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**Sensori Elettronici Biodegradabili
con Polimeri Derivanti dalla
Riqualificazione di Rifiuti Organici**

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List of Acronyms

NMR - Nuclear Magnetic Resonance

FTIR - Fourier Transform Infrared Spectroscopy

TGA - Thermogravimetric Analysis

ChNCs - Chitin Nanocrystals

CsNCs - Surface-Deacetylated Chitin Nanocrystals

C₁₀₀ - Pure chitosan film

C₄₀G₄₀ChNCs₂₀ - Chitosan Film with 40 wt% Glycerol and 20 wt% Chitin Nanocrystals (ChNCs)

C₄₀G₄₀CsNCs₂₀ - Chitosan Film with 40 wt% Glycerol and 20 wt% Surface-Deacetylated Chitin Nanocrystals (CsNCs)

CsNCs₇₀G₃₀ - 70 wt% Surface-Deacetylated Chitin Nanocrystals (CsNCs) and 30 wt% Glycerol

C₅₀G₅₀ - Film 50 wt% chitosan and 50 wt% glycerol

d₃₃ - Piezoelectric Coefficient

PFM - Piezoresponse Force Microscopy

PZT - Lead Zirconate Titanate

BTO - Barium Titanate

PVDF - Polyvinylidene Fluoride

PHB -poly-hydroxybutyrate

DLS - Dynamic Light Scattering (DLS)

XRD - X-ray Diffraction

SEM - Scanning Electron Microscopy

AFM - Atomic Force Microscopy

DFRT - Dual Frequency Resonance Tracking

ROS - Reactive Oxygen Species

PVA - Polyvinyl Alcohol

MRSA - Methicillin-resistant *Staphylococcus aureus*

PCL - Poly(ϵ -caprolactone)

QCSP - Quaternized Chitosan-Graft-Polyaniline

DDS - Drug Delivery Systems

CVG - Chitosan-Vaseline Gauze

ES - Electrical Stimulation

TENGs - triboelectric nanogenerators

GFP - Green Fluorescent Protein

MHA - Hinton Agar

LB - Luria-Betrani

RFID - Radio Frequency Identification

NFC - Near Field Communication

UHF - Ultra High Frequency

PCB- Printed Circuit Boards

PPG - Photoplethysmogram

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Abstract

*This thesis investigates chitosan, a sustainable biopolymer derived from seafood waste, focusing on its conversion into thin films and their potential applications. Chitosan, extracted from crab waste (Maja squinado), was analyzed through comprehensive physicochemical characterization to understand its composition and properties, revealing its exceptional film-forming ability which we then leveraged to develop a scalable method through the use of an automatic applicator for producing chitosan films with precise control over thickness, ranging from ultra-thin layers below 10 μm to films exceeding 200 μm . By modifying the composition of the films, we were able to modulate their mechanical properties across a broad spectrum, achieving tensile strength exceeding 80 MPa and elongation above 60%. Optical transparency was observed in the visible range (400–800 nm), with a distinct UV absorption peak around 200 nm, making them suitable for optoelectronic applications such as UV shielding. Piezoelectric properties were characterized using Piezo Force Microscopy (PFM), with an accurate method implemented to isolate piezoelectric forces from electrostatic ones. By incorporating chitin nanocrystals, the films' crystallinity improved, leading to an increase in the d_{33} coefficient from 9 pm/V to 18.7 pm/V, enabling the creation of fully bio-based piezoelectric soft thin films for sensor and actuators applications. Beyond its potential as an active material, chitosan has also been explored as a passive flexible substrate for electronic devices. For this purpose, we developed a low-cost, environmentally friendly method using an automated cutter, achieving sub-millimeter precision in metal trace patterning, without requiring harsh thermal or chemical treatments. This approach, combined with the films' mechanical properties, biocompatibility and antibacterial activity demonstrated by inhibiting *E. coli* and *S. aureus* growth at concentrations of 10^4 CFU/mL for 72 hours, led to the development of flexible antibacterial smart electronic patches. Specifically, we fabricated working Radio Frequency Identification (RFID) antennas and a Photoplethysmogram (PPG) onto a flexible chitosan-based printed circuit board (PCB) for heart rate monitoring, highlighting the potential of chitosan in sustainable, bio-based electronics for medical and food packaging applications. This research, which began with the extraction of chitosan from crab by-products, culminates in the development of functional, biodegradable, and sustainable electronic devices, demonstrating the value of a circular approach in advancing future technologies.*

Chapter 1 - Chitosan: From Extraction to Film Production and Characterization

Short Abstract:

This chapter presents a versatile technology for producing fully bio-based chitosan films from seafood waste, enabling precise modulation of their properties to suit different applications and supporting medium-scale production. The films can range in thickness from under 10 μm to over 200 μm , with surface areas spanning from a few square millimeters to several hundred square centimeters. The ability to tailor mechanical performance (Young's modulus ranging from 15 MPa to 875 MPa, elongation up to over 60%), solubility, and transparency highlights the potential of these sustainable materials for flexible electronics, biomedical devices, and advanced coating.

1 State of the Art and Research Motivation

The increasing demand for sustainable materials has spurred research into bio-based alternatives to replace traditional fossil-derived polymers^{1,2}. Among these, chitosan—a natural biopolymer derived from chitin—has attracted considerable attention due to its abundance, biodegradability, biocompatibility, and antibacterial properties^{3–5}. Additionally, its reported piezoelectric behavior further enhances its appeal⁶. Recent studies have emphasized chitosan's potential in diverse applications, notably in food packaging^{7–11}, electronics^{12–14} and biomedical field¹⁵ positioning it as a promising alternative to conventional materials.

Chitin, the second most abundant natural polymer, is a biopolymer composed of glucosamine and N-acetyl glucosamine units linked by β -1,4-glycosidic bonds^{1,15}. It is primarily found in the exoskeletons of crustaceans and arthropods, as well as in the cell walls of fungi and yeasts^{1,16}. This polysaccharide plays a crucial structural role in nature, contributing to the rigidity and protection of these organisms. The extraction of chitin from crustacean shells involves a multi-step process: demineralization, deproteinization, and discoloration. Traditionally, these steps rely on chemical treatments, such as dilute acids and alkaline solutions, followed by solvent extraction^{1,16,17}. However, recent advancements in sustainable extraction methods, such as biological fermentation¹⁸ and ionic liquid-based techniques¹⁹, are contributing to a reduction in the environmental impact of chitosan production, aligning with the broader goals of green chemistry and sustainable materials^{1,16,20}. Alternative methods, including the use of deep eutectic solvents (DES), are also being explored to further minimize environmental impact^{16,17}.

Chitosan, derived from the deacetylation of chitin, this process involves the partial removal of acetyl groups from chitin, which increases its solubility in weak acids and makes it a more accessible material for various applications²¹.

Chitosan's ability to dissolve in weakly acidic environments is a key quality that facilitates its processing into films^{22–24}. This characteristic has made it particularly attractive for a variety of manufacturing applications, especially in fields that require materials to be flexible, biodegradable, and easy to fabricate.

Despite these appealing properties, chitosan faces challenges such as its moisture sensitivity and relatively low mechanical stability²⁵. These limitations have driven ongoing research into hybrid materials and the incorporation of additives that can improve chitosan's performance and expand its potential applications^{26–29}. The continuous development of these strategies underscores the growing versatility of chitosan as a bio-based material across diverse industrial sectors, with particular exploration in the electronics field, where its potential is being harnessed for the creation of flexible and biodegradable substrates^{30,31}.

As the electronics industry increasingly focuses on sustainability, the aim is to reduce the environmental impact of devices throughout their life cycle³². This is grounded in four core pillars: (i) eco-design, (ii) the adoption of sustainable materials, (iii) net-zero manufacturing, and (iv) effective recycling^{33,34}. In this context, our research was driven by the potential of chitosan as a sustainable alternative to traditional polymeric substrates in electronic applications. However, our approach did not focus on optimizing its extraction or modifying its inherent properties—tasks typically addressed in chemical research. As engineers, our goal was to investigate the natural properties of chitosan and determine how to leverage these properties for flexible electronic devices. This required a systematic exploration of its fundamental characteristics and limitations, followed by a structured approach to material processing and application.

As a first step, we extracted chitosan from *Granseola* (*Maja squinado*), a crab species found abundantly along the Venetian coasts. Our primary objective was not to refine or optimize the extraction process but to ensure that high-quality chitosan could be sourced from this marine waste. Once successfully extracted, we carried out a series of chemical and physical characterizations (NMR, FTIR, TGA) to compare our chitosan product with commercially available alternatives. This step aimed to verify whether the extracted material met the necessary standards for use in electronic applications.

Given the larger quantities required for further studies, we transitioned to commercially sourced chitosan to facilitate continued experimentation. This decision allowed us to concentrate on processing and enhancing chitosan films, rather than diverting efforts toward perfecting the extraction methodology—an aspect better suited to chemistry experts. With a reliable chitosan source in hand, we focused on developing thin films tailored for electronic applications. Initially, we employed simple casting techniques, but as the research progressed, we adopted a film applicator system. This system allowed precise control over film thickness and surface uniformity, ensuring that the fabrication method was scalable and reproducible.

One of the key findings of our study was that chitosan films readily accepted additives, making them highly adaptable to different application requirements. To improve the mechanical, thermal, and solubility properties of the films while maintaining a fully bio-based composition, we incorporated nanocrystalline chitin and deacetylated chitin nanocrystals—both derived from the same natural source as chitosan. By optimizing the composition of the films, we successfully tuned their crystallinity, mechanical strength, solubility, and thermal stability, allowing for tailored functionalities suitable for flexible electronics.

This research demonstrates the feasibility of using chitosan as a versatile, sustainable material for next-generation electronics. Through the development of chitosan-based solutions, our work contributes to advancing sustainable principles in electronics and shows that chitosan-based materials can be produced at a scalable level, reducing the reliance on fossil-derived polymers.

In the following chapters, we delve into the specific electronic applications of these films, evaluating their performance in real-world use cases and exploring opportunities for further optimization and innovation.

2 Results and discussion

Extraction and characterization of chitosan powders

Chemical Composition of Crab Shells

The chemical extraction of chitin from spider crab, followed by deacetylation to chitosan, was carried out by adapting a procedure described in the literature^{1,16}. The full methodology is summarized in Figure 1.1 and detailed in the Materials and Methods section of this chapter. Chitin in crab shells is naturally bound to proteins, minerals, lipids, and pigments, all of which must be selectively removed to obtain high-purity pulped chitin. To characterize the initial substrate, the ash, chitin, and protein content were quantified.

The ash content was determined following the ASTM E1755-01 Standard Test Method for Ash in Biomass, which defines ash as the residual mass percentage after pre-treated biomass undergoes acid digestion and subsequent drying. The ash content was calculated using **Equation 1**:

$$\%ash = \left(\frac{m_{ash} - m_{cont}}{m_{ar} - m_{cont}} \right) \cdot 100 \quad (1)$$

where %ash represents the mass percentage of ash relative to the dry sample weight at 105°C, m_{ash} is the combined mass of ash and container (g), m_{ar} is the initial mass of the prepared biomass and container (g), and m_{cont} corresponds to the tare mass of the container (g). The analysis revealed that ash accounted for 50% of the total mass, indicating that inorganic components constitute the predominant fraction of the crab shell matrix. Consequently, complete demineralization is a critical step in obtaining pure chitin.

The protein content was assessed through a controlled chemical deproteinization process. The percentage of proteins was derived from **Equation 2**:

$$\%proteins = 100 - \left(\frac{m_{residue}}{m_{biomass}} \cdot 100 \right) \quad (2)$$

Where $m_{residue}$ represents the total dry mass of the protein-free residue, and $m_{biomass}$ refers to the dry weight of the original crab shell sample. Triplicate measurements confirmed that proteins comprised 20% of the total mass.

The chitin content, estimated through conventional chemical extraction, was determined to be 25% of the total shell mass. The remaining fraction of *Granseola* shells consists of lipids, trace elements (such as rubidium, iron, copper, zinc, and bromine), and contaminants including cadmium, arsenic, and lead.

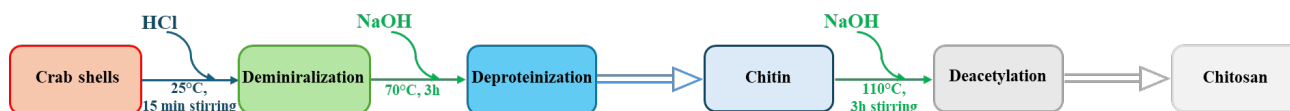


Fig. 1.1. Schematic representation of the standard chemical procedures used for chitin extraction and its deacetylation to chitosan from crustacean shells.

¹H-NMR Analysis of commercial chitin and chitosan and extracted chitosan

The ¹H-NMR spectrum of commercial chitin exhibits distinct signals in the anomeric region, with peaks at 5.33 ppm and 4.95 ppm, corresponding to the α- and β-anomeric protons of the acetylated units, respectively. The H-1 proton of de-N-acetylated units resonates at 4.98 ppm, overlapping with the β-anomeric signal, while the H-1 of acetylated units appears at 4.77 ppm. Additionally, the H-2 proton of deacetylated residues is observed at 3.33 ppm, whereas the remaining ring protons (H2–H6) are found in the 3.5–4.3 ppm region. A prominent peak at 2.53 ppm, attributed to the methyl protons of the acetyl groups, is significantly reduced in the spectra of extracted and commercial chitosan, confirming partial deacetylation in agreement with literature data²⁰.

Despite the deacetylation process, the spectra indicate that complete de-N-acetylation is not achieved upon dissolution in concentrated DCl. This is evidenced by the presence of acetic acid proton signals, which appear at 2.13 ppm for all tested samples.

Compared to the commercial sample, the extracted chitosan exhibits more defined proton signals in the 3.5–4.0 ppm region, particularly a sharper peak at ~3.6 ppm. This suggests a higher degree of chain flexibility or a lower average molecular weight, possibly due to partial depolymerization during extraction. The clearer signal resolution may also reflect a more homogeneous distribution of deacetylated units.

A final consideration concerns the H-1 signals in extracted chitosan, which appear particularly weak. This results in limited reliability when attempting to determine the degree of deacetylation under these experimental conditions, highlighting the need for additional analytical techniques to obtain a more precise characterization. Figure 1.2 illustrates the outcomes of these experiments.

Although the optimization of the extraction process was not the main objective of this study, the NMR analysis offered valuable insights into the molecular characteristics of the extracted chitosan. The observed spectral differences suggest variations in chain structure or degree of polymerization, which may affect its performance in specific applications. Further refinement of the extraction protocol could improve the material's consistency and bring its properties closer to those of the commercial counterpart, potentially enhancing its suitability for use in flexible electronics.

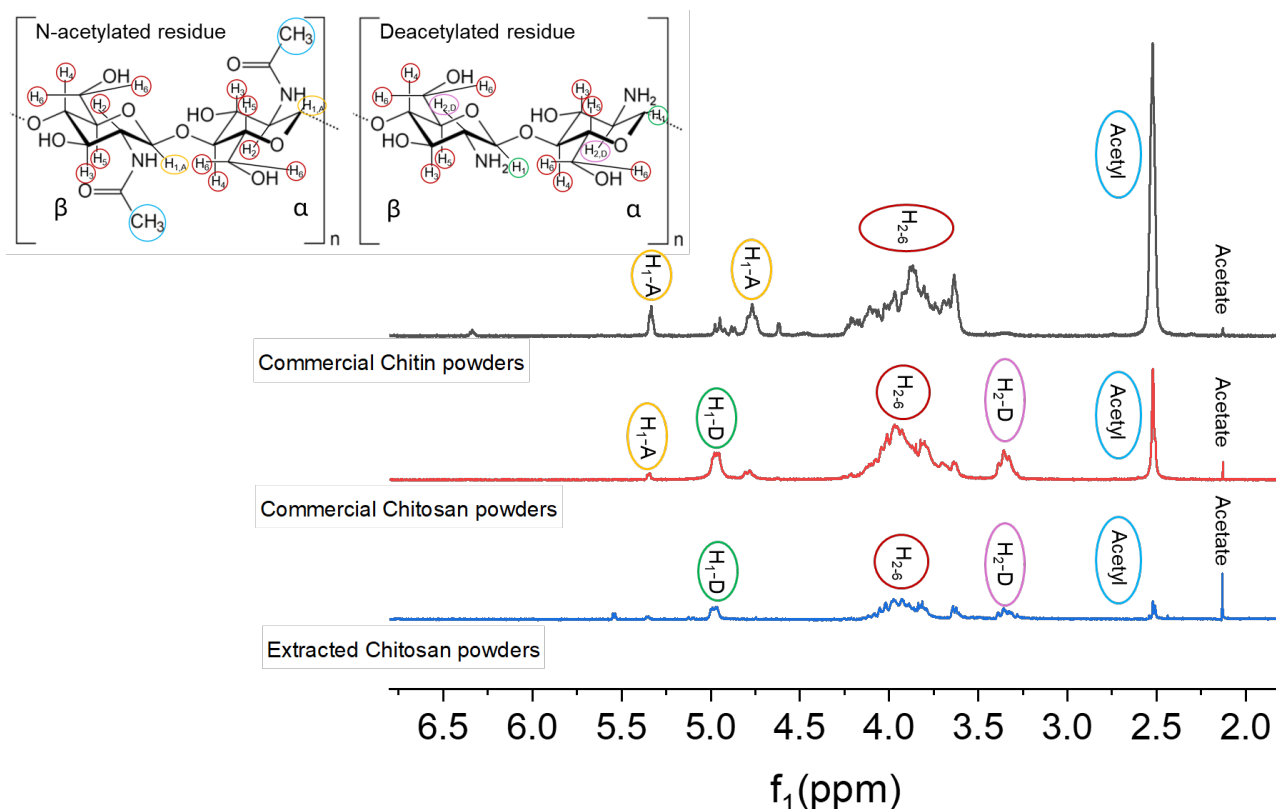


Fig. 1.2. Comparative ¹H NMR spectra of Commercial Chitin Powders (black curve), Commercial Chitosan Powders (red curve), and Extracted Chitosan Powders (blue curve).

FTIR Analysis of Chitin and Chitosan Powders

The FTIR spectra of extracted chitin, extracted chitosan, and commercial chitosan provide valuable insights into the efficiency of the deacetylation process and the purity of the obtained materials.

A key observation is that the extracted chitosan exhibits absorption bands at 1650 cm⁻¹ (Amide I) and 1544 cm⁻¹ (Amide II), which are closer to those of chitin than to those of commercial chitosan. This suggests that while the deacetylation process was effective to a significant extent, it was not complete, leaving a fraction of acetyl groups in the structure. This is further supported by the presence of a notable peak around 1000 cm⁻¹, indicative of residual chitin, and a band at 1400 cm⁻¹, which may be associated with remaining acetyl groups. However, the persistence of these signals does not indicate process failure but rather highlights areas for potential refinement. This is in agreement with ¹H-NMR data, where residual acetyl groups were detected in extracted chitosan (2.5 ppm), though in a reduced amount compared to chitin.

An important aspect is the presence of broad absorption bands between approximately 3800 cm⁻¹ and 3000 cm⁻¹, attributed to O–H stretching vibrations, which are typical of polysaccharides and appear in all samples including commercial chitosan.

However, in this range, another noteworthy feature is the presence of distinct absorption peaks around 3750 cm⁻¹ in both extracted chitin and extracted chitosan, which are absent in the commercial chitosan sample. These peaks may be associated with residual compounds from the extraction process, such as proteins or other organic substances, and suggest incomplete purification compared to the commercial material.

Additionally, signals in the 900 cm⁻¹ and 600 cm⁻¹ regions indicate potential impurities arising from polysaccharides or mineral residues from the crustacean shell; despite these residual impurities, the overall FTIR profile of the extracted chitosan is already approaching that of the commercial reference.

As a final consideration, FTIR analysis confirms that the deacetylation process is progressing in the right direction, though some fine-tuning is needed to further reduce the acetyl content. Additionally, refining

the pulping process could enhance the final material’s purity by minimizing residual organic and inorganic impurities.

Given the larger quantities required for further studies and our focus not on optimizing the extraction process but on material applications, we henceforth employed commercially sourced chitosan to ensure consistency in material properties for our experiments.

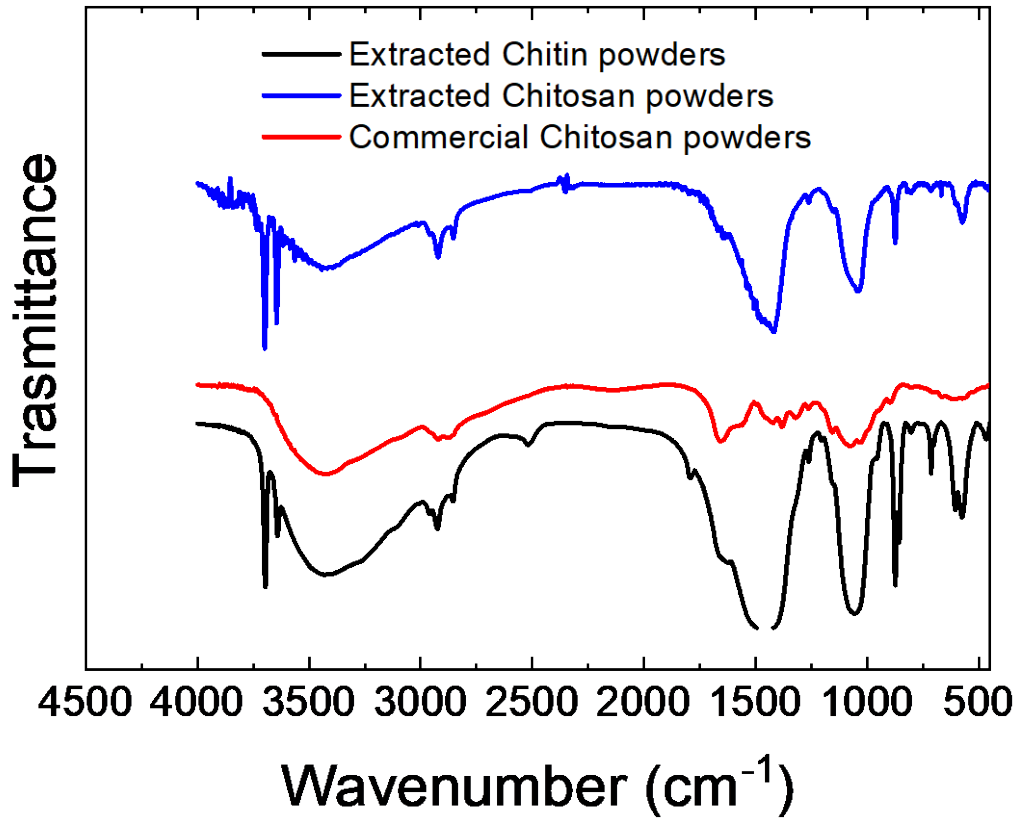


Fig. 1.3. Comparative FT-IR spectra of Extracted Chitin Powders (black curve), Extracted Chitosan Powders (blue curve), and Commercial Chitosan Powders (red curve).

Table 1.1 Assignment of the main FTIR absorption bands in extracted and commercial chitin and chitosan samples, indicating possible structural features and residual impurities.

Wavenumber (cm ⁻¹)	Assignment	Notes
3500–3300	Broad O–H stretching	Indicative of hydrogen bonding, typical of polysaccharides.

~3750	O–H stretching (sharp)	Present in extracted chitin and chitosan; absent in commercial chitosan. May indicate residual organic impurities.
1650	Amide I (C=O stretching)	Present in extracted chitosan and chitin; reduced in commercial chitosan
1544	Amide II (N–H bending)	Present in extracted chitosan and chitin; weaker in commercial chitosan.
1400	C–H bending / residual acetyl groups	Incomplete deacetylation.
1000	C–O–C stretching	Incomplete deacetylation.
900–600	Fingerprint region	Possible impurities from crustacean shell (e.g., polysaccharides or minerals) in extracted samples.

Thermogravimetric Analysis (TGA) of Extracted and Commercial Chitosan powders

Thermogravimetric analysis (TGA) was performed to compare the thermal stability and degradation behavior of extracted and commercial chitosan powders. The thermograms of all samples exhibited three distinct thermal events, corresponding to different stages of mass loss.

The first weight loss event, occurring below 100°C, was attributed to the evaporation of lattice water and physically adsorbed moisture. This step resulted in a mass reduction of approximately 5–10%, consistent with the well-known hygroscopic nature of chitosan and its ability to retain water molecules within its polymeric network through hydrogen bonding.

The second major degradation stage, observed between 200°C and 350°C, corresponded to the thermal decomposition of the chitosan backbone. In this range, a substantial mass loss of 45–55% was recorded, indicating depolymerization and oxidative degradation of the polysaccharide chains. This process involves the cleavage of glycosidic linkages and the breakdown of the polymer into volatile by-products such as carbon dioxide, carbon monoxide, and low-molecular-weight hydrocarbons.

A third degradation peak, observed in extracted chitosan between 120°C and 170°C, was attributed to the residual deacetylation of chitosan during heating. This exothermic event, often overlooked in conventional TGA analyses, is associated with the further elimination of acetyl groups that may not have been completely removed during the initial alkaline deacetylation process. The extent of this residual deacetylation can influence the solubility and charge distribution of chitosan, potentially affecting its functional properties.

Comparing the thermograms of commercial and extracted chitosan, differences were observed, reflecting variations in their chemical composition and structural organization. The extracted chitosan exhibited a slightly broader decomposition range and a marginally lower degradation onset temperature, suggesting a different molecular weight distribution and degree of acetylation. Additionally, the presence of a peak around 400°C in the thermogram of the extracted chitosan (not present in that of the commercial) could be attributable to the presence of saccharide impurities. Furthermore, a higher residual mass above 400°C was observed for the extracted chitosan (40% at 800°C) compared to the commercial sample (30% at 800°C), indicating the presence of a greater amount of inorganic residues or impurities that were not fully removed during the extraction process. These variations directly impact the mechanical strength and solubility properties of the material, which are crucial parameters for its application in bio-based films and coatings.

These results, shown in Figure 1.4., confirm that while the extracted chitosan already exhibits promising characteristics, further optimization of the demineralization process could reduce residual impurities

and bring its properties even closer to those of the commercial material, though this remains beyond the primary scope of this study.

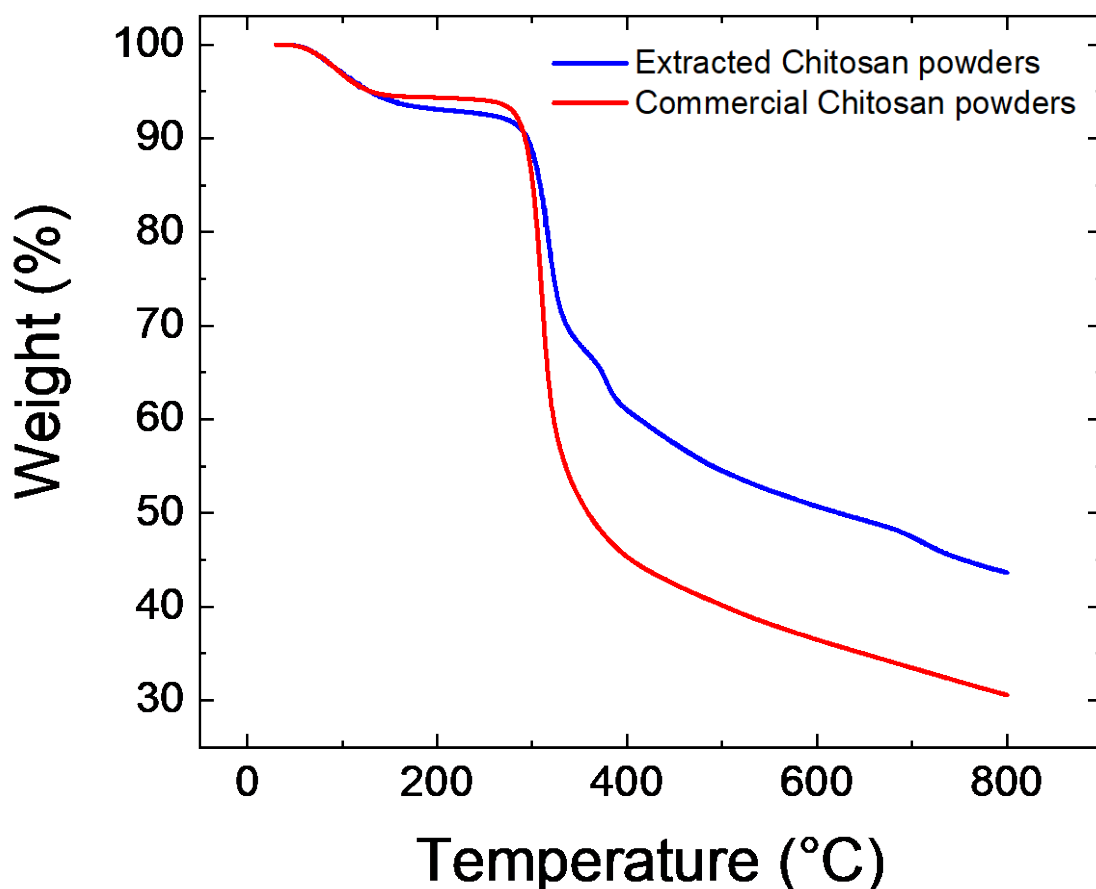


Fig. 1.4. TGA curves of Extracted Chitin Powders (black curve) and Extracted Chitosan Powders (blue curve).

Development of Chitosan-Based Films

Since larger quantities of material were required for subsequent studies, we opted to use commercially sourced chitosan to facilitate continued experimentation. This decision allowed us to focus on processing and enhancing the properties of chitosan films, rather than diverting attention to perfecting the extraction methodology, which had already been validated earlier.

Initially, chitosan films were produced using a straightforward solvent casting technique. Chitosan powder was dissolved at a concentration of 10 mg/mL in a mixture of acetic acid and water (1:99 v/v), and the solution was cast into a Petri dish, left to evaporate under vacuum to form the film. However, to enhance the reproducibility, versatility, and scalability of the process, a more effective method was implemented: the use of an automatic film applicator. This technique, still based on solvent casting, enabled precise control over the film thickness (micrometer scale) and surface area, offering significant improvements in flexibility and efficiency. In this method, the chitosan film-forming solution was applied onto a Kapton substrate, chosen for its smooth surface, which facilitated the easy peeling of the film after solvent evaporation. The thickness of the liquid film was regulated by the passage of a micrometer-adjustable applicator, allowing for precise control over its final thickness, ensuring uniformity of the material (Figure 1.5). By carefully tuning the applicator height, adjusting the solution composition, and varying the material concentration, films with thicknesses from as thin as 10 μm to over 200 μm were obtained after the solvent evaporation. Surface areas can be achieved from a few

square millimeters to several hundred square centimeters. The films produced were smooth, transparent, and highly flexible, with excellent homogeneity, as confirmed by analysis using the Tencor Alpha Step 500 Profilometer. The simplicity and cost-effectiveness of this technology, combined with its high reproducibility, make it an attractive option for scaling up production. An important feature of this fabrication method is the ability to exploit the inherent capacity of chitosan films to accommodate various reinforcing materials, such as TiO_2 , ZnO , carbon black, or pH-sensitive compounds like bromophenol, within their matrix without compromising their mechanical properties (Figure 1.6). Notably, the photocatalytic properties of TiO_2 or ZnO can enhance the antibacterial activity of the films as already observed in previous studies^{35,36}, while the incorporation of pH-sensitive materials allows for the creation of films with tunable properties, such as color changes depending on pH. These capabilities offer exciting prospects for functionalizing chitosan-based films for diverse applications, including antibacterial substrates, pH-sensitive sensors, and conductive films, making them highly promising for use in flexible electronics.

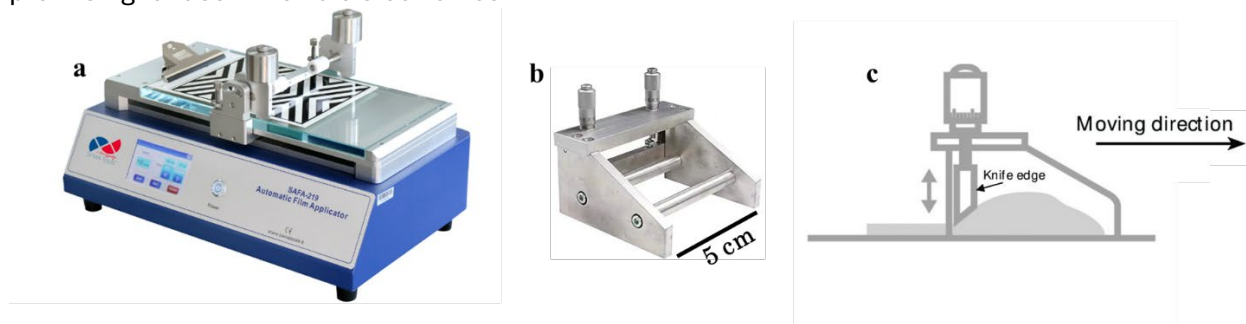


Figure 1.5. (a) Frame of the automatic film applicator used for chitosan film fabrication. (b) Micrometer-adjustable applicator enabling precise thickness control. (c) Schematic representation of the working principle of the system.

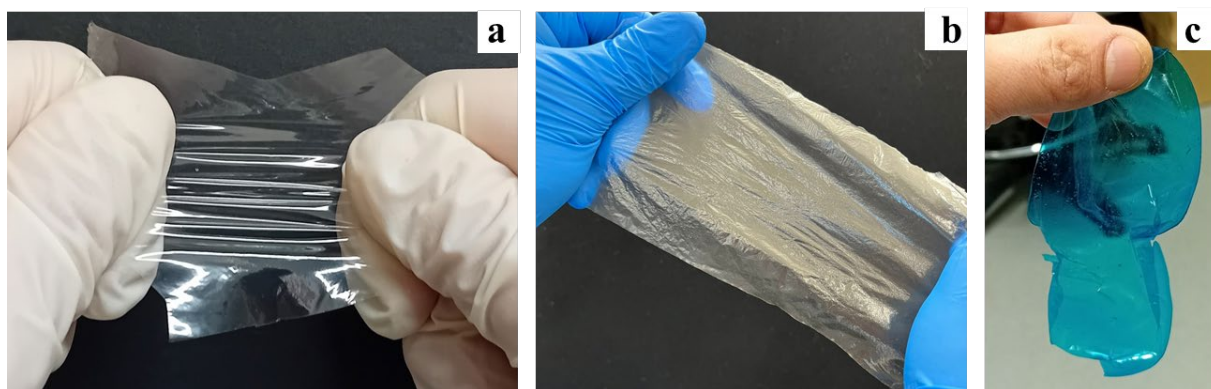


Figure 1.6 (a) Fully biobased chitosan-based nanocomposite elastomer plasticized with glycerol and reinforced with chitin nanocrystals. (b) Large-area pure chitosan film ($\sim 200 \text{ mm}^2$). (c) Chitosan film incorporating bromophenol. These images demonstrate the adaptability of the fabrication technology in producing films with different composition, mechanical performance, optical properties, and dimensions.

Optical Absorption of pristine chitosan films

The optical absorption of neat chitosan films was assessed through repeated measurements, with five independent measurements conducted on the same sample. The absorption spectra of the films were characterized by nearly flat curves in the visible range, between 400 and 800 nm, with a constant value approaching zero, indicating minimal absorption in this wavelength range. In some cases, slightly negative absorption values were recorded, which are not physically meaningful but are a result of

instrumental limitations, such as imperfect baseline correction and normalization inaccuracies due to the high transparency of the samples. These artifacts are commonly observed when dealing with very low-absorbance films and do not affect the qualitative interpretation of the spectral profile³⁷. The overall spectral behavior observed in the visible range is consistent with what is typically reported for chitosan films, which are known to be largely transparent across this region of the spectrum. Notably, a distinct absorption peak was observed around 200 nm, which corresponds to the characteristic UV absorption of chitosan³⁸. This peak is commonly reported in the literature and is attributed to the inherent structure of chitosan and its ability to absorb UV radiation due to the presence of functional groups such as amino and hydroxyl groups within the polymer chain. The absorption behavior of chitosan films in the UV range can be explained by the electronic transitions of these functional groups, which result in a peak around 200 nm. Such properties make chitosan films suitable for applications requiring UV shielding³⁹. The results of these experiments are presented in Figure 1.7.

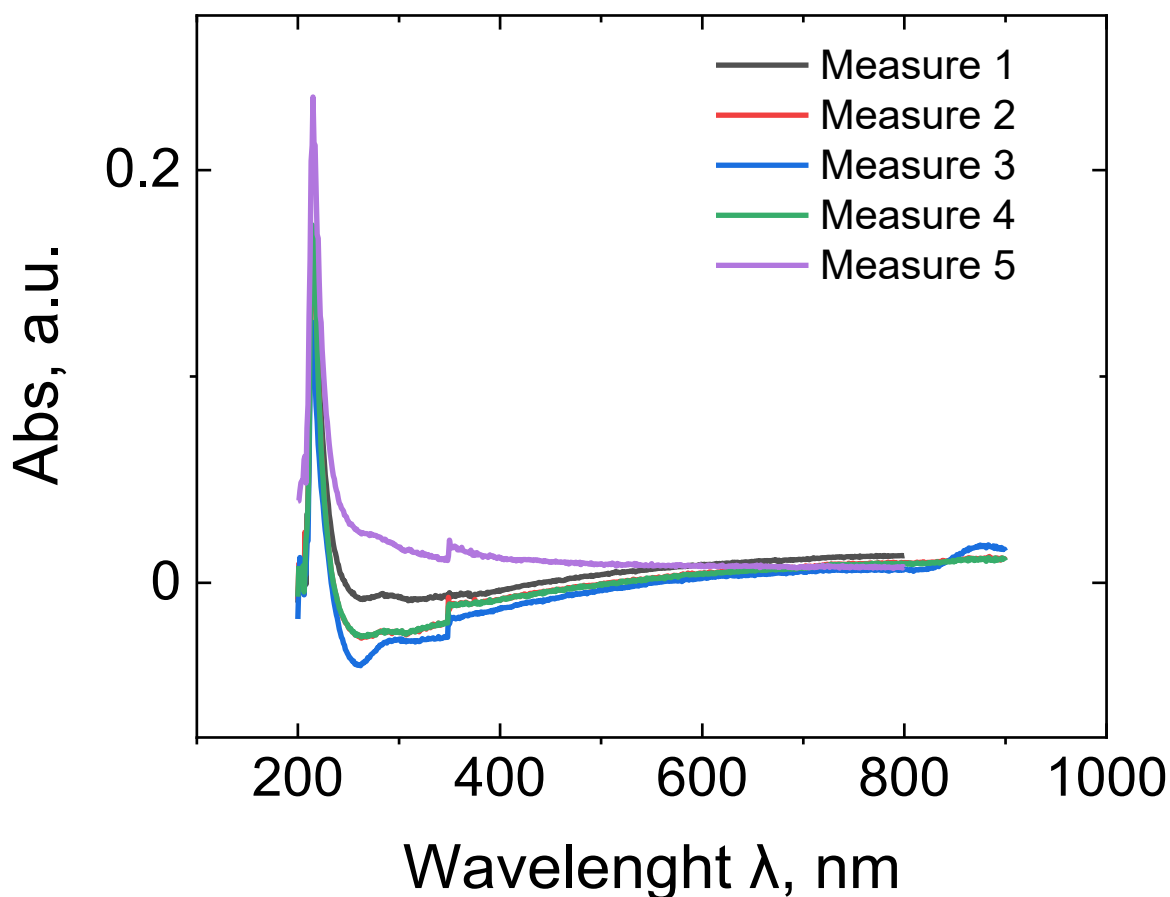


Figure 1.7. Optical absorption of neat chitosan films. The graph refers to five repeated measurements of the same sample. All curves appear flat in the visible range (wavelength between 400 - 800 nm) and with a constant value of approximately zero.

Chitosan-Based Nanocomposite Films

The ability of chitosan films to incorporate reinforcing materials opened the possibility of tailoring their mechanical and functional properties to suit specific applications. Initial tests revealed a critical limitation for pure chitosan films exhibiting excessive water solubility, restricting their potential use in

humid environments. To address this, we focused on developing nanocomposite films with higher crystallinity, which not only enhanced mechanical strength but also significantly reduced solubility. Chitin nanocrystals (ChNCs) and surface-deacetylated chitin nanocrystals (CsNCs) emerged as the ideal reinforcements. Their intrinsic crystallinity contributed to improved film robustness and water resistance, while their shared origin with chitosan^{40,41} ensured seamless integration into the matrix, maintaining a fully bio-based composition. Moreover, the modulation of crystallinity offered additional advantages, including the potential to influence piezoelectric properties, further expanding the scope of applications. Additionally, the incorporation of nanocrystals was expected to enhance the thermal stability of the films, a key factor for applications requiring resistance to elevated temperatures. This strategic approach laid the foundation for the systematic investigation of the mechanical performance, solubility, and thermal stability of the resulting nanocomposite films, as detailed in the following sections.

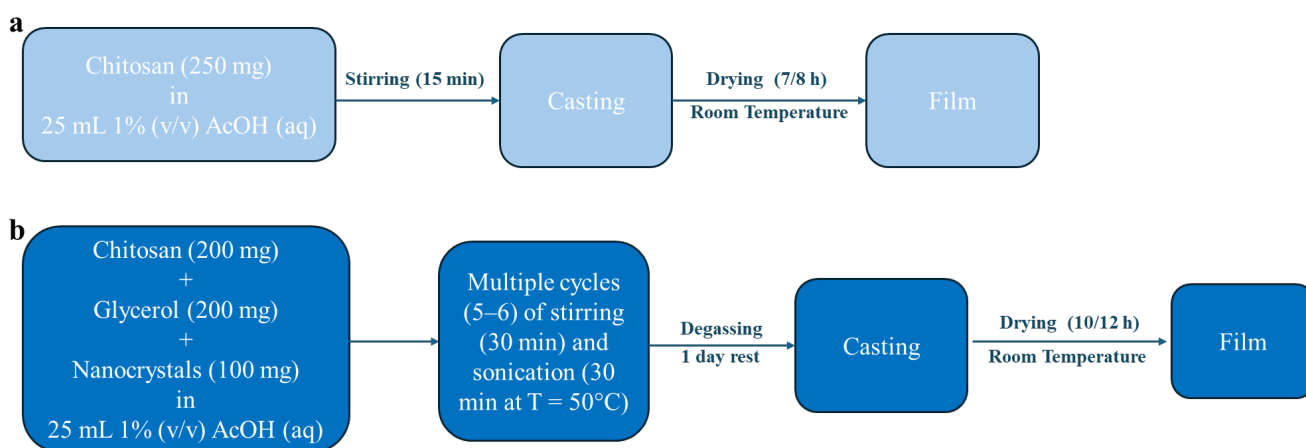


Figure 1.8. Schematic representation of the protocols for the preparation of chitosan-based films: (a) pure chitosan film; (b) composite films containing 40 wt% glycerol and 20 wt% of either chitin nanocrystals (ChNCs) or surface-deacetylated chitin nanocrystals (CsNCs).

Mechanical Characterization

The development of bio-based materials often hinges on their inherent flexibility, ease of processing, and the potential to customize their properties for a wide array of applications. In this regard chitosan has emerged as a promising candidate due to its excellent film-forming ability and ease of incorporation with various additives, making it ideal for the fabrication of composite materials. Our work capitalized on these properties, initially focusing on the incorporation of glycerol, a widely used plasticizer, into the chitosan matrix. This simple yet effective modification enabled the transformation of chitosan into more flexible, elastomeric films. Building on this, we further explored the integration of chitin nanocrystals (ChNCs) and surface-deacetylated chitin nanocrystals (CsNCs), creating fully bio-based composites with enhanced mechanical properties.

By systematically varying the composition of the chitosan-based films, we were able to create a series of materials with tunable mechanical properties. This flexibility in composition provided the opportunity to fine-tune the stiffness, elasticity, and deformability of the films, placing them in a wide mechanical spectrum, from more rigid to highly elastic materials. This property is particularly valuable in applications requiring materials with specific mechanical responses under stress, such as in prosthetics, soft robotics, and biomedical devices.

To investigate the effects of different additives on the mechanical properties of chitosan films, we prepared five distinct compositions, as follows:

- C₁₀₀: Pure chitosan film
- C₄₀G₄₀ChNCs₂₀: Chitosan film with 40 wt% glycerol and 20 wt% chitin nanocrystals (ChNCs)
- C₄₀G₄₀CsNCs₂₀: Chitosan film with 40 wt% glycerol and 20 wt% surface-deacetylated chitin nanocrystals (CsNCs)
- CsNCs₇₀G₃₀: Film composed solely of 70 wt% surface-deacetylated chitin nanocrystals (CsNCs) and 30 wt% glycerol
- C₅₀G₅₀: Reference film composed of 50 wt% chitosan and 50 wt% glycerol

Each composition was tested for mechanical properties, including tensile strength, Young's modulus, and elongation at break. These tests provided essential insights into the material behavior under different stress conditions, showing a clear relationship between composition and the resulting mechanical characteristics. The results of these experiments are presented in Figure 1.8.

Influence of Glycerol on Mechanical Properties

The inclusion of glycerol had a profound impact on the mechanical properties of the chitosan films. The pure chitosan film (C₁₀₀) exhibited high tensile strength (~70 MPa) and low elongation (~8%), indicative of a relatively rigid material. In contrast, the addition of glycerol to the chitosan matrix significantly altered the material's behavior, transforming it into an elastomeric film with the following changes: the tensile strength decreased to approximately 20 MPa, the Young's modulus was reduced to around 15 MPa, and the strain at break increased to 60%. This elastomeric response was marked by a non-linear stress-strain curve, typical of elastomers, where deformation occurs at low stresses and without a clear yield point.

The introduction of glycerol as plasticizers thus conferred increased flexibility and elongation capacity to the films, enabling their potential use in applications where flexibility and deformability are essential. Furthermore, the absence of yield strength in these films points to their suitability for applications involving high levels of deformation, such as in soft robotics and wearable devices.

Role of Nanocrystals: Enhancing Stiffness and Strength

Incorporating chitin nanocrystals (ChNCs) and surface-deacetylated chitin nanocrystals (CsNCs) into the chitosan-glycerol films allowed for further optimization of the mechanical properties. Films incorporating both glycerol and nanocrystals (C₄₀G₄₀ChNCs₂₀ and C₄₀G₄₀CsNCs₂₀) exhibited a balanced combination of stiffness and deformability, offering a tensile strength of approximately 40 MPa, a Young's modulus of around 100 MPa, and a strain at break of more than 40%. These values place the films within the range of biological tissues such as tendons and muscles, which have similar mechanical properties. This makes them highly promising for applications in prosthetics, biomedical implants, and soft robotics, where materials need to replicate the mechanical properties of biological systems. The Results of these experiments are shown in Figure 1.9.

Interestingly, films made with only nanocrystals, such as the CsNCs₇₀G₃₀ film, showed a Young's modulus similar to C₁₀₀, but with a reduced tensile strength (~25 MPa). This reduction is likely due to the limited mobility of polymer chains in the nanocrystal matrix, which restricts the film's ability to deform and causes it to fracture at lower stress levels. Despite this, the mechanical properties of these films are still of interest, especially in scenarios where stiffness is more important than flexibility, such as in structural applications or coatings.

It is therefore evident that by varying the proportions of glycerol and nanocrystals, the mechanical properties of the films can be fine-tuned for specific applications. This opens up possibilities for targeted material design, where the exact balance between stiffness, elasticity, and deformability can be adjusted based on the requirements of the intended application. By continuing to explore the effects of various additives and compositions, these bio-based films hold promise for the development of

innovative materials that combine the best of biopolymer chemistry and material science, ultimately leading to sustainable and adaptable solutions for numerous industries.

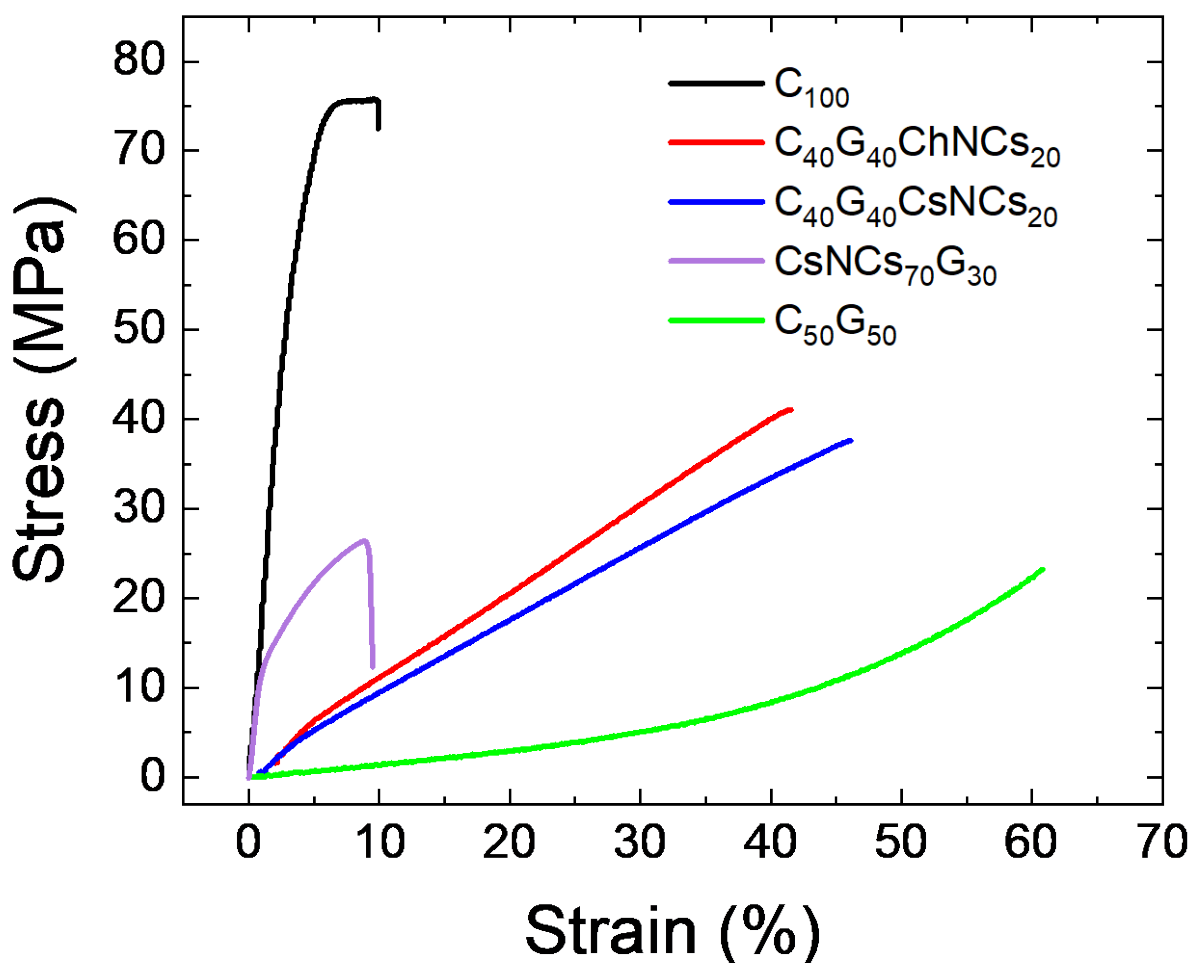


Figure 1.9. Stress/strain curves of C_{100} (black curve), $C_{50}G_{50}$ (green curve), $C_{40}G_{40}ChNCS_{20}$ (red curve), $C_{40}G_{40}CsNCS_{20}$ (blue curve) and $CsNCS_{70}G_{30}$ (purple curve). Nanocrystals enhance materials rigidity, increasing tensile strength and Young's modulus, highlighting their reinforcing. The addition of glycerol improves film deformability by acting as a plasticizer, as evidenced by the decrease in tensile strength due to reduced intermolecular interactions.

Thermogravimetric Analysis (TGA): Composite chitosan films vs chitosan films

Thermogravimetric Analysis (TGA) was performed to examine the thermal stability and decomposition behavior of chitosan-based films with three distinct compositions: pure chitosan (C_{100}), chitosan with glycerol and chitin nanocrystals ($C_{40}G_{40}ChNCS_{20}$), and chitosan with glycerol and surface-deacetylated chitin nanocrystals ($C_{40}G_{40}CsNCS_{20}$).

For the C_{100} film, the TGA data revealed two major weight loss steps. The first, occurring below 100 °C, corresponds to the evaporation of water, with a weight loss of 5-10%. The second, between 200-350 °C, accounted for a significant mass loss of 45-55%, attributed to the decomposition of the chitosan matrix. These findings are consistent with the typical behavior of chitosan films, where initial water loss is followed by the breakdown of the saccharide ring structure.

In films containing nanocrystals, the presence of glycerol influenced the thermal stability. The onset of decomposition was observed already beyond 150 °C, lower than that of pure chitosan, which can be attributed to the increased water content in the matrix. Notably, a distinct weight loss around 300 °C, common to both $C_{40}G_{40}ChNCs_{20}$ and $C_{40}G_{40}CsNCs_{20}$ films, corresponds to the degradation of the chitosan matrix. The films also exhibited an additional weight loss range between 250 and 400 °C, linked to the decomposition of the nanocrystals, with no significant differences between the chitin and surface-deacetylated chitin nanocrystals. These observations align with those found in literature for similar chitin nanocrystals, where the primary decomposition pathway for the polysaccharide occurs between in the same temperature range, with a final complete decomposition above 400 °C⁴⁰. The results of these experiments are shown in Figure 1.9.

These findings underscore key challenges in the fabrication of chitosan-based devices, particularly for applications requiring high-temperature processing. Beyond their susceptibility to water-induced degradation, both pure chitosan and composite films exhibit deformation at temperatures exceeding 80°C, posing limitations for thermal manufacturing techniques. However, mastering the control of crystallinity could offer a pathway to improving thermal stability, providing a potential avenue for expanding the applicability of chitosan-based materials while maintaining their desirable properties.

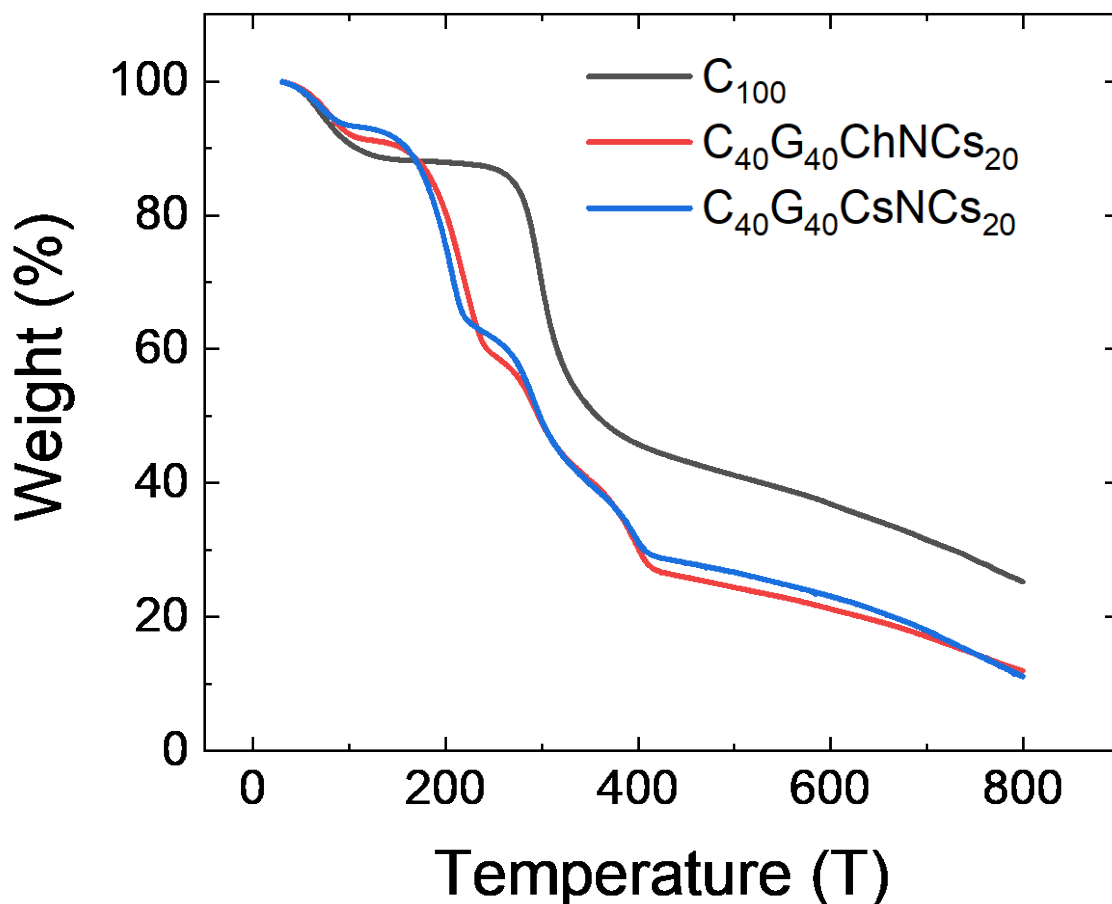


Figure 1.9. TGA curves of C_{100} (black curve), $C_{40}G_{40}ChNCs_{20}$ (red curve) and $C_{40}G_{40}CsNCs_{20}$ (blue curve).

Water Solubility (WS%) tests

The water solubility of chitosan-based films was investigated to assess the impact of different compositions on their dissolution behavior, particularly in relation to the potential for developing

insoluble substrates for specific applications. Three different film compositions were analyzed: C₁₀₀ (pure chitosan film), C₄₀G₄₀ChNCs₂₀ (chitosan film with 40 wt% glycerol and 20 wt% chitin nanocrystals), and C₄₀G₄₀CsNCs₂₀ (chitosan film with 40 wt% glycerol and 20 wt% surface-deacetylated chitin nanocrystals). The underlying hypothesis was that by increasing the crystallinity of the films, through the incorporation of chitin nanocrystals or surface-deacetylated chitin nanocrystals, we could reduce the solubility of the films. This would be particularly valuable in scenarios where water insolubility is required, as pure chitosan films are highly soluble in water, posing a potential limitation for certain applications.

As with the mechanical properties, the goal here was to modulate the characteristics of the films by varying their composition. The water solubility test showed that pure chitosan films (C100) dissolved completely within 24 hours, achieving a Water Solubility (WS%) of 100%, confirming their high solubility in water. In contrast, the films containing nanocrystals, namely C₄₀G₄₀ChNCs₂₀ and C₄₀G₄₀CsNCs₂₀, showed a significant reduction in solubility, with a WS% of 40%. This means that approximately 40% of their initial weight was retained after exposure to water. One plausible hypothesis is that a portion of this retained weight could be attributed to the glycerol, which is more weakly bound to the matrix compared to the chitosan or nanocrystals. This weaker interaction with the chitosan matrix could explain why some of the glycerol remains, even after the dissolution process.

The results show that incorporating chitin nanocrystals, in both native and deacetylated forms, effectively reduced the water solubility of the films. This reduction offers great potential for tailoring solubility in chitosan-based films, allowing for precise control based on application needs. Combined with the tunability of mechanical properties, this highlights the versatility of these bio-based materials for a wide range of uses.

3 Materials and Methods

Extraction of Chitin and Deacetylation to Chitosan

Chitin was extracted from the exoskeletons of *Maja squinado* or *Granseola*, a crab species commonly found along the Venetian coast, using a chemical approach. The procedure began with grinding the crab shells into a fine powder, which was then immersed in an aqueous solution of hydrochloric acid (HCl, 0.5 M, 40 mL/g of shells, Sigma-Aldrich, USA) and stirred at ambient temperature for 15 minutes. The mixture was filtered to remove insoluble material, and the remaining solid was rinsed with water until neutral pH was achieved. The product was then dried under reduced pressure (15 mbar) in an oven overnight.

The demineralized powder was subsequently treated with an aqueous sodium hydroxide solution (NaOH, 1 M, 20 mL/g of solid, Sigma-Aldrich, USA) at 70°C for 3 hours, with continuous stirring. This step facilitated the removal of proteins from the crab shells. After filtration and thorough washing with water until neutral pH, the material was dried under reduced pressure (15 mbar) again for an overnight period. The resulting powder was a white, deproteinized solid.

If needed, a decolouration step can be added to eliminate residual pigments such as carotenoids. Common approaches include the use of mild oxidizing agents like potassium permanganate or hydrogen peroxide. Alternatively, organic solvents such as acetone or a mixture of chloroform, methanol, and water (1:2:4 v/v) may be used. Final steps in the extraction process may involve neutralization, drying, and grinding.

To produce chitosan, deacetylation was performed under heterogeneous conditions. The reaction was carried out at a temperature of 110°C with a sodium hydroxide concentration of 70% (w/v, Sigma-Aldrich, USA) in a solid-to-alkali ratio of 1:10. The mixture was stirred for 3 hours in a flask. Afterward,

the precipitate was washed multiple times with Milli-Q water until neutral pH was achieved, and the solid was separated by filtration.

Physicochemical Characterization of Chitosan Powders

¹H-NMR Analysis

¹H-NMR spectra of extracted and commercial chitosan were obtained using a Bruker UltraShield 300 spectrometer operating at 300 MHz at ambient temperature (298 K). Spectra were recorded over 64 scans with a spectral window of 20 ppm. Samples were dissolved in deuterium chloride solution (DCl, 35 wt% in D₂O) under vigorous stirring at 50°C for 30 minutes before analysis.

FTIR Spectroscopy

FTIR analysis was performed using a Perkin-Elmer Spectrum One FT-IR spectrometer. Samples were prepared by grinding the dry chitosan powders with KBr in a 1:100 (w/w) sample-to-KBr ratio, followed by compression into 5 mm-diameter pellets using a hydraulic press at 10 tons for one minute. Spectra were recorded in the mid-infrared region (4000 cm⁻¹ to 450 cm⁻¹) with a resolution of 4 cm⁻¹ in absorbance mode, averaging 16 scans at room temperature.

Thermogravimetric Analysis (TGA)

Thermogravimetric analysis was performed using a Perkin Elmer TGA 4000. Approximately 10 mg of each sample (both powders and films) were placed in an alumina crucible and heated from 30°C to 800°C at a rate of 10°C/min under a nitrogen atmosphere

Synthesis of Chitin Nanocrystals (ChNCs) and Surface-Deacetylated Nanocrystals (CsNCs)

Chitin Nanocrystals (ChNCs) were synthesized by hydrolyzing chitin flakes (35 mg/mL) in 3M HCl under reflux for 4 hours. The resulting mixture was centrifuged, filtered, and washed with deionized water before being sonicated to obtain a stable colloidal suspension. ChNCs were recovered by adding 1M NaOH, followed by washing and drying at 100°C under vacuum for 24 hours, yielding a final concentration of 11.7 wt%.

For CsNCs, ChNCs (16 mg/mL) were dispersed in 12.5M NaOH and refluxed for 12 hours. After cooling, the mixture was purified similarly to ChNCs and dried, resulting in a final concentration of 15 wt%. Zeta potential analysis of both ChNCs and CsNCs dispersions (pH 4.0, 1 mg/mL) showed values of +54.1 ± 0.7 mV and +55.4 ± 1.1 mV, confirming the stability of the colloidal suspensions.

Fabrication of Chitosan-Based Films

Pure chitosan films were prepared by dissolving 250 mg of chitosan in 25 mL of 1% aqueous acetic acid. Nanocomposite films were obtained by dissolving 200 mg of chitosan and 200 mg of glycerol in 25 mL of acetic acid, followed by the addition of the appropriate amount of hydrated ChNCs or CsNCs to achieve the desired concentration of dehydrated nanocrystals in the final films. The solutions were stirred and then sonicated for 30 minutes at 45°C. Films were cast using a solvent casting method with an automatic applicator (SAFA-219, S.A.M.A. Italia).

Characterization of Chitosan films

Optical Adsorption

The UV-Vis Spectrophotometry tests were carried out with an Agilent Cary 100 UV/Vis Spectrophotometer. The calibration process was carried out using the blank sample holder as a reference, i.e. without the film deposited

Tensile Tests

Stress–strain measurements were carried out on dog-bone-shaped chitosan-based films (4 cm in length, 0.5 cm in width) using an MTS Insight moving beam dynamometer equipped with a 100 N load cell. The gauge length was set to 20 mm, and the crosshead speed was maintained at 0.5 mm s⁻¹. The elastic modulus (E) was calculated from the initial linear region of the stress–strain curve. Each sample was tested in at least five replicates, and the results were reported as mean ± standard deviation.

Water Solubility (WS%)

Water solubility was evaluated cutting the thin films into 2.0 × 2.0 cm² samples. Films were initially dried at 40°C until reaching a constant weight (M₁), then immersed in 50 mL of distilled water at 25 ± 2°C for 24 hours. After immersion, samples were dried at 80°C until a final constant weight (M₂) was obtained. Measurements were performed in triplicate, and WS% was calculated using the following: **Equation 3**:

$$WS(\%) = \left(\frac{M_1 - M_2}{M_1} \right) \cdot 100 \quad (3)$$

4 Conclusions

This study successfully demonstrated the extraction of chitosan from *Maja squinado*, confirming the feasibility of obtaining high-quality chitosan from this marine resource. While characterization results indicated that process optimization could further enhance purity and yield, this was beyond the primary scope of this work. Crucially, we established that the extraction process is viable and can be pursued for larger-scale applications.

To meet the demands of scalability, we developed a highly flexible and reproducible method for producing chitosan films. By implementing an automated film applicator, we achieved precise control over film thickness, ensuring uniformity while allowing the fabrication of films with surface areas ranging from a few square millimeters to several hundred square centimeters. This technology enables the production of films with a broad range of thicknesses, from ultra-thin layers below 10 µm to thicker films exceeding 200 µm, expanding their potential applications across various fields. This adaptability suggests strong potential for industrial applications requiring tunable film properties.

One of the most significant findings of this work is the ability to modulate the mechanical properties of fully bio-based films to suit specific applications. Pure chitosan films (C₁₀₀) exhibited high stiffness, with a tensile strength of approximately 70 MPa and a Young's modulus of 875 MPa, but limited elongation (~8%). In contrast, films containing only glycerol (C₅₀G₅₀) displayed an elastomeric behavior, with a substantial reduction in tensile strength (20 MPa) and Young's modulus (15 MPa), while elongation increased beyond 60%. Films incorporating both glycerol and chitin or surface deacetylated chitin nanocrystals provided an optimal balance between strength and flexibility, achieving a tensile strength of 40 MPa, a Young's modulus of 100 MPa, and elongation exceeding 40%. This tunability enables precise customization of film properties, making them suitable for applications in soft robotics, biomedical materials, and flexible electronics. Additionally, the incorporation of nanocrystals proved effective in modulating the water solubility of chitosan films. Pure chitosan films (C₁₀₀) dissolved completely within 24 hours (WS% = 100%), whereas films containing nanocrystals exhibited significantly reduced solubility. However, the inherent water sensitivity of chitosan-based materials, combined with their tendency to undergo deformation at elevated temperatures, presents challenges for microfabrication processes, particularly in applications requiring high thermal stability. These factors highlight the need for further optimization to develop compositions with tailored properties that

enhance processability while maintaining the desirable functionalities of chitosan films. Furthermore, the optical absorption analysis confirmed that pure chitosan films exhibit high transparency in the visible range (400–800 nm), with minimal absorption. This optical transparency makes chitosan films attractive for optoelectronic applications, such as sensors and transparent substrates. In summary, this work highlights the remarkable versatility of chitosan-based films, demonstrating that their mechanical, solubility, and optical properties can be precisely controlled by adjusting composition. These findings open promising avenues for the development of sustainable, multifunctional materials tailored for a diverse range of applications, spanning flexible electronics, biomedicine, and advanced coatings.

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Chapter 2 - Sustainable Chitosan-Based Elastomer Nanocomposites: Enhancing Piezoelectric Properties for Green Sensors and Actuators

Short Abstract:

We developed a fully biobased piezoelectric elastomer by enhancing chitosan films with chitin and surface deacetylated chitin nanocrystals. The piezoelectric coefficient, d_{33} , reached 18.7 pmV^{-1} for films with 20 wt% chitin nanocrystals, nearly doubling the value of pure chitosan (8.9 pmV^{-1}). An accurate Piezoresponse Force Microscopy (PFM) technique was introduced to enable the decoupling of piezoelectric and electrostatic responses, with d_{33} values precisely quantified. These advances demonstrate the potential of bio-based elastomers for sustainable, high-performance sensors and transducers.

1. Introduction

Piezoelectricity is a fundamental property of certain materials, enabling them to generate an electrical charge when subjected to mechanical stress. This phenomenon arises due to the asymmetric nature of a material's crystal structure, which induces polarization upon deformation. While piezoelectricity is commonly associated with engineered materials, it is also prevalent in natural systems, playing a vital role in the mechanical and electrical functions of living organisms^{1–5}. In plants, piezoelectric properties have been observed in structural components such as silk¹ and cellulose nanocrystals², where they contribute to mechanical stability and responsiveness to environmental forces. Similarly, in animals, piezoelectric behavior is found in tissues, particularly in collagen-based structures, where it plays a crucial role in processes like bone remodeling and tissue repair³. Various proteins and peptides, including glycine crystals and diphenylalanine nanotubes, also exhibit piezoelectric characteristics⁴ that are valuable in bioelectronic applications. A noteworthy example is the M13 phage virus, which, with its helical protein-based structure, can form highly ordered piezoelectric assemblies⁶. Additionally, human skin exhibits an intrinsic dipole moment in the epidermis, which responds to mechanical stimuli, generating bioelectrical signals essential for sensory perception and physiological regulation⁷. A frequently employed metric for measuring piezoelectricity is the d_{33} coefficient, which measures the material's ability to generate electric charge in response to mechanical stress. Specifically, it represents the charge generated along the axis of polarization (typically the z-axis) when the material is subjected to an applied force along the same axis. This coefficient is critical in evaluating and comparing the piezoelectric efficiency of different materials.

The convergence of piezoelectric materials with micro- and nanotechnologies has led to groundbreaking advances in biomedicine, bioelectronics, and healthcare^{8–11}. These innovations range from wearable sensors for continuous health monitoring to precise actuators designed for medical applications. As research progresses in these fields, the demand for biocompatible and mechanically flexible piezoelectric materials has significantly increased^{12–14}. Traditional piezoelectric materials, such as lead zirconate titanate (PZT) and barium titanate (BTO), offer high piezoelectric coefficients^{15–17} (typically in the range of 200–600 pmV⁻¹) but suffer from significant limitations, including mechanical rigidity¹⁸, brittleness¹⁹, and environmental toxicity^{20,21}. Their production and disposal raise serious ecological concerns, which has accelerated the search for sustainable alternatives. Among synthetic piezoelectric polymers, polyvinylidene fluoride (PVDF) is commonly used due to its flexibility^{11,22–25}; however, the fluorine-based chemistry involved creates environmental issues²⁶. The following table presents an overview of d_{33} values for various flexible synthetic polymers, along with their manufacturing methods and measurement techniques.

Table 2.1. Manufacturing technology, measuring piezoelectric technique and piezoelectric coefficient d_{33} values for different soft polymers

Material	d_{33} [pmV ⁻¹]	Measurement technique	Processing	Reference
Silicone rubber + Barium titanate	22.5	Acquisition Voltage circuit	Mixing, moulding polarization and curing.	²⁷
Polyacrylonite	40	-	-	²⁷

Thermosetting silicone + PZT	60	-	-	27
PDMS + PVDF	8	-	-	27
PDMS + poly(siloxane-imide)	0.4 to 6.1 (depending on PI %)	PFM	Polycondensation and chemical imidization	23
PDMS + PI	0.5 pm /V (pure PDMS); from 17 pm/V to 26 pm/V (depending on PI%)	PFM	Polycondensation	24
PVDF + polyacrylonitrile	63	Dynamic mechanical tester	Radical polymerization and freeze-drying processes	22
Chloroprene rubber + 60 vol% PZT	244	-	-	28
Poly(acrylonitrile butadiene) rubber + 80 vol% PMN	33	-	-	28
PDMS + 43 vol % PZT	6.8	-	-	28
PDMS + 8 vol % BCZT	31	-	-	28
PDMS + 50 vol % PZT	101	-	-	28
PDMS + 20-60 vol % PZT	80 - 240	-	-	28
CNA-doped polyurethane foams	244	-	-	28
PDMS + 33–40 wt% poly [(MMA)-co-(DR1-MA)]	27	-	-	28
PDMS + 30 wt% polar polynorbornene	37	-	-	28
Polar smectic bent-core liquid crystal material confined in a biocompatible triblock copolymer (BCLC/SIBSTAR composite).	1 nm/V	Mirau interferometry	0.5 and 1 mm thick elastomer films were made by solvent casting followed by compression molding under 50 atm pressure that was released at 60 μ C, when the liquid crystal component was in the X-phase.	29

While synthetic piezoelectric polymers offer the advantage of flexibility, which expands the potential applications of piezoelectric devices, they often require the addition of toxic ceramic reinforcements, such as PZT, to enhance their properties. Additionally, the production and disposal of these materials raise environmental concerns, due to the energy-intensive processes involved and their non-biodegradability.

In response to these challenges, there has been growing interest in natural, bio-based piezoelectric materials³⁰. These materials often feature well-organized hierarchical structures with low symmetry, allowing for efficient electromechanical interactions^{9,31}. Building on the earlier mention of naturally occurring piezoelectric materials, it is crucial to highlight their systematic characterization in terms of piezoelectric performance. While initial observations have established the presence of piezoelectricity in biological systems such as silk, collagen, and cellulose nanocrystals, their properties have been further investigated using standardized measurement techniques. These biopolymers, despite their relatively lower d_{33} coefficients compared to conventional piezoelectric materials, offer unique advantages due to their intrinsic sustainability and natural availability.

The following table provides a summary of the piezoelectric coefficients (d_{33}) for various biopolymers, highlighting their respective measurement techniques and processing methods.

Table 2.2. Manufacturing technology, measuring piezoelectric technique and piezoelectric coefficient d_{33} values for different bio-polymers.

Material	Piezoelectric coefficient [pmV ⁻¹]	Measurement technique	Processing	Reference
Diphenylalanine (Peptide)	$d_{15}= 60$	PFM	Self-assembly nanotube	5
Collagen fibrils (Protein)	$d_{15}= 1$	-	-	3
Collagen (Protein)	$d_{14}= 12$	PFM	Use of fibrillary rat tail collagen	8
Phage Nano Pillars (PNP)	$d_{33}= 10.4$	Linear motor (periodic load) and output voltage	Self-assembly	6
Cellulose NCs (Polysaccharides)	$d_{33}= 19.3$	-	-	2
Lysozyme (Protein)	$d_{33}= 6.5$	Piezometer	Drop casting	8
Cysteine (Amino acid)	$d_{22}= 11$	-	-	8
Glycine (Amino acid)	$d_{33}= 4.7, d_{16}= 178$	Piezometer	Drop casting	4
Silk thin film	$d_{33}= 56.7$	PFM	Spin coating	1,32
Proline Amino acid	$d_{25}= 27.5$	Impedance/gain phase analyser	Growing of crystals	8,33

As can be observed, this category of biopolymers exhibits relatively modest piezoelectric coefficients, generally not exceeding a few tens of pm/V. However, their exceptional value lies in their origin from renewable and biologically derived sources, making them inherently sustainable and environmentally friendly. Additionally, their fabrication processes are often straightforward, requiring minimal energy input compared to traditional piezoelectric materials. Notably, many of these biopolymers possess a natural propensity for self-assembly, forming hierarchical structures such as nanotubes or nanopillars.

State of the Art in Piezoelectric Chitosan Film

Chitosan, a biopolymer derived from chitin, has garnered significant attention due to its abundance^{34,35}, cost-effectiveness, and desirable properties. The sustainable origin of chitosan, coupled with its biocompatibility³⁶, biodegradability³⁷, and ease of thin-film formation^{38,39}, makes it an ideal candidate for various applications, ranging from biomedical engineering to flexible electronics^{40–42}. Research on the electromechanical properties of chitosan began several years ago⁴³, with recent studies focusing on its performance in thin-film configurations^{44,45}. However, despite its advantages, pure chitosan films exhibit relatively weak piezoelectric properties⁴⁶, with a d_{33} coefficient typically ranging from 5–9 pmV^{-1} ^{30,47}, limiting their application in energy conversion technologies.

To improve these properties, researchers have explored strategies to enhance the piezoelectric response of chitosan-based materials. One approach involves creating composite materials by integrating chitosan with other piezoelectric components. Recent studies suggest that incorporating poly-hydroxybutyrate (PHB) into chitosan films can enhance their crystallinity and, consequently, improve their piezoelectric behavior⁴⁷. Additionally, blending chitosan with nanocellulose structures—such as cellulose nanofibrils⁴⁸ and cellulose nanocrystals⁴⁹—has shown promising results in boosting piezoelectric performance. Another avenue has involved combining chitosan with other piezoelectric materials, like biodegradable glycine⁵⁰, chitin nanofibers, or polyvinylidene fluoride (PVDF)⁵¹, to create flexible and efficient piezoelectric composites. In 2023, a breakthrough was made when optimized neutralization conditions in an alkaline environment led to chitosan thin films achieving d_{33} values of up to 15 pmV^{-1} ²⁵, marking a significant improvement over previous studies.

Despite these advancements, the piezoelectricity of all-natural chitosan elastomers remains underexplored. The ability to fabricate piezoelectric elastomers is particularly exciting for emerging applications in wearable health-monitoring devices^{52–54}, energy harvesting systems, and bioinspired actuators^{55,56}. In our study, we focused on producing elastomers made entirely from natural chitosan, glycerol, and chitin-based nanocrystals. These materials have shown considerable improvements in piezoelectric response, achieving d_{33} values in the range of 15–19 pmV^{-1} . This marks more than a twofold increase compared to pure chitosan films. The enhanced piezoelectricity is attributed to increased crystallinity facilitated by the incorporation of nanostructured chitin components. Notably, unlike conventional techniques that require thermal annealing⁵⁷, mechanical stretching⁵⁸, or electrical poling⁵⁹ to enhance piezoelectric properties, these films exhibit superior performance through a self-organizing nanostructural arrangement, a feature that will be further discussed in the Results and Discussion section. As we move forward in the development of piezoelectric materials, these natural chitosan-based elastomers show promise for use in applications ranging from skin-like sensors and energy scavengers to sustainable, biocompatible electronic devices.

Table 2.3 summarizes the d_{33} values of various chitosan-based materials, highlighting their fabrication methods and measurement techniques.

Table 2.3. Manufacturing technology, measuring d_{33} technique and piezoelectric coefficient d_{33} values for different chitosan-based materials.

Material	d_{33} [pmV^{-1}]	Measurement technique	Processing	Reference
Chitosan thin film (thickness = 15 μm)	15.56	PFM	Solvent casting and neutralization	²⁵
Chitosan film (thickness = 70 μm)	2.54	PFM	Drop casting	⁶⁰

Chitosan pellets (thickness = 1.3 mm)	18.4	PFM	Manual hydraulic pressure	⁴⁵
Chitosan-poly(3-hydroxybutyrate) (CS-PHB) blend thin films (PHB at 13 wt%) (thickness = 40 µm)	5	PFM	Solvent casting	⁴⁷
Chitosan thin film (thickness = 20 µm)	6	Dynamic force sensor	Solvent casting	³⁰
Chitosan-glycine (1:0.8) (thickness= 38 µm)	-	Dynamic pressure using a TIRA shake	Drop casting	⁵⁰
Chitosan - PEDOT nanofibers	-	Pressure and electrochemical system	Electrospinning and H ₂ SO ₄ - treatment for recrystallization	¹⁰
Chitosan film	-	In-house built setup (shaker for dynamic excitation force)	Solvent casting and neutralization	⁴⁶
Chitosan - Glycerol-Chitin/Chitosan Nanocrystals films	15.4 – 18.7	PFM	Solvent casting	Our work

2. Results and Discussion

Synthesis and Structural Characterization of Chitin and Surface-Deacetylated Chitin Nanocrystals

The synthesis of chitin nanocrystals (ChNCs) and surface-deacetylated chitin nanocrystals (CsNCs) was carried out using protocols adapted from the literature, with some modifications to optimize the yield and purity of the nanocrystals. Chitin nanocrystals (ChNCs) were isolated from natural chitin sources, such as crustacean shells, while surface-deacetylated chitin nanocrystals (CsNCs) were obtained by chemically modifying ChNCs through a deacetylation process. This process involves the partial removal of acetyl groups from the chitin structure, which increases the availability of amine groups on the surface of the nanocrystals, making them more reactive and enabling their dispersion within the chitosan matrix.

The characterization of both ChNCs and CsNCs was essential for understanding their size, surface charge, and overall structural integrity. The use of Dynamic Light Scattering (DLS) allowed for the determination of the size distribution of the nanocrystals, with ChNCs measuring an average length of approximately 350 nm and CsNCs slightly smaller at 300 nm. These values suggest that surface deacetylation may lead to a minor change in the crystallinity or size of the nanocrystals, although this effect was relatively small. The Z-potential of both types of nanocrystals was found to be around +55 mV, a value that is typical for nanocrystals with high surface charge⁶¹. This high Z-potential indicates good dispersion stability of the nanocrystals in suspension, which is crucial for the uniform distribution of the nanocrystals within the polymer matrix when fabricating nanocomposite films. Notably, the nanocrystals do not undergo dissolution or redeposition while in solution or during mixing with chitosan. Their colloidal dispersion is stable in acidified aqueous solutions, as those used to form chitosan-based films, for several months.

To further explore the structural integrity of the nanocrystals, Fourier Transform Infrared (FT-IR) spectroscopy was performed. The FT-IR spectra of both ChNCs and CsNCs exhibited characteristic peaks for chitin⁶², including the amide I (C=O stretching) and amide II (N-H bending) bands. These results confirm that both types of nanocrystals retained the fundamental structure of chitin even after the deacetylation process. The degree of deacetylation (DD) was quantitatively determined through CHNS analysis, which revealed a DD of 4.6% for ChNCs and 36.8% for CsNCs. This indicates that the surface deacetylation process was successful in increasing the availability of amino groups on the surface of the nanocrystals, which would enhance their interaction with the chitosan matrix in the subsequent nanocomposite films.

Film Preparation and Characterization

Following the synthesis of ChNCs and CsNCs, a series of nanocomposite films were fabricated by incorporating these nanocrystals into a chitosan matrix. Chitosan was selected as the base polymer due to its unique combination of biocompatibility, biodegradability, and inherent piezoelectric properties. Unlike traditional piezoelectric materials, which often contain heavy metals or non-biodegradable components, chitosan-based films offer a more sustainable and environmentally friendly alternative, making them particularly attractive for biomedical and flexible electronic applications. Furthermore, its ability to form stable transparent films with tunable mechanical and electrical properties further enhances its suitability for these applications⁶³.

To optimize the mechanical flexibility and processability of the films, glycerol was used as a plasticizer. Glycerol interacts with chitosan at a molecular level by disrupting intermolecular hydrogen bonds, thereby increasing polymer chain mobility and imparting improved flexibility to the films. The choice of glycerol as a plasticizer was based on its non-toxic nature, ease of blending, and previous studies indicating its ability to enhance the mechanical performance of chitosan-based films without significantly compromising their functional properties⁶⁴.

A series of nanocomposite films with varying compositions was prepared to systematically investigate the impact of nanocrystals on the physical, mechanical, and piezoelectric properties of the films. The compositions included:

- **C₁₀₀ (pure chitosan film):** A control sample composed solely of chitosan, serving as a baseline reference for evaluating the effects of nanocrystal incorporation.
- **C₄₀G₄₀ChNCs₂₀ (chitosan film with 40 wt% glycerol and 20 wt% ChNCs):** This formulation was designed to assess how the introduction of chitin nanocrystals (ChNCs) influences the structural and functional properties of the chitosan matrix.
- **C₄₀G₄₀CsNCs₂₀ (chitosan film with 40 wt% glycerol and 20 wt% CsNCs):** Similar to the previous formulation, but incorporating surface-deacetylated chitin nanocrystals (CsNCs) instead of ChNCs, allowing for a comparative evaluation of how surface chemistry affects film performance.
- **C_{47.5}G_{47.5}CsNCs₅ (chitosan film with 5 wt% CsNCs):** A lower nanocrystal concentration sample aimed at understanding the minimum threshold required for observable enhancements in piezoelectric and mechanical behavior.
- **C₃₀G₃₀CsNCs₄₀ (chitosan film with 40 wt% CsNCs):** A high-nanocrystal-content film intended to determine whether increasing CsNC loading leads to proportional improvements in film performance or if saturation effects occur.

- **C₅₀G₅₀ (chitosan-glycerol reference sample):** A formulation containing no nanocrystals, included to isolate the impact of glycerol on the film properties and provide a secondary control sample.

These compositions were carefully selected to evaluate the correlation between nanocrystal content, crystallinity, mechanical strength, and piezoelectric performance. By systematically varying the concentration of nanocrystals, we aimed to identify an optimal formulation that balances flexibility, durability, and piezoelectric efficiency. Given that increasing nanocrystal content can enhance crystallinity but may also lead to increased brittleness, these formulations provide critical insights into the trade-offs involved in optimizing chitosan-based nanocomposite films for practical applications.

In particular, from a mechanical perspective, the inclusion of glycerol significantly altered the properties of the films compared to pure chitosan, resulting in a more elastomeric behavior. This was characterized by a non-linear stress-strain curve, with large deformation at low stress and the absence of yield strength. In composite films containing 40 wt% glycerol and 20 wt% ChNCs or CsNCs, a promising balance between stiffness and flexibility was achieved. These films exhibited elongation at break values of approximately 40%, a maximum tensile strength of around 40 MPa, and a Young's modulus of about 100 MPa. Notably, these values closely resemble those found in biological tissues such as tendons and muscles, suggesting potential applications in fields like prosthetics and soft robotics.

The films were cast using an automatic applicator onto glass and Kapton substrates, ensuring a uniform distribution of material across the surface. The slight variation in thickness, ranging from 15 to 40 μm , is consistent with the different compositions of the films, as those containing nanocrystals and glycerol naturally resulted in a slightly increased thickness due to the additional components. The films were characterized in terms of their transparency, flexibility, and stretchability. The flexibility and stretchability of the films were qualitatively assessed by bending and stretching them under different conditions. All films demonstrated the ability to deform easily without breaking, highlighting their potential for applications in flexible electronics and wearable devices.

X-ray Diffraction (XRD) Analysis

X-ray Diffraction (XRD) analysis was performed to investigate the crystallinity of the fabricated films, as structural ordering significantly influences their mechanical, thermal, and functional properties, including their piezoelectric response. The XRD spectra of the samples, shown in Figure 3.1, highlight the differences in crystalline organization among the various film compositions.

The partial crystallinity of pure chitosan films C₁₀₀ (blue curve) is indicated by two distinct peaks at approximately $\sim 9^\circ$ and $\sim 12^\circ$, along with a weaker third peak around $\sim 19^\circ$. These results align with previous studies^{25,65,66}. This diffuse peak indicates a limited but still present degree of structural ordering, which aligns with the semi-crystalline nature of neat chitosan. In contrast, the C₅₀G₅₀ film (green curve) exhibited no distinct diffraction peaks, confirming its entirely amorphous nature.

In contrast, the spectra of C₄₀G₄₀ChNCs₂₀ (black curve) and C₄₀G₄₀CsNCs₂₀ revealed the appearance of well-defined diffraction peaks at approximately 9.5° and 21.0° as previous reported^{67,68}, which correspond to the characteristic crystalline reflections of chitin-based structures. These findings indicate that the incorporation of nanocrystals promotes the formation of more ordered domains within the polymer matrix. The increased crystallinity in these composite films suggests improved molecular alignment, likely driven by intermolecular interactions between the nanocrystals and the chitosan matrix, which promote the formation of ordered domains.

The patterns do not reveal significant differences in crystallinity between C₄₀G₄₀ChNCs₂₀ and C₄₀G₄₀CsNCs₂₀, indicating that both types of nanocrystals contribute similarly to the structural ordering

of the chitosan matrix. This trend suggests that at sufficient concentrations, nanocrystals act as structural templates, facilitating the arrangement of chitosan chains into a more ordered configuration.

Overall, these results confirm that while neat chitosan films exhibit a low degree of crystallinity, the incorporation of nanocrystals promotes the formation of more ordered, crystalline regions within the matrix. This highlights the role of nanocrystals in modulating the structural properties of the chitosan networks.

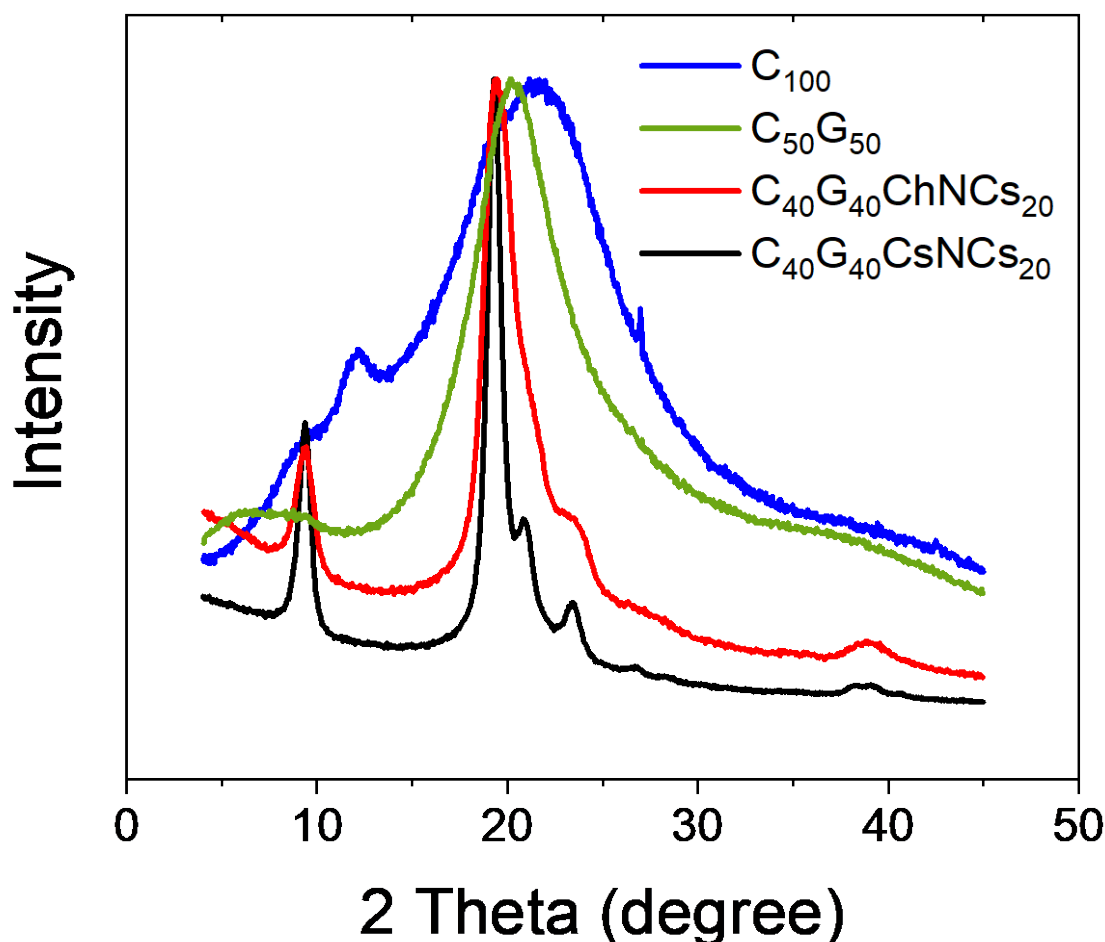


Figure 3.1. XRD pattern of C_{100} (blue curve); $C_{50}G_{50}$ (green curve); $C_{40}G_{40}ChNCs_{20}$ (black curve) and $C_{40}G_{40}CsNCs_{20}$ (red curve).

SEM Analysis

Unlike conventional approaches reported in the literature, our method preserves the pristine chemical structure of the chitosan matrix. Rather than chemically modifying chitosan, we introduce a nanostructured chitin component that, due to its intrinsic similarity to the matrix, ensures high compatibility while simultaneously enhancing the piezoelectric response. The cross-section image of the chitosan–glycerol–chitin nanocrystals thin film, captured using a scanning electron microscope (SEM) (Figure 3.2) , reveals the presence of well-aligned planes within the composite structure. This morphological organization is particularly intriguing, as it suggests a preferential ordering at the nanoscale. While further studies are necessary to fully elucidate the underlying mechanism governing this preferential ordering and dipole alignment, we hypothesize that it may stem from the self-assembly of chitin nanocrystals into chiral nematic structures resembling Bouligand formations. These hierarchical arrangements, commonly observed in crustacean shells^{69–72}, are known to impart

anisotropic properties and could play a key role in directing dipole orientation within the film. If this hypothesis were to be confirmed, it could provide valuable insights into how natural self-organization processes contribute to functional properties in bio-based materials.

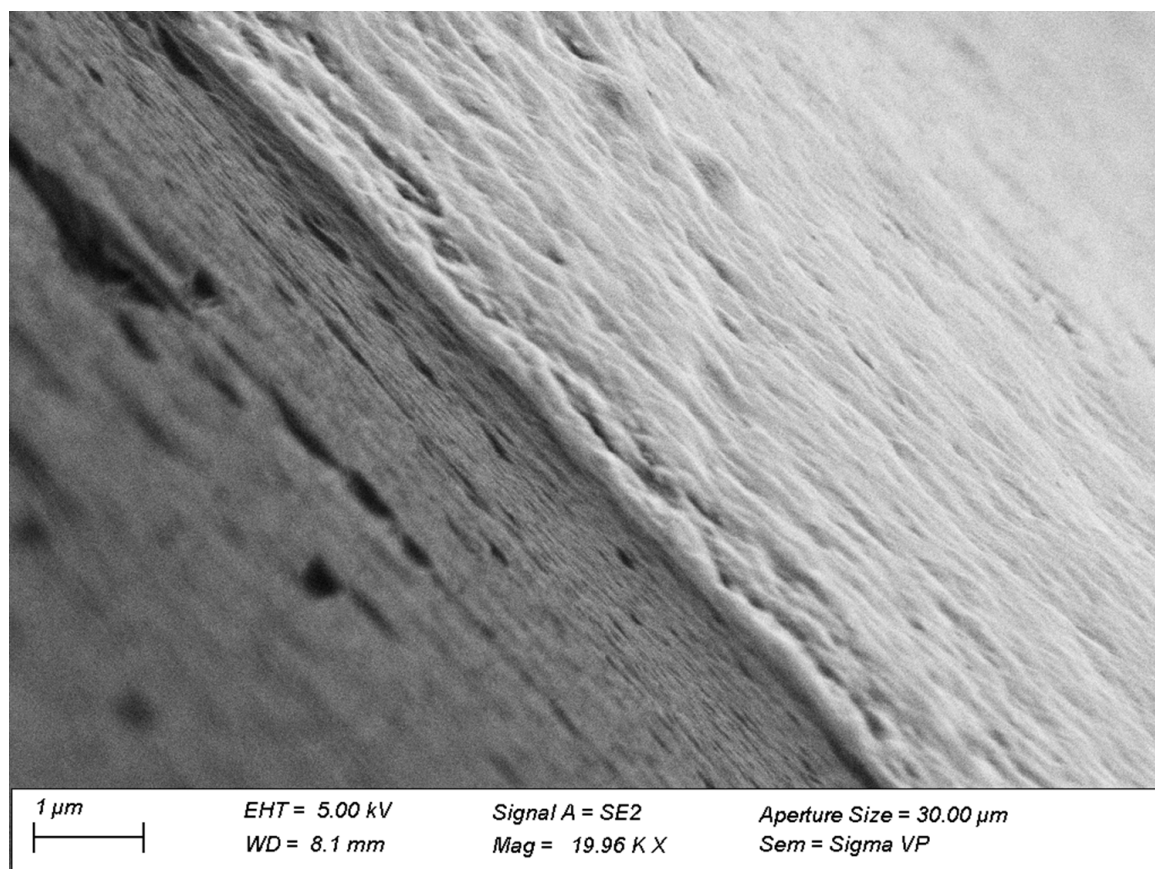


Figure 3.2. Cross section image of chitosan – glycerol – Chitin Nanocrystals thin film taken with a scanning electron microscope

Piezo Force Microscopy (PFM) Analysis of Nanocomposites Chitosan Films

Both Atomic Force Microscopy (AFM) in tapping mode and Piezo Force Microscopy (PFM) were employed to conduct a topographic analysis and assess the electromechanic properties of the films. To minimize the risk of damaging the soft material and ensure high sensitivity for precise topographical characterization, both AFM and PFM were carried out using tips with low spring constants (0.4 N/m and 0.1 N/m, respectively). This approach allowed for the careful exploration of the surface morphology and piezoelectric properties without affecting the integrity of the films.

The topographic analysis of the composite films, specifically $C_{40}G_{40}ChNCs_{20}$ and $C_{40}G_{40}CsNCs_{20}$ (Figure 3.3 (c) and (d)), revealed that the nanocrystals were uniformly distributed within the chitosan matrix, forming intricate networks of interconnected domains. This uniform distribution was consistently observed even at higher concentrations of nanocrystals, as demonstrated in Figure 3.4 (a) for $C_{30}G_{30}CsNCs_{40}$.

Moreover, these structures continued to be visible in the AFM topography images (Figure 3.4 (b) and (c)), which were captured at a larger scan size of $10\ \mu m \times 10\ \mu m^2$ for the $C_{40}G_{40}ChNCs_{20}$ and $C_{40}G_{40}CsNCs_{20}$ films. At this scale, the topographical features associated with the nanocrystal

distribution provide a broader view, contributing to a better understanding and enhanced observation of the material's surface morphology.

The surface roughness of the films was also measured, with pure chitosan exhibiting a smooth surface with roughness of approximately 0.5 nm, while nanocomposite films displayed higher roughness values ranging from 3.0 to 5.0 nm. These roughness variations are likely due to the presence of the nanocrystals, which create surface irregularities as they interact with the polymer matrix.

Out-of-plane Piezoelectric Force Microscopy (PFM) phase imaging was employed to visualize and map the variations in piezoelectric response across the sample surface, providing high spatial resolution. This technique is essential for gaining insights into the electromechanical behavior of the materials, specifically in revealing piezoelectric or ferroelectric domains, as well as their switching behavior.

To generate the necessary electric field, a conductive gold substrate was used, while the conductive PFM cantilever served as the secondary electrode for the measurement process.

In our experiments, the applied AC voltage (V_{AC} bias) was set to 3 V, while the DC voltage (V_{DC}) was fine-tuned to approximately 0.4 V, which is a value consistent with the surface electrostatic potential (V_{EL}) observed across all samples, as detailed in the following sections. This particular DC voltage was carefully selected to minimize the influence of electrostatic forces on the cantilever's oscillations, thereby allowing a more pronounced piezoelectric response to be captured during the measurements. In pure chitosan films (C_{100}), the phase image was homogeneous, with no clearly observable piezoelectric domains. However, in films incorporating 20 wt% of nanocrystals ($C_{40}G_{40}ChNCs_{20}$ and $C_{40}G_{40}CsNCs_{20}$), alternating regions exhibiting a phase shift between adjacent domains were observed. This bimodal phase distribution, with two populations separated by 180° , might initially suggest piezoelectric behavior within the films. However, since the measurements were conducted at a fixed frequency (the cantilever's resonance frequency) and considering the films' soft nature, these variations could stem from subtle alterations in contact forces during the scanning process. To resolve these uncertainties, future investigations could employ Dual Frequency Resonance Tracking (DFRT), a technique that would enable a more precise distinction between the contributions of surface topography and electromechanical properties. Such an approach would offer greater confidence in the interpretation of the observed phase shifts, ensuring that they reflect true piezoelectric effects rather than being influenced by surface morphology or mechanical compliance.

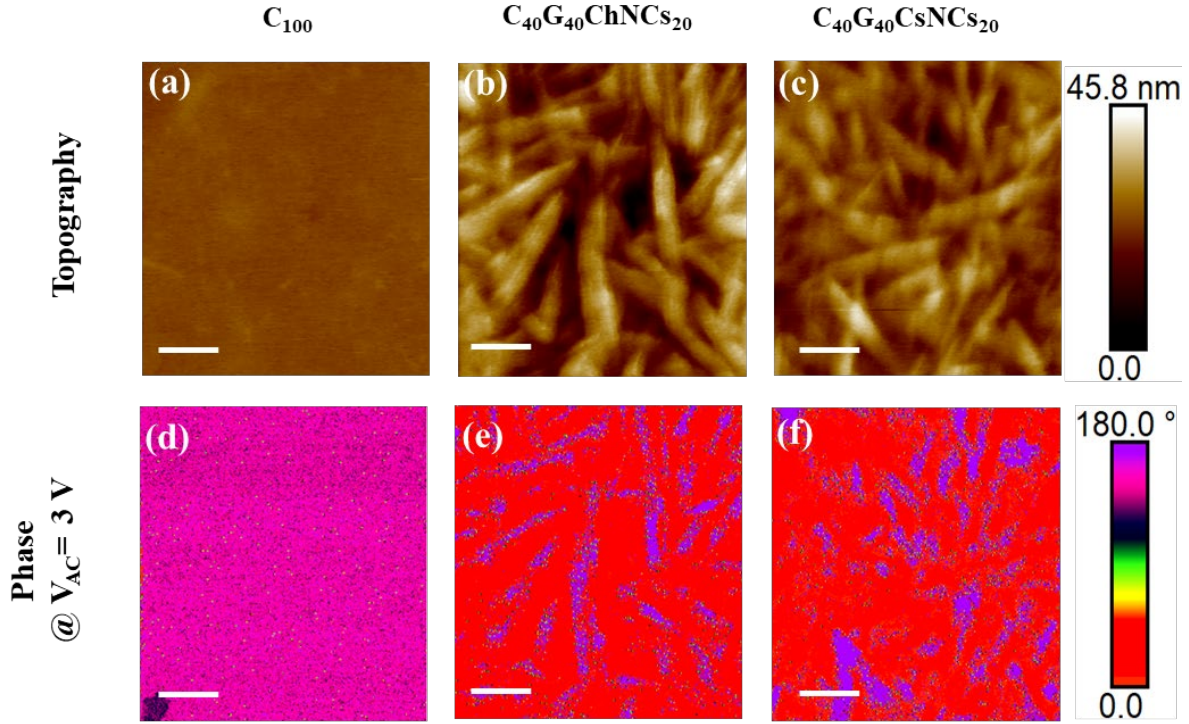


Figure 3.3. PFM images showing the topography (a-c) and phase at $V_{AC} = 3V$ (d-f) for C_{100} , $C_{40}G_{40}ChNCs_{20}$, and $C_{40}G_{40}CsNCs_{20}$. Each subplot shares a scale bar of 200 nm, while the color scales on the right correspond to their respective row.

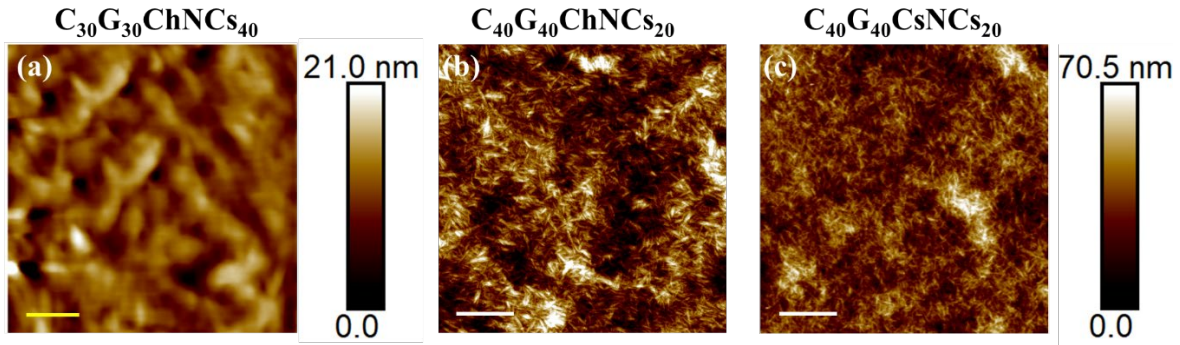


Figure 3.4. (a) PFM topography image of $C_{30}G_{30}CsNCs_{40}$, with a yellow scale bar representing 200 nm. (b, c) AFM topography images of $C_{40}G_{40}ChNCs_{20}$ and $C_{40}G_{40}CsNCs_{20}$, respectively. Both scans cover an area of $10 \times 10 \mu m^2$, with a white scale bar of 2 μm in each plot. The color scales on the right correspond to both images.

Quantification of Piezoelectric Coefficient (d_{33})

In this study, we investigated films composed of different ratios of chitosan, glycerol, and nanocrystals (NCs), with the goal of exploring how the nanocrystal content influences the piezoelectric behavior of the films. The films studied included pure chitosan (C_{100}), a chitosan-glycerol film ($C_{50}G_{50}$) to assess the influence of glycerol on piezoelectric properties, films with 20 wt% of chitin nanocrystals (ChNCs) and 20 wt% of surface-deacetylated chitin nanocrystals (CsNCs) ($C_{40}G_{40}ChNCs_{20}$ and $C_{40}G_{40}CsNCs_{20}$, respectively), as well as those with varying amounts of CsNCs ($C_{47.5}G_{47.5}CsNCs_5$ and $C_{30}G_{30}CsNCs_{40}$). These compositions were chosen to observe how different types and concentrations of nanocrystals, particularly ChNCs and CsNCs, would affect the electromechanical properties and piezoelectric

response of the films. As evident in the composite films, the chitosan-to-glycerol ratio was consistently maintained at 1:1, while the nanocrystal content was systematically varied to isolate and highlight their specific contribution to the piezoelectric properties.

One of the major challenges in using PFM for this purpose is the coexistence of both electrostatic and piezoelectric effects, which can overlap and complicate the interpretation of the results. In typical PFM measurements, the cantilever experiences both electrostatic forces, which arise from capacitive coupling with the sample surface, and piezoelectric forces, which are related to the material's electromechanical response. This dual contribution complicates the interpretation, as both effects can influence the displacement signal recorded by the cantilever.

Capacitive Coupling and Electrostatic Contribution

As illustrated in Figure 3.5 (a), the capacitive coupling between the cantilever and the sample is modeled as a capacitor in our experimental setup. When both a sinusoidal voltage V_{AC} and a constant voltage V_{DC} are applied to the substrate, the cantilever displacement Z is recorded. The displacement Z is influenced by both the electrostatic and piezoelectric effects, with **Equation 4**⁷³:

$$Z = d_{33}V_{AC} + \frac{1}{k} \frac{\partial C}{\partial Z} V_{AC} |V_{DC} - V_{EL}| \quad (4)$$

describing the total displacement. In this equation, k is the cantilever spring constant (0.1 N/m), and $\frac{\partial C}{\partial Z}$ represents the capacitive coupling between the cantilever and the sample, which varies with the cantilever displacement Z . The total displacement Z is thus composed of two components: the piezoelectric contribution, which depends on V_{AC} and is independent of V_{DC} , and the electrostatic contribution, which becomes more pronounced as the V_{DC} moves away from the electrostatic potential V_{EL} .

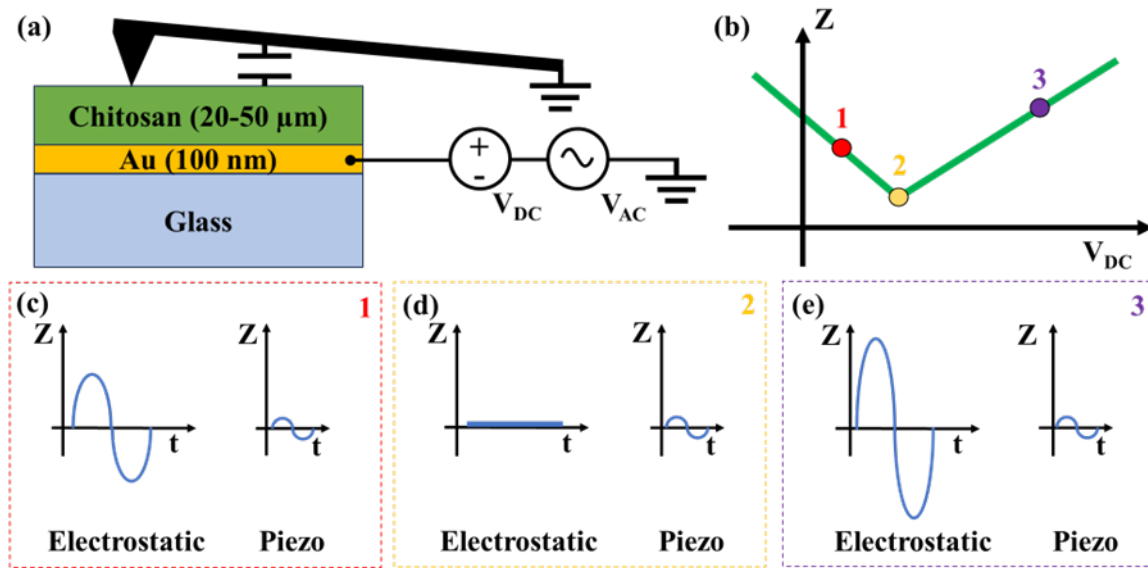


Figure 3.5. (a) Sketch illustrating the grounded PFM cantilever interacting capacitively with the sample, where an AC and DC voltage are applied to the gold electrode. (b) Variation of the biopolymer's

displacement in response to the applied VDC. (c-e) Analysis of the displacement Z, highlighting the separation of electrostatic and piezoelectric contributions at three selected points

Electrostatic and Piezoelectric Interactions

Figure 3.5 (b) shows that when the displacement Z is plotted as a function of V_{DC} , the curve takes on a V-shape, with the minimum occurring at $V_{DC}=V_{EL}$, where the electrostatic forces are exactly counterbalanced by the applied V_{DC} . At this point, the displacement is solely due to the piezoelectric contribution. At values of V_{DC} that deviate from V_{EL} the electrostatic influence increases, leading to larger displacements. This is most clearly seen in Figure 3.5 ((c), (d) and (e)); where larger distance between V_{DC} and V_{EL} result in larger displacement responses. Given this, accurate determination of the piezoelectric coefficient d_{33} requires careful extrapolation of the displacement Z as a function of V_{AC} , measured at $V_{DC}=V_{EL}$ to avoid contributions from electrostatic forces (Figure 3.6). Interestingly, in previously reported techniques, d_{33} was determined by keeping $|V_{DC} - V_{EL}|$ constant^{73,74}.

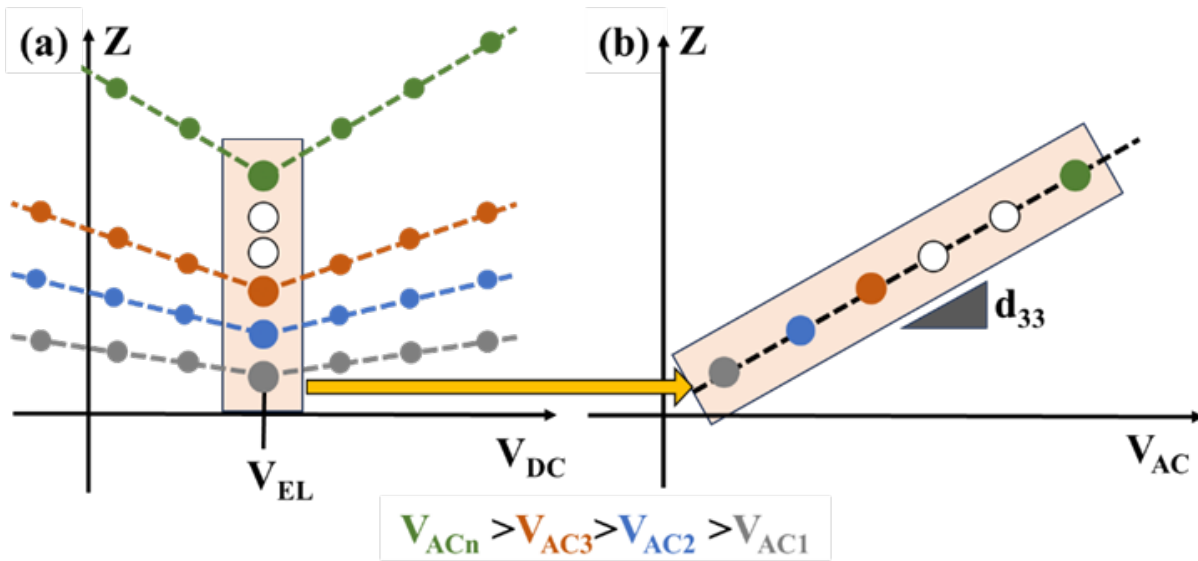


Figure 3.6. (a) Sketch illustrating plot of Z vs V_{DC} for various V_{AC} . (b) Focus on d_{33} extraction mechanism.

Estimation of Piezoelectric Coefficient d_{33}

To determine the piezoelectric coefficient d_{33} we performed a series of experiments in which we swept V_{DC} from -1.75 V to 1.75 V in increments of 0.25 V, while simultaneously measuring the displacement Z for various V_{AC} values ranging from 0.5 V to 3 V. The results of these tests for the compositions C_{100} , $C_{40}G_{40}ChNCs_{20}$, and $C_{40}G_{40}CsNCs_{20}$ are presented in Figure 3.7. As seen in the displacement vs. V_{DC} curves, each exhibits a V-shape, with higher values of V_{AC} leading to larger displacements

To account for any variations in the cantilever resonance, we normalized the displacement values by the resonance quality factor Q of the cantilever and the amplification factor of the lock-in amplifier. The precise value of V_{EL} for each V_{AC} was determined by fitting the displacement Z as a function of V_{DC} , and identifying the point where the descending and ascending slopes of the displacement curve intersect. This allowed us to accurately determine $V_{DC}=V_{EL}$, the point where electrostatic forces are perfectly counterbalanced. Using this value, we interpolated the experimental points of displacement Z as a function of V_{AC} and extrapolated the piezoelectric coefficient d_{33} . Measurements were repeated at five different points for each sample to ensure accuracy, and the resulting mean and standard deviations are shown in Figure.

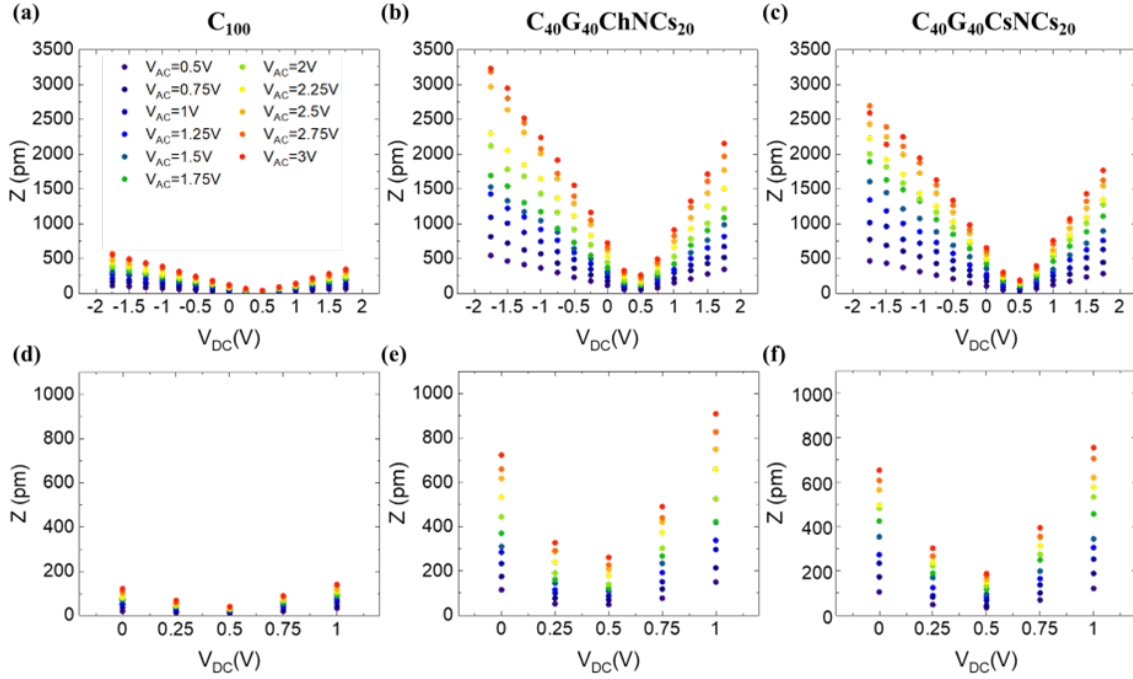


Figure 3.7. (a-c) Graphs and (d-f) zoomed-in views illustrating the displacement Z as a function of V_{DC} (-1.75 to 1.75 V, with 0 to 1 V in the zoomed-in views) for different V_{AC} (0.5 to 3 V) for C_{100} (a,d), $C_{40}G_{40}ChNCs_{20}$ (b,e), and $C_{40}G_{40}CsNCs_{20}$ (c,f). The data shown are prior to normalization by the cantilever resonance curve's quality factor (Q)

Results and Analysis

The obtained values for d_{33} highlight the strong influence of nanocrystal content on the piezoelectric properties of the films (Figure 3.8). Specifically, the pure chitosan film (C_{100}) exhibited a d_{33} of 8.9 pm/V, whereas the introduction of nanocrystals led to a marked enhancement in piezoelectric response. Films containing 20 wt% of ChNCs ($C_{40}G_{40}ChNCs_{20}$) and 20 wt% of CsNCs ($C_{40}G_{40}CsNCs_{20}$) displayed d_{33} values of 18.7 pm/V and 15.4 pm/V, respectively, demonstrating a nearly twofold increase compared to C_{100} .

$C_{47.5}G_{47.5}CsNCs_5$, with only 5 wt% CsNCs, exhibited a d_{33} value close to zero, similar to the $C_{50}G_{50}$ control film. This suggests that at low nanocrystal concentrations, their distribution within the polymer matrix is insufficient to generate a detectable piezoelectric effect. In contrast, $C_{30}G_{30}CsNCs_{40}$, containing 40 wt% CsNCs, reached a d_{33} of approximately 13 pm/V, a value comparable to $C_{40}G_{40}CsNCs_{20}$.

Interestingly, the piezoelectric response did not continue to increase proportionally with the concentration of nanocrystals. For example, the $C_{30}G_{30}CsNCs_{40}$ film, which contained 40 wt% nanocrystals, did not exhibit a significantly higher d_{33} value than the films with lower nanocrystal content. This suggests that there is an optimal concentration of nanocrystals beyond which further increases in nanocrystal content do not result in a proportional enhancement of piezoelectricity.

The results indicate indeed that nanocrystals enhance piezoelectric performance, but only when their concentration exceeds a certain threshold. Films with 20 and 40 wt% of NCs exhibit superior piezoelectric behavior, whereas lower concentrations do not contribute meaningfully to electromechanical performance.

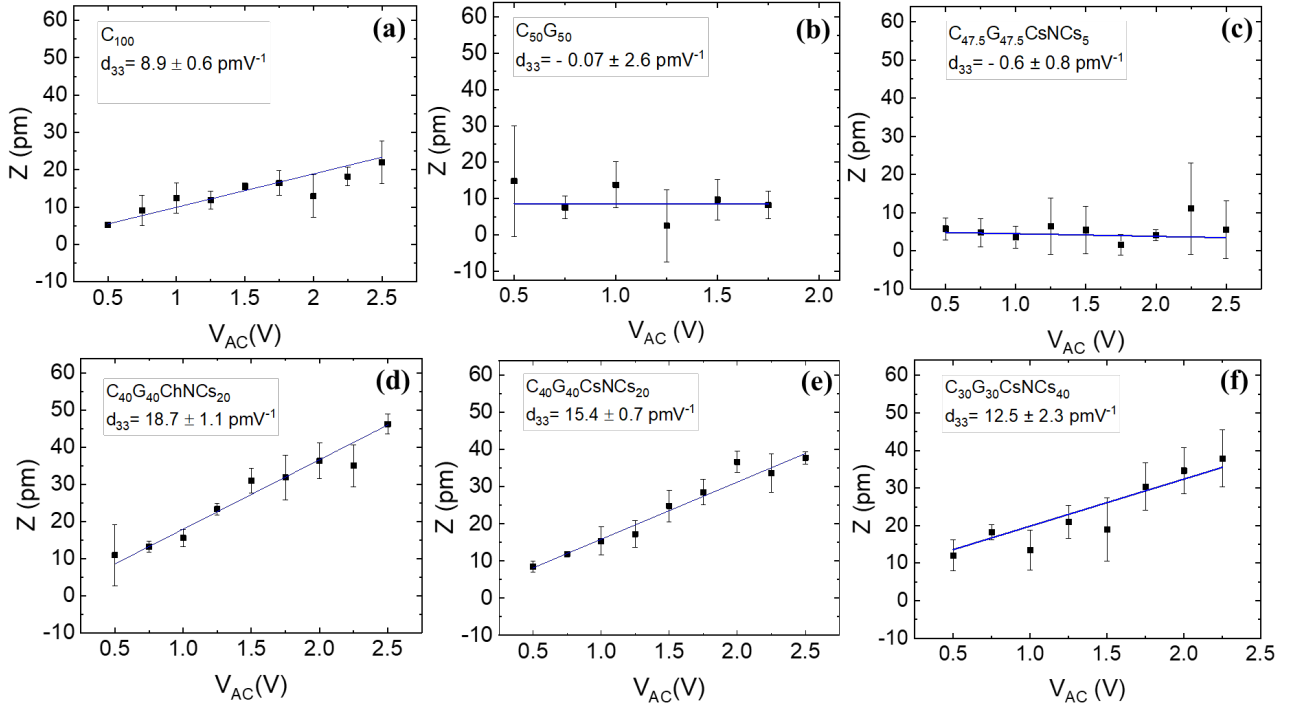


Figure 3.8. Average on 4 points and piezoresponse Z as a function of V_{AC} fitted linearly for (a) C_{100} ; (b) $C_{50}G_{50}$; (c) $C_{47.5}G_{47.5}CsNCs_5$; (d) $C_{40}G_{40}ChNCs_{20}$; (e) $C_{30}G_{30}CsNCs_{40}$; (f) $C_{47.5}G_{47.5}CsNCs_5$. The slope of the curves, hence d_{33} , is printed on the graphs.

Calibration and Comparison with negative control Gold sample

The piezoelectric coefficient d_{33} for pure chitosan films obtained in this study is consistent with values reported in previous literature, thereby validating our experimental approach. However, the key novelty of our work lies in the development of a fully bio-based, elastomeric thin film that combines piezoelectric and mechanical properties in a sustainable and flexible manner, as opposed to the rigid, non-elastomeric films typically studied in existing literature. This flexibility offers a significant advantage for practical applications, particularly in bioelectronics and flexible sensors. Furthermore, our methodology allows for the fine-tuning of both piezoelectric and mechanical properties of these films by simply varying their composition, while maintaining robust piezoelectric performance even at higher nanocrystal content.

To validate the reliability of our method for extracting d_{33} we applied the same analysis technique to a gold (Au) reference sample, known for its non-piezoelectric properties. The resulting d_{33} value for gold was approximately 1.6 pm/V , which is close to the detection limit of the PFM instrument, confirming that gold can be considered non-piezoelectric under our measurement conditions (Figure 3.9). Additionally, the method was calibrated using a lithium niobate (LiNbO_3) standard, adjusting the cantilever's sensitivity to match the known piezoelectric coefficient for this material. The calibration results, shown in Figure 3.10, further confirm the accuracy and effectiveness of our approach in extracting reliable piezoelectric coefficients.

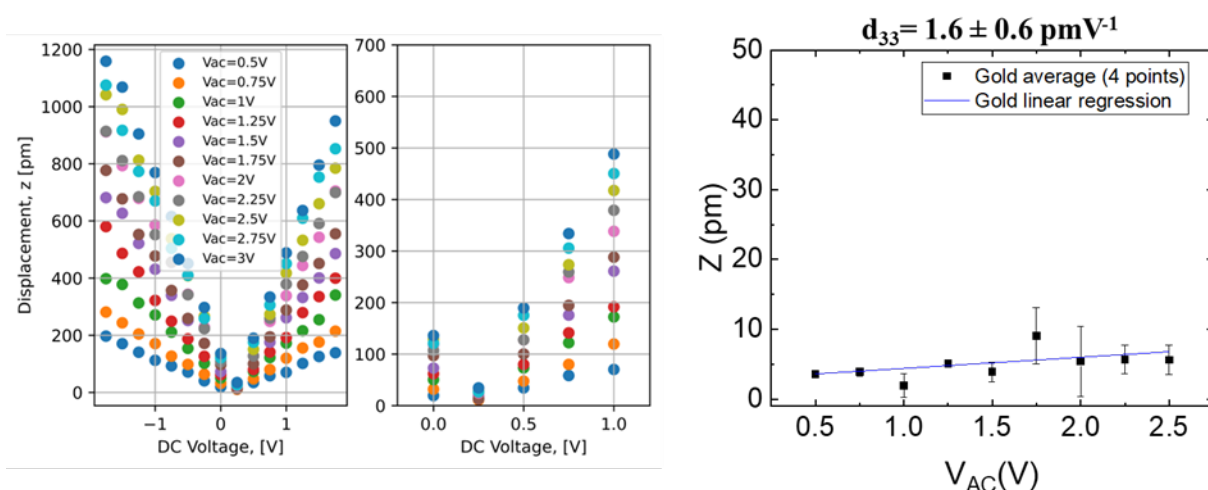


Figure 3.9. PFM Analysis results of a gold negative control sample. On the left a graph showing Z vs V_{DC} for different V_{AC} . On the right the calculated d_{33}

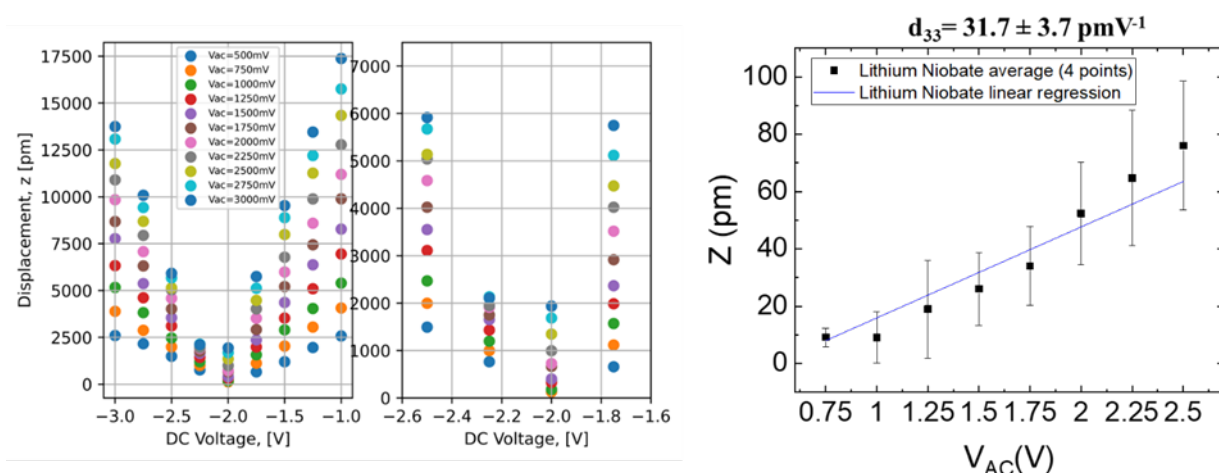


Figure 3.10. PFM Analysis results of Lithium Niobate sample. On the left a graph showing Z vs V_{DC} for different V_{AC} . On the right the extracted d_{33} used for the calibration process.

3. Materials and Methods

Materials

High-molecular-weight chitosan, glycerol (MW 92.09 g/mol), chitin flakes, and acetic acid (99 wt%) were obtained from Sigma Aldrich. Deionized water was used for all preparations.

Synthesis of Chitin Nanocrystals (ChNCs) and Surface-Deacetylated Nanocrystals (CsNCs)

ChNCs and CsNCs were synthesized following a modified version of a previously reported method. Chitin flakes (35 mg/mL) were hydrolyzed in a 3M HCl solution under reflux at 100 rpm for 4 hours. The mixture was then cooled, centrifuged (4700g, 15 min), filtered, and repeatedly washed with deionized water. The resulting precipitate was dispersed in water and subjected to sonication (Branson Digital

Sonifier, 40% amplitude, 10 min) to obtain a stable colloidal suspension. ChNCs were recovered by adding a 1M NaOH solution to induce precipitation, followed by washing and drying at 100°C under vacuum for 24 hours. The final concentration of ChNCs in the gel was determined as 11.7 wt%.

For CsNCs synthesis, ChNCs (16 mg/mL) were dispersed in 12.5M NaOH and refluxed under constant stirring (100 rpm) for 12 hours. The mixture was then cooled, purified following the same steps as for ChNCs, and dried to determine the final concentration (15 wt%).

To evaluate the colloidal stability of the nanocrystals, zeta potential analysis was performed. ChNCs and CsNCs were dispersed in acidified water (pH 4.0, 1 mg/mL) and analyzed, yielding values of $+54.1 \pm 0.7$ mV and $+55.4 \pm 1.1$ mV, respectively. These values confirm the stability of the dispersions, as zeta potentials in the range of ± 40 to ± 60 mV typically prevent aggregation⁶².

Fabrication of Thin Films

Six different composition of films were prepared:

- C₁₀₀ (pure chitosan film)
- C₄₀G₄₀ChNCs₂₀ (chitosan film with 40 wt% glycerol and 20 wt% ChNCs)
- C₄₀G₄₀CsNCs₂₀ (chitosan film with 40 wt% glycerol and 20 wt% CsNCs):
- C_{47.5}G_{47.5}CsNCs₅ (chitosan film with 5 wt% CsNCs)
- C₃₀G₃₀CsNCs₄₀ (chitosan film with 40 wt% CsNCs):
- C₅₀G₅₀ (chitosan-glycerol reference sample):

Pure chitosan films were obtained by dissolving chitosan (250 mg) in 25 mL of aqueous acetic acid (1% v/v). For nanocomposite films, solutions were prepared by dissolving 200 mg of chitosan and 200 mg of glycerol in 25 mL of acetic acid (1% v/v), followed by the addition of the appropriate amount of hydrated ChNCs (855 mg) or CsNCs (667 mg) to reach the desired 20% or 40 % NCs concentration.

The films were cast using a solvent casting method with an automatic applicator (SAFA-219, S.A.M.A. Italia). After stirring and sonication (30 min), the solutions were deposited onto substrates. For XRD analysis, Kapton sheets were used as casting substrates to facilitate film removal. For AFM/PFM analysis, films were cast directly onto conductive gold-coated glass slides (100 nm gold layer, 2.5×3 cm²). In this case, films were applied to cover approximately $\frac{3}{4}$ of the slide, leaving a portion of the gold surface exposed to provide electrical contact for PFM measurements.

XRD Analysis

X-ray diffraction (XRD) patterns were recorded using an Empyrean Series 3 diffractometer (Malvern Panalytical). The system was set to operate in Reflection-Transmission Spinner 3.0 mode, using Cu K α 1 radiation ($\lambda = 1.540598$ Å) at 40 kV and 40 mA. The analysis was performed in continuous scanning mode over the 5°–40° range.

AFM Analysis

The experiments were performed using a Bruker Dimension Icon system (Bruker Corporation, Billerica, MA, USA) equipped with a silicon tip mounted on a silicon nitride cantilever (SCM-PIC-V2, Bruker). The probe featured a nominal spring constant of 0.4 N/m, a length of 115 μ m, and an approximate free resonance frequency of 70 kHz. Measurements were carried out in tapping mode.

PFM Analysis

Piezoelectric force microscopy (PFM) measurements were conducted using a Bruker Dimension Icon instrument equipped with a Platinum-Iridium coated conductive silicon probe (SCM-PIC-V2, Bruker). The probe had a nominal spring constant of 0.1 N/m, a length of 450 μm , and a free resonance frequency of approximately 10 kHz. Measurements were performed in contact mode at 40 kHz, corresponding to the resonance frequency of the tip when in contact with the sample surface. An alternating voltage of 3 V was applied during scanning to acquire amplitude and phase images.

The piezoelectric coefficient (d_{33}) was determined through calibration using a standard lithium niobate sample. The calibration process involved applying an extraction method to determine tip sensitivity relative to the known d_{33} values of lithium niobate, as shown in Figure S5.

d_{33} Extraction Procedure:

For each sample, measurements were performed at four different points. The following steps were taken for each point, with biasing applied at various values of V_{DC} and V_{AC} :

1. The displacement-frequency curve (Z-f) was recorded for each V_{DC} value in the range of [-1.75, 1.75] V, with a step of 0.25 V (11 V_{DC} values in total). The maximum value of the Z-f curve, **max{Z-f}**, along with the quality factor, was obtained after interpolation.
2. The **max{Z-f}** values were plotted against their corresponding V_{DC} values, after normalizing for the average quality factor Q .
3. Steps (1) and (2) were repeated for different V_{AC} values, ranging from 0.5 V to 3 V, with 0.25 V increments.
4. The electrostatic voltage (V_{EL}) and the corresponding Z value were determined by performing a linear fit of the Z versus V_{DC} data for each V_{AC} value. V_{AC} values of 2.75 V and 3 V were excluded from the analysis due to observed non-linear effects (refer to Equation 1).
5. The Z values obtained from step (4) were plotted against the corresponding V_{AC} values.
6. The procedure outlined in steps (1) to (5) was repeated for each of the four measurement points on each film type, and the results were linearly interpolated to determine the d_{33} coefficient (the slope of the fitting line).

4. Conclusions

In this study, we successfully developed an innovative bio-based piezoelectric elastomer by enhancing the crystallinity of chitosan thin films through the incorporation of chitin and surface-deacetylated chitin nanocrystals. The structural investigation via X-ray diffraction (XRD) allowed us to correlate crystallinity enhancements with piezoelectric performance, as assessed through Piezoresponse Force Microscopy (PFM). The results demonstrate a significant increase in the piezoelectric coefficient, with the highest recorded value reaching 18.7 pm/V for films containing 20 wt% chitin nanocrystals (ChNCs), compared to 8.9 pm/V for pure chitosan films. Based on our results, it appears that there is a certain concentration of nanocrystals at which the piezoelectric response reaches its maximum enhancement,

beyond which further increases may not result in significant improvements. This suggests that the dispersion and interaction of nanocrystals within the chitosan matrix play a crucial role in optimizing performance therefore identifying the optimal concentration could be key to achieving the best results. Beyond these performance optimizations, this work also introduced a comprehensive and accurate method for assessing the piezoelectric properties of soft films, effectively isolating piezoelectric contributions from electrostatic effects through PFM. The combination of remarkable piezoelectric performance, optical transparency, biocompatibility, and sustainable production through biowaste upcycling highlights the potential of these bio-based elastomers for next-generation transducers and sensors. These materials open new possibilities for applications in soft robotics, prosthetics, advanced human-machine interfaces, and the Internet of Things, paving the way for sustainable, high-performance piezoelectric solutions. As further enhancements are made, it is conceivable that the piezoelectric properties of these chitosan-based elastomers could approach those of synthetic polymers like PVDF, offering a potential shift towards replacing traditional materials in certain applications. This would not only foster the development of more efficient bio-based piezoelectric materials but also substantially lessen their environmental footprint, ushering in a new era of sustainable, eco-friendly alternatives in piezoelectric technology.

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Chapter 3 - Chitosan-Based Antibacterial Smart Patches for Medical and Food Packaging Applications

Short Abstract:

This study presents the development of antibacterial smart patches on chitosan-based substrates, confirmed biocompatible through cytotoxicity tests and capable of inhibiting the growth of *E. coli* and *S. aureus* at concentrations up to 10^4 CFU/mL for up to 72 hours. Using an automatic cutter, sub millimetric resolution metallic traces were fabricated, enabling the realization of Radio Frequency Identification (RFID) antennas (Near Field Communication (NFC) and Ultra High Frequency (UHF) and a Photoplethysmogram (PPG) on a flexible Printed Circuit Board (PCB) for heart rate monitoring. These innovations highlight the potential of antibacterial chitosan-based electronics for sustainable medical and food packaging applications, enabling eco-friendly, high-performance flexible devices.

1. State of the Art

Antibacterial Properties of Chitosan and Its Derivatives

Chitosan, a biopolymer derived from chitin, which is predominantly extracted from the shells of crustaceans, has garnered significant attention for its biocompatibility, biodegradability, and notable antimicrobial properties. These attributes make it an ideal material for diverse applications, ranging from biomedical uses like wound healing to food packaging and water purification^{1–4}. Numerous studies have demonstrated that chitosan solutions, films, and gels exhibit broad-spectrum antibacterial activity, inhibiting both Gram-positive and Gram-negative bacteria, including pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus cereus*, *Bacillus subtilis*, and *Klebsiella pneumoniae*^{5–8}.

The antibacterial mechanisms of chitosan are not yet fully understood and likely involve multiple interconnected processes⁹. A primary mechanism is attributed to its cationic nature, which facilitates interaction with negatively charged bacterial membranes. This interaction, influenced by factors like molecular weight, degree of deacetylation, concentration, and pH, disrupts the structural integrity of the bacterial cell wall, increasing its permeability and causing intracellular leakage, which ultimately leads to bacterial cell death^{10–12}. Additionally, chitosan's ability to chelate essential metal ions, such as calcium and magnesium, critical for bacterial survival, further compromises bacterial cell function by destabilizing cell walls and hindering vital cellular processes⁹. Moreover, chitosan can interfere with bacterial gene expression interacting with DNA and RNA, inhibiting gene transcription and protein synthesis, thereby impairing the bacteria's ability to replicate and thrive¹³. Another crucial mechanism is the generation of reactive oxygen species (ROS), which induce oxidative stress in bacterial cells, enhancing chitosan's bactericidal effects^{9,14}.

In addition, the synergy between chitosan and other biopolymers, such as polyvinyl alcohol (PVA), cellulose, starch, and alginate, improves both the mechanical and antimicrobial properties of the films, making them more suitable for use in hygiene-sensitive environments^{15–18}. For example, chitosan-PVA systems with benzalkonium bromide, a quaternary ammonium compound, have shown promising antibacterial properties against Methicillin-resistant *Staphylococcus aureus* (MRSA) in both in vitro and in vivo studies¹⁹. In wound care applications, chitosan-based nanofibrous dressings, particularly those electrospun with poly(ϵ -caprolactone) (PCL) and quaternized chitosan-graft-polyaniline (QCSP), have demonstrated superior antibacterial, antioxidant, and electroactive properties, which significantly improve wound healing compared to conventional treatments²⁰.

Additionally, integrating additives such as silver (Ag), oxides, metal oxides (e.g., ZnO, TiO₂), and graphene, enhances the efficacy of chitosan by boosting antibacterial and physical properties and by broadening their use in food packaging, medical dressings, and coatings for electronics^{9,11,21,22}.

These bioactive compounds not only provide antimicrobial effects but also offer anti-inflammatory and wound-healing benefits, making chitosan-based dressings highly suitable for advanced wound care applications²³.

Antibacterial Electronic Smart Patches for Medical Applications

Antibacterial smart patches have emerged as one of the most promising innovations in the medical field, blending advanced materials with real-time monitoring technologies to improve the management of various medical conditions. These wearable devices are designed not only to monitor physiological parameters but also to prevent and treat infections, offering solutions for continuous health monitoring and infection control. Initially, the concept of antibacterial smart patches was largely focused on infection management, but it has now evolved to incorporate a range of therapeutic approaches, including drug delivery systems (DDS) and tissue regeneration.

Antibacterial Sensors and Drug Delivery Systems

Today, the concept of antibacterial in smart patches is closely linked to the idea of drug delivery systems (DDS). One compelling example can be found in Ref (24), which examines how smart patches can facilitate the transdermal delivery of drugs, permitting their direct uptake through the skin²⁴. These systems enable drugs to be delivered directly into the skin, offering a non-invasive alternative to injections and improving both patient comfort and compliance.

Ref (25) further elaborates on stimuli-responsive DDS, which provide on-demand drug delivery in a closed-loop manner²⁵.

These systems respond to changes in the body's internal conditions, such as fluctuations in temperature or pH, to release drugs when and where they are most needed. This highly personalized form of treatment helps to ensure that drugs are administered precisely, enhancing therapeutic efficacy and reducing the risk of side effects by targeting only the affected areas and improving both patient comfort and compliance.

A further development in this space is the use of implantable smart antibacterial patches, which combine controlled drug release with antibacterial agents. Ref (26) describes such a system that allows healthcare providers to monitor the infection in real time and adjust treatment as necessary. This implantable device is equipped with wireless technology, enabling continuous therapeutic monitoring and ensuring that patients receive optimal care for deep infections, a breakthrough for managing severe medical conditions²⁶.

Electrical Stimulation for Wound Healing: A Drug-Free Approach

Beyond drug delivery, electrical stimulation represents another innovative approach in smart patches, focusing on tissue regeneration while also offering antibacterial benefits. This approach focuses on promoting healing through electrical currents rather than drug administration, which is especially beneficial in cases of chronic wounds or impaired tissue regeneration.

Kim et al explored how bioelectronic systems use electricity to stimulate the selective excitability of opportunistic pathogens, helping to eliminate infections without relying on antibiotics²⁷. This method is an effective alternative to conventional antimicrobial treatments, offering a potential solution for reducing the risk of antibiotic resistance while promoting infection control.

Another relevant study highlights the use of antibacterial conductive hydrogel electrodes in flexible patches²⁸. These electrodes can be printed on flexible substrates and stimulate wound healing by applying gentle electrical currents, which not only accelerate tissue regeneration but also prevent bacterial growth at the wound site, ensuring a drug-free approach to infection prevention and healing.

Role of Chitosan in Wearable Electronic

Chitosan has recently gained attention as a promising material for electronic applications due to its biodegradability, flexibility, and biocompatibility. In a recent study Ahmad et al. have explored its potential as a substrate for stretchable and flexible electronic devices, particularly in wearable sensing applications²⁹. Chitosan-based films have been successfully integrated with conductive nanomaterials, such as silver nanowires, to develop biodegradable and recyclable electronic devices. Additionally, chitosan's piezoelectric properties have been leveraged in the development of flexible and wearable patches for physiological strain sensing, demonstrating its potential for real-time health monitoring and biomedical applications³⁰. In addition, biocompatible chitosan-based electronic skins have been investigated for their ability to conform to human skin while maintaining reliable electrical performance, further supporting their application in next-generation bioelectronics^{31,32}.

Recent studies have also explored chitosan's role in advanced bioelectronic applications, including wound healing and self-powered sensing technologies. In a much-cited study a novel wound dressing combining Chitosan-Vaseline gauze (CVG) with a flexible electrical stimulation (ES) device to treat diabetic wounds was explored³³. Another study developed a porous chitosan/PVA/carbon nanotube hydrogel as a highly sensitive, wide-range wearable pressure sensor with antibacterial properties³⁴. Additionally, triboelectric antibacterial electronic skin (e-skin) is attracting attention for its self-powered capabilities, high sensitivity, and adaptability to curved surfaces. In this context, chitosan has been

increasingly utilized in the development of biocompatible, biodegradable electronic skins for triboelectric nanogenerators (TEGs)³⁵. Chitosan enhances the e-skin's transparency and self-powered sensing abilities, and when integrated with gold electrodes, chitosan-based TEGs have demonstrated rapid voltage responses, highlighting their potential applications in healthcare, wearables, and human-machine interactions³⁶.

Smart Patch for Food Packaging

The use of smart packaging is emerging as an innovative solution to enhance food safety and reduce waste. Current technologies for monitoring food quality include colorimetric, electrochemical, and electronic sensors capable of detecting volatile gases associated with food spoilage, such as ammonia (NH₃) and hydrogen sulfide (H₂S), which are typical markers of meat, dairy, and vegetable degradation. The most advanced solutions combine these technologies with wireless communication systems based on NFC (Near Field Communication), allowing consumers to check food freshness via smartphones³⁷.

Role of Chitosan in Food Packaging

Beyond electronics, chitosan is widely studied as a sustainable material for food packaging due to its natural antimicrobial properties and biodegradability^{3,38}. Its ability to form films with excellent mechanical strength and barrier properties makes it a viable alternative to conventional petroleum-based packaging materials³⁹. Chitosan-based composite materials have been explored for their ability to extend food shelf life while maintaining environmental sustainability, offering an innovative approach to reducing plastic waste in the food industry⁴⁰.

Building on all these considerations, our work has focused on producing smart antibacterial patches using chitosan films, taking advantage of their adjustable mechanical properties, the ability to pattern metal traces, and the potential to integrate electronic functionalities. This approach aligns with a green, low-cost production process, exemplified by the use of a cutter, thereby establishing a new concept of antibacterial smart patches where the antimicrobial effects are primarily derived from the inherent properties of chitosan itself. The next section will delve into the Results and Discussion, where we will present our findings on the performance and potential applications of these chitosan-based smart patches.

2. Results and Discussion

Cytotoxicity Test

The cytotoxicity of the produced chitosan-based films was evaluated using MRC-5 cells, a human lung fibroblast line derived from normal embryonic tissue. These cells are widely used in biomedical research due to their stability, predictable growth patterns, and resemblance to primary fibroblasts. MRC-5 cells were cultured under standard conditions, and cytotoxicity tests were conducted by seeding cells in multiwell plates. After cell adhesion, chitosan discs were introduced into the medium. Cell viability was assessed at different time points using a luminescence-based cell viability assay with a microplate reader (Figure 3.1 a and b). As further demonstrated in Figure 3.1 c, the MRC-5 cells exhibited no morphological signs of cytotoxicity.

These results testify to the biocompatibility of chitosan films: a characteristic that makes them suitable for applications involving direct contact with the human body.

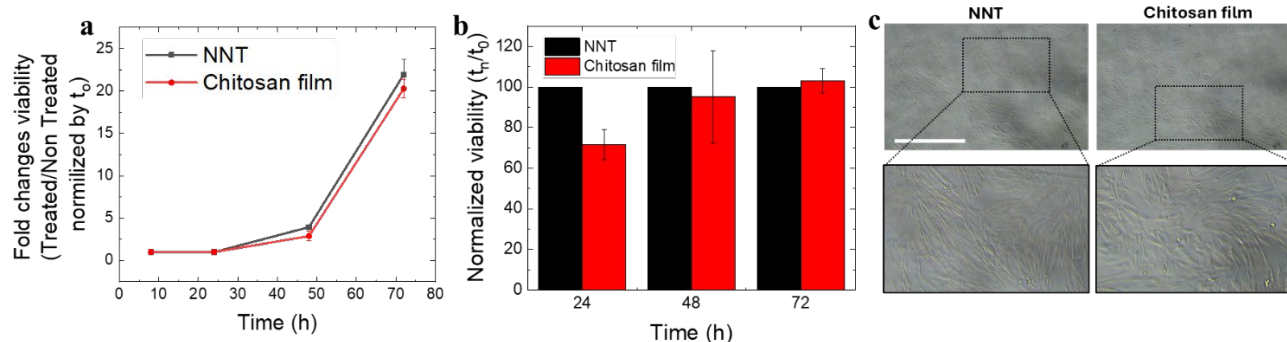


Figure 3.1. Growth curve of MRC-5 cells lines treated with biofilms at 8, 24, 48 and 72 hours. Cell viability was measured using the CellTiter-Glo Assay, with dots representing the mean and error bars showing the standard deviation (SD). (a) Viability of MRC-5 cell lines treated with biofilms at different time points (24, 48, and 72 hours), as calculated using the CellTiter-Glo Assay. The graph shows the relative cell viability of treated cells compared to non-treated controls (NNT) at each time point. (b) Each bar represents the mean fold change, while error bars represent SD across three replicates.

Time-dependent antibacterial activity of chitosan films against *E. coli* and *S. aureus* was tested by spotting (10 μ L) bacterial dilutions (10^6 to 10^1 CFU/mL), within the concentration range of 10^3 to 10^6 CFU/cm² typically found on healthy human skin, and potentially increasing to 10^8 to 10^9 CFU/cm² during states of infection or inflammation, onto MHA plates and allowed to dry. Chitosan films were placed over the dried bacterial spots for the treatment group, while control plates were left without films. Plates were incubated at 37°C in aerobic conditions, and bacterial growth was observed at 24, 48, and 72 hours. (c) Representative brightfield images of MRC-5 cells after 72 hours of treatment with biofilms. Scale bar is 300 μ m.

Antibacterial Tests

Antibacterial Assays and Bactericidal Effect Test

The results from the antibacterial spot assays against *E. coli* and *S. aureus* are shown in Figure 3.2.(a). While bacterial concentrations ranging from 1 to 10^8 CFU/mL were tested, only the results for concentrations between 10^2 and 10^5 CFU/mL are presented for clarity.

The experiments were conducted by depositing 10 μ L of bacterial suspensions at various concentrations onto sterile Petri dishes containing Mueller-Hinton Agar. Sterile disks of chitosan film (1 cm in diameter) were then placed over the bacterial spots, and the plates were incubated at 37°C for 24 hours. The spot assay, a method for evaluating antimicrobial activity by observing bacterial growth inhibition at the site of the sample, was used to assess the antimicrobial effects of the chitosan films.

Both *S. aureus* and *E. coli* showed inhibition by the chitosan films, but with notable differences between the two bacteria. For *S. aureus*, the data revealed that lower bacterial concentrations (1 to 10^4 CFU/mL) led to more significant inhibition by the chitosan film, as evidenced by smaller colonies compared to the control. At higher concentrations (10^5 to 10^8 CFU/mL), although inhibition was still observed, it was less pronounced, suggesting that the film's effectiveness decreases as bacterial concentration rises.

Similarly to *S. aureus*, inhibition was also observed in *E. coli* at lower concentrations (1 to 10^3 CFU/mL), where colonies under the film were noticeably smaller than in the control. However, at 10^5 CFU/mL, the bacterial spot reached 10.5 mm, larger than the 8 mm observed in the control, suggesting that the motile *E. coli* were able to spread beyond the film's boundary. A similar pattern was seen at 10^4 CFU/mL, where the spot expanded to 10.4 mm, compared to 7.5 mm in the control. At lower concentrations (1 to 10^3 CFU/mL), no notable spread beyond the chitosan film edges was observed. However, the motility of *E. coli* enabled it to proliferate more in regions where the film did not directly contact the agar, further emphasizing the difficulty of achieving homogeneous inhibition.

Control experiments revealed that the commercial chitosan solution (10 mg/mL) exhibited clear inhibition for both *S. aureus* and *E. coli*, especially at 10^2 to 10^5 CFU/mL, where bacterial growth was significantly reduced compared to the positive controls (bacterial solution alone). Negative controls, consisting in untreated bacterial spots remained uncontaminated, confirming the reliability of the experimental conditions.

The comparison between the test chitosan films and the commercial chitosan solution revealed that the inhibition observed with the films was less pronounced than that of the commercial solution, yet markedly stronger than the control. At 10^3 CFU/mL, the positive control exhibited robust bacterial growth, while the growth under the chitosan film was markedly reduced. This trend continued at 10^2 and 10 CFU/mL, where the chitosan films effectively limited bacterial proliferation for both *S. aureus* and *E. coli*.

After removing the chitosan films and incubating for an additional 24 hours, both *E. coli* and *S. aureus* exhibited renewed bacterial growth, as seen in Figure 2.1 (b), indicating that the films had a bacteriostatic, rather than a bactericidal effect.

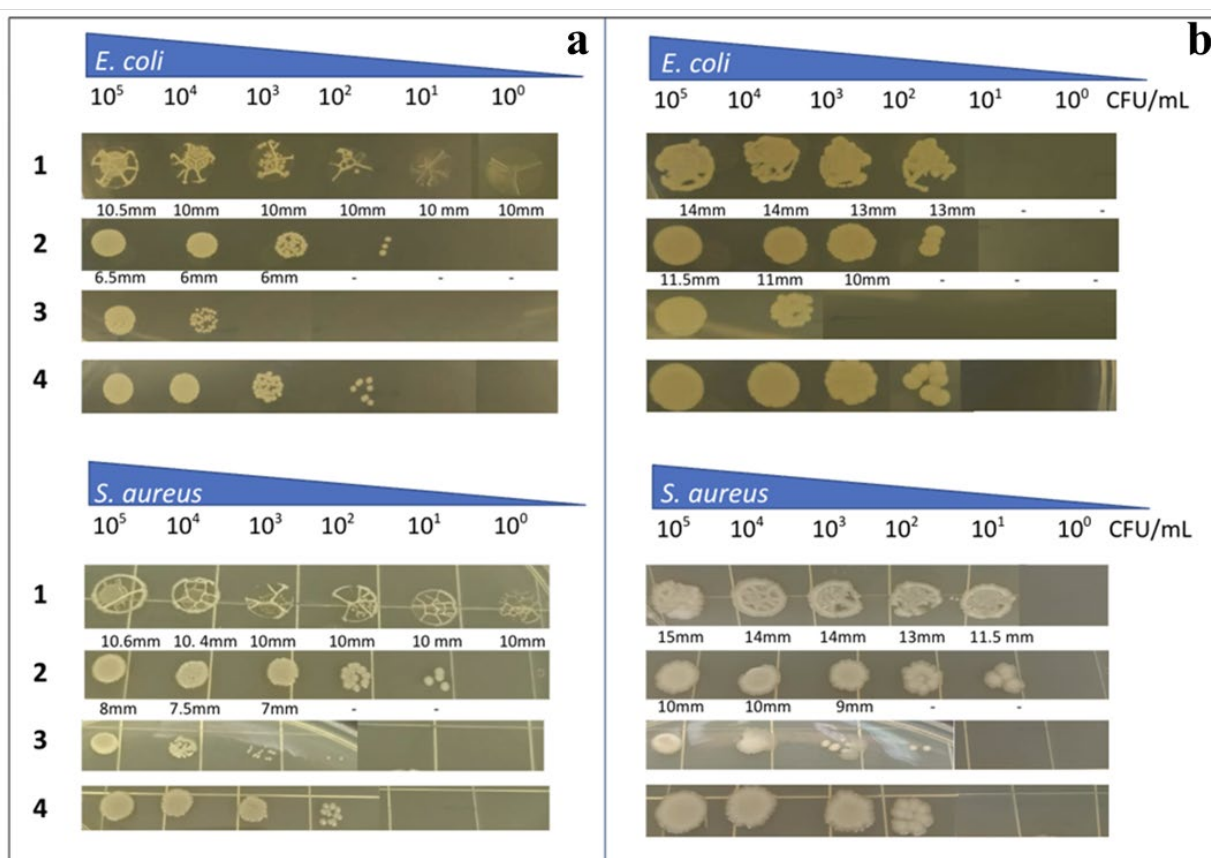


Figure 3.2. (a) Evaluation of the antibacterial properties of chitosan films against *E. coli* and *S. aureus* using a spot assay after 24 hours of incubation. (b) Assessment of the bacteriostatic effect of chitosan films on *E. coli* and *S. aureus* after an additional 24 hours of incubation following the removal of the films. The renewed bacterial growth observed in both *E. coli* and *S. aureus* indicates that the films exhibited a bacteriostatic effect, rather than a bactericidal one.

The antibacterial activity is analyzed under four different conditions: (1) Bacterial spots initially in contact with chitosan films, (2) Untreated bacterial spots, (3) Bacterial spots exposed to a 10 mg/mL commercial chitosan solution, and (4) Bacterial spots treated with a 0.1 M acetic acid solution.

Phenotypic Analysis with Green Fluorescent Protein (GFP)-Expressing *E. coli*

Since *E. coli* was observed moving around the chitosan film and continuing to grow, we hypothesized that the bacteria under the film and those moving around it might exhibit different phenotypes. To investigate, we used GFP-expressing *E. coli* 25922 under identical experimental conditions, where GFP (Green Fluorescent Protein) serves as a marker to visualize bacterial activity through green fluorescence.

As shown in Figure 3.3, distinct phenotypic differences were observed. The *E. coli* colonies under the chitosan film did not express GFP (a,b), while those growing around the film did (c,d), suggesting that the chitosan film creates a stress environment that inhibits GFP expression. However, when these colonies were streaked on fresh media, they resumed GFP expression, confirming that the suppression was reversible and not a permanent genetic change. In addition, the streaking of colonies on Mueller-Hinton Agar (MHA) and Luria-Betrani (LB) + Ampicillin plates confirmed that the GFP gene was not lost, further supporting the notion that the observed inhibition of GFP expression was a stress response rather than a genetic alteration as shown in Figure 3.3 (e-h).

After removing the chitosan films and incubating for 24 more hours, all bacteria, including those that had been under the film, expressed GFP again. This indicates that GFP expression was inhibited under the chitosan film due to stress but was restored when the film was removed (Figure 3.3 (i-l)).

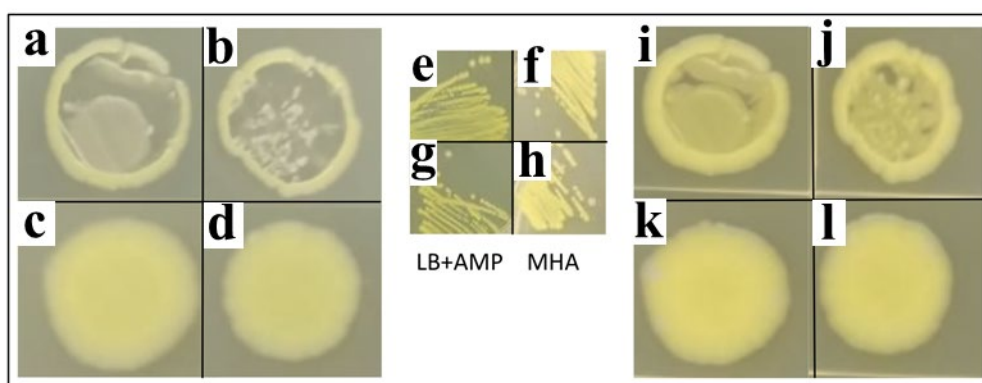


Figure 3.3. GFP Expression in *E. coli* Exposed to Chitosan Films. (a) Bacterial spots (10^5 CFU/mL) exposed to chitosan film immediately after removal. (b) Bacterial spots (10^4 CFU/mL) exposed to chitosan film immediately after removal. (c, d) Bacterial spots (10^5 and 10^4 CFU/mL) after chitosan film exposure. (e, f) Streaking of colonies from the areas outside the chitosan film on LB + Ampicillin and MHA media. (g, h) Streaking of colonies from the regions under the chitosan film on LB + Ampicillin and MHA media. (i) Bacterial spots (10^5 CFU/mL) after 24 hours of incubation post-film removal. (j) Bacterial spots (10^4 CFU/mL) after 24 hours of incubation post-film removal. (k, l) Bacterial spots (10^5 and 10^4 CFU/mL) after 48 hours of incubation.

Time-Dependent Antibacterial Activity

The antibacterial effect of chitosan films on *E. coli* and *S. aureus* was also monitored for 72 hours at various bacterial dilutions. At the 10^2 CFU/mL dilution, the chitosan film's inhibitory effect was most prominent. Only four colonies were visible under the film, with minimal growth over the 72-hour period, while the control plates exhibited continuous bacterial growth, forming a flat lawn. At higher concentrations ($\geq 10^3$ CFU/mL), bacterial motility around the chitosan film's edge was observed, indicating a bacterial response to the antimicrobial environment (Figure 2.3.A).

For *S. aureus*, at the 10^2 CFU/mL dilution, no colonies were seen under the film, indicating complete inhibition. At the 10^3 CFU/mL dilution, the control plates showed about 10 distinct colonies, while the colonies under the chitosan film were too small to count at the 24-hour mark and showed minimal growth. At higher concentrations ($\geq 10^4$ CFU/mL), bacterial growth was observed under the film, but it remained less extensive compared to the controls. The colonies in the control plates expanded by

approximately 1.5 times over 72 hours, while the colonies under the chitosan film showed minimal growth at the 72-hour mark (Figure 2.3.B).

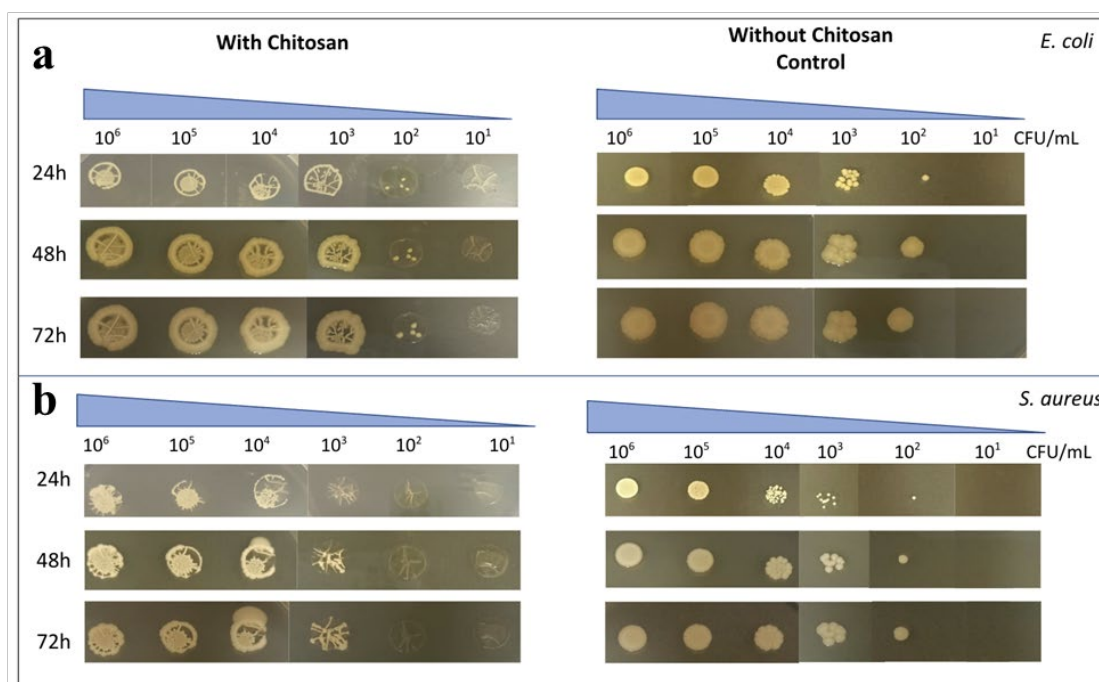


Figure 3.4. Bacterial Growth of *E. coli* and *S. aureus* in the Presence and Absence of Chitosan Films Over 72 Hours. Serial dilutions of bacterial cultures (ranging from 10^6 to 10^1 CFU/mL) were spotted ($10\ \mu\text{L}$) onto MHA plates and left to dry for about 10 minutes. Chitosan films were placed over the bacterial spots in the experimental group, while the control group remained without films. The plates were then incubated aerobically at 37°C , and bacterial growth was monitored at 24, 48, and 72 hours. (a) *E. coli* growth patterns with and without the chitosan film. (b) *S. aureus* growth patterns with and without the chitosan film.

In conclusion, the findings from this study highlight the antibacterial properties of chitosan films, which primarily exhibit a bacteriostatic effect against *E. coli* and *S. aureus*. The spot assay results demonstrate that chitosan films inhibit bacterial growth without killing the cells, as evidenced by the regrowth of bacteria after the films were removed.

The use of GFP-expressing *E. coli* provided further evidence of the stress caused by the chitosan film. Under the film, bacteria did not express GFP, indicating that the chitosan environment suppresses gene expression due to stress. However, when streaked on fresh media, the bacteria regained GFP expression, suggesting that the suppression was reversible.

The results also indicated that chitosan films are more effective at inhibiting bacterial growth at lower bacterial concentrations, particularly at 10^2 CFU/mL. At higher concentrations, bacterial motility around the edges of the film was observed, suggesting an attempt to circumvent the antimicrobial effect. These findings point to the potential for chitosan films to act as long-lasting antimicrobial agents, but also suggest that further optimization of film properties (e.g., thickness, concentration, or the addition of other antimicrobial agents) may be necessary to enhance their efficacy at higher bacterial concentrations.

Overall, the chitosan films exert a bacteriostatic effect by inducing stress in bacterial cells, which inhibits their growth and gene expression. Further studies should focus on the specific stress pathways involved, the potential for bacterial resistance, and the practical applications of chitosan films in areas such as antimicrobial packaging, wound dressings, and surface coatings.

Patterning Metallic Traces on Chitosan Thin Films

The use of an automatic cutter, which can selectively cut copper while preserving the underlying chitosan film, proved highly effective for the microfabrication of metallic traces on chitosan films, achieving sub-millimeter resolution ideal for intricate patterns such as those used in RFID antennas and PCBs. The precision attained is critical for applications where the integrity of metallic traces directly influences the performance of the final device. Compared to traditional techniques, such as photolithography or metal deposition methods like evaporation and sputtering, this cutting approach offers several distinct advantages. In addition to being cost-effective and simple, it is environmentally friendly, avoiding the use of hazardous chemicals and protecting the chitosan substrate from exposure to high temperatures. By sidestepping aggressive chemical or thermal treatments, the cutter preserves the delicate chitosan material, ensuring both the film and the final product remain intact. Moreover, this method does not require complex fabrication facilities, making it a sustainable and accessible solution for the production of flexible electronics.

To thoroughly assess the capabilities of the cutting technique for patterning metallic traces on chitosan films, a series of systematic experiments was conducted. The investigation focused on determining the minimum width of the copper traces that could be reliably fabricated, as well as the smallest pitch: the spacing between adjacent metallic features that could be consistently maintained. By examining these critical parameters, the study aimed to establish the resolution limits of the method and assess its potential for applications requiring finely structured conductive patterns.

Figure 3.5 presents the results of the systematic experiments conducted to evaluate the cutting technique for patterning metallic traces on chitosan films. The images show the CAD designs alongside the optical microscope images of the fabricated traces across three distinct tests.

In the first experiment, the focus was on determining the minimum achievable trace width while maintaining adequate spacing between individual traces. Rectangular metallic traces were fabricated with varying widths of 1.2 mm, 1 mm, 800 μm , 600 μm , 400 μm , and 250 μm . Each trace seamlessly transitions into a square (3 mm per side) at the end, simulating a continuous, fine metallic wire terminating in a larger contact pad, as would be the case in structures designed for electrical connections to larger pads. As shown in Figures 3.5 a) and d), the correspondence between the CAD design and the fabricated traces is exceptionally high. Even with the thinnest traces, down to 250 μm , the integrity of the copper was preserved, with no deformation or geometric alteration, particularly at the junction between the 250 μm trace and the copper pad. This demonstrates that the cutting technique successfully maintains both the trace geometry and the integrity of the conductive connection.

The second experiment investigated the minimum achievable pitch between adjacent rectangular metallic features of the same 1.5 mm width. Traces were fabricated with varying spacings of 1.5 mm, 1 mm, 750 μm , 500 μm , and 250 μm . As depicted in Figures 3.5 b) and e), even with the smallest pitch of 250 μm , the traces were fabricated without any distortion, maintaining the precise geometry as designed in the CAD model. This highlights the cutter's capability to maintain excellent resolution even in tightly packed patterns.

In the third experiment, both the minimum trace width and pitch were examined simultaneously. Rectangular traces of decreasing width (1.5 mm, 1 mm, 750 μm , 500 μm , and 250 μm) were placed at progressively smaller spacings (1.5 mm, 1 mm, 500 μm , and 250 μm). The results, shown in Figures 3.5 c) and f), reveal a strong correspondence between the CAD design and the fabricated traces, although slight deformation was observed at the thinnest traces with the smallest pitch. This deformation indicates that the limits of the cutter's capabilities were reached when working with traces as thin as 250 μm and a pitch of 250 μm .

In conclusion, these experiments demonstrate that the cutting technique is capable of achieving sub-millimeter resolutions with remarkable precision. It is evident that the method can produce regular and defect-free structures even at sizes below 500 μm , underscoring its potential for applications requiring high-resolution patterning of conductive traces.

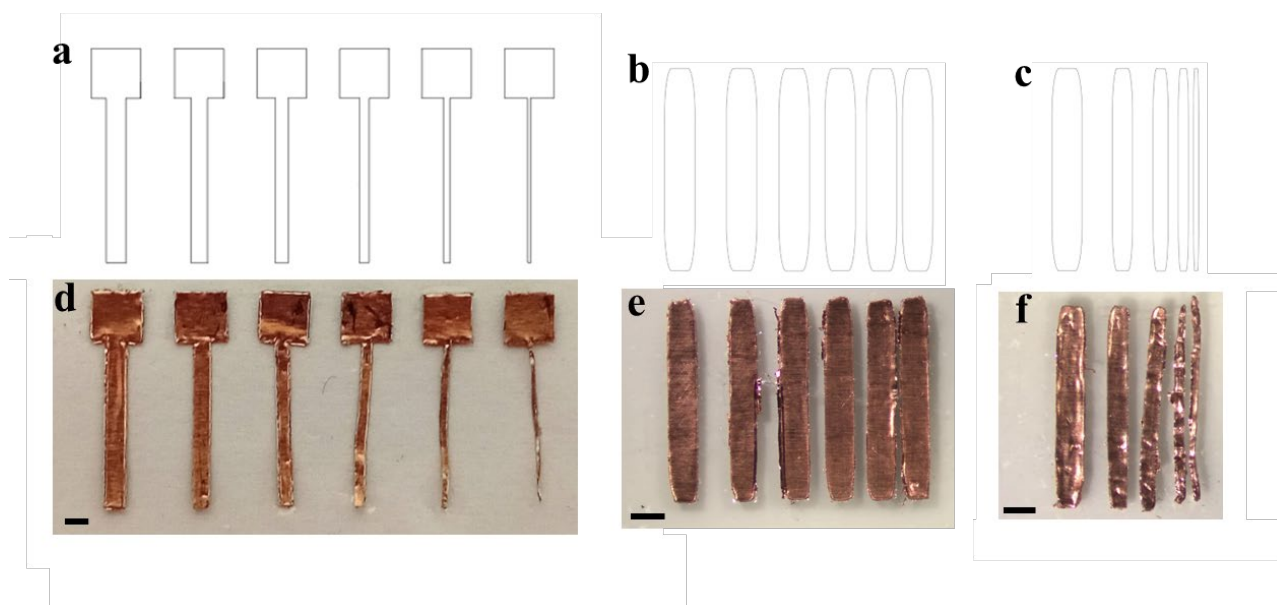


Figure 3.5. Evaluation of the cutting technique for patterning metallic traces on chitosan films. (a, d) CAD design and corresponding optical microscopy image of traces with widths of 1.2 mm, 1 mm, 800 μm , 600 μm , 400 μm , and 250 μm . (b, e) CAD design and optical microscopy image of traces with a fixed width of 1.5 mm and pitches of 1.5 mm, 1 mm, 750 μm , 500 μm , and 250 μm . (c, f) CAD design and optical microscopy image of traces combining varying widths (1.5 mm, 1 mm, 750 μm , 500 μm , and 250 μm) with progressively smaller pitches (1.5 mm, 1 mm, 500 μm , and 250 μm). For all optical microscopy images (d, e, f), the scale bar is 1 mm.

Development of Antibacterial Smart Patches for Medical and Food Applications Using Chitosan Thin Films

The ability to tailor the mechanical properties of chitosan films, combined with their biocompatibility and antibacterial characteristics, makes them ideal for a wide range of applications in medical and food packaging sectors. These properties allow for seamless contact with irregular or curved surfaces, while providing a safe and hygienic interaction, which is crucial in fields such as physiological parameter sensing and food preservation. In this context, we focused on developing flexible electronic devices, including RFID antennas and flexible PCBs, utilizing the cutter-based patterning technique previously described. To enhance the durability and functionality of the produced devices, all electronic components, including RFID antennas, were encapsulated in an additional thin layer of chitosan. This not only ensures mechanical stability and protection from environmental factors, but also contributes to maintaining the antibacterial properties of the devices.

In the following subsections, we will discuss the production, characterization, and testing of RFID antennas, followed by the development of a flexible PCB and its application in a plethysmograph for cardiac activity monitoring.

RFID Antennas on Chitosan for Smart Sensing Applications

In this work, we developed RFID antennas on chitosan substrates using a cutter-based patterning technique. Two types of RFID technologies were explored: Near Field Communication (NFC) and Ultra-High Frequency (UHF) RFID, each distinguished by its coupling mechanism. NFC operates via magnetic coupling (inductive), enabling short-range communication, while UHF RFID relies on electromagnetic coupling (radiative), allowing for longer read distances.

These RFID-based devices can be integrated into medical and food packaging applications, enabling wireless monitoring of physiological parameters or the preservation state of perishable goods. The

biocompatibility and antibacterial properties of chitosan further enhance their suitability for such applications.

Design, Fabrication and Characterization of an NFC Antenna on Chitosan

To design an NFC antenna operating at 13.56 MHz, we first needed to estimate its inductance (L), a key parameter for setting the antenna's resonance frequency. To do this, we used the empirical formula (**Equation 5**) for spiral antennas:

$$L = 31.3 \cdot \mu \cdot N^2 \cdot \frac{a^2}{8a + 11c} \quad (5)$$

where μ is the permeability of the medium, N is the number of turns, $a = (r_{in} - r_{out})/2$, and $c = r_{out} - r_{in}$ is the spacing between turns. However, this formula is only an approximation, based on ideal conditions such as free space. In reference to the Figure 3.6, our antenna have: $r_{out} = 10$ mm ; $r_{in} = 3.75$ mm.

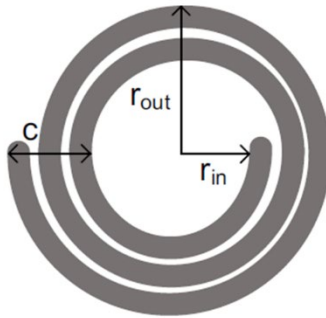


Figure 3.7. Schematic design of a coil NFC antenna.

In our case, the antenna was fabricated on chitosan, with 30- μ m-thick copper traces. The width of the traces was chosen to be 750 μ m, with a corresponding pitch (trace-to-trace distance) of 750 μ m. This choice was guided by the sub-millimetric resolution achievable with our cutter-based fabrication technology. These factors altered the actual inductance, as the properties of the substrate and the copper conductors introduce parasitic effects that were not considered in the formula.

Therefore, the formula was used only as a starting point to define the antenna's geometry. After fabricating the antenna, we experimentally measured the real inductance using a Vector Network Analyzer (VNA). This measurement provided us with an actual inductance value of 0.2 microhenry (μ H), which was crucial for fine-tuning the antenna's performance.

Once we had the actual inductance value, the next step was to choose a matching capacitor to achieve resonance at 13.56 MHz. The resonance condition for an LC circuit is given by:

$$f = \frac{1}{2\pi\sqrt{LC}}$$

Therefore the chosen tuning capacitor was 680 pF, which was used in the circuit. Fine adjustments were made during testing to ensure optimal resonance at 13.56 MHz. This process—starting with a theoretical approximation, followed by real-world measurement of inductance and capacitor tuning—resulted in a properly tuned NFC antenna for 13.56 MHz operation.

Given that these flexible antennas are designed for wearable medical applications and food packaging, they must function effectively even when in contact with curved surfaces. To assess this, we conducted tests using a Vector Network Analyzer (VNA) to determine whether the resonance frequency would change under bending conditions.

The antenna was tested in a flat position and then bent with four different radii of curvature, down to a radius of 0.5 cm. The S11 parameter (reflection coefficient) was measured as a function of frequency. As shown in Figure 3.8 (a), the coil-only configuration exhibited a resonance frequency around 3 GHz, while the antenna with the tuning capacitor (Figure 3.8 (b)) was properly tuned to 13.56 MHz.

Crucially, the performance of both configurations remained unchanged across all bending angles tested. The resonance peak, whether with or without the capacitor, stayed stable in magnitude and frequency position, demonstrating that the antenna can maintain its performance even when bent. This highlights that while the capacitor is essential for tuning the frequency to 13.56 MHz, the antenna remains well-suited for flexible electronics applications, including those involving curved surfaces.

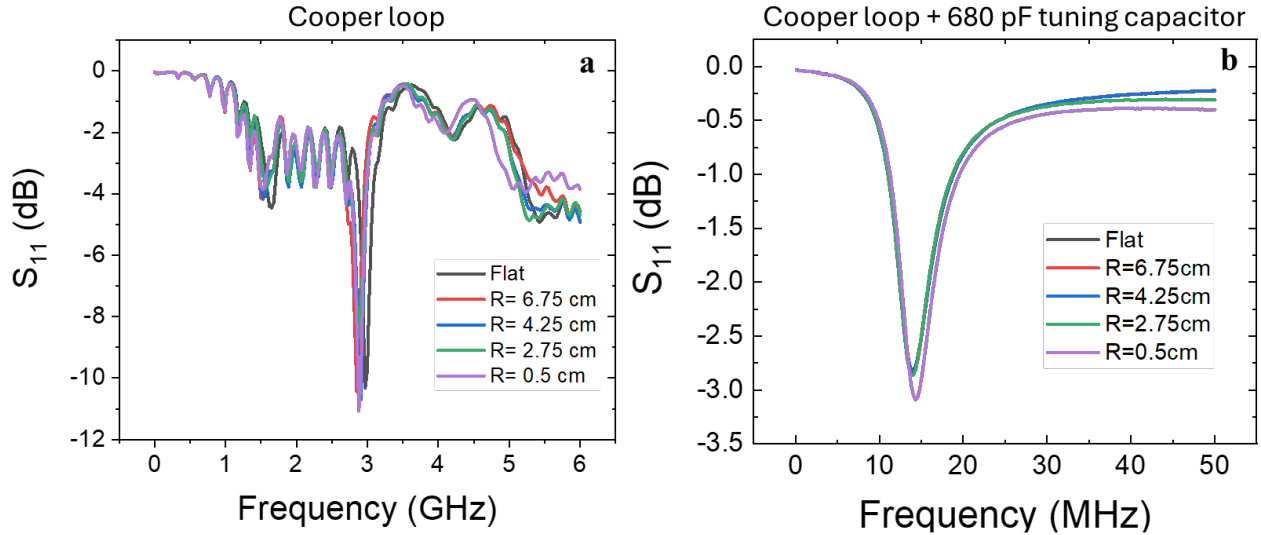


Figure 3.8. Reflection coefficient (S_{11}) as a function of frequency for the flexible NFC antenna under different bending conditions. (a) Coil-only configuration and (b) coil with a 680 pF tuning capacitor. Measurements were taken in a flat position (black curve) and with radii of curvature of 6.75 cm (red), 4.35 cm (blue), 2.75 cm (green), and 0.5 cm (purple).

Once the fabrication technique was established and the antennas were characterized and tested, further experiments were conducted to explore miniaturization possibilities. Based on the previously demonstrated resolution achievable with the cutter, we investigated the minimum possible trace width for the antenna coil, reducing it from the tested 750 μm .

To this end, antennas with coil trace widths of 600 μm , 500 μm , and 450 μm were designed and fabricated, with a corresponding pitch (trace-to-trace distance) equal to the trace width in each case, as shown in Figure 3.9. The results confirm that this technique allows for the fabrication of lightweight antennas with acceptable uniformity down to 450 μm . However, at this limit, a slight loss of consistency in trace definition was observed.

These findings demonstrate the versatility of this fabrication method in achieving coil antennas with resolutions below half a millimeter. Further optimization of the process could potentially enhance trace uniformity, pushing the resolution limits even further.

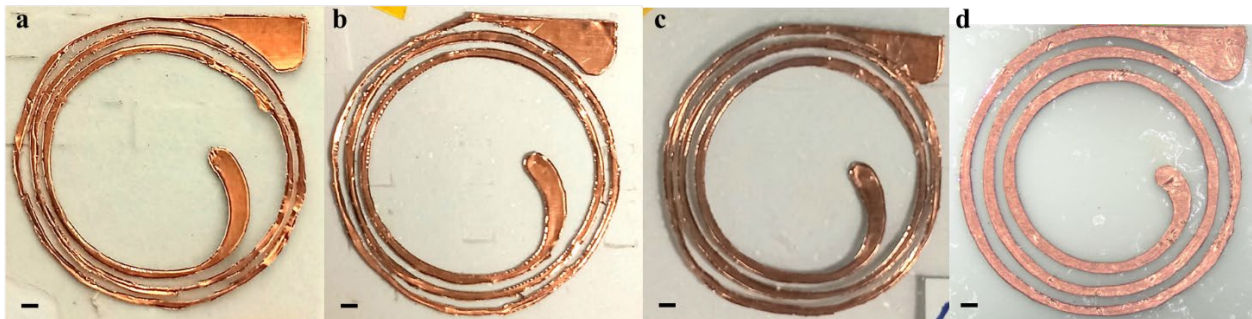


Figure 3.9. Antennas with coil trace widths of (a) 450 μm , (b) 500 μm , (c) 600 μm , and (d) 750 μm , each with a corresponding pitch equal to the trace width. Scale bar: 1 mm for each image.

Modular fabrication approach and Testing of the NFC Antenna

After confirming the antenna's resonance at 13.56 MHz, the next step was to test the actual functionality of the device. The antenna was designed as a copper coil on a chitosan substrate, with a tuning capacitor integrated into the system. To validate the functionality of the antenna, we mounted different NFC ID chips onto the antenna, specifically the ST25DV16KC-IE6S3 and ST25TV02KC-TFH3 chips from STMicroelectronics. These chips were chosen for their compatibility with NFC systems, and their function was tested to confirm the antenna's performance.

Once the chips were mounted, we tested the system using the ST25 NFC RFID Tap version app, which allows a smartphone to function as an NFC reader. This app interacts with the antenna, enabling us to query the NFC chips and ensure proper communication between the antenna and the chips.

The mounting of the NFC chips and the tuning capacitor followed a modular approach. Rather than directly soldering the components onto the antenna coil, we employed adhesive copper commas (we define them as such due to their shape), produced through a patterning technique using a cutter to shape copper foil. These copper commas were first attached to a Kapton substrate, where the chip and capacitor were soldered onto the commas using lead-free solder paste (specifically EEYUIHF Sn42Bi58 solder paste). A hot air reflow system was then applied to complete the soldering process.

After the components were successfully soldered to the copper commas (while still attached to the Kapton), the Kapton substrate was carefully peeled away. One pad of the copper commas, now holding the chip and capacitor, was attached to the coil's pad on the chitosan substrate using the adhesive copper properties. The second pad of copper comma, with the chip and capacitor, was connected to the other pad of the coil through a second adhesive copper comma, ensuring continuity.

To prevent short-circuiting the coil of the antenna with this second copper comma, a thin layer of chitosan film was used as an insulating layer, leaving only the second copper comma exposed at both ends. This creates a multilayer structure that provides effective electrical insulation while maintaining the flexibility of the antenna. This unique approach may prove useful in other applications, such as PCB design, where multiple layers are required without short-circuiting.

This modular design offers several advantages:

- Minimizing copper waste: If a soldering error occurs, only the small copper commas need to be discarded, saving the bulk of the copper used in the coil.
- Chip versatility: Different chips with varying sizes and functionalities can be easily mounted on the same coil, allowing for quick adaptation to different use cases. The geometry of the copper commas can be optimized to fit the specific chip.
- Reusability of the coil: The antenna coil can be reused multiple times by simply replacing the mounted chip, allowing for significant flexibility in testing and adaptation.
- No heat treatment of the loop substrate: By soldering the components to the copper commas on Kapton, delicate substrates like chitosan are not exposed to high temperatures, preserving the integrity of the material.

Once the modular components were integrated, a final layer of chitosan was applied over the entire structure to encapsulate the device. The completed system was then tested with the ST25 NFC RFID Tap version app, successfully reading the chips and confirming the functionality of the entire device.

This modular assembly approach can be observed in Figures 3.10 (a).

In Figure 3.10 (b), the image of a functional copper antenna on a chitosan substrate, built using the modular approach, is shown applied in a conformal manner to human skin, demonstrating the potential utility of such a device in wearable medical applications.

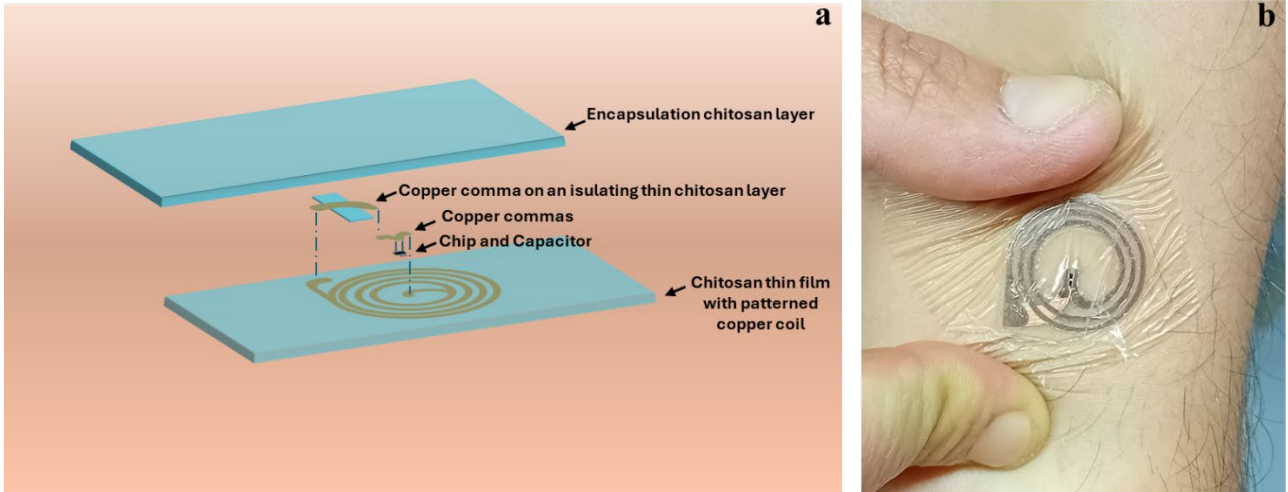


Figure 3.10. (a) Exploded schematic illustrating the modular assembly approach for fabricating copper NFC antennas on chitosan films. (b) Photograph of a functional copper NFC antenna on a chitosan substrate applied conformally to human skin, highlighting its potential for wearable medical applications.

Design, Fabrication, and Characterization of UHF Antenna on Chitosan

The UHF RFID antennas were fabricated using the same selective cutting technique employed for the NFC antennas, in which 30 μm -thick copper tape was precisely patterned on the chitosan substrate. The antenna geometry was based on a reference design from the Asygn database, which was optimized for the Asygn AS3213L.3 and Asygn AS3212.5 RFID chips. Given our intention to use these specific chips, we made slight adjustments to the design, such as rounding the corners and introducing softer bends in the serpentine shapes, to ensure compatibility with the flexible antenna concept. This approach allowed us to maintain the chip's impedance matching while adapting the antenna to the flexibility requirements, resulting in a folded dipole antenna with a T-match network at the pad level. The final configuration was chosen to improve impedance matching and make the antenna more compact. In RFID systems, maximum power transfer between the antenna and chip is achieved when the impedance of the radiating element is the complex conjugate of the chip impedance. Given that the selected chips have an impedance of $Z_{\text{chip}} = 23 - j213 \Omega$, the antenna geometry was adjusted to ensure optimal electromagnetic coupling and compactness.

Impedance Measurements and Power Transmission Coefficient (τ)

Once fabricated, the antenna was characterized using a Vector Network Analyzer (VNA). The real and imaginary components of the antenna impedance were extracted to calculate the power transmission coefficient (τ), a key parameter for evaluating impedance matching. τ is defined as (**Equation 6**):

$$\tau = \frac{4R_{\text{chip}}R_a}{|Z_{\text{chip}} + Z_a|^2} \quad (6)$$

where Z_{chip} and R_{chip} correspond to the chip impedance and resistance while Z_a and R_a are obtained from VNA measurements. A τ value close to 1 indicates efficient power transfer, ensuring optimal antenna performance.

Measurements were conducted across a frequency range from 750 MHz to 980 MHz, with particular focus on 866 MHz, the target resonance frequency of the antenna. The results demonstrated a τ value near unity in the vicinity of 866 MHz, confirming effective impedance matching.

Given the flexible nature of the chitosan-based antenna, additional tests were performed to evaluate its behavior under mechanical deformation. The antenna was subjected to controlled bending at different radii of curvature, ranging from a flat configuration to a 2.75 cm radius. The τ values remained nearly

unchanged across all bending conditions, indicating that the antenna retained its optimal performance even when conforming to curved surfaces. This result highlights its suitability for applications requiring integration onto irregularly shaped objects.

To further investigate real-world applicability, the antenna was tested in direct contact with water-filled containers. As expected for UHF RFID antennas, the presence of water significantly affected performance, causing a sharp drop in τ . This is due to the strong absorption of UHF signals by water, which disrupts the electromagnetic coupling between the antenna and the chip. These findings reinforce the known limitations of UHF RFID in high-dielectric environments while demonstrating the robustness of the fabricated antenna in standard operational conditions. The results of these experiments are shown in Figure 3.11.

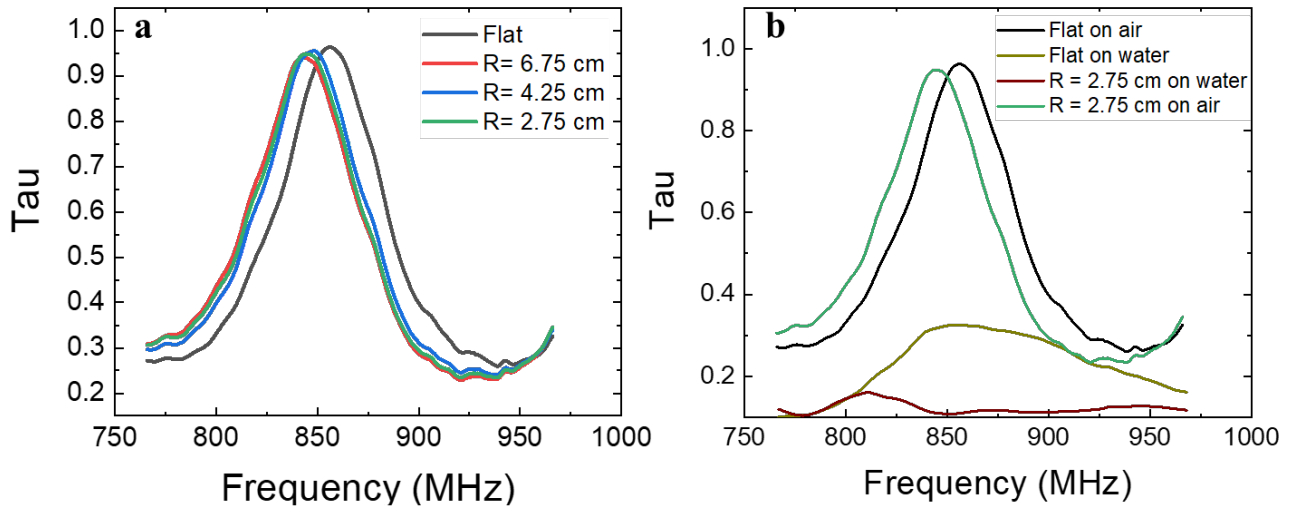


Figure 3.11. (a) Transmission coefficient (τ) vs frequency for the UHF antenna at different radii of curvature: Flat, $R = 6.75$ cm, 3.25 cm, and 2.75 cm. (b) Transmission coefficient (τ) vs frequency for the UHF antenna in flat configuration and at $R = 2.75$ cm, measured in air and on water.



Figure 3.12. Optical image of an UHF copper antenna on chitosan substrate. The scalebare is 5 mm

Testing the UHF Antennas with a Commercial Wireless Reader

To integrate the RFID chip with the antenna, the same modular approach previously described for NFC antennas was employed. The chip was first soldered onto two copper commas placed on a Kapton substrate. These were then detached and adhered to the antenna's T-match pads by exploiting the adhesive nature of the copper tape. Unlike the NFC antenna, only two copper commas were required, and no additional chitosan insulation layer was necessary.

The fabricated UHF RFID antennas were then tested to evaluate their reading performance using a SENSOR ID reader. The tests were conducted in a custom experimental setup designed to minimize electromagnetic interference. The antenna-chip system was suspended using clamps made of insulating material and mounted on a mechanical arm, which allowed precise adjustments of the distance between the tag and the fixed reader. The maximum readable distance was recorded as the point at which the reader could still detect the tag.

In addition to the read range, the Received Signal Strength Indicator (RSSI) was measured. This parameter, expressed in decibel-milliwatts (dBm), indicates the strength of the signal reflected by the tag, with more negative values corresponding to weaker signals. The RSSI was also expressed as a percentage. Measurements were carried out in three different configurations:

0° configuration – The antenna-tag system was parallel and directly facing the reader.

90° configuration – The tag was oriented perpendicularly to the reader's plane.

180° configuration – The tag remained parallel but was rotated by 180° relative to the reader.

The results obtained are summarized in the table below:

Table 3.1: Performance of the UHF RFID antenna in different configurations. Reading distance and RSSI values are shown for 0°, 90°, and 180° orientations. The maximum read range was 62 cm at 0°, with the strongest signal reflected.

Configuration	0°	90°	180°
Reading distance (cm)	62	40	46
RSSI (dBm)	-70.7	-64.3	-59.3

These results show that the maximum read distance exceeded 60 cm in the 0° configuration, where the tag was directly facing the reader. This orientation also exhibited the most negative RSSI values, indicating a stronger reflected signal. The 90° and 180° configurations resulted in reduced read distances, with the 90° setup showing the lowest range due to the misalignment between the tag and the reader's antenna. Given that the tests were performed with the NXP chip rather than the Asygn chip for which the antenna was optimized, it is likely that the actual read range could be further improved with the intended Asygn chip. Overall, these findings confirm that the fabricated flexible RFID UHF antenna performs effectively in different orientations, maintaining a reliable communication range suitable for practical applications.

For these preliminary tests, instead of using the Asygn chips for which the antenna was originally designed, NXP SL3S1203FTB0,115 chips were employed. This choice was driven by practical considerations: the NXP chips, being simple identification chips without integrated sensors, were easier to mount and more cost-effective for initial experiments. Importantly, the impedance of the NXP chip ($Z = 25 - j237 \Omega$) is relatively close to that of the Asygn AS3213L.3 and AS3212.5 chips ($Z = 23 - j213 \Omega$). As a result, while these tests provide a valid performance assessment, it is reasonable to expect that the optimized antenna could achieve even greater read distances when paired with the intended Asygn chips.

Development and Testing of a PPG Sensor on a Chitosan Substrate for Wearable Medical Application

The innovative approach to flexible printed circuit boards (PCBs) based on chitosan, combined with the cutting-edge technology for sub-millimetric trace fabrication and the excellent biocompatibility, antibacterial properties, and flexibility of the chitosan films, opens up significant potential for the development of wearable medical devices. One such device is a photoplethysmograph (PPG), designed

to acquire heart rate data simply by placing a finger on the device. This non-invasive monitoring technique leverages the variation in blood volume that modulates the reflected light, offering valuable insights into a person's cardiovascular health. The traces on the PCB are carefully shaped using the selective cutting process to ensure smooth, rounded designs, which are in line with the design rules for stretchable electronics. This design ensures the device is not only functional but also adheres to the principles of flexibility and durability required for wearable applications. The light emitted by a red LED is modulated by variations in the blood circulating under the skin, which is reflected by the finger and subsequently detected by a photodiode. The signal-to-noise ratio (SNR) is enhanced by the use of an operational amplifier configured as a transimpedance amplifier. The construction of the PPG device involved integrating commercial silicon electronic components onto the chitosan-based PCB. In particular, as shown in Figure 3.13, the circuit consists of a red LED, a photodiode, and the operational amplifier. These components were soldered onto the flexible substrate using a reflow soldering process. To prevent damage to the chitosan substrate from high temperatures, Kapton masks were used to shield the areas of the PCB that didn't require heating, ensuring only the components that needed to be soldered were exposed to the heat. The LED was driven with an alternating voltage at 30 Hz, and the resulting signals were collected using an oscilloscope. A post-processing algorithm was developed to analyze the data, with a sampling rate of 150 Hz to ensure adequate resolution for the heart rate signal. The code filters the signal, removes any baseline drift (referred to as "dark light" noise), and applies a low-pass filter to isolate the relevant frequency components of the PPG signal. The heart rate acquisition works based on the principle of photoplethysmography (PPG), where the volume of blood flowing through the finger causes variations in the amount of light reflected by the tissue. These variations are then detected by the photodiode and amplified by the operational amplifier. By simply placing a finger on the LED, the device is able to acquire real-time heart rate data, which can be visualized in graphical form. As shown in Figure 3.14, the data from the PPG device successfully reveals key physiological features, such as the systolic peak, which corresponds to the maximum pressure generated during the systolic ejection phase of the heart cycle, and the diastolic notch, which marks the closing of the aorta. The data, represented in Figure 3.14, clearly shows the time series of the heart rate cycles. The frequency analysis, shown in Figure 3.14 (c), reveals a dominant peak at approximately 1 Hz, corresponding to a heart rate of about 60 beats per minute in this case.

This approach proves effective for heart rate monitoring and sets the stage for the development of new medical devices that not only monitor heart rate but also integrate antibacterial properties for long-term skin contact. The success of this photoplethysmograph opens the door to a new frontier in the creation of wearable, flexible, and biocompatible medical sensors.

In the near future, we envision combining this RFID-based technology with PPG sensors to create wireless monitoring systems that can be easily read by a smartphone app. Moreover, by implementing algorithms for signal classification, we could differentiate between various activities—such as standing, running, or jumping—based on changes in the PPG signal. This would lead to more personalized health monitoring solutions.

A further step forward would involve creating multi-sensing platforms, integrating the photoplethysmograph with other sensors, such as temperature sensors and pulse oximeters. These devices, which share similar circuit architectures and principles, could be integrated into a single wireless system, offering comprehensive and continuous monitoring for users.

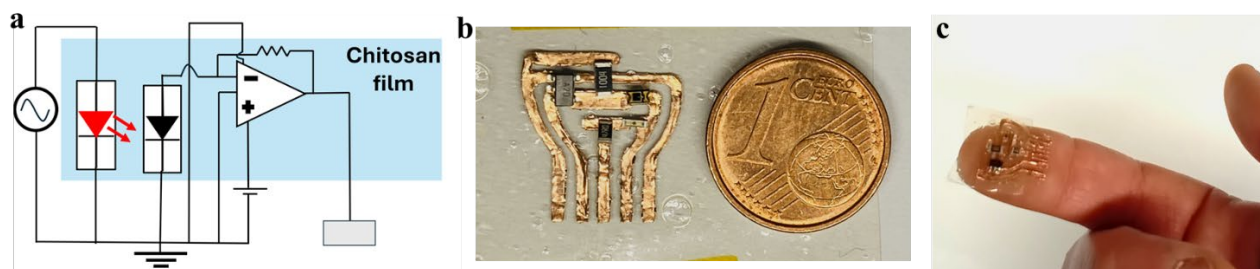


Figure 3.13. (a) Representative circuit of the plethysmograph on flexible chitosan PCB (b), (c) Optical images of the device.

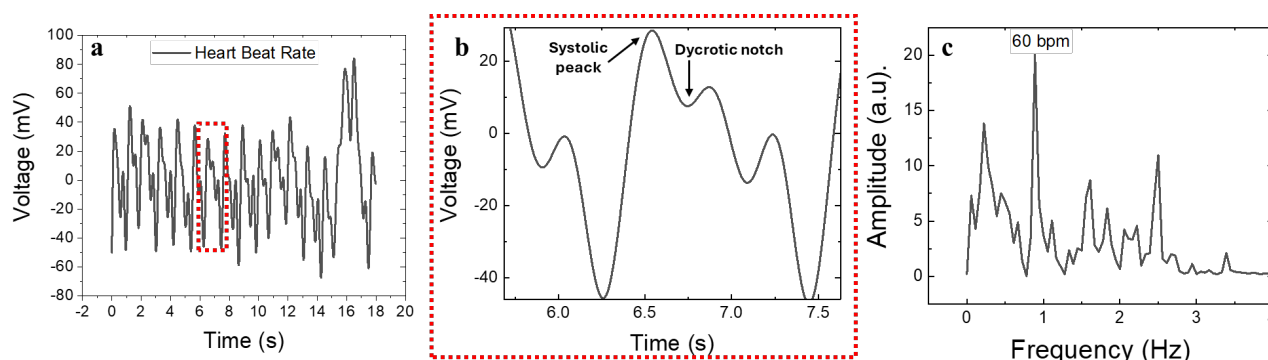


Figure 3.14. (a) Measurement results during recording on the finger. (b) Magnified view of the red dashed box; (c) FFT of the signal.

3. Materials and Methods

Chitosan Film Production and Characterization

The chitosan films were produced using a solvent casting method. Chitosan powder (10 mg/mL) was dissolved in a slightly acidic aqueous solution consisting of acetic acid and water (1:99 v/v). The solution was stirred at 400 rpm and subjected to sonication for 30 minutes to ensure complete dissolution of the chitosan. Once the solution was homogenous, it was cast onto Kapton substrates using an automatic film applicator (SAFA-219, S.A.M.A. Italia, Italy). The thickness of the liquid film was carefully regulated using a micrometer-adjustable applicator, ensuring precise control over the film's thickness. After the solvent had evaporated, films with micrometer-scale thickness and surface areas up to several hundred cm² were obtained. The uniformity of the film thickness and surface quality was further confirmed by profilometric analysis using the Tencor Alpha Step 500.

Cytotoxicity Test: Cell Viability Assay:

MRC-5 cell lines were cultured at 37°C and 5% CO₂. For the growth curve, 2,000 cells/well were seeded, and for the cytotoxicity assay, 8,000 cells/well were seeded in 96-well plates. After cell adhesion to the plate, 4 mm diameter discs of chitosan were placed into the medium. Cell viability was measured using the CellTiter-Glo Assay (Promega) with a BioTek Synergy H1 plate reader at time points of 8, 24, 48, and 72 hours.

Antibacterial test on chitosan film

Preparation of Bacterial Suspension

A single colony of *Escherichia coli* 25922 (*E. coli*) and *Staphylococcus aureus* 6538 (*S. aureus*), two widely recognized reference strains for antibacterial testing, was inoculated into 10 mL of sterile Mueller Hinton Broth (MHB). The culture medium was prepared using 2 g/L beef infusion solids, 17.5 g/L casein hydrolysate, and 1.5 g/L starch (all from Sigma Aldrich, Darmstadt, Germany). The bacterial cultures were incubated in a shaker at 37°C with continuous agitation at 150 rpm for 18 hours. Since *E. coli* is Gram-negative and *S. aureus* is Gram-positive, their inclusion allows for an assessment of antibacterial properties across distinct bacterial classifications.

Spot Assay for Evaluating Antibacterial Activity

A spot assay was carried out to determine the antimicrobial effects of chitosan films against *E. coli* and *S. aureus*. Bacterial suspensions were diluted in a stepwise manner from 1 CFU/mL to 10⁸ CFU/mL. A 10 µL volume from each dilution was deposited onto sterile Petri dishes containing 25 mL of Mueller-Hinton Agar (MHA), which had been supplemented with 17 g/L bacteriological agar (Sigma-Aldrich, Darmstadt, Germany). After allowing the drops to dry for approximately 10-15 minutes, sterile chitosan films (1 cm in diameter) were placed over the bacterial spots. The plates were then incubated at 37°C for 24 hours. As a positive control, a solution of commercially available low molecular weight chitosan (10 mg/mL, Sigma-Aldrich, Darmstadt, Germany) was applied to the bacterial colonies, along with 10 µL of bacterial suspension and 0.1 M acetic acid. The negative controls consisted of the chitosan film alone, 0.1 M acetic acid, and the 10 mg/mL chitosan solution without bacterial inoculation.

Assessment of Bacteriostatic and Bactericidal Effects

To distinguish between bactericidal and bacteriostatic properties of chitosan films, an additional incubation step was implemented. After 24 hours of initial incubation, the films were carefully removed from the agar plates, which were then re-incubated at 37°C for another 24 hours. If bacterial colonies resumed growth following film removal, the action of chitosan was classified as bacteriostatic, indicating growth inhibition rather than bacterial eradication. Conversely, if no bacterial regrowth was observed, the effect was deemed bactericidal, implying that chitosan had induced irreversible bacterial damage or death during initial exposure.

Time-Dependent Antibacterial Assessment

To evaluate the antibacterial activity of chitosan films over time, bacterial suspensions of *E. coli* and *S. aureus* were serially diluted from 10⁶ to 10¹ CFU/mL, and 10 µL of each dilution was spotted onto MHA plates. Following a drying period of 10-15 minutes, chitosan films were placed over the bacterial spots in the experimental group, while control plates were prepared without chitosan films. All plates were incubated aerobically at 37°C, and bacterial growth was monitored at 24, 48, and 72 hours. Growth inhibition was evaluated by comparing colony formation on the chitosan-treated plates with that on the control plates, assessing bacterial reduction over time.

Microfabrication of Metallic Traces

To integrate metallic traces onto the chitosan films, adhesive copper strips were applied in a rectangular shape onto the chitosan substrate. Using the Silhouette Cameo 4 cutter (Silhouette America, USA), the copper strips were precisely cut to create the metallic patterns, based on CAD designs. After the cutting process, the excess copper was removed, leaving behind the desired metallic traces integrated onto the chitosan film.

Characterization of Metallic Traces

The quality of the metallic traces was assessed using an optical microscope (Zeiss Stemi 305, Carl Zeiss AG, Germany), which allowed for high-resolution imaging of the chitosan films and the embedded metallic traces.

Fabrication of Smart Patches

The fabrication process of both RFID antennas and flexible printed circuit boards (PCBs) for the plethysmograph was based on a common set of materials and methodologies. The metallic traces were obtained from 3M™ EMI Copper Foil Shielding Tape 1181 with Electrically Conductive Acrylic Adhesive (3M, USA), a self-adhesive copper tape with a width of 2.5 cm and a thickness of 30 µm. The components were soldered using BEEYUIHF Lead-Free Solder Paste, Sn42 Bi58 (BEEYUIHF, China), which was applied via a syringe dispenser to ensure precise deposition on the designed contact pads. The soldering process was carried out using a XY-brand heat gun (Brand, Country), ensuring controlled reflow while minimizing thermal exposure to the underlying biopolymer substrate.

RFID Antennas

The NFC antennas were fabricated by soldering ST25DV16KC-IE6S3 and ST25TV02KC-TFH3 chips (STMicroelectronics, Switzerland) onto the copper coil structures. These NFC antennas were subsequently read using the smartphone application ST25NFC Tap (STMicroelectronics, Switzerland). For the UHF antenna, the SL3S1203FTB0,115 chip (NXP USA Inc., USA) was mounted onto the copper serpentine structure. The resulting RFID tag was then interrogated using an RFID reader from SensorID, Italy.

Photoplethysmograph (PPG) Fabrication

The photoplethysmograph (PPG) was assembled by soldering commercial electronic components onto the patterned copper traces. Specifically, a red LED (IN-S85CS5R, Inolux, USA), a photodiode (TEMD7000X01, Vishay Semiconductors, USA), and an operational amplifier (ADA4505-1, Analog Devices, USA) were used to construct the circuit.

4. Conclusions

In conclusion, this research has successfully developed antibacterial smart patches on chitosan-based substrates, demonstrating their potential for both medical and food packaging applications. These patches combine the mechanical properties essential for flexibility and durability with the added benefit of biodegradability, making them suitable for a wide range of practical uses. Biocompatibility was confirmed through cytotoxicity tests, while antibacterial tests demonstrated that the chitosan films effectively inhibited the growth of both *E. coli* and *S. aureus* at bacterial concentrations up to 10⁴ CFU/mL. Furthermore, the antibacterial action persisted for up to 72 hours, making these smart patches particularly well-suited for wound healing applications, where infection prevention and real-time monitoring are critical.

Beyond medical applications, these antibacterial smart patches also hold great promise in food packaging, where their ability to prevent bacterial contamination can help extend the shelf life of food products. This dual functionality highlights their versatility, positioning chitosan-based smart patches as a sustainable solution for both healthcare and food safety.

Moreover, the use of an automatic cutter enables the production of metallic traces with sub-millimetric resolution on these substrates, facilitating the fabrication of highly miniaturized functional devices. This capability has led to the successful realization of a photoplethysmograph (PPG) for heart rate measurement on a flexible PCB, as well as RFID antennas (both NFC and UHF). The modular design approach used for the antennas ensures minimal material waste and allows for component reuse, further expanding the potential of chitosan-based substrates in flexible electronics.

The development of antibacterial smart patches on chitosan not only marks a significant step forward in wearable medical devices and food packaging but also offers a sustainable, biocompatible, and highly versatile solution for next-generation applications in healthcare and food safety.

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Coclusions and Future Perspectives

Conclusions

This thesis explores the potential of chitosan, a bio-based material derived from seafood industry by-products, with a focus on its fabrication into thin films and their advanced applications. Starting from the extraction and characterization of chitosan from crab waste (*Maja squinado*), we conducted a preliminary physicochemical analysis to understand its composition and structure. This provided the foundation for a deeper investigation into its remarkable film-forming ability, positioning chitosan as a promising candidate for flexible electronics.

Subsequently, we developed a versatile method for producing chitosan films using an automated applicator, also considering the scalability of the process for large-scale production. This approach enabled high uniformity, reproducibility, and control over film thickness, ranging from ultra-thin layers below 10 μm to thicker films exceeding 200 μm , with surface areas varying from a few square millimeters to several hundred square centimeters. By adjusting the composition, we successfully modulated mechanical properties across a wide range, achieving a tensile strength exceeding 80 MPa and elongation above 60%.

A key outcome of this research was the precise characterization of the piezoelectric properties of chitosan thin films using Piezo Force Microscopy (PFM). We implemented a method that allowed us to isolate piezoelectric forces from electrostatic ones and measure the d_{33} coefficient with high accuracy. Through this technique, we detected an increase in d_{33} up to 18.7 pmV^{-1} by incorporating chitin nanocrystals into the chitosan matrix enhancing the films' crystallinity. This advancement led to the creation of a fully bio-based piezoelectric soft thin films, greatly expanding the potential of chitosan as an active substrate for electronic devices, including high-performance sensors and transducers.

Further characterization highlighted both the strengths and limitations of the films. Among the challenges were their sensitivity to humidity, water-induced degradation and relatively low thermal stability, which could hinder microfabrication. To address these challenges, we developed a green and innovative patterning technique that overcomes issues related to traditional metal deposition methods. Our approach, utilizing a low-cost automated cutter, enables the selective patterning of metal traces onto chitosan with sub-millimeter precision, ensuring defect-free structures even at dimensions below 500 μm .

Nevertheless, the films exhibited highly promising characteristics, including optical transparency in the visible range (400–800 nm), along with a distinct absorption peak around 200 nm, indicative of UV absorption, making them suitable for optoelectronic applications, including UV shielding

Moreover, they demonstrated biocompatibility, confirmed through cytotoxicity tests with human lung fibroblasts, and antibacterial activity, effectively inhibiting the growth of *E. coli* and *S. aureus* at concentrations up to 10^4 CFU/mL for 72 hours.

Building on the antibacterial properties and mechanical flexibility of chitosan films, crucial for biomedical and smart packaging applications, as well as the method developed for precise metal trace patterning, we created smart electronic patches. These include Ultra High Frequency (UHF) and Near Field Communication (NFC) Radio Frequency Identification (RFID) antennas, fabricated using a modular approach that minimizes material waste and allows for component reuse, and tested with commercial RFID readers. We also assembled a Photoplethysmogram (PPG) sensor on flexible printed circuit boards (PCBs) for heart rate monitoring, showcasing chitosan's potential as a versatile material for sustainable, bio-based electronic technologies.

In summary, our work demonstrates that chitosan is a remarkably versatile material, with significant potential for advanced applications in flexible electronics and medicine. Its ability to modulate mechanical and piezoelectric properties through the incorporation of bio-based additives, coupled with its biocompatibility and antibacterial activity, makes it an ideal candidate as both a passive and active substrate in electronic devices. Our research journey, beginning with the crab as its source, culminates

in the creation of functional, biodegradable, and sustainable electronic devices, emphasizing the importance of a circular approach to the development of future technologies.

Future Perspectives

Looking ahead, several key areas of development remain crucial for advancing chitosan-based technologies. One of the main challenges is the inherent water sensitivity of chitosan materials, coupled with their tendency to deform at elevated temperatures. These limitations present obstacles in microfabrication processes, especially in applications requiring high thermal stability. Therefore, further optimization of chitosan compositions is essential to enhance their processability while maintaining the material's desirable properties.

Moreover, a deeper understanding of chitosan's piezoelectric behavior on a larger scale would benefit from mesoscale measurements, offering greater insight and correlation into the phenomenon. This could lead to the realization of a fully functional piezoelectric device, paving the way for the integration of a fully bio-based polymer into sensor and actuator technologies.

A key next step in the development of antibacterial smart patches is to leverage our advancements in flexible PCBs and RFID antennas to create a wireless multi-sensing platform. By integrating various sensors, such as pulse oximeters, temperature sensors, and the PPG for healthcare applications, alongside pH and gas sensors for packaging for food monitoring, this platform would enable continuous, real-time monitoring of both physiological and environmental parameters.