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Review Article

Exploring the Therapeutic Potential and Pharmaceutical Applications of Avian Egg Shell Membrane: A Comprehensive Review

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Abstract

The eggshell membrane (ESM) is a beneficial biomaterial derived from egg industry byproducts. This detailed overview discusses ESM's complicated structural composition and molecular chemistry, along with its applications in several sectors. ESM is made up of collagen, glycosaminoglycans (GAGs), and proteins from the eggshell matrix. This review investigates extraction approaches, such as mechanical, chemical, and enzymatic procedures, and discusses the multiple issues connected with solubilizing ESM proteins. ESM offers a wide range of therapeutic applications, including tissue regeneration, wound healing, joint health, osteoarthritis symptom reduction, and improved skin wound recovery. ESM has anti-inflammatory, antioxidant, and antibacterial characteristics, which make it a key component in pharmaceutical compositions. ESM also plays an important role in electric devices and next-generation batteries, providing potential future opportunities for sustainable energy solutions. This review focuses on the diverse applications of ESM and possible developments, emphasizing its importance as a promising biomaterial in the fields of medicine and pharmaceuticals.

Keywords: Egg shell membrane (ESM), biomaterial, structural characteristics, molecular characteristics, separation techniques, solubilization techniques, safety assessment, tissue engineering,

1. Introduction

The eggshell membrane (ESM) contributes to about 10% of the overall weight of an avian egg (Wu 2021). The eggshell is composed of 95% calcium carbonate (Athanasiadou 2019), whereas the organic matrix, which contains proteins, glycoproteins, and proteoglycans comprises about 3.5% (Wu 2021, Athanasiadou 2019). ESM also contains cross-linked collagens (types I, V, and X), glycosaminoglycans (GAGs), eggwhite proteins (e.g., ovotransferrin and lysozyme), and eggshell matrix proteins (such as ovocalyxin-36) (Wu 2021, Athanasiadou 2019, Hincke et al. 2011, Zhong 2009). Figure 1 shows the various components of the eggshell.

Considering the wide range of ESM uses in industries, such as food, cosmetics, pharmaceuticals, and engineering, comprehending its positional and structural complexity is critical. The primary goal of this review is to provide a full explanation of the structure and composition of ESM, as well as its isolation methods, solubilization procedures, safety assessment, and diverse uses. Our goal is to employ existing knowledge and integrate new discoveries to highlight the diverse potential of ESM as a biomaterial. We seek to promote its proliferation throughout industries by assessing existing patterns and forecasting future changes.

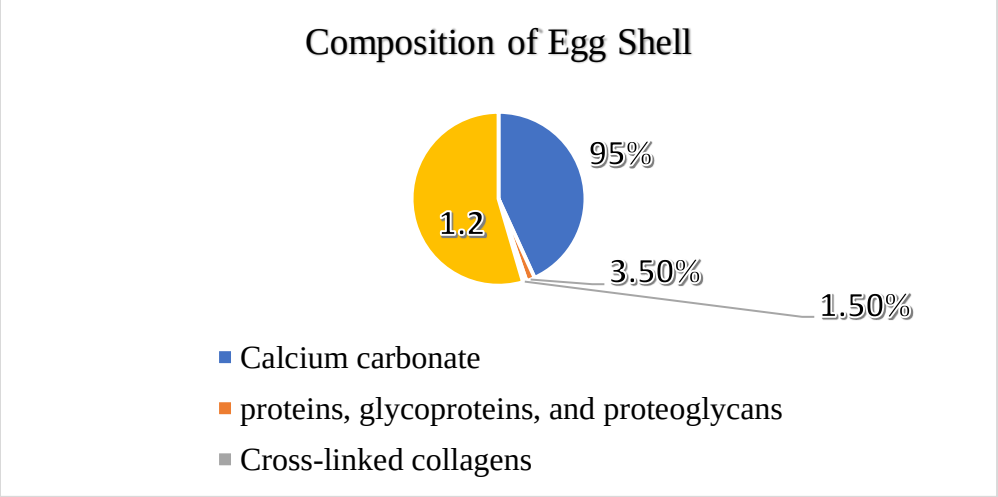

Figure 1: The figure shows the composition of egg shell.

1.1 Layers and Structure of ESM

The ESM is the intermediate layer between the mammillary layer and albumen, with a unique fiber organization. This peculiar structure of ESM promotes eggshell mineralization while inhibiting egg white mineralization (albumen) (Park et al. 2016). ESM consists of three levels: the outer, inner, and limiting layers. These layers form a spiral design (Shi, Kovacs-Nolan, et al. 2014a).

(i) Outer Shell Membrane

The outermost layer (50-70 μm) forms a bud-like structure on the mammary knob. It connects the ESM and the eggshell. Furthermore, fibers weaves between the inner shell layer and the outer shell membrane in a distinct pattern that excludes the air cell region (Hamilton 1986).

(ii) Inner Shell Membrane and the Limiting Layer

The surface roughness of the inner shell membrane, which is interlaced with outer shell membrane fibers, varies, with the limiting layer surrounding the egg white directly. The limiting layer is distinguished by fiber knobs, and the diameter differential across the membrane decreases from the outermost layer to the limiting membrane (Baláž 2014). Figure 2 depicts a schematic representation of various layers of an eggshell.

Moving forward, we will investigate the complex protein composition of ESM, shedding light on the main components that contribute to its structural integrity and functional capabilities.

1.2 Composition of Protein in the ESM

ESM is mostly made up of protein-based fibers (80-85%), with about 500 different kinds of proteins (Strelec et al. 2023) Collagens, which make up 10% of the ESM, serve as the basic structural underpinning, with collagen I and V having distinct distributions in the inner and outer layers. Collagen X, which is found in both levels, is supposed to hinder mineralization, undermining the notion. In addition, fibronectin, a dimeric glycoprotein activator or binding protein, is also present (Matsuoka et al. 2019).

1.3 Additional Components in the ESM

In addition to the major components, the ESM contains osteopontin, which provides binding sites for cells and ions such as calcium (Ca++), as well as several serine and threonine-phosphorylation binding sites (Herman et al. 2023). Beyond proteins, it contains $CaCO_3$, uronic acid, sialic acid, and trace amounts of saccharides (M Pillai, Saha, and Tayalia 2023, Kavarthapu and Malaiappan 2019).

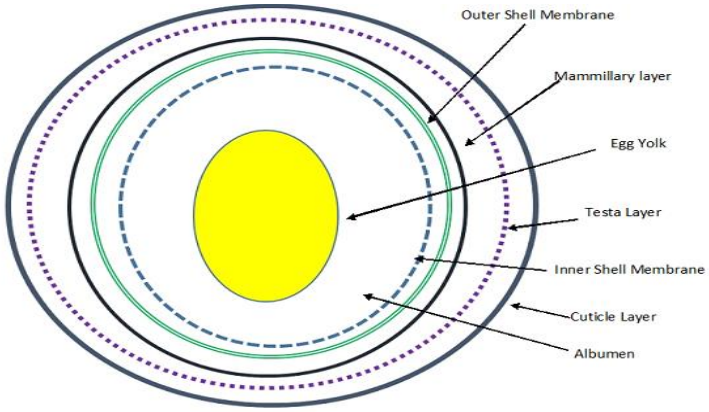

Figure 2: This figure represents a schematic illustration of different layers of Egg Shell.

2. Isolation and Solubilization of the ESM

2.1 Isolation Methods for ESM

ESM can be isolated from eggshells by using three methods: mechanical, chemical, or enzymatic. The mechanical method entails separating the inner part of the ESM and its membrane from the eggshell manually. Whereas, the chemical method involves using acids like hydrochloric acid or EDTA to break down the structure of eggshells, and subsequently extracting the ESM (Zhao et al. 2019, Li et al. 2020, Mensah and Jo 2021).

2.2 Challenges and Solubilization Techniques

As there are complex bonds among various proteins, such as Chorioallantoic Membrane Extracted Proteins (CREMPs), keratins, desmosines, and hydroxylysinonorleucine, pose issues for ESM dissolution in aqueous solutions. The solubility of ESM proteins (SEP) must be enhanced to unlock its full utilization potential. Moreover, to prevent the breakdown of heat-sensitive fibrous proteins during SEP preparation, temperature control (50–70 °C) is also necessary (Han et al. 2023, Kulshreshtha et al. 2020, Kulshreshtha et al. 2022).

2.3 Applications of SEP

SEP has a wide range of applications, such as biomaterials for tissue regeneration (Jia et al. 2012), efficient purification methods (Zhang et al. 2016), optimization of collagen extraction, and antioxidant properties (Mohammadi et al. 2016). Ongoing research aims to explore SEP applications across diverse fields, with a focus on tailoring SEP for specific practical uses.

3. Application of ESM as an Innovative Biomaterial

3.1. Osteoarthritis Management

Osteoarthritis (OA) is the most common cause of joint disorders, and it has a substantial impact on overall quality of life. Multiple studies on ESM, as a traditional therapy for connective and joint tissue disorders, show promising results in reducing stiffness, joint discomfort, and excessive cartilage turnover caused by strenuous physical exercise. In a double-blind research test, postmenopausal women (aged 45-70) were given 500 mg of a commercial medication called Natural ESM (NEM®) every day for two weeks. This, combined with frequent alternate-day activity, resulted in decreased cartilage turnover. Consumption of the ESM product assisted rapid recovery from exercise-induced joint problems (Shi, Kovacs-Nolan, et al. 2014a).

3.1.1 Clinical Studies and Findings

i) Clinical Trial with BiovaFlex

In a clinical double-blind study, the therapeutic efficacy of a supplement named BiovaFlex was investigated. A placebo and BiovaFlex (450mg) were given at random to 88 OA patients. At day 5, patients felt better with BiovaFlex (Hewlings, Kalman, and Schneider 2019). These data strongly suggest that ESM hydrolysate has the potential to be a helpful dietary supplement for relieving

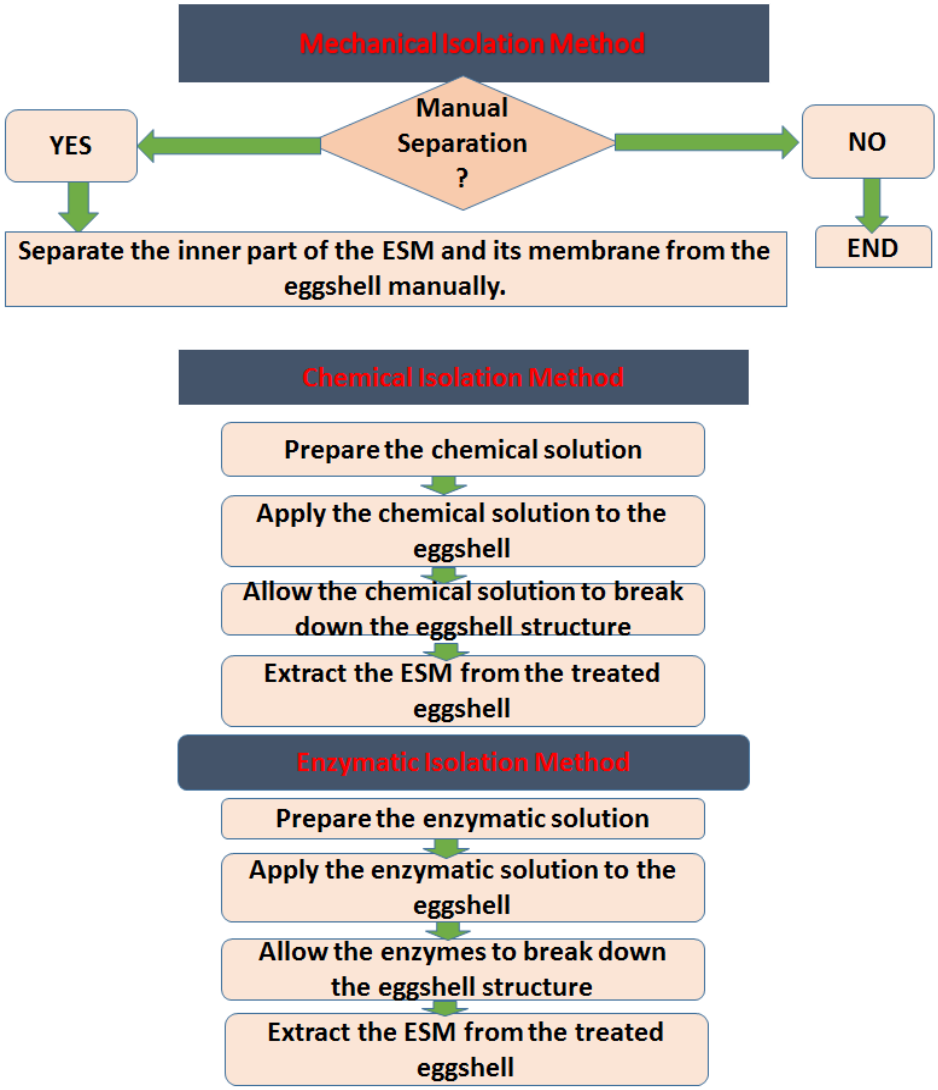

Figure 3: This flowchart outlines the decision-making process for choosing between mechanical, chemical (acidic treatments), and enzymatic extraction methods based on the integration level of the limiting membrane and inner portion ESM into the ES.

osteoarthritis symptoms and improving mobility in those suffering from the condition.

ii) Additional Placebo-Controlled Trials.

In another clinical trial, 150 OA patients aged 45 to 70 were administered ESM powder (300 mg) OD for 12 days. Patients who received ESM supplements reported improved well-being (Kiers and Bult 2021).

iii) Contributions to Overall Joint Health

Besides from its role as a dietary supplement, ESM contributes considerably to joint health in additional ways. A strong and flexible material, similar to meniscus, was developed to encourage cell growth and repair. ESM/silk fibroin hydrogels have been shown to support human articular chondrocyte cells, indicating that they could be utilized like cartilage substitutes in tissue engineering applications in the future (Pillai et al. 2018, Adali, Kalkan, and Karimizarandi 2019).

3.2. An Effective Biomaterial for Skin Wounds

Different studies on mice prove that solubilized ESM helps in the healing of wounds by synthesizing type III collagen. This can potentially

reduce facial wrinkles and improve skin (Ohto-Fujita et al. 2019).

3.2.1. Cost-Effective Solution:

Another study used processed ESM powder (PEP) in a mouse wound splinting model. Researchers discovered that PEP hastened wound healing by activating matrix metalloproteinase (MMP) activity in dermal fibroblasts and mouse skin, resulting in increased MMP-2 protein levels. Furthermore, this stimulation increased the production of alpha-smooth muscle actin (Ahmed, Suso, and Hincke 2019, Vuong et al. 2018). These findings highlight PEP's potential as a cost-effective way to promote efficient wound healing.

3.2.2. Promising Application in Tympanic Membrane Perforation Treatment:

A randomized clinical trial found that using ESM patching for tympanic membrane perforation improves healing time (Jung et al. 2017, Choi 2014). This highlights the significance of ESM patches in enhancing the healing process.

3.2.3. Physicochemical Enhancement through Inorganic Compounds for Optimal Healing:

The enhancement of physic-chemical processes improves the healing process. Organic ingredients are combined with ESM, which improves the healing process by addressing important aspects like hydrophilicity. Copper-containing bioactive glass nanocoatings (Cu-BG)/ESM film increases angiogenesis *in vivo* and promotes epidermal layer formation. Additionally, a regulated release of Cu^{2+} ions inhibits bacterial development and efficiently avoids wound infections (Li et al. 2016).

3.2.4. Synergistic Healing with ESM and Silver Nanoparticles:

ESM-Silver synergy improves reepithelialization, formation of granulation tissues, and overall wound healing through cell proliferation and inhibiting inflammation (Liu et al. 2017). These findings suggest the versatility of ESM's role in the wound healing process and shaping innovative therapeutic approaches.

3.2.5 ESM and Chitosan in Wound-Dressing Films

The combination of ESM and chitosan-forming film improves the antibacterial activity in wound dressing and provides a diverse approach to therapeutic intervention (Li, Ma, et al. 2019).

3.3. ESM Impact on Gut:

ESM supplements improved gut health in mice by preventing colon shortening, intestinal inflammation reduction, restoration of epithelial cell integrity, and modulating gut flora (Jia et al. 2017). In an in vitro study, ESM showed inhibitory effects on lipopolysaccharide-induced inflammatory cytokine synthesis, which ameliorates Caco-2 cell proliferation through the mechanism of up-regulation (Jung et al. 2017). Moreover, ESM limits dysbiosis by enhancing bacterial diversity, regulates the expansion of Th17 cells, and minimizes the absolute counts of pathogenic bacteria like *Escherichia coli* and *Enterobacteriaceae*. The use of ESM, in combination with a high-fat diet, led to not only reduced circulating triglyceride levels but also decreased total cholesterol content in hepatocytes in mice. Hence, genes related to lipid metabolism and microbial composition were modified (Ramli et al. 2020). In addition, ESM hydrolysate suppresses pro-inflammatory cytokine IL-8 secretion in vitro and showed efficacy in the colitic mouse model. Furthermore, the research attributed the inflammation alleviation in the colitic-mouse model to the IL-6-mediated pathway and enhanced apoptosis of T-cells to restore immune homeostasis in the gut (Shi, Rupa, et al. 2014).

3.4. Anti-Inflammatory and Antioxidant Activities of ESM

The antimicrobial activity against skin pathogens can specifically be improved by utilizing innovative techniques, such as cryogrinding and homogenization, to unlock the potential of submicron-sized ESM (Kulshreshtha et al. 2020). An aqueous extract of ESM stops inflammation by modulating T-cell signaling and reducing pro-inflammatory TNF-α, supporting ESM's anti-inflammatory and immunomodulatory properties (Benson, Ruff, and Jensen 2012, Vuong et al. 2017).

Table 1: The table shows different application of ESM and their key findings.

Application	Key Findings
Joint Health	• ESM alleviates stiffness, joint pain, and cartilage turnover.
	• Clinical trials with Natural ESM (NEM®) and BiovaFlex show improved knee function and mobility.
	• ESM contributes to joint health in tissue engineering applications.
Wound Healing	• Solubilized ESM boosts type III collagen synthesis, enhancing skin elasticity.
	• Processed ESM Powder (PEP) accelerates wound closure and stimulates MMP activities.
	• ESM patching reduces healing time in tympanic membrane perforation treatment.
Gut Health	• ESM exhibits diverse impacts, including suppressing inflammation, improving microbial diversity, and regulating lipid metabolism.
	• ESM hydrolysate relieves inflammation in colitic mice models.
Anti-Inflammatory and Antioxidant Activities	• Cryo-grinding produces submicron-sized particles with enhanced antimicrobial activity.
	• Aqueous extract of ESM reduces pro-inflammatory cytokine TNF-α.
	• ESM hydrolysates show anti-lipopolysaccharide and antioxidant activities.
Bacteria Control	• Copper-containing bioactive glass/ESM exhibits efficient antibacterial activity.
	• ESM combined with silver nanoparticles enhances antibacterial properties
Biomineralization	• ESM acts as a substitution for CaCO3 nano-crystals, influencing crystal growth in biomineralization processes.
	• ECM proteins derived from ESM influence calcitic biomineralization.
Immobilization	• ESM serves as a versatile material for biosensor development, supporting enzyme immobilization.
	• ESM modifications with polyethyleneimine enable urea biosensor development.
Tissue Engineering	• ESM enhances nerve tissue regeneration and improves mechanical properties in 3D tissue engineering.
	• ESM-based constructs mimic heart valve leaflets and vascular intima surface
Food Packaging	• ESM-derived gelatin enhances mechanical and barrier properties in edible films.
	• Solubilized ESM Protein (SEP) composite films exhibit satisfactory properties.
Biosorbent Activities	• ESM proves effective in adsorbing diverse substances and shows potential in environmental remediation

Similarly, acetic acid-treated ESM fractions have anti-inflammatory properties, inhibiting lipopolysaccharide and IFN-γ-induced inflammation for effective skin inflammation management (Yoo et al. 2014). As the degree of hydrolysis increases, ESM hydrolysates gain powerful radical scavenging and antioxidant properties in a variety of manner with greater efficacy (Yoo et al. 2014, Shi, Kovacs-Nolan, et al. 2014b, Jain and Anal 2017, Nagamalli et al. 2017). These findings support the anti-inflammatory and anti-oxidant properties of ESM.

3.5. ESM for Bacteria Control

ESM nano-composites, which have been treated with inorganic chemicals, such as copper-containing bioactive glass or ESM, demonstrate their efficiency through the prolonged release of Cu^{2+} ions. This distinct characteristic makes ESM

suitable for bacterial control in a variety of sectors (Li et al. 2016). ESM, when coupled with silver nanoparticles, improves bacterial control through a synergistic mechanism (Liu et al. 2017, Li, Cai, et al. 2019).

Metal-oxide integration provides potent antibacterial activity under visible light exposure. When ESM nano-composites are mixed with metal-oxides, like CuO-ZnO, they exhibit antibacterial action. Notably, *E. coli* is specifically targeted by the establishment of an axial p-n junction (Preda et al. 2020).

3.6. ESM as a Substitution in Biomineralization: Beyond $CaCO_3$ Nano-Crystals

ESM appears as a substitute for $CaCO_3$ nano-crystals in the process of biomineralization, which involves the deposition of inorganic ions within organic matrices or scaffolds, demonstrating its function in biomineralization (Kulshreshtha et al. 2020). ECM proteins produced from ESM, notably the 46 proteins linked with membrane fibers, play an important role in the biomineralization process and regulate calcitic biomineralization (Rose-Martel, Smiley, and Hincke 2015). Some research suggests that Type X collagen, the predominant component of non-mineralized ESM, inhibits cellular mineralization, while others imply that it modulates mineralization (Kirsch, Swoboda, and von der Mark 1992). Recent research indicates that ESM is an appropriate biotemplate for the crystal formation and repair process. It improves flower-like agglomerates of hydroxyapatite crystals and influences the kind of CaCO3 polymorph during shell repair in the snail (Helix aspersa) (Zhang et al. 2011, Fernández et al. 2016). When ESM is treated with sodium trimetaphosphate, phosphate groups are placed onto the surface of type I collagen, which promotes mineralization by producing calcium phosphate crystals. Similarly, treating ESM with polycarboxylated increases the mineralization process by increasing the number of places on the surface where CaCO3 mineralization can occur. These findings strengthen its function in crystal formation (Xu, Neoh, and Kishen 2010, Arias et al. 2020).

3.7. ESM for Immobilization: Versatility in Biosensor Development

ESM is insoluble in water but has great permeability to both water and air. This distinguishing feature of ESM makes it a suitable biomaterial for immobilization and biosensor applications. ESM can immobilize enzymes of many functional groups, including oxidases (D-amino oxidase, glucose oxidase), hydrolases (urease, myrosinase), and antioxidant enzymes (catalase, tyrosinase) (D'Souza et al. 2013). When coated with a particular polymer (polyethyleneimine), ESM obtains a positive charge, acting as a magnet for urease molecules and forming a sensitive urea sensor (potentiometric urea biosensor) (Zhang et al. 2015). Scientists coupled gold nanoparticles with porous carbonized ESM to create a sensor capable of detecting horseradish peroxidases with great accuracy and sensitivity (Li, Wang, et al. 2017a). These applications demonstrate the importance of ESM in biosensor development due to its unique qualities of enzyme immobilization and sensing goals.

3.8. ESM for Tissue Engineering

i) Nerve Tissue Regeneration

In nerve tissue engineering, ESM effectively played a major part in nerve regeneration (Golafshan et al. 2017, Golafshan, Kharaziha, and Alehosseini 2018). Scientists have created multilayer ESM hydrogels that resemble heart valve leaflets, not only in form and function but can also be employed in artificial heart valve replacements (Li, Bai, et al. 2017).

ii) Improved Mechanical Properties for 3D Tissue Engineering

ESM powder, when combined with collagen proteins for 3D tissue engineering, improves cellular adhesion and proliferation during cell regeneration (Rønning et al. 2020).

iii) Vascular Graft: Mimicking Natural Blood Vessels

In tissue engineering, an ESM/thermoplastic polyurethane vascular graft replicates the vascular intima surface. This arrangement encourages the growth of endothelial cells and the replication of natural blood arteries (Yan et al. 2020).

3.9. ESM for Food Packaging

ESM offers edible, safe and environment-friendly films as packaging materials that protect food from oxygen, carbon dioxide, lipids, fragrance, flavors, and moisture (Umaraw et al. 2020). ESM-chitosan combination may aid in food preservation by increasing the mechanical strength and barrier properties of edible films (Mohammadi et al. 2018).

i) SEP for Composite Films

SEP combines with other proteins, such as soybean protein, to produce composite films with increased mechanical strength, barrier characteristics, water resistance, and hydrophobicity(Li et al. 2020). These examples demonstrate ESM's promising involvement in the production of protein-based edible films, thereby preserving food.

3.10. ESM as a Biosorbant

ESM shows biosorbent activity due to its unique property of absorbing inorganic substances through a chemical alteration mechanism (Xin et al. 2018, Al-Ghouti and Khan 2018, Mirzaei and Javanbakht 2019, Zhao et al. 2021, Li, Wang, et al. 2017b, Bessashia et al. 2020, Parvin et al. 2019).

4. Safety Evaluation of ESM

It is critical to ensure the safety of ESM products because of the variety of applications. A thorough review was performed to measure its mutagenicity and cytotoxicity. The analysis revealed that human cells were not harmed by the administration of 100 micrograms of ESM. Furthermore, when it was administered to the animals at a dose of up to 5000 micrograms per plate or fed to them at a rate of up to 2000 milligrams per kilogram of body weight per day for three months, no cytotoxic or mutagenic effects were found in their cells (Ruff et al. 2012).

5. Future Perspective

ESM is gaining popularity, particularly among scientists looking for improved ways to store energy. These materials may become the next big thing after lithium-ion batteries. They are investigating the ESM to assess its utility for saving energy in novel ways. Interestingly, scientists are converting ESM into a unique type of carbon for energy conservation. However, several issues remain to be resolved. For instance, they must up-scale the project for real-world application, and keep costs to a minimum, while achieving and maintaining optimum functionality. Furthermore, to employ ESM in devices such as phones and laptops, their durability and efficacy must be ensured in every condition. To tackle this challenge, diverse experts must collaborate and come up with innovative solutions. Notably, ESM has the potential to significantly alter how we store energy. With further discoveries on the way, we may envision a world in which our energy-saving solutions are not just better, but also preferable for the environment (Park et al. 2016).

Conflict of interest

The authors declare that they have no conflicts of interest to disclose.

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Study Approval

NA.

Consent Forms

NA.

Authors Contributions

AAK conceptualized the study, SUS and HK did the literature search, collected relevant articles,

AAK and SUS analyzed the articles, SUS and HK wrote the initial manuscript, AAK, SUS, and HK refined the final manuscript.

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