days of incubation. So, beyond the intrinsic antibacterial activity of CS and LZ, the LZ-NPs showed a sustained antibacterial activity that resulted in about a 2-log decrease in the number of viable *S. epidermidis* compared to plain CS nanoparticles, and showed a full in vitro cytocompatibility towards murine fibroblasts.

It is well known that the development of small interfering RNA (siRNA)-controlled-release NPs may improve the therapeutic efficacy of RNA interference (RNAi) by prolonging their release to allow long-term gene silencing. In 2014 Shi et al. [35], proposed an NP platform with sustained siRNA-release properties, which can be self-assembled using biodegradable and biocompatible polymers and lipids, with an excellent silencing efficacy. The temporal release of siRNA from the NPs continued for over one month. When tested in vitro on luciferase-expressed HeLa cells and A549 cells after short-term transfection, the siRNA NPs showed greater sustained silencing activity than lipofectamine 2000-siRNA complexes. More importantly, the NP-mediated sustained silencing of prohibitin 1 (PHB1) generates more effective tumor cell growth inhibition in-vitro and in vivo than the lipofectamine complexes.

Morganti and coworkers [36] are working on the formulation of complex chitin–hyaluronan nanoparticles as a multifunctional carrier to deliver anti-aging active ingredients through the skin. They are evaluating in vitro its antioxidant capacity, anti-collagenase activity, and metalloproteinase, and anti-inflammatory mediator release. These copolymeric nanoparticles are able to entrap different kinds of active ingredients and release them at different times, depending on the productive process adopted and fine size of the micro/macro particles designed [37,38]. It is interesting to underscore how these nanoparticles, based on the use of chitin nanofibrils and obtained from crustacean waste, support the industrial sustainability [39] and respect the indications of the in-progress green economy [40].

3.2. Non-Woven Tissue

The current direction in the research area of non-woven tissue is to create a scaffold that mimics the structure and function of the native extracellular matrix (ECM) (Figure 6). The best scaffold for clinical use is one that has both structural integrity and allows for normal cellular function and interaction [41]. There is growing evidence that nanofibers amplify certain biological responses, such as cellular contact guidance and differentiation [42]. According to the medical application, i.e., wound dressing, small-diameter vascular graft, several parameters must be analyzed, such as: material selection, scaffold design, porosity, mechanical properties, fiber morphology, and cytocompatibility. Chitosan (CS) is a natural chitin-derived polysaccharide that is extensively used as a biomaterial in different engineering applications due to its low cost, large-scale availability, antimicrobial activity, biodegradability, and biocompatibility [43–46]. Besides nanoparticles, many authors have focused their studies to evaluate in vitro the biocompatibility of new co-polymer based on chitosan.

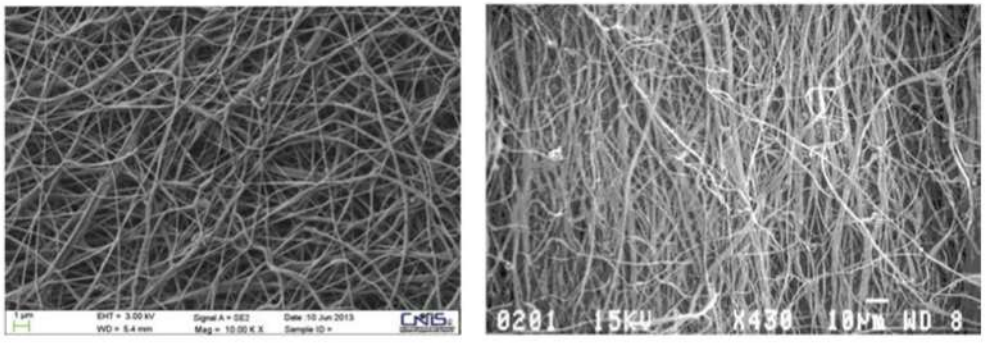

Figure 6. Similarity between the structure of Chitin Nanofibril scaffold (left) and skin ECM (right). From Morganti, P. et al. [4].

In 2013 Wang et al. [13], evaluated the biocompatibility of an electrospun chitosan/collagen complex as a scaffold in vascular tissue engineering to support the cell adhesion, proliferation, and phenotypic expression of endothelial cell markers by PIECs in-vitro. The chitosan/collagen scaffold showed endothelial cell adhesion, and did not adversely affect cellular function. In addition, the chitosan/collagen scaffold (w/w; 50/50, 20/80), in particular, showed the highest potential for vascular tissue engineering.

Naseri and coworkers [47], developed electrospun chitosan/polyethylene oxide-based randomly oriented fiber mats, reinforced with 50 wt % chitin nanocrystals (ChNC) for wound dressing. The results showed that the electrospun porous random mats comprising ChNC were free from any defects because of a homogeneous dispersion of ChNC in the chitosan matrix, indicating good chemical compatibility between the matrix and the chitin. The addition of chitin nano-crystals improved the moisture stability of these mats and facilitated water-mediated cross-linking processes. The cross-linked nanocomposite fiber mats with 50 wt % chitin nano-crystals had a high surface area (35 m^2 g^{-1}), a high tensile strength of 64.9 MPa and modulus of 10.2 GPa, and were at the same time flexible, and, therefore, were considered as beneficial for wound healing. The water vapor transmission rate of these mats was between 1290 and 1548 g m^{-2} day^{-1}, and was in the range for injured skin or wounds. The compatibility towards adipose-derived stem cells of the electrospun fiber mats confirmed their potential use as wound dressing materials [32]. Many other associations have been tested in the last few years. Enrione et al. [48] designed a gelatin/chitosan/hyaluronic acid biopolymer using a thermophysical approach for use in tissue engineering. The gelatin/chitosan/hyaluronic acid biopolymeric scaffold was made by applying a modification of the method described by Liu et al. [49]. Its thermal characterization was performed using differential scanning calorimetry (DSC), and its physical characterization by gas pycnometry and scanning electron microscopy. The effects of the gelatin (Ge) content and cross-linking on the thermophysical properties were evaluated by means of a factorial experiment design (central composite face centered). The Ge content was the main factor that affected the thermophysical properties (microstructure and thermal transitions) of the scaffold for tissue engineering, which were studied by seeding skin cells on the biopolymers. Different amounts of Ge did not affect cell attachment, while the cell growth rate increased linearly with the decrease in the Ge content. This relationship, together with the thermophysical characterization, can be used to design scaffolds for tissue engineering. Finally, the authors concluded that a Ge stock solution of 0.8% was adequate to formulate Ge/Ch/Ha-scaffolds to seed fibroblasts.

Different types of chitin- and chitosan-based wound dressing materials are commercially available. Chitin and chitosan in the form of composites, gels,

nanofibers, films, non-wovens, and scaffolds have been used to regenerate wounded tissues. Nowadays, commercial products are available for topical application in wound repair based on chitin nanofiber polymers. Our research group [50] tested in vitro a novel combination of chitin nanofiber/lignin in different ratios for their anti-inflammatory and wound repair activity in experimental models of human keratinocytes. For the evaluation of the anti-inflammatory effect of chitin /lignin nanofibers, the IL-8, IL-1α and TNF-α expressions were analyzed on human keratinocytes treated with lipopolysaccharide of *P. aeruginosa* (LPS). Also evaluated were the expression of the beta-defensin 2 (hBD-2) and metalloproteinases 2 and 9 (MMP-2 and -9), which are well known to be involved in the mechanisms of tissue regeneration. The polymer tested significantly reduced the pro-inflammatory cytokines that were induced by LPS in human keratinocytes and modulated the expression of MMPs and hBD-2. This suggests that the association may improve the ability of chitosan/chitin polymers in their scaffold function.

Bacterial cellulose also seems to be a promising material for the construction of polymers in a nano, micro, and macro scale to use in wound dressing. It is composed of a pure cellulose nanofiber mesh that is spun by bacteria. Its high water content contributes to its biocompatibility [51]. Recently, Harkins et al. [52], tested a novel composite containing chitosan and cellulose for its antimicrobial activity, absorption of anticoagulated whole blood, and anti-inflammatory activity through the reduction of tumor necrosis TNF-α and IL-6, and the biocompatibility with human fibroblasts. The composites tested inhibited the growth of both Gram-positive and -negative microorganisms, such as *Escherichia coli* (ATCC 8739), methicillin-resistant *Staphylococcus* (ATCC 33591), and vancomycin-resistant *Enterococcus faecalis* (ATCC 51299) by 78, 36, and 64%, respectively. In addition, they showed no toxicity *vs*. fibroblasts responsible for the formation of the connective tissue matrix. The composites proved to be a good absorbent for anticoagulated whole blood and were able to maintain a moisture balance for wound healing. For successful tissue repair, several factors, including blood clotting and cellular survival are necessary. Therefore, the dressing material should possess anti-inflammatory activity, since proinflammatory cytokines (TNF-α and IL-6) contribute to the inflammation in chronic wounds, which stalls and prevents them from proceeding to the proliferative phase of tissue regeneration. The significant reduction in TNF-α and IL-6 by stimulated macrophages obtained with the Cel + CS composites clearly indicates their biodegradability, biocompatibility, and non-toxicity.

Advances in medicine have led to a significant increase in life expectancy but significant advances have also been made in cosmetics. The increasing proportion of women and men interested in skin rejuvenation has created a rapidly growing demand for anti-aging remedies to rejuvenate photo-damaged skin. Wrinkling, slackening, and irregular pigmentation, and symptoms of age-associated skin

damage, are in fact influenced by environmental factors, particularly lifetime sun exposure. In the last few years, different techniques for rejuvenation, such as injections with fillers and bio stimulating agents for wrinkle treatment, correction of scars, and soft-tissue augmentation, have been proposed. To obtain beauty and wellness inside and outside, there has been an increased demand from plastic surgeons for new effective medical devices and procedures, and an increased use of cosmeceuticals and nutricosmetics. Morganti and coworkers [53] developed and studied a new medical device to treat facial lines and body contours by balancing the skin-cell turnover and metabolism. To this purpose, block-polymer nanoparticles (BPN) of linoleic acid–rich phosphatidylcholine nanocomplexed with hyaluronan and chitin nanofibrils (PHHYCN) were formulated by encapsulating into them cholesterol, creatine, caffeine, melatonin, vitamins E and C, and the amino acids glycine and arginine. The BPN quickly re-establish the skin-barrier function thanks to their high content in linoleic acid and phosphatidylcholine. The phosphatidylcholine-fatty acids contained inside the BPN contribute to balancing the disturbed composition and organization of the lipids at the level of the epidermal keratinocytes, and consequently of corneocyte lamellae, while the high content of linoleic acid contributes to reintegrating the reduced level of ceramide 1, a structural and stabilizing component of the stratum corneum. These BPN seem to be useful in improving the activity of permanent fillers, making them useful as an anti-aging remedy in plastic surgery. This innovative biostimulating medical device may be used for wrinkle treatment and skin rejuvenation, as well as an adjuvant in soft-tissue augmentation and stretch-mark corrections.

4. Conclusions

Developments in the field of nanotechnology and the increasing interest for the environment have promoted the formulation of new nano-polymeric materials based on natural raw materials, and/or biodegradable and biocompatible synthetic polymers to use as innovative strategies in the fields of medicine, pharmacology, agriculture, and cosmetology [54–56]. Thus, the increased use of natural polysaccharides, such as chitin derivatives and lignocellulosic polymers, can reduce greenhouse emissions and improve our way of living.

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Clinical Activity of Innovative Non-Woven Tissues

Tommaso Anniboletti, Marco Palombo, Simone Moroni, Agostino Bruno, Paolo Palombo and Pierfrancesco Morganti

Abstract: Tissue engineering and the study of engineered materials are ever-expanding disciplines for the development of technological applications, focused on the reconstruction of mammalian tissue. In this study, we explained the surgical problems connected with the wound healing of burned skin, reporting the solutions recovered by the use on non-woven tissues (that we called MAVI dressing) made prevalently by chitin nanofibril (CN)-binding Ag^+ ions. Chitin-based materials and their derivatives, in fact, are receiving increased attention in tissue engineering because of their unique and appealing biological properties, useful to support the skin anatomical structure and its physiological functions. To this purpose, the in vivo results obtained on 30 patients will be shown by photographic images. The biocompatibility and the histological immune responses are shown by these advanced medications connected with the modulating effectiveness of the cytokine cascade. The obtained results recovered in vitro on a culture of keratinocytes and fibroblasts, were confirmed by the quick regenerative activity shown on people affected by burns of the first and second grade. In conclusion, these medications have shown that, while CN seems to be a natural polymer of choice for rapidly regenerating the burned skin, Ag^+ ions, bonded to its fiber structure in very low concentration, possess a sufficient antibacterial effectiveness to control the skin microbiological growth without showing side effects. Therefore, this hybrid biomaterial (chitin nanofibril/Ag nanoparticle composite) can be used for manufacturing advanced medications, solving both the problem of increasing its effectiveness for wound healing, and to slow down the bacterial growth connected with the wounded and burned skin. Moreover, the use of these advanced medications reduces the cost/h of plastic surgeons and the ancillary costs, shortening the time of the wound healing process.

Regenerative medicine has developed extremely rapidly during the last few years, so that the ideas, aspirations, and expectations of cell biologists, material scientists, engineering chemists, biochemists, and of course dermatologists and plastic surgeons have flourished [1–3]. Thus, naturally occurring nanostructures and biomaterials useful for the life science sectors have been a source of inspiration for new nanotechnological designs and to make innovative building blocks

and produce products. The biological use of raw biomaterials for wound dressings, such as the natural chitin/chitosan polymers and nanocomposites from fishery waste and the lignocellulosic compounds such as lignin from plant biomass [4–6], has shown that these natural ingredients possess interesting cicatrizing and reparative activity on wounded skin and organs [7–9]. In addition these compounds, totally biodegraded from environmental chitinases and human chitotriosidases [10], have been shown to be non-toxic, skin-friendly and environmentally-friendly, being catabolized to molecules normally used from all the living cells. Among the chitin derivatives, chitin nanofibril (CN) represents the patented purest crystalline form of chitin [11]. This crystalline polymer has been used to make block copolymeric nanoparticles which, embedded into nanoemulsions and non-woven tissues, may release the entrapped ingredients at different skin layers and different times [12–14]. These nanoparticles may be regarded as interesting carrier for gels and emulsions, and important potential filler materials for the enhancement of the physical and mechanical properties of the polymer matrices used to produce the non-woven-tissue that we called Mavi dressing as the name of the factory that furnished the free sample-test for this study [15].

On the other hand, they are useful to entrap active ingredients and Ag-ions that, when embedded into CN electrospun fibers, assume structural similarities compared to the native extracellular matrix (ECM). The obtained Ag-non-woven tissue made of chitin nanofibers (CN) and chitosan (CS) has shown the capacity to reduce the burden bacteria of infected skin (Table 1), and to enhance the reparative capacity of the skin (Figure 1), showing interesting antibacterial activity together with wound healing effectiveness [16,17]. The consensus within wound therapy recommends, in fact, that modern wound dressings should preserve the skin humid environment, while creating a barrier against mechanical stress and secondary infections [18]. Moreover, on the one hand, advanced dressings have to absorb wound exudate and reduce potential microorganism growth, while on the other hand they have to be safe and a non-irritant. Other important properties of these non-woven tissues are their acceptability to the patient and cost per unit [19]. It must be remembered that wound infections are the most serious complications related to burn injuries that affect up 1% of the worldwide population each year. This is the reason that wound therapy represents one of the challenging areas in drug product development; in the USA more than 6.5 million patients are involved annually, with an estimated cost of treatment per year of US$25 billion [20].

While the use of biomaterials for the treatment of wounded and burned skin has revealed to a huge potential for skin repair [21–24], natural fibers, such as CN, form an interesting option for most widely-applied polymers in medical technology, because of their capacity to form scaffolds, mimicking the structure and organization of the extra-cellular matrix (ECM) [25,26].

Table 1. Antibacterial activity of CN-Ag non-woven tissue [27].

Sample	Bacterial Growth (CFU/g)
Agar + culture of bacteria from bioburden skin tissue (CB)	10^7
Agar + CB+ CS − CN nanocomposite film	10^5
Agar + CB + CS – CN − AG nanocomposite film	10^3

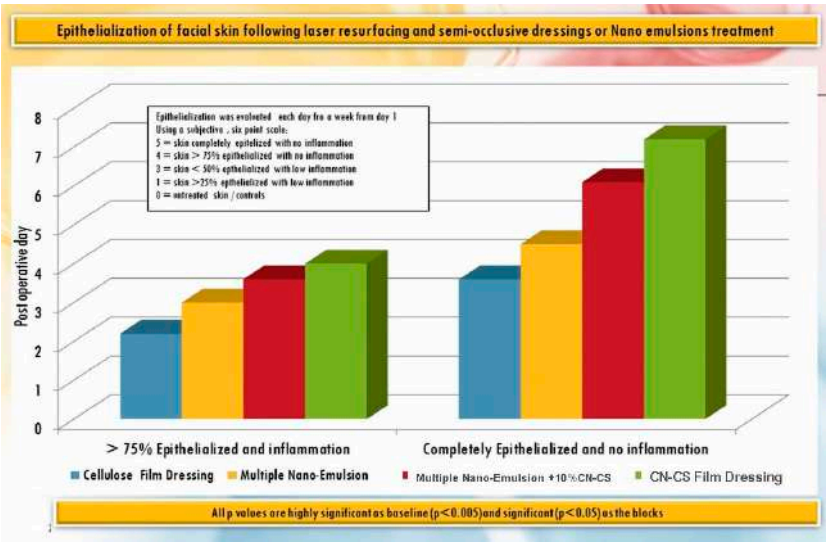


Figure 1. Effect of CN-CS dressing type on the epithelialization of facial skin following laser resurfacing. The epithelialization was evaluated daily during a week beginning from the 1st post-operative day. The appearance of the facial skin was as follows: >25% of the epithelialized skin with low inflammation (1st post-operative day), <50% of the epithelialized skin with low inflammation (3rd), 75% of the epithelialized skin with no inflammation (4th), completely epithelialized skin with no inflammation (5th) [27].

Assesment of antibacterial activity of Naonocomposite CN-CS and Cn-Cs-Ag films after 18 h incubation at 36 °C on agar Containing the Culture of Bacteria taken from Bioburden Skin Tissue. However, only recently the nature of the cellular environment, required for optimal tissue repair and regeneration, has been understood. A major consideration in tissue engineering is, in fact, the first pursuit of scaffolds that provide an architecture on which seeded cells are directed to proliferate and differentiate to form new tissues [27]. This tissue-engineering approach allows the production of new extra-cellular matrices which, resembling the native ECM, replace or regenerate the injured tissues. The creation of this engineered matrix that mimics the ECM requires a scaffold that, serving as a cell carrier, provides structural

support until native tissue is again formed in vivo [28–30]. The pore size of a scaffolding system is, in fact, one of the more important structural design parameters, as it influences cellular infiltration, spreading, intercellular communication, and the transport of nutrients and metabolites [28–32]. On the other hand, the stiffness and mechanical properties of the used substrate, acting as a physical support for the transport of cell signals, influence cellular behavior such as adhesion, spreading, motility, survival, and differentiation.

Together with the physicochemical characteristics of these matrices, the concept of the slow-release of antiseptic agents has been accepted, which, delivering antimicrobial ingredients without being detrimental to the healing process, impedes the micro-organisms proliferation. Wound infections, in fact, continue to be an important complication of chronic wounds in terms of patient morbidity and medical resources. Based on these findings, bioactive, antiseptic ingredients and materials for in situ tissue regeneration play an important role in the control of the skin microenvironment. Over time, many products have been manufactured, particularly in the burns field. In superficial partial-thickness burns, some authors suggest the use of paraffin gauze because there is a low risk of infection. This medication tends to dry out with burn exudate, causing pain in the dressing change and impairment in movement. In deep partial-thickness burns, the incidence of infection is higher than in superficial partial-thickness burns, thus the use of medications that can prevent infection is advocated. The use of silver-containing dressings is therefore recommended. Chitin is a natural, high-molecular-weight linear polymer of β-(1,4) linked *N*-acetylglucosamine (*N*-acetyl-2-amino-2-deoxy-D-glucopyranose) units. Chitosan, a copolymer of glucosamine and *N*-acetyl glucosamine units linked by 1–4 glucosidic bonds, is a cationic polysaccharide obtained by a partial (~60%) alkaline deacetylation of chitin, Industrial chitin nanofibrils (CNs) [4–9] have been shown to be a 1:1 copolymer of N-acetyl glucosamine and glucosamine. However, the role of chitin and chitosan are amazing, attracting increasingly more attention due to their biological and physicochemical characteristics, so that different scientific papers and patents have been published [33,34].

These natural polymers are biocompatible, biodegradable, and nontoxic, showing anti-inflammatory, anti-microbial and hydrating actives, and, therefore, have good biocompatibility and positive effects on wound healing. Previous studies have shown that chitin-based dressings can accelerate the repair of different tissues, facilitating wound contraction and regulating the secretion of inflammatory mediators and innate immunity, depending on their dimensions [35]. Thus, mean-sized chitin has shown pro-inflammatory activity, while small-sized chitin has shown an anti-inflammatory function activating both TNF and IL-10 in macrophages. For this purpose, chitin nanofibrils have a mean dimension of 240 $\times$ 7 $\times$ 5 nm [36].

Chitosan and chitin provide a non-protein matrix for 3D tissue growth and activate macrophages stimulating cell proliferation and a specific hierarchical tissue organization [33,34]. Moreover, they also have a hemostatic activity, which helps in natural blood clotting and blocks nerve endings, hence reducing pain. Both chitosan and chitin gradually depolymerize to release *N*-acetyl-β-D-glucosamine, which, initiating fibroblast proliferation, helps ordered collagen deposition. Finally, the stimulating synthesis and increased production of natural hyaluronic acid at the wound site helps with faster wound healing and scar prevention. This the probably reason why the different CN-Ag-Lignin/PEO non-woven tissues made by electrospinning or casting technologies, have been shown to have interesting anti-inflammatory, anti-microbial [27,36] and cicatrizing [37] activities, without revealing toxic side-effects, being skin-friendly and environmentally-friendly [35–38].

Chapter 1

Burns are a complex skin lesion that are recognized in different depth degrees involving the skin extensive body surface with underlying tissue destruction. In consideration of the histological depth of the loss of tissue, three burn degrees are reported [39].

1st degree burn: only the epidermis is involved with raising of the stratum corneum, dermal edema and vascular dilatation. It heals spontaneously in seven days (Figure 2).

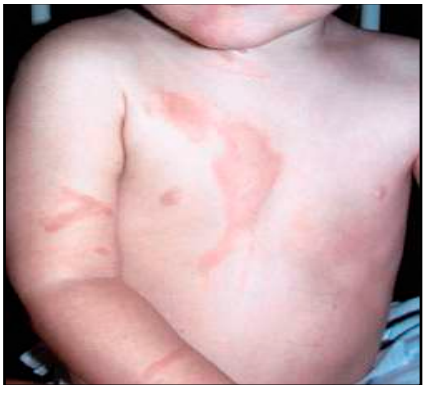


Figure 2. Clinical case of a child with first degree burn on the chest and the right arm.

Superficial 2nd degree burn: in this case the destruction of the epidermis and the superficial layer of the dermis is observed with the development of blisters and a detachment of the epidermis due to the pressure of the transudate fluids. This type of burn is characterized by erythema, edema, blisters and intense pain. Skin is pink,

warm, and painful to touch. Bleeds easily and clears the acupressure. The hairs are present. It heals spontaneously in 15 days (Figure 3).

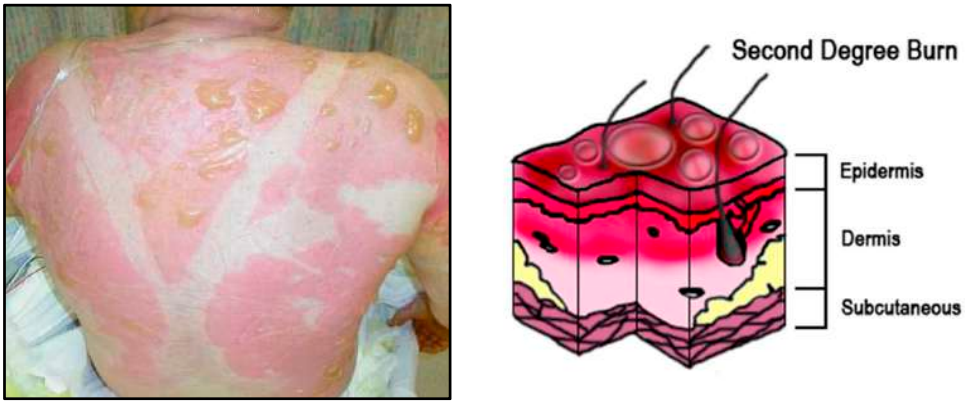


Figure 3. (**Left**): schematic skin section of superficial second-degree burn; (**Right**): clinical case of a patient with superficial second-degree burn on the back due to scald.

Deep 2nd degree burn: Damage that involves the epidermis, dermis, and many superficial neural structures, causes pain and burning. The de-epithelialized areas appear whitish, with eschar expression of more serious damage not uncommon. Under the blisters the skin is pale or bright red (dermal edema and vasodilatation) and it is not painful to touch. Heals in 3–4 weeks with frequent scars and often requires surgical treatment (Figure 4).

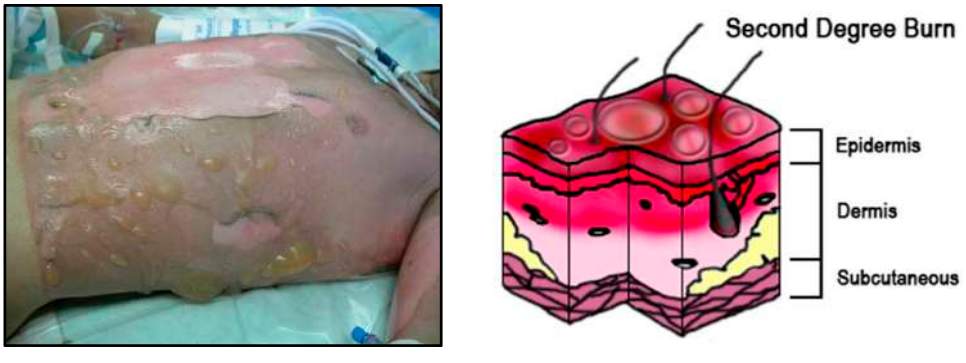


Figure 4. (**Left**): Schematic skin section of a deep second-degree burn; (**Right**): clinical case of a patient with deep second-degreeburn on the trunk.

Third 3rd degree burn: complete destruction of epidermis and dermis is observed. In more severe cases, the exposure of muscles, tendons and bones is possible. If the burning agent is fire or a hot body, the area of necrotic eschar looks blackish or brownish and is dry, cold, hard, painless and non-bleeding. If the agent is a scald, the necrotic area appears whitish or gray in color and of soft consistency. The presence of thrombosed vessels in waxy skin is a pathognomonic sign of this injury. Pain is absent because of the destruction of nerve endings, while only sensitivity to deep pressure is preserved. The hair, even when present, can easily be removed. The treatment is surgical and it heals with scarring (Figures 5 and 6). The goal of the surgical treatment in burns is to prevent invasive local infection and sepsis, to avoid that the burn damage becomes deeper, to get the best skin coverage as quickly as possible, to avoid hypertrophic scars and scar contractures, and to obtain complete healing in the shortest time and with the least number of operations. The operations consist in the removal of all necrotic tissue, which is extended up to the achievement of a vital plane.

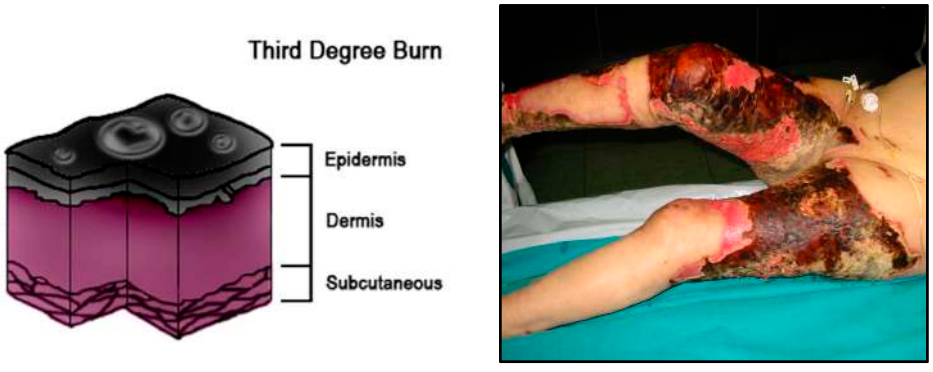


Figure 5. (**Left**): schematic skin section of third-degree burn; (**Right**): clinical case of a patient with third degree burn on the legs due to fire.

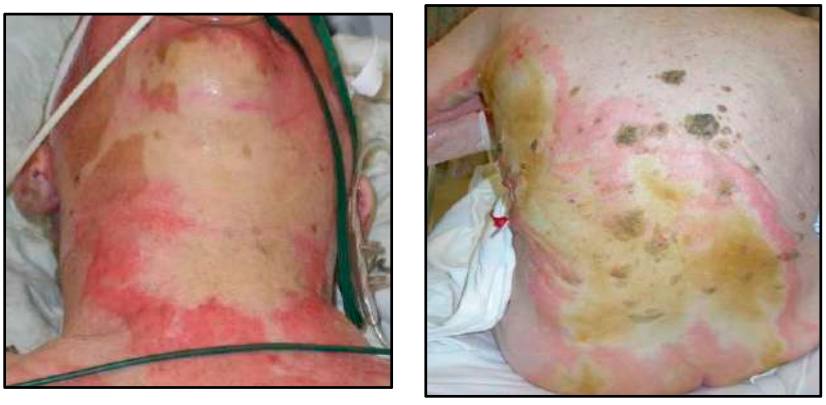


Figure 6. (**Left**): third-degree burn of the back due to scald; (**Right**): third-degree burn of the neck due to scald.

Based on the burn depth, a superficial tangential, a deep tangential and a fascial escharotomy—depending on the depth of the of necrotic tissue removal—can be distinguished. The superficial tangential escharotomy consists of the removal of the superficial dermis, until the dermal capillary plexus is reached; dermal plexus bleeding indicates the achievement of the vital plane on which the skin graft can be performed. The deep tangential escharoctomy is carried out at different levels of depth, sacrificing the entire dermis and hypodermis; the unviable dermis (hard and gray) also does not provide a substrate for skin grafting, but healthy fat looks shiny and yellowish and contains blood vessels that allow a high possibility that the graft will take. In the fascial escharotomy, the excision of necrotic tissue is done to the muscolaris fascia; the subcutaneous tissue is brown, necrotic and hemorrhagic, while the fascial smooth surface is a well-vascularized recipient site (perforating arteries) and allows skin grafts to take easily. Obviously, the main disadvantages of this procedure are the permanent cosmetic deformity (because the fat does not regenerate), risk of nerve and superficial tendon damage (skin denervation and permanent loss of sensation), and bone and tendon exposure. The autologous skin graft is the only definitive method to cover a loss of substance. The donor sites are, in order of preference: the upper and anterior-lateral thigh, the anterolateral area of the legs, upper arms, abdomen, back, chest and buttocks (Figure 7).

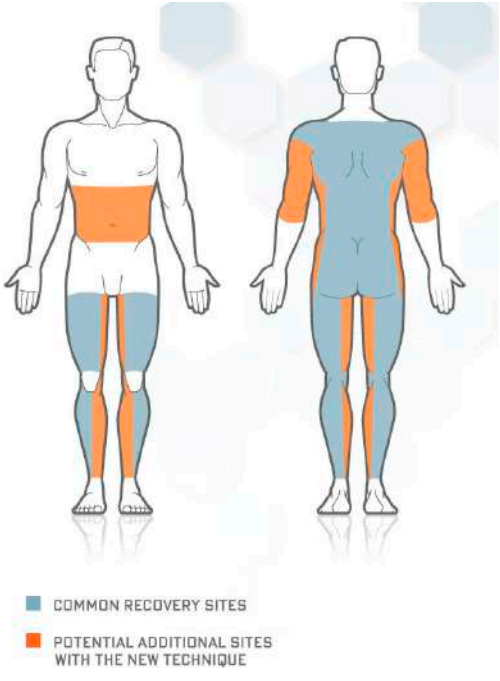


Figure 7. Schematic representation of full and split-thickness skin graft donor sites (Source: by the courtesy of World Press Media).

To take the dermal-epidermal area, skin grafts are used: Brown dermatome (electric or air) electric Padgett or Zimmer dermatome, and a range of freehand knives. Autografts are taken with a thickness of 0.010 to 0.012 cm and applied in a laminar or meshed structure. The mesh graft in the skin produces a series of perforations in a geometric pattern, to allow the expansion of the skin by increasing the initial size. In this way a limited donor area can cover a wider receiver area. The dermal-epidermal skin graft is prepared with mesh graft in ratio of 1 to $1\frac{1}{2}$ or 1 to 3, until even, in some cases, 1 to 6 or 1 to 9.

The skin grafts, both laminar and meshed, lie on the vital area (after adequate and careful hemostasis), are regularized and adapted to the loss of substance, and anchored using mechanical staplers (small grafts and those made in the face are fixed with thin sutures and moulage) (Figures 8 and 9). The meshed skin grafts are used in patients with large burns and limited donor sites. The presence of the "breaks" in the structure of the graft allows excellent drainage of the serous and blood collections that can be formed under laminar skin grafts.

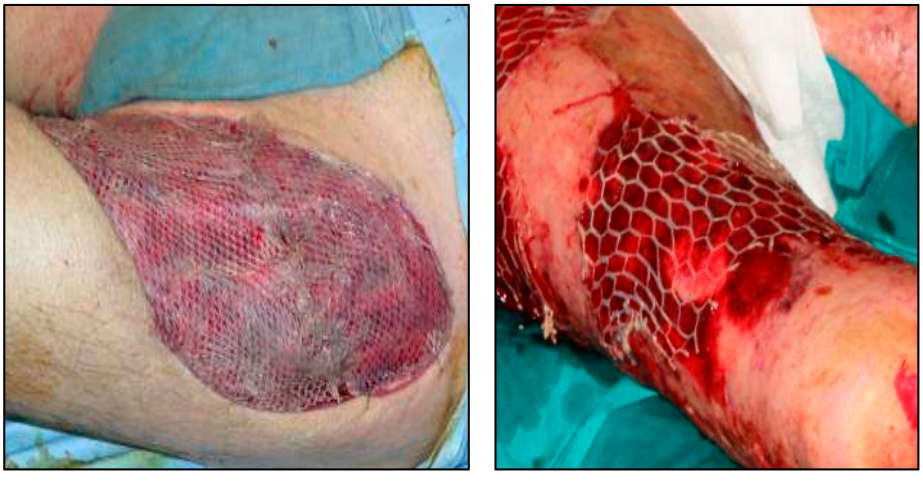


Figure 8. Skin grafts prepared with mesh graft in ratio of 1 to 1:1.5 (**Left**) or 1:6 (**Right**).

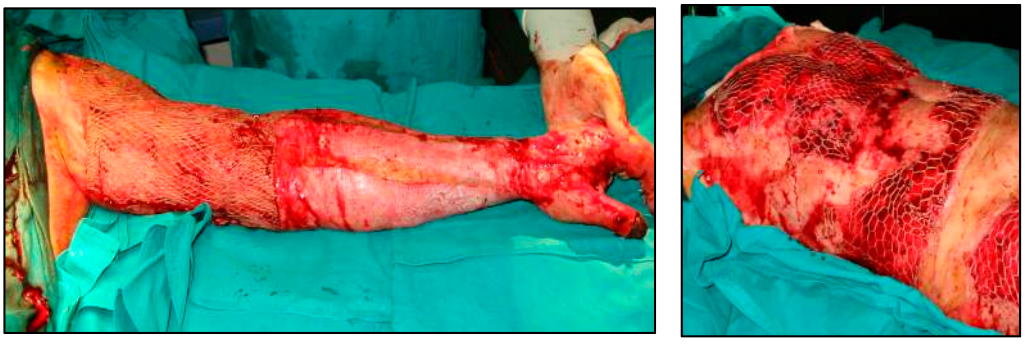


Figure 9. Clinical cases of patients with large burns and limited donor sites treated with meshed split-thickness skin grafts.

Ideal cutaneous wound repair should involve skin regeneration, bringing all the anatomical and physiological capabilities back to normal, without scarring. Unfortunately, wound healing in adult mammals too often results in a fibrotic normal or abnormal scar formation. Abnormal scarring that leads to hypertrophic scar or keloid formation that also invades the healthy tissue seems to be due to a persistent skin-barrier perturbation [29,30]. Thus, the prevention of unsatisfactory scarring begins before the treatment by the selection of both the dressings to be used and the post-operative care. A key part of the preoperative discussion is informed consent, which requires adequate information for the patient so that she/he can to make a decision regarding the procedure, indicating the procedure selected with the relative

risks and benefits. Naturally, with regard to scarring, the patient should be aware of potential scarring that may occur considering which areas of the body can be prone to this unusual wound contraction, as well as she/he has to know that smoking, alcohol and certain medications such as retinoids, may have negative impact on the final scar [31]. However, scarring, which cannot be prevented but controlled, is the inevitable final stage of wound healing. To what degree the resultant scar affects functional and cosmetic outcomes is dependent on early and consistent treatment. This is the reason why our group dedicates great attention to the control of the location and depth of injury, together with the pretreatment and cleansing of the burned/wounded skin, and the relative selection of the non-woven tissues to be used.

Chapter 2

Since natural polymers are an interesting option for most widely-applied fibers in composite non-woven technology, this study aimed to use an innovative non-woven tissue made prevalently by CN, functionalized by the use of marine collagen peptides, lignin and Ag^+ ions.

This innovative engineered composite, proposed as the most promising polymer matrix in wound-dressing development, has been shown in vitro to possess interesting antibacterial properties (Figure 10) connected with good cell adhesion and proliferation capabilities to guide cell differentiation [32].

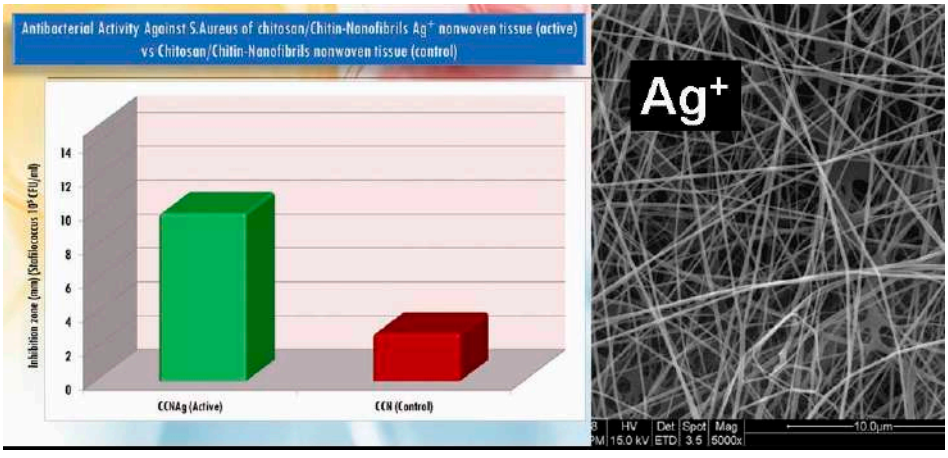


Figure 10. Antibacterial activity of chitosan/chitin fibers Ag^+ treated.

It is interesting to underline that this medical dressing is composed of two different layers. The outside layer (blue in color and composed of polypropylene of pharmaceutical grade) slows down the transcutaneous perspiration, increasing the cicatrizing activity of CN and the bactericidal power of the Ag^+ ions cross-linked to

the chains of the CN-polymer. The inside very thin layer, in direct contact with the wound and composed of natural dispersible materials, mimics the ECM architecture, influencing the cell life and reproduction. The methodology to crosslink Ag^+ to crystal Chitin, before obtaining the non-woven tissue by electrospinning, gave the possibility not only to reduce the toxic effects of the metallic ions (nano-concentrated) at the level of the skin cells, but also to enhance the fibers' anti-inflammatory and bactericidal activity [36–38].

The aim of the study was to use these innovative, biodegradable, and skin-friendly non-woven tissues to facilitate skin repair, serving as either temporary or permanent replacements for burned tissue.

In the treatment of superficial second-degree burns and split-thickness skin graft donor sites, the goal is to achieve complete re-epithelialization. Thus, the effectiveness of MAVI dressing in the healing of split-thickness skin graft donor sites was evaluated on superficial second-degreeburns.

After local institutional ethical committee approval, we collected data from ambulatory patients of our burn center from June 2014 to August 2014.

Exclusion criteria were admitted patients, full-thickness burns, and operated patients that came to the ambulatory facility for postoperative follow up.

Out-patient treatment was selected according to the classification of the American Burn Association: a second-degreeburn with TBSA (total burn surface area) less that 15% in adults (10% in children), burns not involving eyes, ears, face, hands, feet or perineum; burns not derived from electrical injuries, not associated with inhalation, not in poor-risk patients.

The burn depth was clinically assessed. No systemic antibiotics were used. Patient's burns were diagnosed as completely healed up when re-epithelisation was complete in all affected areas. For each patient, we collected the age, sex, cause of burn (scald or flame), type of dressing used and the days required for complete healing (healing time). We used three different preparations of MAVI dressings.

MAVI n.1 was utilized in 10 patients, treating the coverage of split-thickness skin graft donor sites in four patients and superficial second-degreeburns in six patients.

(MAVI n.1 is made of CN-Ag-lignin-PEO and collagen peptides made by electrospinning).

MAVI n.2 was utilized in 10 patients, treating the coverage of split-thickness skin graft donor sites in seven patients and superficial second-degreeburns in three patients.

(MAVI n.2 is made of CN-Ag-lignin-PEO made by electrospinning).

MAVI n.3 was utilized in 10 patients, treating the coverage of split-thickness skin graft donor sites in two patients and superficial second-degreeburns in eight patients.

(MAVI n.3 is made of CN-Ag-lignin-chitosan made by casting technology).

A prospective randomized clinical study was performed on 30 burn patients to investigate the effectiveness, safety and tolerability of MAVI dressings.

The MAVI dressing was applied on superficial second-degree burns and split-thickness skin graft donor sites in patients aged between seven and 71 years old, the mean average age was 43.6. The cause of the burn was scalding in 83% of cases and fire in the remaining 17%. The donor sites and the superficial second-degreeburn wounds were assessed on postoperative days one, five, 14, 21, and long-term for infection, hyperemia, pruritus, pain, exudate level, and adherence to the wound bed (Table 2).

Table 2. Different preparations and applications of MAVI dressings.

	Characteristics	Patients	Donor Sites/Second-Degree Burns
MAVI I	CN-Ag-lignin-PEO and collagen peptides	10	4/6
MAVI II	CN-Ag-lignin-PEO	10	2/8
MAVI III	CN-Ag-lignin-chitosan	10	7/3

At the follow-up visits, the donor sites and the superficial second-degree burn wounds were assessed again for pruritus and pain, patient comfort and convenience for the doctor. Touch-pressure, thermal and pain sensibility tests were performed preoperatively and on postoperative follow-up together with the assessment of color and texture of the re-epithelialized areas.

In all patients, re-epithelialization was completed between five and 13 days (mean eight days) after the application of the MAVI dressing.

Six patients out of 30 required pain killers over the first three days after burns with no significant differences between the type of dressing used.

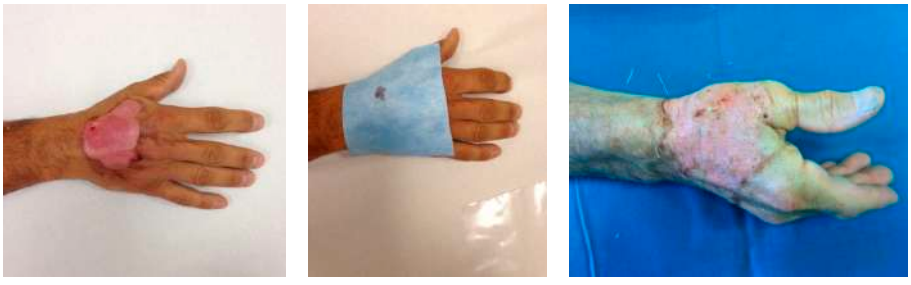
There were neither significant differences between donor sites and the superficial second-degree burn wounds regarding pain, hyperemia, pruritus, exudate, and final appearance (color and texture).

The areas dressed with MAVI completely healed within 5–13 days in a significantly higher proportion than the traditional dressings, showing during the whole study less incidence of exudates and peri-lesional erythema.

The aesthetic outcome of the treated lesions after healing was significantly better for the MAVI dressing and it required very few renewals of the medication during the first week of treatment. The high interval time between dressing changes reduced the amount of medication, patient suffering, overall costs and human resources, according to the reported following cases.

After the application of MAVI n.1 we observed the complete re-epithelialization of the split-thickness skin graft donor sites in three patients and a healing delay due

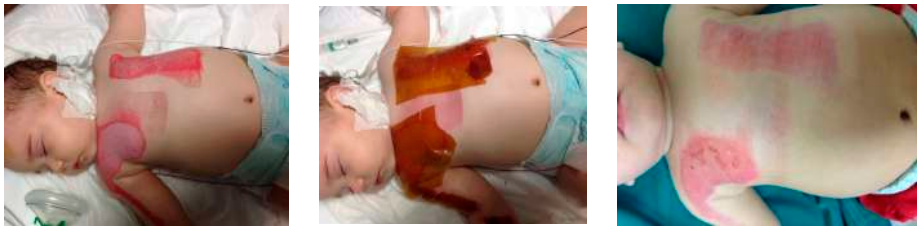
to exudate in one patient; and complete superficial second-degree burn healing in four patients (Figure 11).



(**a**) (**b**) (**c**)

Figure 11. Clinical case n°1: (**a**) superficial second-degree burn on the dorsum of the right hand; (**b**) Application of MAVI dressing number 1; (**c**) Complete re-epithelialization after five days post-burn.

After the application of MAVI n.2, we observed the complete re-epithelialization of the split-thickness skin graft donor sites in five patients and a healing delay due to exudate in two patients; complete superficial second-degree burns healing in two patients and the failure of the procedure in one patient due to infection. After the application of MAVI n.3 (Figure 12), we observed the complete re-epithelialization of the split-thickness skin graft donor sites in all patients, and complete superficial second-degree burns healing in seven patients.



(**a**) (**b**) (**c**)

Figure 12. Clinical case n°2: (**a**) Second-degree burn of the chest and right shoulder; (**b**) Application of Mavi dressing n°3; (**c**) Complete skin re-epithelialization after six days post burn.

Chapter 3

The self-renewing property of the epidermis plays a major role during wound healing. Within moments of wounding, keratinocytes not only inform each other

that the barrier has been broken, but also communicate with the dermis and the local immune system about the urgent need to repair the gap, maintaining a constant flow of information.

The purpose of such complex signaling cascades is to regulate gene targets during wound healing, thus controlling the keratinocyte activation cycle.

The most common initiator of keratinocyte activation is Interleukin-8 (IL-8), while defensine-2 (Figures 13 and 14 [36,37]) was found to be closely associated with the progression of wound healing.

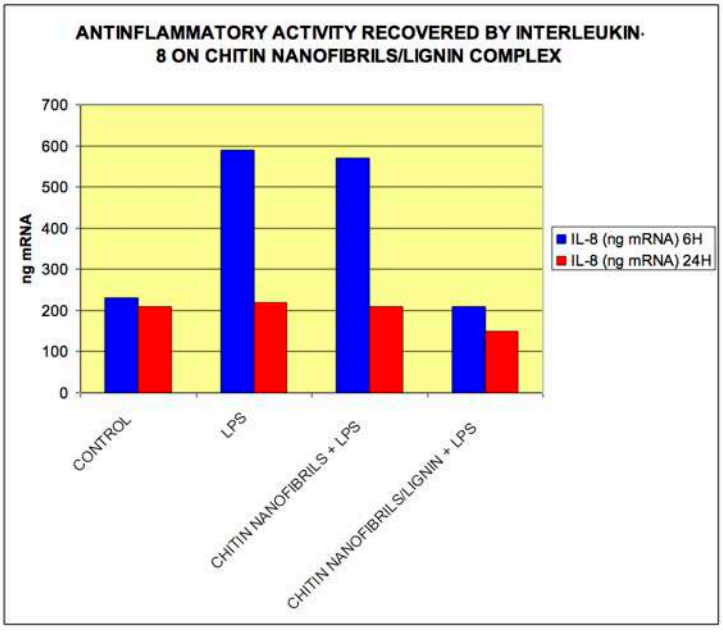


Figure 13. The figure illustrates gene expression modulation in human cell lines of pro-inflammatory cytokines in HaCaT keratinocytes. The cell lines were treated with: Control = untreated cells; LPS (Lipopolysaccharide of *P. Aeruginosa*) = pro-inflammatory substance; LPS + the Complex Chitin Nanofibrils/Lignin; LPS + Chitin Nanofibrils [37].

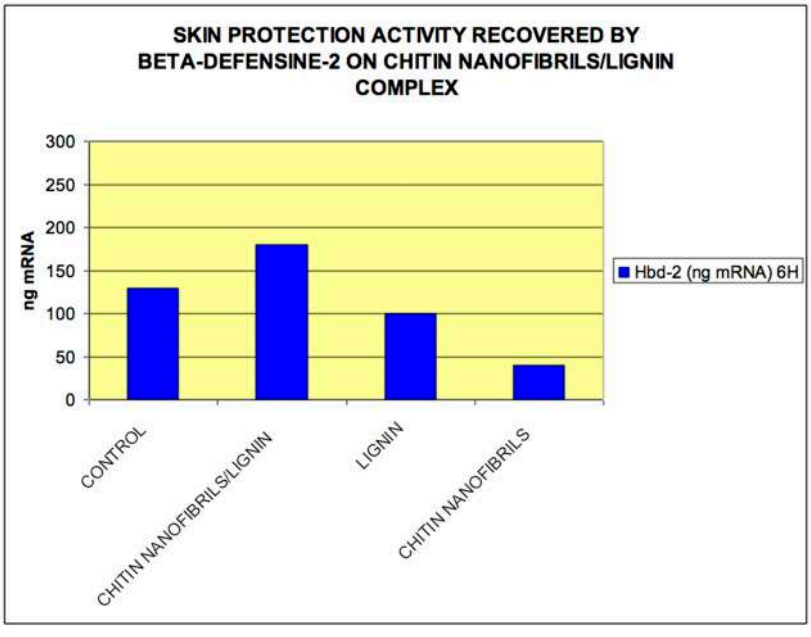


Figure 14. The figure illustrates the modulation of the gene expression of beta-defensin in human keratinocyte HaCaT cell lines. The cell lines were treated with: Control = untreated cells; Lignin; The Complex Chitin Nanofibrils/Lignin; Chitin; An over-expression in the values of these markers is an indication of tissue regeneration activity [37].

Therefore, IL-8 and defensine 2 appear to be accurate prognostic markers of wound repair.

The in vitro results of our studies on keratinocytes cultures were in agreement with the in vivo studies. An interesting modulating activity was observed on both IL-8 and defensine-2 on the non-woven tissue made by the natural fibers embedded in keratinocyte cultures.

On the other hand, the in vivo double-blind study made on the burned and wounded skin of a patient-group recovered in hospital notably accelerated the skin repair and cicatrizing activities of the same non-woven tissues. Moreover, due to the bactericidal and anti-fungal activity of the Ag-metal-ions, capable of regulating microorganisms invasion, it was possible to keep the dressing in place for one week, sensibly reducing the general cost of the in-hospital treatment.

It is interesting to underline how the obtained nano-fibers, made prevalent by the polymer chitin nanofibril bound to lignin and peptides, establishing each with other a stable ionic bonds, show an interesting skin-modulating activity on the synthesis of defensines and interleukines released from keratinocytes, also modulating the

metalloproteinase activity [37]. Moreover, Ag^+ ions, strictly bound to the Chitin chains, gave an interesting antibacterial activity to the medical dressing.

By this approach it seems desirable to use these innovative, chitin-based, non-woven tissues for medical purposes, due to their particular effectiveness in mimicking the size and arrangement of native collagen, accelerating the normal repairing activity of the skin, altered for burn or wound healing. Additionally, the electrospun scaffolds, obtained by a layer-by-layer method for their high surface-to-volume ratio and interconnecting pores, seem capable of facilitating cell adhesion and the formation of cell–cell junctions, accelerating the cicatrizing process [35–39]. These nano-fibrous scaffolds, in fact, seem to induce favorable cell—ECM interactions, increasing the cell proliferation rate, maintaining the cell phenotype, supporting cell differentiation, and promoting in vivo-like three-dimensional matrix adhesion [36–38]. Moreover, they probably activate cell-signaling pathways, by providing the chemical and physical stimuli to cells necessary for faster skin-repairing activity. The sequential, multi-layering electrospinning of the different natural polymers employed in combination with natural peptides, surely enhanced the mechanical integrity and dimensional stability of the electrospun meshes, while the nanostructured Ag^+ silver probably maintained the skin microbiota in equilibrium, impeding the excessive growth of the opportunistic microorganisms [36,37].

These innovative medical dressings seem to represent new, cost effective, non-woven tissues and procedures to be used for skin regeneration and tissue transplantation. Their activity, compared to the traditional non-woven tissues normally used in our hospital, was shown to be more effective in a shorter time to regenerate the skin affected by burns of the first and second degree. It is interesting to underline the capacity that these non-woven tissues have to prevent microbiota growth for the right period of time, extending the medication period. In conclusion, the MAVI dressing seems to be a safe and effective dressing for the re-epithelialization of skin graft donor sites and superficial second-degree burn wounds, showing higher activity than traditional dressings.

The goal of such a treatment strategy based on the use of innovative, bioengineered, non-woven tissues, which are totally biodegradable and environmentally-friendly, is, in fact, to act as smart band-aids, to replace altered or senescent resident cells and reestablish the anatomy and physiology of burned/wounded skin [40,41]. Well-designed randomized clinical trials will involve our group in the next phase in order to scrutinize the true potential of these natural polymers for regenerative medicine.

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Conflicts of Interest: We declare hat Paolo Palombo, Marco Palombo, Simone Moroni, Agostino Bruno and Tommaso Anniboletti have no conflicts of interest. Pierfrancesco Morganti works as the Head of the R&D Centre of Nanoscience, MAVI SUD, Srl, Italy.

Abbreviations

CS	Chitosan
CN	Chitin nanofibrils
S. aureus	Staphylococcus aureus
CFU	colony forming unit
CB	bio-burden skin bacteria
PEO	polyethylene oxide
Ag	Silver

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EU Cosmetic Regulation: Quality Enhancement of Consumer and Environment Protection, Market Development

Sonia Selletti

Abstract: The purpose of this article is to outline the main features of the recent European recast of the Cosmetics Regulation (Regulation EC No. 1223/2009), focusing on the core aims of the legislator: harmonization throughout member states (hence the choice of a Regulation as the appropriate legal instrument) and the reduction of administrative burden and ambiguities, to enhance the protection of human health and the environment, thus fostering quality in the market to the benefit of consumers, who may rely on strengthened in-market controls. Specific attention is also paid to the justification of claims, in order to lead consumers to make informed choices based on clear, transparent, and honest claims, counting on a set of 'common criteria' laid down in the specific Regulation (EC) No. 655/2013. This framework makes room for innovation in cosmetic research, since it regulates the use of nanomaterials in cosmetic products, respecting the environment.

1. Introduction

A revision of regulations governing cosmetics has recently taken place in Europe thanks to the (EU) Regulation no. 1223, adopted on 30 November 2009, which came into force for all member states on 11 July 2013 [1].

The new regulation is a re-casting of the rules on cosmetics adopted with directive 76/768—which was the object of as many as 65 amendments—and is a fundamental step in the enhancement of the quality of cosmetics, both in terms of consumer safety and market development. Although consumer safety has taken a place of pride in the legislator's production, the latter has made clear that quality and safety are the main pro-competitive elements, holding equal prominence, which may be beneficial to market development [2].

2. Main Features of the Regulation

Although the Regulation appears at times to be a harmonized coding of the previous regulations, it does in fact sum up almost 40 years' experience in the application of the rules in question, taking this experience to heart and thus setting the stage for an even more effective protection. Thus, the issue of safety is a lynchpin

concept to such an extent that Article 3 prescribes that "cosmetic products made available on the market shall be safe for human health when used under normal or reasonably foreseeable conditions of use...". From this initial indication, one may well understand the kind of responsibility that companies are required to shoulder if they intend to place cosmetic products on the market.

The safety of cosmetics is pursued using a number of different "tools":

- the Regulations are accompanied by Annexes that list the substances that are subject to prohibitions or restrictions to safeguard health (these lists may be modified over time based on scientific advancements),
- for the purpose of imputing cosmetics on to the market, they must be subjected to a safety assessment (the EU Commission on 25 November 2013 issued the specific guidelines for the performance of an appropriate safety evaluation) [3].
- cosmetic production must take place while complying with good manufacturing practices,
- to ensure supervision and guarantee compliance with the obligations indicated in the Regulations, the figure of the 'responsible person' has been introduced in Article 4 (for example, the importer is, according to the law, a responsible person),
- a system has been introduced that enables the identification of the supply chain in order to guarantee the traceability of the cosmetics, supported by the institution of a centralized notification procedure through an EU portal managed by the Commission (so called CPNP—Cosmetic Products Notification Portal) and the identification of the subjects that operate in various capacities in the production process (manufacturer; importer; distributor) that have been assigned specific obligations,
- reinforced surveillance procedures have been outlined, to be implemented by the authorities with the aim of curtailing the counterfeiting that even afflicts the cosmetic sector and may be detrimental to the consumer's health; this system is based on the principle of cooperation between the various authorities and envisages the active participation of the responsible person and other subjects involved in the production process,
- customer protection is also achieved through a cosmetic vigilance program that involves the reporting and collection of information on undesirable effects; a specific reporting of serious undesirable effect (SUE) has been introduced and is ongoing in each member state,
- the Regulation also envisages a special protection related to the claims made by cosmetics to ensure that the consumer can make informed decisions based on objective and not deceitful elements; this has meant the introduction of "common criteria" (with EU Commission Regulation No. 655 of 10 July 2013) to "inform end users about the characteristics and quality of products" seeing

as these are "essential in order to differentiate between products" and help to "stimulate innovation and foster competition" [4,5].

- concerning the environment, special recommendations have been expressed by the Unfair Commercial Practices Directive (UCPD) Guidance Document to encourage industries to make good and accurate environmental claims, avoiding the use of "green claims" regarding the composition and process of products if not well-documented or not documented at all. Greenwashing is in fact a coined expression to underline the act of potentially misleading consumers regarding the environment practices of a company or the environmental benefits of a product or service. According to the revision of the UCPD Criteria Guidelines on Environmental Claims [6] completed on May 25, 2016, a definition for "environmental claims" has been provided (the expressions "environmental claims" or "green claims" refer to the practice of suggesting or otherwise creating the impression (in the context of a commercial communication, marketing, or advertising) that a product or a service is environmentally friendly (i.e., it has a positive impact on the environment) or is less damaging to the environment than competing goods or services. This may be due to, for example, its composition, the way it has been manufactured or produced, the way it can be disposed of, and the reduction in energy or pollution which can be expected from its use. When such claims are not true or cannot be verified, this practice can be described as "greenwashing"). Furthermore, a couple of main principles address the behavior of traders that: (i) must, above all, present their environmental claims in a specific, accurate, and unambiguous manner; and (ii) must have scientific evidence to support their claims and be ready to provide it in an understandable way in case the claim is challenged [7].

3. Innovation

The theme of innovation in a cosmetic context, often referred to by the legislator, offers scope for a brief consideration of the room that the regulations allow for innovative products, both in terms of the research and development of molecules, formulae, and technologies, as well as new kinds of products.

Thus, research is stimulated by the fact that the restrictions already imposed on the use of certain substances do not curtail the development and research of other substances, formulae, and technologies, the use of which will then have to be further investigated through the safety assessment procedures that the manufacturer is responsible for carrying out. The manufacturer is therefore fully entitled to head down innovative paths, being fully aware of the parameters that the company is required to comply with.

In relation to product types, it should be recalled that the legislator has underlined the need to hold firm on a clear demarcation between cosmetics and

similar health products (medicines, medical devices, biocides, food integrators) in an attempt to avoid overlapping classifications relative to so-called borderline situations so that the correct sector regulations that apply can be identified.

Thus, by way of example, plenty of discussions and comparisons have been held on the cosmetic—or other—nature of certain products such as teeth whitening chewing gum, mascara that enhances eyelash growth, and adhesive patches used to fight unsightly body fat deposits (or cellulite) or to improve hair growth. There are many other interesting examples that have been assessed in an EU Commission manual on borderline products (Manual on the Scope of Application of the Cosmetic Regulation EC No. 1223/2009, November 2013), which has turned out to be a very useful tool not only for those seeking to interpret the regulations but also, and especially, for those engaged in product research and development [8].

The issue of innovation and development in the field of cosmetics finds its natural source in the very definition of the cosmetic product, which, as we know, is based on what one may term a binary system, which refers to the application site of the cosmetic product (external surfaces of the human body: epidermis, hair and hair follicle, nails, lips, external genital organs, teeth, mouth mucous) and the functions that are prevailingly or primarily performed (cleaning, perfuming, change of appearance, protection, maintenance, correction of body odors). Thus, the function of cosmetics, despite being established at a legal level, leaves plenty of scope for research, development and innovation in terms of functions such as "protection" and "maintenance", onto which one may graft the most advanced cosmetic qualities that are in a position to establish said products as functional cosmetics, even if they are also recommended in other specific or complementary contexts such as therapeutic contexts (while maintaining the prohibition of boasting therapeutic effects).

The easiest example, and also the most significant one, is found in the dermatological context, where the treatment for relevant pathologies is not only based on innovative medicinal products, but also on the contribution provided by functional cosmetics that enable the patient to improve their quality of life from every point of view. Thus, the cosmetic product plays an important complementary role to the therapy.

4. Nanomaterials

It is worth spending a few words on the issue of "nanomaterials", which have been specifically regulated by the European Cosmetics Regulation No. 1223/2009 as well as from a legal point of view, as an explicit acknowledgement that these ingredients are undergoing considerable development.

The Regulation has introduced several articles with implications for products containing nanomaterials, starting with the definition of a nanomaterial, for the purposes of the Cosmetics Regulation, provided under Article 2.1 (k) as "an insoluble

or bio persistent and intentionally manufactured material with one or more external dimensions, or an internal structure, on the scale from 1 to 100 nm". While it is the responsibility of the manufacturer (under the aegis of the responsible person) to decide whether any ingredients they are using are nanomaterials (to this end, it is important to acquire proper information from raw material suppliers), the Regulation states (Article 16) that a high level of protection of human health should be ensured for any cosmetic products containing nanomaterials. Moreover, the Regulation requires that a specific evaluation be carried out, as part of the safety assessment, in order to determine any toxicological effects due to particle sizes, including nanomaterials (i.e., Annex 1—Cosmetic Safety Report). To help assessors evaluate nanomaterials appropriately, the Scientific Committee on Consumer Safety (SCCS) has published a report entitled Guidance on the Safety Assessment of Nanomaterials in Cosmetics [9,10].

Both the authorities and consumers shall be specifically informed about the presence of nanomaterials in cosmetic products: the former by means of a centralized notification procedure (cosmetic products that contain nanomaterials will need to be notified six months before the product is placed on the market), and the latter by means of the product label (all ingredients present in the form of nanomaterials shall be clearly indicated in the list of ingredients followed by the word 'nano' in brackets, so as to enable consumers to make informed decisions).

There are some exemptions for nanomaterials intended to be used as colorants, preservatives, and UV filters, which should be listed in Annexes IV to VI in order to be permitted for such uses: these are never subject to the nano-notification requirements, irrespective of the size of the ingredients, since the positive listing in the Annexes supersedes the need for nano-notification. Products containing ingredients listed in Annex III (list of substances which cosmetic products must not contain except subject to the restrictions laid down) in the form of a nanomaterial need not be notified.

Conflicts of Interest: The author declares no conflict of interest.

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The Patent Protection of Nanotechnological Inventions: The European View

Claudio Germinario

Abstract: The patent protection of nanotechnological innovations represents an exciting challenge for all those in the specialized circle. The scope of the present work is to highlight how the protection of nanotechnological inventions implies a new interpretation and application of the general requirements of patentability. By relying on the Case Law of the Board of Appeal of the European Patent Office, the author will illustrate with examples how the requirements of novelty, inventive step, industrial applicability, clarity, and sufficiency of disclosure apply in the nanotechnological platform.

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1. Introduction

What is a patent?

The patent is a "contract" involving three parties: inventor, authority, and public. The inventor makes the innovation and applies for a patent; the authority grants the patent, which confers exclusive rights to the inventor (i.e., a monopoly for the commercial exploitation of the invention); and the public is allowed, and must be enabled, to use and exploit the invention after expiry of the patent terms, normally 20 years. Therefore, the very concept of "patent" represents the compromise of two opposite interests: the inventor's interest and the public's interest.

2. The Patent Application

It is fundamental to diffuse the culture and knowledge of these innovative, environmentally friendly nanomaterials and nanotechnologies by scholastic teaching, together with the use of newspapers, television, and any other mean of mass media communication.

The patent application is a written document normally consisting of two sections: (i) the description of the invention and (ii) the claims.

(i) The description discloses the invention in all its technical aspects. The description may contain specific examples of concrete invention embodiments and make reference to drawings. In order that the public's interest (i.e., the possibility of exploiting the invention after expiration of the patent) is safeguarded, the patent application shall disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the field, i.e., the public.

(ii) The "claims" are the legal section determining the extent of the protection conferred by the patent or patent application.

2.1. The Basic Principles of Patentability

In order for an invention to be patentable, it must be novel, involve an inventive step and be susceptible to industrial application.

Additionally, as seen above, the invention must be clearly and sufficiently disclosed.

An invention is considered to be new if it does not already form part of the state of the art; in other words, if it was not made available to the public by means of a written or oral description, by use, or by any other way before the date of filing the patent application.

An invention is considered to involve an inventive step if it is not obviously derivable from the state of the art and it is considered to have industrial application; if it can be made or used in any kind of industry, including agriculture.

3. Patentability in the Nanotech Field

This section evaluates how the usual requirements of novelty, inventive step, industrial applicability, clarity, and sufficiency of disclosure apply to inventions in the nanotechnology area [1].

First, it is necessary to identify an accepted and shared meaning of "nanotechnological invention". It seems there is a consensus in the scientific and patent literature, on the point that "nanotechnology" is not simply the science of the infinitesimally small (i.e., at least one dimension of less than 100 nm), but rather, the science of the infinitesimally small accompanied by at least one new property (physical, chemical, pharmaceutical, etc.) directly derived or caused by the nano-size.

Under this definition, a patentable nanotech invention may be reasonably considered as a patentable "selection-invention". A selection invention is an invention based on the recognition of a sub-range extracted from a previously known larger range, with said sub-range causing a technical effect not recognized or attributed to the whole larger range.

3.1. Novelty

If, prior to the filing date of the patent application seeking protection for an invention, the same invention had already been described in a written publication, oral presentation, or public prior use, then the invention no longer fulfils the requirement of novelty necessary for patentability. For example, if a natural product, such as chitin, is generically described in a scientific publication, with its chemical structure and some characterizing parameters, then this product will be excluded from subsequent patent protection since it is lacking novelty.

However the question is whether the same principle also applies when we move from the macro scale to the nano scale. For instance, if the invention consists of "nanocrystals" or "nanofibrils" of chitin, can this "nano-form" of chitin be considered novel over the chitin described in the prior art?

An indirect answer can be found in the case law of the Board of Appeal of the EPO, which laid down general principles in assessing the novelty of polymorphisms, but that also find application in the field of nanocrystalline substances.

For instance, the Board of Appeal recognized the novelty of a substance either in crystalline or in amorphous form, although the very same substance was known the prior art in a different physical form (either amorphous or crystalline, respectively) [2–4]. The Board of Appeal also recognized the novelty of Aspartame type IIa, whcih is a specific polymorphic form of aspartame, over Aspartame III, which is another polymorphic form of the same aspartame dipeptide [5].

By translating the same principles, a substance in nano-form, such as nanocrystals or nanoparticles, may be considered novel, i.e., a different entity, over the same substance in macro-size form. This is indeed the case of the chitin nanofibrils (or chitin-whiskers), over the normal amorphous chitin or chitin long fibers obtained by electro-spinning. The same principles have been applied in assessing the novelty of composite materials, comprising a nano-sized particulate phase of a given chemical compound.

In a first decision [6], the Board acknowledged the novelty of a composite material comprising a layer of nanocrystalline nickel obtained by electrodeposition, on the basis of the size of the nanocrystals (less than 11 nm). Prior art documents disclosed essentially identical materials, yet comprising a layer of crystalline nickel having micro/macro-size crystals (>100 nm).

In a second decision [7], the Board of Appeal was faced with the problem of evaluating the novelty of a cigarette filter, consisting of a cellulose acetate tissue comprising nanoparticles of TiO_2 of size less than 100 nm as a photodegrating agent. A prior document disclosed essentially identical cigarette filters, but comprising TiO_2 particles of a much larger size, about 500 nm (pigment grade).

4. The Board Recognized the Novelty of the Invention

4.1. Inherency

The situation may prove even more intriguing when the down-scaling to nano-size confers to the "known" substance some novel properties, either chemical, physical, or biological.

In the macro world, it is a generally accepted rule that the discovery of a novel property of a known substance is unable to restore the novelty of this substance. It is in fact considered that the novel property was inherent to the substance itself.

In other words, if the novel property had been searched for in the substance, it would have been immediately revealed.

This does not necessarily happen to nanomaterial, since the novel properties characterizing the material in the form e.g., of a nanoparticle or a quantum-dot may directly result from the infinitesimal size of the material and, accordingly, could not be detected in the same material at the macro scale, even if it was purposely looked for. For this reason, the inherency approach, and its impact on the novelty of macro materials, no longer applies when we move from the macro to the nano scale.

4.2. Inventive Activity

The claimed subject matter is considered to involve an inventive activity if, for the skilled person, it is not obviously derivable from the state of the art.

This requirement, applied to nanoscience, raises a number of questions, specifically in consideration of the interdisciplinary nature of the nanotech platform. For example, which should be the reference technical field for inventions overlapping different technical fields, such as biotechnology, electronics, and semiconductors? Also, who should be the skilled person?

The case law of the EPO in the biotechnological field already offers some answers, which can be extended to the nanotech field.

The Board has recognized in many cases in the biotech field that the skilled person is indeed represented by a team of persons, each of whom is an expert on a different aspect of the same technical field. Furthermore, in the nanotech platform it is likely that this fictitious person will be embodied by a team of persons, each of whom is an expert on a different technical field: for instance, gene technology and semiconductors. Yet, this multidisciplinary approach raises the bar in assessing the requirement of inventiveness, making this requirement more difficult to meet. In fact, what seems to be inventive to one single "expert", may prove obvious when evaluated in the light of the combined knowledge of two or more experts.

A further question is whether the miniaturization of an object can in itself endow said object with an inventive step. In the macro domain, the answer is in the negative. The Board of Appeal has already set forth [8] that the easy miniaturization of devices or equipment is suggested by the well-known industrial need of optimizing space and materials.

However, moving into nano-size can be regarded as an obvious step only if the necessary tools and technology are available to the skilled person. It is well known that the top-down method has technical limits. Overtaking these limits by an alternative top-down method or by novel bottom-up methods could never a priori be regarded as obvious.

Moreover, as already seen above, miniaturization to the nano scale is very often accompanied by a novel and unpredictable effect. Under these circumstances,

the result of miniaturization should always be considered inventive, regardless of the apparent obviousness of the means used to achieve the result.

5. The New Technical Effect Directly Caused by the Nano-Size

There are in the patent literature many examples of "novel properties" or "novel effects", caused by miniaturization, that justified the patentability of nano-substances or nanomaterials already known in macro-form.

For instance, chitin nanofibrils show, in addition to optimal mechanical properties, a surprising resistance to hydrolytic enzymes as compared to natural amorphous chitin. See Table 1 (kindly provided by Prof. Pierfrancesco Morganti—Mavi Sud S.r.l.).

Table 1

	CHITIN NANOFIBRILS		Weight variation		CHITIN NATURAL		Weight variation	
	INITIAL	FINAL	g.	%	INITIAL	FINAL	g.	%
CELLULASE	0.10028	0.10021	-0.00007	0.07%	0.10060	0.10004	-0.00056	0.56%
LYSOZYME	0.09999	0.09991	-0.00008	0.08%	0.10010	0.09900	-0.00110	1.09%
PECTINASE	0.10110	0.10109	-0.00001	0.0099%	0.10234	0.10120	-0.00114	1.11%
AMYLASE	0.10031	0.10029	-0.00002	0.02%	0.10905	0.10305	-0.00600	5.5%
COLLAGENASE	0.02563	0.02560	-0.00003	0.12%	0.10958	0.10008	-0.00950	8.67%

This unexpected property makes chitin nanofibrils a very promising material in dermatology and cosmetic surgery, for protecting wounds or damaged skin or as filler in the treatment of wrinkles, showing a high resistance to hydrolytic endogenous enzymes.

Thank to this novel effect, chitin nanofibrils have been claimed for use in dermo-cosmetic surgery and in therapy [9]. They have been claimed in complexes with negatively charged polymers for use as carriers for medicament [10], for the preparation of films comprising anti-bacteria metals, as well as for many cosmetic and therapeutic applications and food supplementation or preservation [11].

Other examples include nano-constructs with pharmacological activity comprising an inorganic metallic nanoparticle with an amphiphilic polymeric coating and an active antiviral peptide bound thereto [12]. The nanoparticles used as a carrier of the medicament enable an effective transportation of the active molecule through the cellular membrane and directly into the cell cytosol, thus escaping the endosome pathway and accordingly the lysosomal degradation, i.e., an unpredictable behavior caused by the nano-structure.

Yet another example is the "antireflective coating for use in photolithography" of Advanced Micro Devices Inc. [13,14]. An antireflective Titanium Nitride (TiN) layer with a thickness between 25 and 40 nm minimized the percent of reflected radiation to less than 5%. Thickness values below 25 or above 40 nm, as disclosed in the prior art, resulted in a significant increase of the reflected radiation up to 30%, as illustrated in Figure 1.

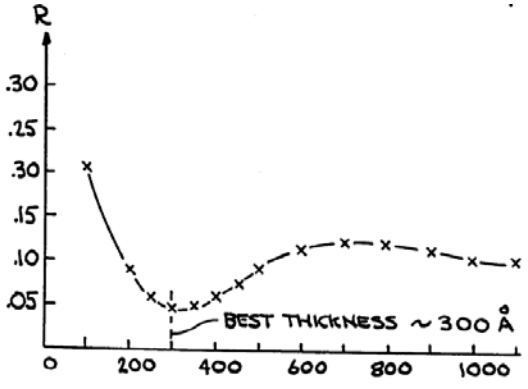

Figure 1. Reflected radiation of about 5% for 25 to 40 nm (i.e., 250–400 Å).

An additional case [15,16] (Rohm and Haas Company) related to a coating composition comprising a dispersion of copolymer particles having a size range of 20 to 70 nm. The miniaturization of the particles to 70 nm or less, although achieved by "normal" methods, was able to endow the claimed coating with an unpredictable optimal effect, namely with excellent transparency and clarity, where the effect was lost for larger particle-sizes. The closed prior art document described comparable coating compositions comprising polymer particles spread along a much broader range (10–1000) and preferably along the range of 100–500 nm. Accordingly, the Board of Appeal recognized the novelty and the inventive step involved in the selection of the range of 20–70 nm over the coating compositions described in said prior document.

6. The Reasonable Expectation of Success

Another important factor playing a role in evaluating the existence of an inventive step is the "reasonable expectation of success". This principle, which was developed and frequently applied in the biotechnology field, may give a strong support to inventiveness in any pioneer field such as nanotechnology. In fact, the prejudicial effect, for the patentability of an invention, represented by an earlier suggestion of the invention in the prior art depends on the circumstances. In a traditional technical field, all procedures and tools needed for realizing a "suggestion" are normally known and available to the skilled person and thus the results easily

predictable. Under these circumstances, putting to practice the suggested teaching of a prior art document is always accompanied by a high expectation of success, and does not involve any inventive merit.

In reverse, in an emerging field in which the technology is not necessarily available and the procedures are not always standardized or reproducible, the result of an action is hardly predictable. In this situation, the realization of a "suggestion" is not always accompanied by a "reasonable expectation of success". Thus, achieving the desired result may prove inventive even though it is theoretically suggested.

For example, the above discussed decision [6] related to a composite material comprising a layer of nanocrystalline nickel of less than 11 nm, obtained by electrodeposition. As many as eight prior documents apparently suggested the same process of the invention, but in the context of microcrystalline structures (i.e., in the order of 100- to 1000-fold larger). Under these circumstances, the Board found that "...the skilled person had no obvious reason to foresee that the prior teaching could still be successfully extrapolated to structures smaller by at least two orders of magnitude, if not with the benefit of hindsight". In other words, the skilled person could not have any expectation of success when putting to practice the prior art teaching on nano-size level.

7. Sufficiency of Disclosure

The patent application describes the invention in a manner sufficiently clear and complete for it to be carried out by the skilled person. This requirement means that the skilled person should be able to repeat the invention, essentially over the whole ambit of the claim, based on the technical teaching given by the application (corroborated by the common general knowledge), and the realization of the invention, with the achievement of the declared effects of the invention, may not depend on chance. These conditions are essential in the patent protection of nanotech inventions.

In fact, when the technical effect achieved by, and characterizing, the invention strongly depends e.g., on the (nano) size of the claimed material or on the delicate conditions necessary to obtain said material and to realize the invention, the requirement of sufficiency of disclosure plays a decisive role and leaves no room for practices broadly shared in other technical fields, such as arbitrary generalization of the real invention or the extension of the protection to non-investigated equivalents.

Coming back to the antireflective coating for use in photolithography of decision T 453/97 (Note [14] and Figure 1) the effect characterizing the invention was the percentage of reflected versus incident radiation of less than 5%. This effect was quickly lost outside the very limited thickness range of 25 to 40 nm. Therefore, any arbitrary generalization of this range would make the realization of the invention and the achievement of the characterizing effect impossible.

The same applies to the concept of "equivalents", and strongly suggests that in the nanotech field there appears to be no room for any form of speculation or arbitrary extension of the scope of protection to cover embodiments theoretically "equivalent" to the real invention, but not yet investigated. This means that the need for a sufficient number of examples and experimental results substantiating all described embodiments of the invention is felt in the nanotech field more than in any other technical field.

For instance, in a case relating to an electro-magnetic transducer [17], one element of the invention was a semiconductor layer produced by molecular beam epitaxy using, as a doping element, atomic (N°) nitrogen radicals.

A similar method was described in a prior art document which illustrated with all experimental data an embodiment making use of molecular (N2°) nitrogen radicals, but which also cited as an alternative the use of atomic (N°) radicals, though without any support of examples or experimental results. Lacking any experimental confirmation, the Board considered that the use of atomic (N°) radicals in the prior art document was a merely a speculative attempt to disclose not yet investigated subject matter and accordingly disregarded this prior art teaching.

8. Metrology

A specific aspect of the requirement of "sufficiency of disclosure" is metrology. For the scientist, the definition of metrology is "the science of weights and measures" [18], thus including procedures, techniques, instruments, sensors, or any other tool for measuring.

On the contrary, for the patent attorney, "metrology" refers to an important aspect of patentability, namely the requirement of "sufficiency of disclosure" which enables the "repeatability" of the invention by the skilled person. Thus, metrology is indeed a condition for patentability.

To meet this requirement, the expert, supported by the technical teaching in the patent specification, optionally integrated by common general knowledge, should be able to put to practice the invention, in addition to achieving the technical effects the invention is said to produce. It goes without saying that the skilled person must be aware of all necessary instructions for monitoring the parameters characterizing the invention and for measuring the results obtained, in order to understand whether or not he/she has actually achieved the declared effect of the invention.

In a traditional technical field all analytical procedures, tools, and techniques are usually a part of the common general knowledge, and thus they need not be exhaustively described in the patent specification. However, this is not always the case in a pioneer field, such as nanotechnology.

In fact, nanotechnological inventions, like all inventions in any emerging fields, are often characterized by non-conventional features and effects: by unusual

parameters, e.g., nano-size, monoatomic layers; by unusual processes, e.g., epytaxis, spattering, mono-atomic deposition; by unusual effects, e.g., detection of a single analyte molecule or detection of fluctuation of a specific index. Under these circumstances, the patentability of an invention strongly depends on the detailed description in the patent text of all technical aspects falling within the concept of "metrology".

This requirement has been clarified in many decisions of the Board of Appeal of the EPO. In a very recent case [19], the patent claimed: (i) "A silica glass for EUV lithography containing TiO, characterized in that the fluctuation of the refractive index (Δn) is at most 2 $\times$ 10-4 within an area of 30 mm $\times$ 30 mm in each of two orthogonal planes".

The validity of the patent was contested in an opposition procedure based on the following opponent's arguments: " ... that the patent did not provide sufficient information on the measurement of the parameters appearing in the claims: i.e., 'the fluctuation of the refractive index (Δn) ... '". Therefore, the skilled person repeating the invention did not know for sure whether the obtained glass fell within the scope of protection of the claims.

Another decision [20] related to a patent protecting "A silica gel, characterized, inter alia, by the feature represented by the silanol-group density of 6–20 ... " in combination with other parameters.

Yet, the patent specification taught an incomplete and erroneous method for determining and measuring the silanol-group density cited in the claim. Nor could the skilled person find any more reliable teaching in the prior art documents. For this reason, the Board revoked the patent, affirming that: " ... an erroneous method of measurement or determination of an essential parameter of the claimed product unavoidably results in a lack of sufficiency of disclosure of the claimed product ... ".

The same principles were recently confirmed by still another decision [21] relating to an adsorbent article, whose absorbent capability was defined in the patent claims by totally inconsistent values produced by an arbitrary test method.

The Board revoked the patent, arguing that: " ... the purpose of a parameter contained in a claim is to define an essential technical feature of the invention. ...The method specified for determining that parameter should therefore be such as to produce consistent values, so that the skilled person will know, when he carries out the invention, whether what he produces will fall within the scope of the invention".

9. Clarity

This requirement ensures that the claims define the object of the protection and are clear, concise, and based on the description.

A simple question arises here: which is the most suitable claim format, for a nanotech invention, that is able to meet the requirement of clarity without unduly reducing the scope of protection?

In fact, there exists no claim form specific to nano-invention, but there are claim forms which are certainly more suitable than others, as illustrated below.

10. Product-by-Process Claim

At the nano scale, it may prove difficult to properly define an invention, product, substance, or material by way of its structural features. It may be easier and safer to identify the invention by way of its preparation process, using wording such as: "product obtainable by the following preparation process comprising ... ". For this reason, the product-by-process claim is extensively used to define inventions in the nanotech platform.

For example, the "nanocrystal metallic material" object of decision T0915/00 [6] was defined as being " ... obtainable by a process comprising the electrodeposition of the material on a substrate in a solution containing ions of the metal characterized ... by passing a D.C. current ... at pulse intervals ... ".

The meaning recognized by the Board of Appeal of this type of claim is quite different when the invention is on the macro scale or in the nano field. At the macro scale, there is a generally recognized principle according to which a new process for preparing a known product is unable to restore the novelty of the obtained product.

Yet, at the nano scale, it is difficult to prove that even minor modifications in the process parameters are neutral as regards the structural features of the obtained product. This has been the position taken by the Board of Appeal in more than one decision.

In the aforementioned decision [6], Nickel nanocrystals with the size of 11 nm were obtained by electrodeposition by passing in an ion solution a D.C. current at pulse intervals. These nanocrystals have been considered different from Nickel nanocrystals of nearly the same size obtained by electrodeposition by passing in the ion solution a D.C. current having the same characteristics, but applied continuously. The Board accepted the appellant's argument that the different process resulted in a different crystalline structure, though having the same size.

By applying the same criteria, the Board acknowledged in a subsequent decision [22] the novelty of a nanocrystalline product obtained by sputtering versus the same nanocrystalline product obtained by electrodeposition.

In an earlier case [23], a device having a specific surface topography was defined by means of its preparation process involving epitaxial growth. The Board recognized the novelty of the device, accepting that the process gave rise to peculiar characteristics.

11. Functional Definitions

Another frequently used claim type for the characterization of nanotech inventions is the functional claim, which defines the claimed compound by way of its functional properties. As seen above, the miniaturization to the nano scale is frequently accompanied by a novel specific property. Accordingly, it is sometime easier and clearer to characterize the miniaturized material by way of its novel property caused by the nano-size rather than to characterize the material by way of its structural features, which eventually produce the novel property. For this reason, the functional definition of the invention is extensively used in this field.

12. Terminology

A final aspect of clarity is the clarity of the terminology used to describe the invention. In a pioneer multidisciplinary area, the technical meanings of the terms are not necessarily standardized and equally recognized by the experts of the different overlapping fields. This circumstance may render the interpretation of the claim, and accordingly the interpretation of the scope of protection, subjective and unpredictable. Therefore, all nanotech patent applications should contain a self-standing "glossary" clearly explaining the meaning, for the purpose of the invention, of all technical terms used in the claims. This glossary should be intended not only to facilitate the examination of the application, but, with a view to the future, to enable the national courts to make reliable decisions on the question of patent infringement.

Conflicts of Interest: The author declares no conflict of interest.

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Subject Index

Glossary and Abbreviations

Acetic acid: a two-carbon, carboxylic acid, colorless and with a pungent odor, which is the most essential component of vinegar.

Acetyl Glucosamine: molecule composed of glucose with a chain of acetyl groups linked to an amino group.

Advanced medications: non-woven tissues or specialized tools for medical purposes, produced by innovative molecules and methodologies.

Agglomerate: collection of weak, la-bound particles or an aggregated mixture of the two in which the resulting external surface area is similar to the sum of the surface areas of the individual components.

Aggregate: particle comprising strongly bonded or fused particles in which the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components.

Alginates: water-solubile and anionic, linear hetero-polysaccharide composed of two different monomers (beta-D-mannuronic acid and alpha-L-glucuronic acid, (1--4)-beta-linked).

Allograft: transplantation of cells, tissues, or organs from a nonidentical donor of the same species into a recipient.

Angstrom: one-tenth of a nanometer.

Antimicribial agent: substance that inhibits the growth of microorganisms such as bacteria, fungi, or protozoans.

Antioxidant: molecule capable of inhibiting the oxidation of other molecules.

Autograft: transplantation of cells, tissues, or organs from one part of the body to another in the same individual.

Allotrope: forms of an element with different arrangements of atoms.

Amylase: enzyme that is present in human saliva for beginning the chemicals process of digestion and catalyzing the breakdown of starch into sugars.

Angiogenesis: growing new blood vessels by sprouting existing ones.

Anti-inflammatory: activity of a specialized network of compounds that slow downs and regulates the inflammatory cascade of cytokines.

Antimicrobial: activity of specialized compounds to modulate the growth of pathogens and microorganisms.

Antioxidant activity: protective activity performed by ingredients such as A, E, and C vitamins and enzymes to protect the skin and human body from environmental aggressions.

Anthocyanin: large group of red-blue plant pigments(flavonoids) with antioxidant properties, occurring mostly in flowers and fruits.

Apoptosis: also known as programmed cell death, is the process that deletes cells from a population in a deliberate manner without eliciting an immune response.

Arginine: polar, semi-essential amino acid involved in many metabolic processes. It is one of the constituents of NMF, natural moisturizing factor of the skin.

Artificial polymer: polymer man-made by synthesis.

Attract: move closer together, opposite charges (positive and negative) and opposite magnetic poles(north and south) are attracted to each other.

Bacterium: unicellular prokaryote microorganism, classified as a lower form of life.

Bioavailability: the presence of active molecules or products where they are needed in the body to be most effective.

Bio-based: material derived from plant, living matter, or renewable biological resources.

Biodegradable plastic: plastic compound that undergoes biodegradation via naturally-occurring microorganisms, such as bacteria, fungi, and algae.

Biodegradation: chemical dissolution of materials by bacteria or other biological means.

Bio-economy: economy based on the use of agricultural and industrial by-products with low consumption of water and energy, which safeguards social necessities.

Bioethylene: obtained from plant biomass, via bio-ethanol, represents a chemically identical alternative of ethylene from petrochemical feedstocks. Its synthesis reduces the production of greenhouse gas emissions (GHG), retaining a biodegradable compound.

Biomass: natural source of raw materials remaining from the agricultural and industrial processes used in the production of bio chemicals regarded nowadays as an alternative to petrochemical technologies.

Bio-mineralization: the process by which living organisms produce minerals to harden or stiffen existing tissues.

BIOBOARD: EU project that deals with the development of sustainable, protein-based paper and paperboard coating systems to increase the recyclability of food and beverage packaging materials.

Biocompatibility: properties of materials that, being biologically compatible, do not elicit any local or systemic responses from a living system.

Bio-composite: biopolymers or synthetic polymers reinforced with natural fibers.

Biodegradability: the ability of organic substances and materials to be broken down in carbon dioxide, methane, and water through the enzymatic action of microorganisms into a specific period of time and environment.

Biodegradable: material capable of disintegration by biological means.

Biodegradable compostable packaging: specific type of biodegradable and compostable package that may be intended for the composting of organic waste. The resulting compounds may be used as fertilizer.

Biodegradable (non**-**compostable**) packaging:** packaging that can be degraded in the presence of microorganisms, releasing CO2, water and methane into the atmosphere under certain time, heat, humidity, and oxygen conditions.

Biodiversity: represents the variety of different types of life found on earth, measuring all the organisms found in its ecosystems.

Bioinformatics: interdisciplinary field that addresses biological problems through the use of computational techniques.

Biological environment: complex of physical, chemical, and biotic factors in which life forms can exist, positively acting upon an organism or an ecological community.

Biomass waste: is represented by all the by-products that remain in the environment after agricultural and industrial production.

Biomaterials: defined as a material or combination of materials, synthetic or natural in origin, that can be used to flair, replace, or model tissues and organs *in vitro* and *in vivo.*

Biomaterials science: multidisciplinary field involving materials, mechanical, and biomedical engineering; molecular cell biology clinical medicine; histology; bioethics; and all the disciplines connected with the life.

Biomimetic production: green methodology that, looking to the productive methods adopted from animals and plants, makes ingredients and products with the use of enzymes without using chemicals.

Biomimetic product: product made by green methodologies with low consumption of water and energy, and use of waste products as raw materials.

BIO-MIMETIC: EU project based on the use of lignin and chitin nanofibrils, obtained from biomass, to produce innovative household products and cosmetics through the use of enzymatic technologies with low consumption of water and energy.

Biomimicry: imitation of some systems in nature used to solve human problems.

Bionanomolecules: naturally occurring building blocks, recognition modulae, and machines, such as ribosomes.

Bionanotechnology: use of biological principles for nanotechnological applications; an overlap between biology and nanotechnology with the prevalent use of biomolecules of natural origin obtained from agricultural and industrial waste, as an alternative to chemicals that are petrol-derived.

Bioplastic: whole family of materials that are bio-based, biodegradable, or both, derived from renewable biomass sources, such a fishery's, plant waste, or microbiota.

Biopolyester: as *cutin*, this is a compound composed of hydroxy and epoxy acids, functioning as a barrier between the aerial parts of higher plants and their environment.

Biopolyethylene: polymer made through the use of renewable feedstocks that emits less greenhouse bases when compared to fossil-based polyethylene. Its production helps reduce greenhouse gas emissions, but the polymer is not biodegradable like polyethylene.

Bioprocess: any process that uses complete living cells or their components to obtain desired products.

Biorefinery: facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals.

Biosynthesis: production of chemicals that are enzyme-catalyzed by living organisms.

Biotechnology: any technological processes and applications that use biological systems or living organisms to make products.

Blow Molding Process: most advanced method for processing plastic bottles. Transparent bottles are made of PET, while opaque ones are made of HDPE or PL.

Bottom-up: process based on the formation of complex structures through the coordinated assembly of simple building blocks.

Bulk material: material ordered, stored, issued, and sold by weight.

Burn: severe skin damage, caused by heat, electricity, chemical, or irradiation, where many of the cells affected die. It is categorized by severity as first, second, and third degree, when the skin turns white and loses sensation.

Carbohydrates: polymers of simple sugar molecules that are bonded together.

Carrageenan: family of high molecular weight-sulfated polysaccharides obtained by extraction from some categories of the algae class.

Casting Technology: low- or high-pressure process that allows one to develop, under controlled condition, thin films by using natural/man-made fibers or other special materials.

CBC: productive process characterized by three main bio transformations of lignocellulosic materials: production of saccharolytic enzymes, hydrolysis of cellulose and hemicellulose to sugar, and fermentation of next se and pentose sugars.

Cell cycle: series of events that takes place in a cell, leading to its division and duplication.

Cell culture: process in which cells grow under controlled conditions, outside their natural environment.

Cell differentiation: process in which a precursor cell acquires its final specialized character.

Cell membrane: biological membrane that separates the interior of all cells from the outside environment.

Cell signaling: process in which cells communicate with each other or are stimulated or inhibited by extracellular signals.

Cell viability: method to control the state of cells, to determine their possibility of living or dying , after a specific treatment made *in vitro* by cellular cultures. Cell viability tests might calculate the effectiveness and safeness of many active ingredients.

Cellular uptake: cellular mechanisms regulating the endosomial trafficking of materials into the cells to determine their delivery effectiveness.

Cellulase: enzyme that catalyzes the hydrolysis of the glucose 1,4 bond to form polysaccharides.

Cellulose: structural linear polysaccharide consisting of repeating cellobiose units made by two glucose units connected via befa-1,4 glucosidic bond. This natural polymer provides tensile strength in the cell walls of plants.

Catabolism: set of pathways that break down molecules into smaller units, releasing energy.

Catechin: photochemical compound with antioxidant property found in high concentration in green tea and other vegetables, such as grapes, cocoa, etc.

Chemical structure: molecular geometry reporting the arrangement of the chemical bonds of the chemical compound.

Chemicals: terminology used to indicate chemical products man-made by petrochemical processes.

Chemoattractant: chemical substance that induces a cell or organisms to migrate towards it.

Chitinase: digestive enzyme that breaks down glycosidic bonds in chitin.

Chitosan: partially de-acetylated and water-soluble form of chitin.

Chitin: a natural polymer obtained from crustacean waste, formed by many molecules of glucosamine and acetyl glucosamine linked by covalent bonds.

Chitin dressing: non-woven tissue made prevalently by chitin micro/nano fibers.

Chitin nanofibrils: pure crystal-like fibrils of chitin having a mean dimension of 240x7x5 nm obtained from a patented methodology.

Cicatrization: physiological process of wound healing producing a scar tissue.

CN: Chitin Nanofibrils. The purest Crystallin portion of the raw material chitin.

CNC: Crystallin cellulose nanofibers. It is the purest part of the cellulose polymer.

Co-extrusion: process that involves the extrusion of two or more materials through a single fiber due to plastic fiber production.

Collagen: protein rich in glycine and proline that represents the major component of the ECM of animals (about 30% of all the body proteins) and confers tensile strength to the skin tissue.

Collagenase: enzyme that breaks collagen peptide bonds, often destroying their structures.

Colloidal quantum dots: semiconductor nanocrystals between ~1 and 10 nm, which are transitioning between bulk solids and molecules. Their different emission colors are size-dependent.

Competitiveness: ability and performance of a company to supply goods and services in a given market, in relation to and in competition with other companies.

Composite: building material consisting of two or more constituent materials with different physicochemical characteristics that are different from the individual components used.

Compost: result of disintegration and aerobic biodegradation of a product that can be used as a fertilizer.

Compostability: capacity of an organic material to be transformed into compost through the composting process, following the EU directive 94/62/EC on Packaging & Packaging waste.

Compostable: material that meets D6400 composting standards set by American Society of Testing and Materials (ASTM). It has to (1) break down to carbon dioxide, water, and inorganic compounds and biomass at a rate similar to paper; (2) disintegrate into small pieces within 90 days; and (3) leave no toxic residue.

Compostable polymer: plastic material that is capable of undergoing biological decomposition in a compost site as part of an available program. However, biodegradation and disintegration rate are the points that constitute the difference between biodegradable and compostable.

Copolymer: chemical compound of high molecular weight produced by polymerization of two or more monomers together.

Cosmeceuticals: terminology used to try to define cosmetic products made and controlled by more strict pharmaceutical methodologies.

Cosmesis: preservation or restoring of body beauty.

Cosmetic regulation: rules under which cosmetics are produced, claimed, and sold in different countries.

Covalent bond: chemical bond characterized by the sharing of one or more electrons between two atoms.

Cross section: shape obtained cutting transversally an object.

Crustacean waste: represents ~45% of the discarded by-material from the crustaceans' food. It is made prevalently by chitin embedded in calcium and magnesium carbonate.

Cyclodextrines: crystalline, non-hygroscopic, and cyclic oligosaccharides derived from starch, used as solubilizing and stabilizing agents.

Cytocompatibility: or cytological compatibility refers to the cytological effects produced by the temporary interaction between cells and the material of various polymers and surface topographies.

Cytokines: large group of proteins, peptides, or glycoproteins secreted by specific cells as signaling molecules to mediate and regulate the immune system, the inflammation cascade, and hemopoiesis.

Cytology: medical and scientific study of cells.

Cytotoxicity: degree to which an agent has specific toxic effects on the cells.

Kraft process: it consists of a chemical pretreatment of wood at 155/175 C by the use of a solution of Na2S/NaOH that allows the delignification of the raw material.

Dalton: unit used for indicating mass on an atomic or molecular scale. One Dalton is approximately equal to the mass of one proton or one neutron.

Defensin: family of antimicrobial peptides found in animals and plants capable of killing or inactivating Gram-positive and Gram-negative bacteria, fungi (including yeasts), parasites, and even enveloped fit uses such as HIV.

Degradable: general term used to describe all polymeric materials that disintegrate through a range of physical and chemical processes.

Detergent: surfactant or mixture of surfactants that have *cleaning properties in dilute solutions.* According to the ingredients used, the products refer to laundry or dish detergents or human cleaning agents.

Disaccharide: carbohydrate formed when two monosaccharides undergo a condensation reaction.

Disintegration: breakdown of a material into small and separate fragmentation.

Drug delivery: method or process of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals.

EC: European Economic Commission.

ECM: Extra cellular matrix. The gel composition of cellular components comprising the Hyaluronan-water network containing collagen and elastic fibers.

Ecological economics: economy embedded and supported by natural systems so that goods and processes have to be made and organized by low consumption of water and energy and by the use of agricultural and industrial waste as raw materials.

Ecosystem: systems of plants and animals that, living all in a perfect equilibrium in our planet, permit the maintenance of its biodiversity.

EDTA: ethylene-diamine tetra-acetic acid.

Effectiveness: capacity of the ingredient/product to show its efficacy.

Elagitannins: any group of tannins, related to ellagic acid, occurring in various tanning extracts.

Elastic fibers: bundles of proteins found in an extracellular matrix of connective tissue, characterized by great elasticity.

Elastin: small tropoelastic polymers used to form composite materials.

Electromagnet: metal core made into a magnet by passing a current through a wire wrapped around it.

Electron: negatively charged subatomic particle that moves around the nucleus of an atom; it acts as a carrier of electricity.

Electrospinning: technology that uses electrical charges to draw micro/nano fibers from a liquid. Using this method, it is possible to create nanofibrous scaffolds for tissue engineering.

Electrospun: non-woven tissue resulting from the electrospinning of fibers.

ELISA: enzymatic biochemical technique used for diagnostic tools, mainly in immunology, to detect the presence of an antibody or an antigen in a sample.

Environment: air, water, plant and animals that live around us.

Environmentally-friendly: ingredient/product that does not produce noxious waste.

Enzymes: macromolecules able to accelerate or catalyze bio-chemical reactions.
Epidermis: skin outer covering, composed of keratinocyte cells, that creates a waterproof barrier that is self-repairing and continually renewed.
Epigalochatechin: major Cathechin content with an antioxidant and cancer preventive capacity, accounting for 59% of the total catechins in green tea.
EPO: European Patent Office.
Epidermal growth factor (EGF): mediator implicated in keratinocyte migration, fibroblast proliferation and differentiation, and granulation tissue formation.
EU: European Union. It represents the Union of 27 different countries.
EuroPaBio: European Association for Bioindustries.
European Patent Office: EU office for applying and searching issues, grants, rules, and methodology for patents.
EVA: poly(ethylene-co-vinyl acetate). Polymer used to produce bi-layer sheets to enhance the barrier properties of food packaging.
Extrusion coating/lamination: coating of a molten web of synthetic resin onto a substrate material to form thin hot films.
Dermis: relatively thick layer of connective tissue beneath the epidermis that is composed of an Extracellular Matrix (ECM) gel rich of hyaluronic acid and water, into which collagen and elastic fibers are embedded.
FABS: Food, Agriculture, Fisheries, and Biotechnology. A flagship initiative for EU-China Cooperation on Research and Innovation on Food, Agriculture, and Biotechnologies.
FDA: Food and Drug Administration. The Health Ministry of the United States of America.
Feed stock: any bulk raw material or fuel required for an industrial process.
Fiber: structure of polymer that closely can mimic the extracellular matrix and may be used for tissue engineering and other medical applications, particularly when in nanoscale and of natural origin.
Fiber processing: methodology to produce natural or man-made fibers.
Fiber strength: any bulk raw material or fuel required for an industrial process.
Fiber structure: architectural organization of a fiber.
Fibroblast: cells that, when present in the dermis, synthesize the ECM framework.
Filler: reinforcing material for fiber nanocomposites, employed to improve the desired properties or simply reduce the cost of a nanocomposite.
Financial capital: plays an important role in our economy by reflecting the productive power of the other types of capital.
Flat die extrusion: common process and arrangement for cast film, extrusion coating, and extrusion laminating.
Flavonoids: large family of polyphenols with a common chemical structure and antioxidant properties, synthesized by plants.
Flexible packaging: package or container made of flexible or easily yielding materials that, when filled or closed, can be readily changed in shape.
Food packaging: package to provide protection, tampering resistance, and special physical, chemical, or biological needs. It plays a fundamental role in the maintenance of food benefits prior to consumption.
Force: push or pull that tends to charge the motion of an object.

FP7: Seventh Framework Program. The EU program of research finished at the end of the year 2013.

FTIR: Fourier Transform Spectroscopy analysis. It offers quantitative and qualitative measurement S for organic and inorganic samples, identifying chemical bonds and functional groups of molecules.

Furfural: colorless, oily liquid with an aromatic odor, obtained from cellulosic waste.

Gallotannins: class of hydrolisable molecules obtained by the condensation of the carboxylic group of Gallic acid. It acts as a cytoprotective agent in oxidatively stressed cells.

GDP: Gross domestic product. It represents all manufacturing products machines and services made by a country.

Gene Regulatory Network (GRN): combination of different regulators that communicate with each other and with other particles with and in the cell that control the level of gene expression in mRNA and in proteins.

Gene therapy: use of a gene to obtain a therapeutic, prophylactic, or diagnostic effect.

GHGs: greenhouse gas emissions. It is the gas that traps heat in the atmosphere.

Glycine: the simplest non-polar amino acid of human body. It can fit into hydrophilic or hydrophobic environments because of its minimal chain compose of only one hydrogen bond.

Glycan: generic term for any sugar, in free form or attached to another molecule, that is used interchangeably with carbohydrate.

Glucosamine: natural occurring amino sugar compound found in the human body, especially in the fluid around joints, where it appears as acetyl glucosamine.

Glycolic acid: smaller alpha hydroxy acid found in some sugar-crops, used in cosmetic dermatology as peeling and skin moisturizing agent.

Glycobiology: study of the structure, function, and biology of carbohydrates.

Glicomics: systemic study of all glycan structures in a biological system.

Glycopeptide: peptide having one or more covalently attached glycans.

Glycoproteins: protein with one or more covalently attached glycans.

Glycosaminoglycans: polysaccharide side chains of proteoglycans of free complex polysaccharides composed of linear disaccaride repeating units, each composed of a hexosamine and a hexose or hexuronic acid.

Glycosilation: enzyme-catalyzed covalent attachment of a carbohydrate to a polypeptid, lipid, polynucleotide, or another carbohydrate.

Gram-negative bacteria: bacteria, such as gonococci and meningococci, that cannot retain the violet stain after the decolonization step. They are especially found in the gastro-intestinal tract, are responsible for many diseases, and are resistant to many antibiotics.

Gram-positive bacteria: bacteria, such as staphylococci and streptococci, produce a positive result in the gram stain test (crystal violet), appearing purple-colored under a microscope.

Graphene: man-made material representing a new class of nanoparticles, known as nanotubes. Made by an atomic structure of carbonic atoms, nanotubes are about 200 times stronger than steel by weight.

Gravity: force of attraction between all objects; the amount of attraction depends in part on the mass of the objects and the distance between the objects.

Green economy: economical way of producing goods based on the low consumption of energy and water through the use of agricultural and industrial by-products as raw materials, while respecting the environment and society.

Green washing: *green sheen*, in which green marketing is deceptively used to promote the perception that an organization's products, aims, or policies are environmentally-friendly.

Guar gum: hydrocolloidal galactomannan structurally comprising long and straight chains of (1--4)-alpha-D-mannopyranosyl units linked together by (1--4)-beta-D-galactopyranosil units by (1--6) linkages.

HDPE: high density poly(ethylene).

Hexosamine: hexose with an amino group in place of the hydroxyl group at the C-2 position. Common examples are the N-acetylated sugars, such as N-acetyl glucosamine.

Hexose: six-carbon monosaccharide topically with an aldehyde at the C-1 position and hydroxyl groups at the all other positions.

Homogenization: methodology to reduce the size, for example, of an emulsion by reducing the size of its particles, distributing them equally throughout the suspension to render it uniform.

HPCS: hydroxy propyl chitosan. Chitosan enriched by propulsion chains.

Human capital: people's health, skills, and motivation, which are required for productive work.

Human physiology: study of how normal human tissues and organs function mechanically, physically, and in terms of biochemical functions.

Hyaluronan: mammals' natural polymer composed of repetitive molecules of glucuronic acid and acetyl glucosamine, which are capable of attracting and bonding hundred molecules of water.

Hydrogels: when derived from natural polymers, they are used biologically in stem cell engineering applications, as they are components resembling the ECM structure or exhibit properties similar to the matrix components in native tissues.

Hydrolisis: chemical process involving the breaking of a bond in a molecule using water.

Hydrolitic enzymes: complex of catalytic proteins that uses water to break down substrates in their simplest units.

Hydrophilic polymers: substrates containing polar or charged functional groups, which dissolve or become swollen in water.

Hydrophobic polymers: substrates which, in water, often cluster together to form micelles. Hydrophobic is often interchanged with lipophilic, i.e., ***fat-loving.***

Hydroxyapatite: naturally occurring mineral form of calcium phosphate and an essential component of normal bone and teeth.

IgE: Immune globulin E. An immune antibody protein that is produced by the immune system and acts as an antibody to activate allergic reactions.

IL-1 (interleukin-1): cytokine with pro-inflammatory activity.

IL-8 (interleukin-8): cytokine with pro-inflammatory activity.

IL-10 (interleukin-10): cytokine with antiinflammatory and immunosuppressive activity.

IL-12 (interleukin-12): involved in the differentiation of T cells.

IL-5 (interleukin-5): a T cell-derived glycoprotein that stimulates eosinophil production and activation.

IL-6 (interleukin-6): both pro-inflammatory and anti-inflammatory cytokine.

Imunostimulants: substances that stimulate the immune system by inducing activation or increasing activity of immune cells.

Interleukins (IL): small signaling proteins produced by a variety of cell types that participate in the body's defense system regulating many aspects of inflammation and immune and autoimmune responses.

Injection molding: manufacturing process for producing gods or parts of goods by injecting material into a mold.

Inventiveness: ability to think and/or realize new ideas and methods.

Ionic strength: measure for quantifying the concentration of ions or dissolved chemicals in a solution.

Iridescence: lustrous color caused by differential refraction of light waves, which change with the angle of view.

ISO: International Standardization Organization.

Keloid: irregular and shaped scar of fibrous tissue formed over the wound during the repairing process.

Keratinocyte: cell with the capacity to synthesize the keratin filament proteins, which give the epidermis its toughness. They are embedded in the skin and covered by lipid lamellae.

Kinetic: movement energy, i.e., the energy that possess an object because of its motion.

Kraft process: process for convention of wood into wood pulp, consisting of cellulose fibers. It consists of the treatment of wood chips with a mixture of sodium hydroxide and sodium sulfite that breaks the bonds that link lignin to the cellulose.

Lectin: protein that specifically recognizes and binds to Glycans without catalyzing a modification of the glycan.

Levulinic acid: organic compound classified as a ketoacid and produced from plant biomass, has pharmaceutical, food, cosmetic, and agricultural applications.

Lightness: perceived quality of color by which the object appears to reflect or transit the incident light.

Lignins: one of the main classes of materials that , with a structure rich in polyphenols, provides structural support for the plant, giving strength, rigidity, and resistance to environmental stresses.

Lignin derivatives: unlike lignins, they may be used in the pharmaceutical and cosmetic sectors because of their water solubility.

Lignosulfonates: compounds extracted from waste liquid of plant biomass at the end of sulfite-pulping process.

Lignocellulosic Feedstocks: inedible plant material known as biomass with polymeric structure composed principally of natural biopolymers built up by three main constituents: cellulose, hemicellulose, and lignin.

Lipectomy: surgical excision of subcutaneous fatty tissue.

Liquefaction: process by which a gas is concentrated to a liquid.

Liquid nitrogen: nitrogen in a liquid state at an extremely low temperature. Zit is produced industrially by fractional distillation of liquid air.

Lyophilization: or freeze-drying procedure: dehydration process used to preserve a perishable material.

Lysozyme: enzyme that, as part of the innate immune system, is characterized by the ability to break down the bacteria cell wall.

Lotus effect: occurring in the lotus plant, water is repelled by the surface of the leaves, rolling off and collecting dirt in the process.

Macro- : large scale.

Macromolecules: very large molecules, with a diameter ranging from ~100 to ~10,000 angstroms, commonly created by polymerization of smaller subunits (monomers).

Macrophage: specialized defensive cells that phagocyte the investing pathogens and help activate lymphocytes to mediate the sophisticated adaptive immune system responses.

Manufactured capital: comprises material goods or fixed assets, such as tools, machines, buildings, and other forms of infrastructure that contribute to the production process but do not become embodied in its output.

Matrix: composition of one or more polymers to be reinforced by the use of inorganic or organic materials called fillers.

MEM medium: Minimal Essential Media is one of the most commonly used cell culture media for *in vitro* studies.

MEMS: microelectromechanical systems.

Metrology: science of measurement that includes all the theorical and practical aspects of measurement.

Micro-: prefix meaning one-millionth (0.000.001).

Micron: one-millionth of a meter.

Microarray: also known as a biochip. Collection of microscopic DNA spots attached to a solid surface.

Microbe: microscopic living organism, such as bacterium, fungus, protozoan, or virus.

Microbiome: all of the genetic material of a microbial community sequenced together.

Microcapsule: mall sphere with a uniform wall around it.

Microspheres: spherical micro particles with diameters in micrometers ranging from 1 to 1000 millimicrons.

Mitogen: chemical substance that stimulates cell division.

Monosaccharide: basic unit of the carbohydrates.

Montmorillonite : very soft phyllosilicate group of minerals that forms a clay swelling in water, possessing a high cation-exchange capacity.

Nano-: prefix meaning one-billionth (0.000 000 001).

Nanobiomaterial: any material, surface, or construct that interacts with biological systems, having at least one dimension between 1 and 100 nm in size.

Nanobiotechnology: application of nanotechnological principles and tools to biology.

Nanocellulose: highly crystalline cellulose nanofibers, present in natural plant bodies that are useful as reinforcing agent in nanocomposites.

Nanochitin: known also as chitin whisker, chitin nanofibril, or chitin crystallite.

Nanocomposites: polymer-matrix composites in which nano-sized inorganic or organic fillers are distributed homogeneously, to give them more mechanical, thermal, and chemical resistance.

Nanocrystal: any nanometer-sized crystal.

Nanofibre: nano-object, flexible or rigid, with similar external dimensions in the nanoscale, whose third dimension is significantly larger.

Nanomaterials: material with at least one external dimension of approximately 1–100 nanometers.
Nanomedicine: application of nanotechnology in medicine.
Nanometer (mm): unit of length in the metric system, equal to one billionth of a meter.
Nani-object: material with one, two, or three external dimensions in the nanoscale.
Nanoparticle: particle of any shape with all three external dimensions between 1nm and 100 nm.
Nanoplate: Nano-object with one external dimension in the nanoscale, whose two other external dimensions are significantly larger.
Nanorod: solid nanofiber.
Nanoscale: size range from approximately 1 nm to 100 nm.
Nanoscience: study of particles on an atomic or molecular scale, whose size is measured in nanometers.
Nanotechnology: application of scientific knowledge for processing, separation, consolidation, and deformation of materials by one atom or by one molecule**.**
Nanotube: miniaturized cylindrical carbon-atoms structure measuring nanometers. It has composed of hexagonal graphite molecules attached at the edges.
Nanowire: electrically conducting or semi-conducting nanofiber.
Natural capital: any stock or flow of energy and matter that yields valuable goods and services.
Natural fibers: all the fibers found in nature.
Natural polymer: all polymers of natural origin.
n-CHITOPACK: EU research project to produce biodegradable and compostable food packaging.
Neuroprotective activity: the ability to exert protective activity against cytotoxic agents.
NIR: (near-infrared) simple instrument useful for direct measurements without the necessity to prepare samples.
nm: nanometer. 1 nanometer is a billionth of a meter.
NMR: powerful technique that can be used for qualitative and quantitative measurements of chemicals.
Non-woven tissue: tissue made by fibers organized randomly.
Nutricosmetics: cosmetic products made by using raw material utilized as food or food components.
OECD: Organization for Economic Co-operation and Development. An international organization helping governments tackle the economic and social governance challenges of a globalized world.
Oligomer: molecule consisting of few monomer units in contrast to a polymer that consists of an unlimited number of monomers.
Oligosaccharides: important class of polymeric carbohydrates found in all living entities.
Organosolv process: the treatment of biomass to extract lignin by using solvents/water at 180–200 C and a working pressure between 2 and 5 bars.
Osteocalcin: non-collagenous, small protein, secreted by fibroblast, found in bone and dentin.

Osteopontin: multifunctional protein molecule, part of the mineralized Matrix. It is considered a bridge between cells and minerals and as a key cytokine regulating tissue repair.

Particle: minute piece of matter with defined physical boundaries.

Packaging: wrapping material around a consumer item that serves to contain, identify, describe, display, promote, and otherwise make the product marketable and keep it clean.

Patent novelty: based on the novelty requirements necessary to protect an original invention by a Patent.

Patent protection: protection by the law of the intellectual property connected with any original invention.

PBAT: poly butyrate adipate terephthalate. A biodegradable copolymer used for plastic bags and wraps, as an alternative to polyethylene.

PBLG: poly(benzyl-L-glutamate), a biodegradable and biocompatible polymer utilized in fields of drug delivery systems, tissue engineering, and biomedical materials for safety and effectiveness.

PBS: polybutylene succinate. A thermoplastic biodegradable polymer resin of the polyester family, produced from a petrochemical-base such as nice vid and 1,4 butanediol.

PCL: Polycaprolactone. A natural nanocomposite used to replace traditional polymers for packaging applications.

PE: polyethylene.

Pectin: a structural colloidal heteropolysaccharide contained in the primary cell walls of terrestrian plants.

Peptide: short polymer composed of two or more amino acids.

PGA: poly**(**Glycolic acid**).** Common polymer used as a scaffold biomaterial.

PEG: polyethylen-glycol. It may be considered a water-soluble carrier acting as humectant, lubricant, binder, and coupling agent, depending upon its concentration and grade of polymerizations.

PEOX: polyethylene oxide. A water-soluble sinthetic polyester available in a range of molecular weights, used as carrier or component in biomedical fields.

Permeability: property or state of being permeable, i.e., how easily liquid and gas pass through something.

PET: polyethylenteraphtate. A colorless, thermoplastic, crystal-like resin used to make Food packagings.

PHA: polyhydroxy alkanoates. Biodegradable, thermoplastic polymers used for packaging productions.

PHB: semicrystalline biodegradable polymer with properties similar to polypropylene.

Pholyphenol: class of organic chemicals characterized by the presence in their molecules of large multiples of phenol structural units.

Photolithography: process that involves manipulating material through the use of light and processing geometric shapes on a mask on the surface of a silicon wafer.

Protonic crystals (PhCs): periodic optical structures that confine or control the emission and propagation of light.

PLA: poly (lactic acid) is a biodegradable and biocompatible thermoplastic polymer obtained from plant biomass.

PLGA: poly(DL-lactic-co-glycolic acid). A biocompatible and biodegradable polymer exhibiting interesting properties, used to fabricate devices for drug delivery and tissue engineering applications.

Polyacrilic acid: synthetic high molecular-weight polymers of acrylic acid repeated units, frequently used as emulsifying, stabilizing, and controlling releases for pharmaceutical and cosmetic active agents.

Polylactil esters: non-degradable polymers, transparent and elastic, that are resistant to breakage. They have a broad range of properties that make them suitable for supporting various cell cultures as the Extra Cellular Matrix (ECM).

Polyethylene: made from polymerization of ethylene. The most thermoplastic commodity used in consumer products. It possesses good elasticity and resistance to stress but it is not biodegradable. It is also a gelling agent for anhydrous hydrocarbon oils and fatty esters.

Poly(butylene adipate-co-terephtalate): biodegadable, thermoplastic polymer with excellent balanced properties useful for improving both UV and heat resistance.

Poly(butylene succinate): thermoplastic**,** polymer resin of the polyester family useful for Eco-Smart Food packaging.

Poly(ethylene terephthalate): thermoplastic polyester formed from ethylene glycol through a direct esterification process. It possesses Bret electrical resistance and low moisture adsorption.

Poly(gamma- benzyl l-glutamate): amphiphilic biodegradable cationic bioplymer.

Poly(lactic acid): see PLA.

Polyamide: high molecular weight polymer in which amide bounds occur among many molecules of amine. These bounds may be either natural or synthetic.

Polycaprolactone: natural nanocomposite used to replace traditional polymers in packaging applications.

Polycyanoacrylate: potential lysosomotropic, non-toxic carrier for producing materials to close wounds.

Polyester: generic name for a manufactured fiber in which the fiber-forming substance is any long chain synthetic polymer.

Polyethylene glicol: polyglycol composed of several molecules of condensate ethylen glicol.

Polymer: chemical compound made of small molecules arranged in a single repeating structure for a larger molecule.

Polymer composite: combination of a polymer and synthetic or natural inorganic filler. Fillers are employed to improve the desired properties or simply reduce the cost.

Polymerization process: any process in which relatively small molecules, called monomers, combine chemically to produce a very large, chainlike network.

Polyolefin: any class of thermoplastic polymers produced from a single olefin.

Polypropylene: thermoplastic polymer for producing films for packaging, textile, and plastic items for medical and laboratory use.

Polysaccharide: combination of 9 or more monosaccharides, linked together by glucosidic bonds.

Polystyrene: thermoplastic, synthetic resin of polymerization styrene of variable molecular weight.

Polyurethanes: class of thermoplastic polymeric materials with a wide range of properties that make them a good candidate as functional material for applications in biomedical science. They are produced by the condensation reaction of a polyisocianate and an hydroxyl-containing material, such as lignin.

POSS: polyhedral oligomeric sesquioxane. A novel chitosan nanocomposite.

Poyhydroxyalcanoate: biodegradable linear polyesters produced in nature by bacterial fermentation of sugar or lipids.

PP: polypropylene. Polymer of propylene.

PPG: polypropylene glycol.

Pro-anthocyanidin: class of polyphenols found in nature in a variety of plants, also known as condensed tannins, with very powerful antioxidant properties.

Product innovation: creation and subsequent introduction of a good or service that is either new or an improved version of a previous good or service.

Productive process: system of defined chemical or mechanical steps to create an object involving the use of raw materials, machinery, and power.

Proteins: essential components of organisms, participating in every process within cells together polysaccharides and nucleic acids.

Proteoglycan: any protein with one or more covalently attached glycosaminoglycan chains.

Proteomic: large-scale study of proteins to control their structures and functions.

PS: polystyrene

Pyrolysis: thermochemical decomposition of organic materials at elevated temperatures in the absence of oxygen.

PVP: poly(vinyl pyrrolidone), film-forming, hair fixative resin; a dispersant and adhesion promoter.

Quantum: smallest amount of physical quantity of energy that can exist independently.

Quantum dot: crystalline nanoparticle that exhibits size-dependent properties due to quantum confinement effects on the electronic states.

Raw material: any inorganic or organic material, natural or man-made, used to produce goods or energy.

REACH: actual EU Regulation on registration, evaluation, authorization, and restrictions of all the chemicals produced or imported.

Recyclability: ability of a material to be captured and separated from a waste stream for conversion or reuse.

Regenerative Medicine: study and realization of tools, non-woven tissues, and special bio-materials capable of helping natural healing processes to speed up the regrowth of damaged tissues.

Release: active ingredient in a single short to obtain an effect over a defined timespan. It is necessary to measure, for example, the effectiveness of a drug.

Renewable material: resources that have the potential to be replaced over time on a daily basis using agricultural and industrial by-products.

Resorption: process of losing substance or tissue to prove its destruction, disappearance, or dissolution by a biochemical activity.

Safeness: property of a product that defines it as safe.

Salicylic acid: ortho-hydroxybenzoic acid. It is a white powder of acrid taste and discolored by light, used in dermatology as a peeling agent.

Scaffold: 3D structure made of synthetic or natural biocompatible materials that, degraded over time, leaves only the integrated tissue in its place.

Scaffold protein: assembles the cell signaling-cascade, localizing and enhancing its efficacy.

Scar hypertrophic: cutaneous condition characterized by the deposit of an excessive amount of collagen, which produces a raised scar, which is different from a keloid scar.

Sebum: oily, lipid-containing substance secreted by the sebaceous glands of the skin.

Self-assembly: arrangement of molecules without outside guidance.

SEM: Scanning Electron Microscopy.

SHF process: Separate Hydrolysis Fermentation. A two-step process of the biomass. In the first step the lignocellulose is hydrolyzed to sugars using enzymes, while in the second step, the sugars are fermented to ethanol with the help of yeasts.

Skin-friendly: terminology used to define a product that is effective on skin and does not show negative side-effects.

Silver nanoparticles: spherical nanoparticles of silver between 1nm and 100nm.

Slurry: thin mixture of insoluble substances with a liquid, as water or oil.

Smart textile: textile possessing innovative and interesting properties.

Social capital: comprises all the different cooperative systems and organizational frameworks that people use to live and work together.

Solvolysis: chemical reaction in which the solvent, such as water or alcohol, is one of the reagents and is present in great excess so that it is required for the reaction.

Somatic cell: any cell that makes up an organism, except for a reproductive cell.

Somatic-cell therapy: use of manipulated cells or tissues to obtain biological effects.

SSCF process: simultaneous saccharification and co-fermentation. It is a process carried out by genetically engineered microbes used for Xylose-rich lignocellulosic materials.

SSF process: process in which hydrolization and fermentation of sugars take place in the same vessel, unlike SHF.

Starch: polysaccharide from grain, relatively low-cost and highly degradable, used to produce hard and flexible plastic biomaterials.

Stem cells: undifferentiated, self-renewing cells that carry on dividing to generate a mixture of different cell progeny. They are required wherever there is a recurring need to replace differentiated cells that cannot divide themselves.

Strenght: tensile strength of a polymer that quantifies how much an elongating stress will endure before its failure.

Surface: outmost or uppermost layer or area of something.

Surface tension: surface film caused by the attraction of the liquid molecules to each other.

Sustainable economy: use of raw materials obtained from agricultural and industrial by-products by processes, possibly *green,* with low consumption of water and energy.

Swelling: a time-dependent volume increase of a polymer, caused by stress changes, an increase of water content, or a combination of both.

Synthetic polymer: polymer hand-made by a process of synthesis.

T-cells: or T-lymphocytes are a type of linphocyte that plays a central role in cell-mediated immunity.

Tannic acid: natural substance widely found in natgalls and other plant parts.

Tannins: class of lower molecular weight, water-soluble polyphenols present in many plant foods, commonly used in wine circles, as well as in medicinal applications.

Thermochemical conversion: application of heat and chemical processes in the production of energy products from biomass.

Thermoforming: polymer material fed in sheets with thicknesses in the range 50–300 microns for packaging production. The method consists of heating the material in its glass transition temperature, below its melting point, to obtain a softened sheet that under vacuum is transformed to the desired shape.

Thermosetting: known also as thermoset. It describes a prepolymer material that cures irreversibly by means of heat or a chemical reaction.

Thiocidolysis: last step of the lignin degradation process, catalyzed by peroxidase enzymes.

Tissue engineering: multidisciplinary field focused on the development and application of knowledge in chemistry, physics, engineering, and life and clinical sciences to the solution of critical medical problems.

Tissue-engineered: cells or tissues that have been modified to be used for repairing, regenerating, or replacing human tissue.

TNF-alpha: tumor necrosis factor. It is a non-glycosilated protein acting as a cell signal.

Tobramycin: aminoglycoside antibiotic, derived from *Streptomices tenebrarius,* used for different bacterial infections.

Top-down: process based on the miniaturization of a complex system through the sizing-down of its components.

Toxicology: studying or detecting the detrimental effects of a substance.

Tumor necrosis factor-beta (TGF-beta): key mediator for fibroblast migration and proliferation, granulation tissue formation, increased collagen synthesis, and neovascularization.

Ultrasound: energy input used during the thermochemical conversion of lignocellulosic biomass into liquefied depolymerized products.

um: millimicron. One-thousandth micron; 1 nanometer.

UNEP: United Nations Environment Program. The voice for the environment in the United Nations systems that delivers analysis and interpretation of data and information about biodiversity.

UV: ultraviolet rays. Electromagnetic radiation with a light wave length from 400 nm to 100 nm.

Viscoelasticity: property of materials that exhibit both viscous and elastic characteristics when undergoing deformation.

Viscosity: quality of being viscous. The coefficient of viscosity is the ratio of the tangential frictional force per unit area to the velocity gradient perpendicular to the direction of flow of a liquid.

Wavelength: distance between the high points of a wave.

Washing: act of person to apply water or some other liquid for the purpose of cleaning.

Waste: any material unused and rejected as worthless , unwanted, or unusable.

Wet spinning: manufacturing process for creating polymeric fibers through continuous electrospinning.

WHEYLAYER: EU project based on the use of natural protein to make biodegradable containers.

WHO: World Health Organization. A workforce of some 8,500 people representing more than 150 nationalities with the mission to provide global leadership in public health.