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Review

Avian eggshell membrane as a material for tissue engineering: A review

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ABSTRACT

Eggshell membrane (ESM) has recently gained interest for various applications in the field of biomedical engineering, materials science and environmental engineering. It is a routinely generated waste material which makes it easily available and an affordable biomaterial. ESM is a protein-rich, thin, fibrous membrane composed of collagen and hyaluronic acid, a composition similar to that found in human tissues. The physicochemical properties of ESM make it suitable for tissue engineering applications such as regeneration of skin, bone, cartilage, tympanic membrane, nerve and blood vessels. Further, ESM has been used either as nanoparticles or as a platform to deliver nanoparticles for various therapeutic applications. The review discusses the intrinsic structural and chemical properties of ESM, the techniques to isolate ESM, the various forms in which it has been used and its varied tissue engineering and nanomedicine applications, thereby highlighting its potential as an ideal natural biomaterial for biomedical applications. It also highlights the challenges to the utility of ESM and the unmet needs.

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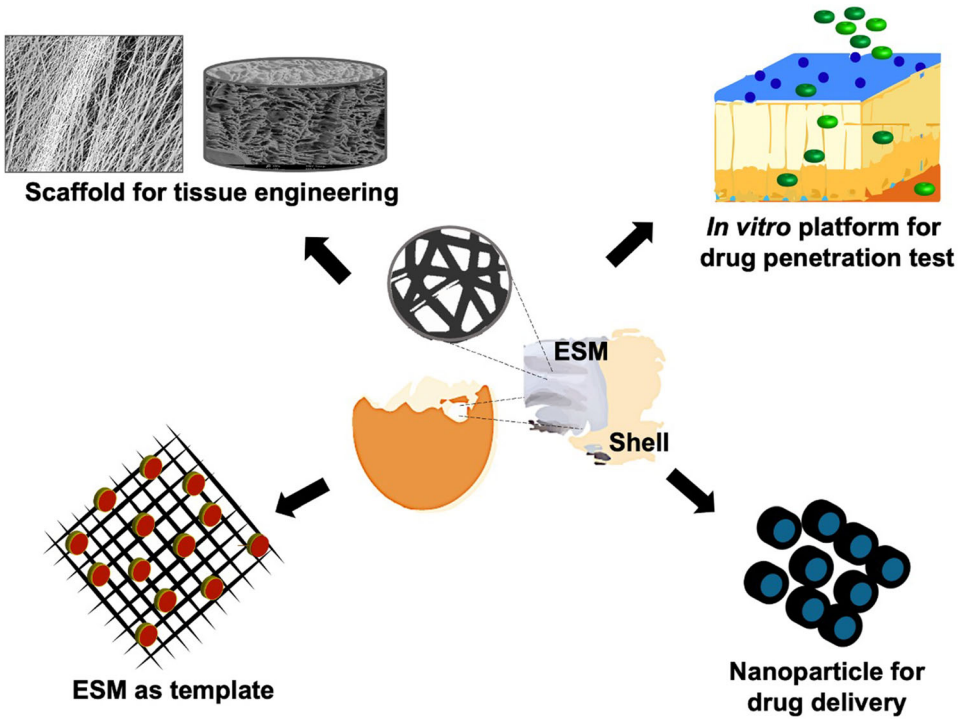
GRAPHICAL ABSTRACT



Introduction

Avian eggs are a popular food worldwide, with over 10^{12} eggs consumed globally per year. Eggshell and eggshell membrane (ESM) are discarded as waste by households and industries. The eggshell is a hard, outermost covering composed of calcium and phosphate. ESM is a protein-rich membrane that underlies the shell and around the egg white, and is a source of a variety of biomolecules such as proteins, glycosaminoglycans (GAGs), monosaccharides, and lipids [1]. Since ESM is a natural extracellular matrix (ECM) of the egg, it has been found to possess great potential as a material for various biological applications.

ESM has been used as a traditional wound dressing by the Chinese population for a long time. In 1982, it was identified as a suitable biocompatible material for cell culture studies, and in 1984, it was first used as a scientifically proven burn wound dressing [1]. In Japan, it is still widely used by sumo wrestlers to treat injuries. ESM is also sold as a dietary supplement for treating osteoarthritis [2]. ESM is semipermeable in nature owing to its fibrous network-like structure, large surface area, porosity, good mechanical strength, biocompatibility, and biodegradability. It is inexpensive, easy to procure, eco-friendly, and non-toxic, and the functional groups of its constituents can be chemically modified to enhance its properties [1].

Recently, ESM has been increasingly used in water purification, heavy metal ion removal, enzyme immobilization, and tissue engineering. Although ESM has been used historically, a publication in 1995 by Wu et al. first demonstrated the application of ESM as a biomaterial owing to its structure and composition [2, 3]. Previous review articles on avian eggs have focused on the materials science uses of ESM [1, 3] and tissue engineering applications of either solubilized ESM [2] or different components of eggs [4]. Shi et al. described diverse biomedical and materials science applications of ESM with a lesser focus on the tissue engineering aspect and a major

focus on its isolation, solubilization, and safety [5]. Jana et al. discussed the biomedical applications of various waste-derived biomaterials, including ESM and its isolated proteins, with a focus on xenografts [6]. This review discusses the applications of different forms of ESM in tissue engineering and nanomedicine and intends to provide a comprehensive understanding of ESM to readers by including studies on structural and functional characterization, chemical composition, and applications of ESM.

Structure of ESM

The ESM structure was first discovered in 1957. ESM is a semi-permeable, double-layered, collagen-based fibrous matrix between the eggshell and egg white. It acts as a physical barrier to the entry of microbes due to its mesh-like structure [1]. It is structurally and functionally equivalent to the ECM of mammalian tissues and is impregnated with calcium carbonate [2]. It is composed of three layers (Fig. 1a): the outer membrane, inner membrane, and limiting membrane. The outer membrane remains attached to the tips of the calcified eggshell and has knob-like structures that provide a rough texture. The inner membrane lies between the outer and limiting membranes and has a smooth texture with compact bundled fibers. The limiting membrane is the innermost membrane, adjoining the inner membrane and remaining in contact with the egg white [3].

The outer and inner membranes adhere closely to each other, except at the blunt end of the egg, where they can be distinguished by the air space [7]. The thicknesses of the outer and inner membranes are 50–70 μm and 15–30 μm, respectively [1, 3]. The limiting membrane is the thinnest layer, measuring between 0.09 and 0.15 μm [7]. It surrounds the egg white and appears as particles that fill the spaces between the fibers of the inner membrane [3, 8]. The diameter of collagen fibers is 1–7 μm in the outer membrane and 0.1–3 μm in the inner membrane [1, 3]. Collagen fibers in the inner layer are more densely packed than those in the outer layer (Fig. 1b), with a pore size of approximately 5 μm, which ensures the permeability of gas and water [1]. The

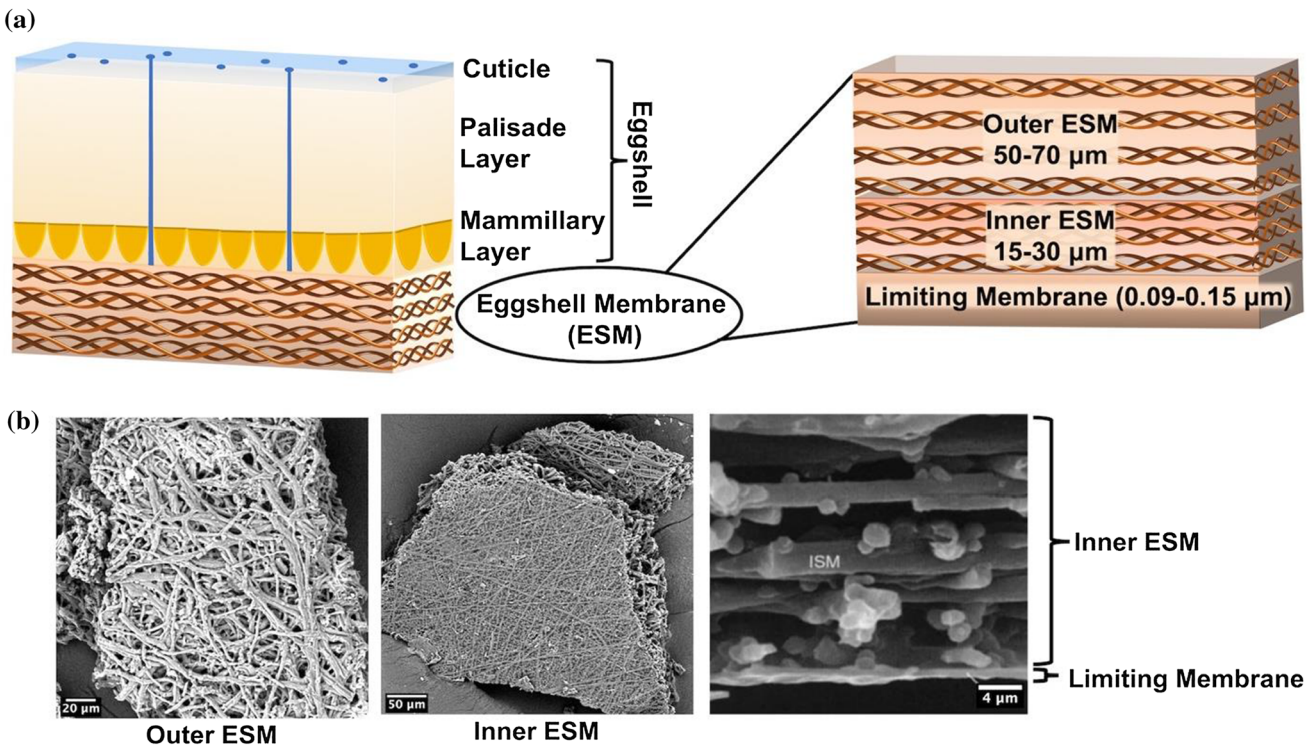


Figure 1 Structure of eggshell membrane (ESM). **a** Schematic cross-sectional representation of eggshell and ESM. **b** Scanning electron micrographs showing the microstructure of outer and inner ESM, and limiting membrane (scale bar = 20, 50 and 4 μm, respectively). Picture courtesy for outer and inner ESM—Cell and Tissue Engineering Laboratory, Indian Institute of Technology Bombay. Image for limiting membrane has been adapted with permission from Balaz et al. [3].

fibers are randomly oriented and are made of fibrils that have a collagen-rich inner core and a glycoprotein-rich outer mantle [2, 3]. Often, the mantle of adjacent fibers coalesces to form a branched appearance [7]. The inner and limiting membranes can be easily separated by mechanical force, but the outer membrane is firmly embedded in the eggshell and can be isolated by harsh acidic treatment with acetic acid, hydrochloric acid, ethylenediaminetetraacetic acid, or the dissolved air flotation method [3]. ESM plays a vital role in the mineralization of eggshells, while preventing the mineralization of egg whites. Hence, its outer layer is also slightly mineralized, which manifests as knob-like structures of calcite deposits [1, 3].

Composition of ESM

ESM constitutes 80–85% of the organic content, which includes C (36–47.5%), H (5–6.8%), N (11–15.3%), O (12.0%), and S (2–3.0%) (Fig. 2a) [9, 10]. Among the structural proteins, collagen is the most abundant in ESM [7]. While the outer ESM is rich in collagen I, the inner ESM contains collagen I and V at a ratio of 100:1. Collagen X is present in both layers [2, 3]. Other structural proteins include keratin, laminin, agrin, ovoglycan, desmozine, isodesmozine, ovoglycoprotein and ovocalyxin-36 [11]. CREMPs (cysteine-rich eggshell membrane proteins) form disulfide linkages between collagen domains, providing strength to the structure [2] while simultaneously making ESM water-insoluble [3]. Lysyl oxidase, an enzyme, provides maturation and stabilization to collagen via crosslinking [8]. Proline, glutamic acid, and glycine are the most abundant amino acids in ESM [12], whereas tryptophan is the least abundant amino acid [13].

It also contains lysozyme, gallinacin, gallin, mucin, ovocalyxin and ovomucoid that provide antimicrobial properties. Egg white proteins such as ovalbumin, ovotransferrin, and clusterin are also highly abundant in ESM and provide cytoprotective and chaperone-like functions [10]. The distribution of proteins in different compartments of the egg is as follows: > 500 in eggshell and ESM, 148 in egg white, 137 in vitelline membranes, and 316 in egg yolk [3]. The total protein content of ESM is 21.5 ± 2.8 mg [9]. The presence of amine and amide groups, exhibiting peaks at 1545 and 1655 cm^{-1}, and hydroxyl groups, depicting peaks at 3200–3500 cm^{-1}, was confirmed by FTIR (Fig. 2b) [9, 10].

Sugars, such as glucose, galactose, and mannose, represent 70% of the carbohydrate content in ESM. Minor quantities of fructose, xylose, glucosamine, and galactosamine have also been reported [7]. GAGs such as hyaluronic acid (HA), dermatan sulfate, and chondroitin sulfate are present in ESM [2, 3]. Small quantities of lipids have also been isolated from ESM, including mono-, di-, and triglycerides, free fatty acids, cholesterol and its ester counterparts, sphingomyelin, lecithin, lysolecithin, and cephalin [7].

The inorganic content of ESM is largely composed of calcium carbonate [2, 3]. Calcium ions interact with acidic functional groups of proteins, resulting in calcite formation, contributing to the mechanical strength of the membrane [2]. Other inorganic elements in ESM include trace amounts of potassium,

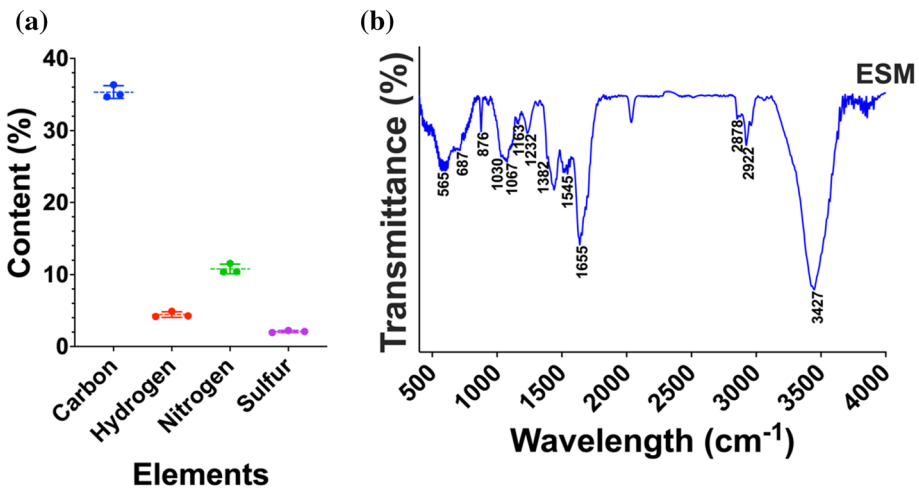


Figure 2 Chemical characterization of ESM. **a** Elemental composition of ESM powder. **b** FTIR spectrum of ESM. (Adapted with permission from Saha et al. [9]).

sodium, zinc, manganese, copper, aluminum, and boron [7].

Tissue engineering relevant properties of ESM

Physical properties

The Hen ESM is a soft substrate with a density of 1.358 g/cm^3 [10]. The weight and nitrogen content of ESM decreases with increase in age of hens. However, the percentage of total lipids remains constant. ESM thickness also tends to decrease with increasing age and varies among different breeds of hens [14]. Torres et al. reported that the Young's modulus of hen ESM ranges from 5.5 to 235 MPa, depending on environmental conditions such as humidity, temperature, and strain rate [15, 16]. Due to a high protein content, its thermal stability is low, with denaturation beginning at 50–55 °C owing to collagen degradation, as depicted by DSC analysis [3]. XRD analysis shows that ESM has a semi-crystalline nature at low temperatures due to the presence of collagen and calcite but transforms into a crystalline material at higher temperatures [13].

Zeta-potential of ESM decreases from 10 to − 21 mV, with an increase in pH from pH 2 to pH 11. This property allows the production of different types of nanoformulations using ESM [13]. ESM is a transparent membrane with a transmittance above 80%, making it an ideal wound dressing for skin and corneal wounds. Mensah et al. reported that the porosity of ESM is ∼ 56% with a fluid absorption capacity of ∼ 230% enabling it to absorb wound exudate [17]. Moreover, the outer ESM can serve as a barrier to moisture loss and pathogen invasion owing to its interwoven fibrous structure, whereas the inner membrane, being hydrophilic, can interact with cells in wounds and promote healing [18]. ESM of various bird species, such as hens, ducks, quails, and turkeys, has been found to possess suitable dielectric properties for producing capacitors [19]. Although some properties of ESM tend to be similar across various bird species, a few properties can vary greatly (Table 1).

Antioxidant properties

Oxidative stress is defined as an imbalance between reactive oxygen species generation and cellular antioxidant defense production. Solubilized ESM has been reported to exhibit antioxidant properties by scavenging free radicals and preventing DNA damage [23, 24]. Similarly, enzymatic hydrolysates of ESM also possess antioxidant activity, as proved by in vitro and in vivo studies (Fig. 3a) [25–27].

Anti-inflammatory properties

It has been established that ESM can reduce the expression of various inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, both in vitro and in vivo, through nuclear factor Kappa light chain-activator (NFκB) pro-inflammatory pathway [29]. Vuong et al. discovered that ESM, both in its powder and extract forms (carbohydrate fraction), exerts an immunomodulatory effect on macrophages and monocytes by decreasing NF-κB activity, TLR-4 and ICAM-1 mRNA transcription, CD44 expression, and pro-inflammatory cytokine production, while increasing IL-10 secretion [30]. Kulshreshtha et al. showed that powdered ESM can reduce accumulation of nitric oxide (NO) in a dose-dependent manner in RAW 267.4 macrophages, as depicted in Fig. 3b [28].

In a similar manner, ESM fraction with > 10 kDa proteins has been shown to suppress NO formation [31]. Benson et al. investigated the anti-inflammatory effects of soluble ESM (SESM) on peripheral blood mononuclear cells and observed that it reduces lymphocyte proliferation and secretion of pro-inflammatory cytokines such as IL-6, IFN-γ, and TNF-α [32]. In a subsequent study, SESM blended with -Nerium oleander leaves and Aloe vera extract was found to enhance the activation factors in natural killer cells and the production of cytokines involved in wound healing [33].

ESM has also been clinically proven to reduce symptoms such as joint stiffness and pain in osteoarthritic conditions [34, 35]. Oral supplementation of ESM powder in rats has been shown to reduce the levels of IL-1β and TNF-α while increasing IL-10 levels, thereby establishing the mechanism underlying the anti-inflammatory activity of ESM in osteoarthritis [36]. Kiers et al. reported that ESM could drastically reduce knee joint pain and stiffness and improve the overall quality of life of patients with osteoarthritis when supplemented orally [37]. Furthermore, Jia et al. demonstrated the anti-fibrotic role of ESM by showing that ESM hydrolysate

Table 1 Difference in properties of ESM between different bird species

Physical parameters	Hen ESM	Ostrich ESM
Thickness	0.008 ± 0.0018 cm [3]	0.14 ± 0.04 cm [20]
Young's modulus	235 MPa in air at 20 mm/min [15]	5.04 MPa in air at 10 mm/min [21]
Thermal degradation by TGA	250 °C [3]	310–460 °C [21]
Porosity	56% [22]	21.23% [21]
Elemental composition	C = 36–47.5%, N = 11–15.3%, H = 5–6.8%, S = 2–3%, O = 12% [9, 10]	C = 47.03%, N = 15.25%, H = 6.74%, S = 3.86%, O = 27.12% [21]

reduced TGFβ-1 induced pro-collagen expression in human hepatocytes and attenuated the hyperactivity of liver enzymes and oxidative stress [38].

Antibacterial properties

The presence of lysozyme has been reported to impart antibacterial properties to ESM, as confirmed by microsequencing and western blotting [39]. Lysozyme is also present in high concentrations in most mammalian fluids [40]. Raw ESM possesses better antimicrobial activity than autoclaved or solubilized ESM [41]. However, ESM hydrolysates prepared by chemical treatment exhibit varying degrees of antibacterial activity against various pathogens [31]. A study showed that ESM in particle form is more effective against gram-positive microorganisms, such as *S.aureus* (Fig. 3c), than against gram-negative microbes, namely *P. aeruginosa* [28].

Biomimicry properties

Natural biomaterials are known to enhance cell attachment, ECM synthesis, interaction with host tissues, and angiogenesis [42]. ESM, which is also a natural biomaterial, has a well-defined molecular structure, biocompatibility, and a composition similar to that of human ECM [34, 35]. The diverse constituents of ESM make it ideal for tissue regeneration [2]. ESM contains collagen I, V, and X, with collagen type I being the major constituent, which is also predominant in the human ECM. SEM imaging has proven that ESM has randomly oriented collagen fibers with irregular lumps of calcite deposits (Fig. 3d) [9, 15].

HA in ESM aids in the formation of hydrogen bonds with other constituents, and imparts stiffness and hydrophilicity. In humans, an essential part of the ECM is HA, which aids in shock absorption and lubrication of synovial joints. HA also acts as a scavenger of free radicals, modulates inflammatory cells, and participates in bone mineralization and osteogenesis [2].

Fibronectin is a glycoprotein that exists in the dimeric form of two closely resembling polypeptides, each of which is a monomer consisting of three modules arranged in a manner that allows proteins to bind along the length of the monomer. Module 3 contains arginine-glycine-aspartate (RGD) peptide sequences that play a key role in wound healing by facilitating the attachment and migration of cells via interactions with integrins on cell membranes [2].

Osteopontin is a phosphorylated glycoprotein expressed in human bones and other cell types such as endothelial cells, macrophages, and smooth muscle cells. Osteopontin also contains RGD motifs, and regulates physiological processes such as bone mineralization, inflammation, and tissue remodeling. Calcium carbonate is a key inorganic element in bone mineralization and wound healing [2]. Thus, the constituents of ESM demonstrate its suitability for tissue engineering applications.

Isolation and processing of ESM

Isolation of ESM

Isolation of ESM from eggshells is difficult because the outer membrane is strongly integrated into the mammillary cone of the eggshell. ESM can be isolated

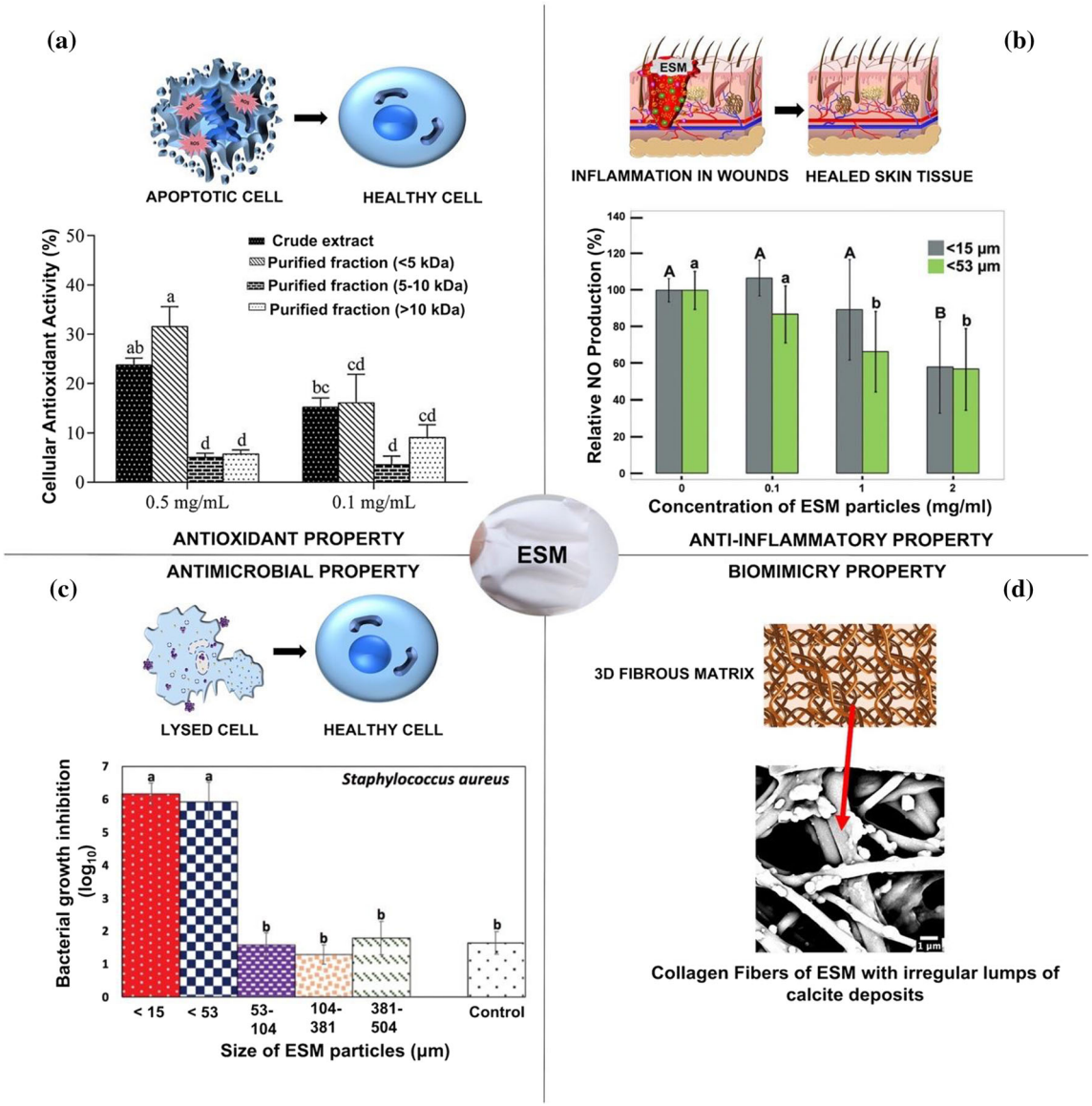


Figure 3 Tissue engineering relevant properties of ESM. **a** *Antioxidant property*—cellular antioxidant activity of crude extract and purified fractions of solubilized ESM measured via DCFH-DA dye in Caco-2 cells. **b** *Anti-inflammatory property*—Quantification of nitric oxide production by Greiss assay in LPS stimulated RAW 264.7 macrophages following treatment with different sized ESM powder. **c** *Antimicrobial activity*—Quantification of growth inhibition of *S.aureus* by different sized ESM powder. **d** *Biomimicry property*—Scanning electron micrograph of ESM showing randomly-oriented collagen fibers with calcite deposits (scale bar = 1 µm). Reproduced with permission from Ref. [9, 26, 28].

from eggshells, either mechanically or chemically. The mechanical or manual detachment method is widely employed [43]. Dissolved air flotation (DAF) is another method that allows ESM to be separated from eggshells. Ground eggshell waste is placed in the separation unit of the DAF instrument, followed by the injection of an air–water mixture from the bottom of the unit. The dissolved air causes ESM to float up, whereas heavier eggshell particles settle at the bottom [44]. Herein, 96% of the membrane and 99% of calcium carbonate can be retrieved within two hours [3]. Hydrochloric acid, acetic acid, sulfuric acid, and ethylenediaminetetraacetic acid are used for chemical isolation [43]. Furthermore, the passage of eggshell fragments through a series of processes involving an aqueous environment heated by steam followed by separation in a cyclone is a commercially used method [45].

Processed forms of ESM

Since ESM can serve as an organic matrix that promotes cell proliferation and ECM synthesis, it has been used in different forms to develop appropriate scaffolds for various tissue engineering applications (Fig. 4).

Native ESM

Native ESM has been used to develop scaffolds in different forms such as raw ESM (RESM), autoclaved ESM (AESM), and powdered ESM. In a study by Yi et al., it was found that RESM showed slight cytotoxicity towards NIH3T3 cells owing to the presence of a harmful mucilaginous layer, which was eliminated after autoclaving the membrane [46]. Powdered ESM prepared by cryo-chilling and subsequent grinding via ball milling [47], mortar and pestle [48], or micronizing in a cyclone vortex [49] has been successfully used for various tissue engineering applications [50]. Powdered ESM has been used as a filler to enhance the physical and biological properties of composite scaffolds owing to its higher surface area than the native membrane, while retaining its microfibrous structure [48, 49]. Pillai et al. incorporated powdered AESM in silk fibroin-polyvinyl alcohol scaffolds and found that as the concentration of powdered AESM increased, the surface area, porosity and mechanical strength of the scaffold also increased [48]. Bio-cellular glass–ceramics supplemented with ESM powder as a source of calcium phosphate have been used in bone and dental tissue engineering [51]. ESM has also been used for topical application in cosmetic formulations due to its rich collagen content, wherein ESM powder was dispersed in cream, body lotion, face mask, gel, and foundation formulations and tested on human skin. These formulations significantly reduced wrinkles, blemishes, acne and dry skin, proving the anti-aging properties of ESM [47].

To probe the role of ESM as a dietary supplement for maintaining healthy joints and connective tissues, Ruff et al. investigated the toxicological effects of powdered ESM, and found it to be cytocompatible up to 100 µg in keratinocytes and non-genotoxic against auxotrophic bacteria, even at high concentrations (5000 µg). Additionally, rat studies revealed that consumption of powdered ESM for 90 days did not show any signs of toxicity, suggesting its safety for human use [52].

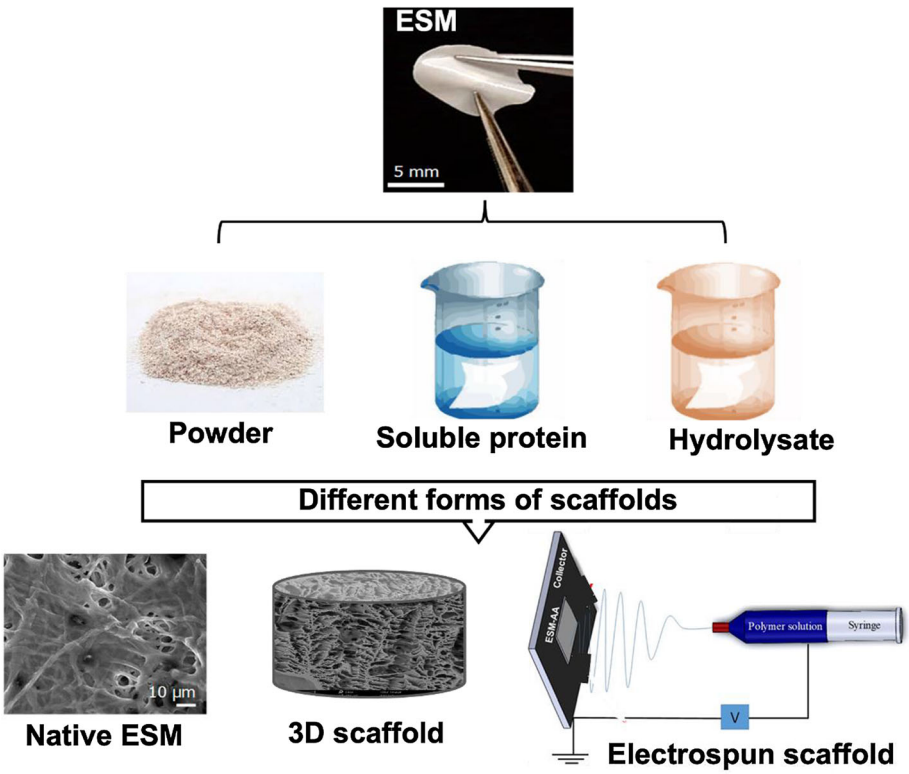


Figure 4 Different forms of processed ESM for tissue engineering applications: native ESM, powdered ESM, solubilized ESM, and ESM hydrolysate. These various forms can either be used as it is or used to develop scaffolds. Reproduced after permission from Makkar et al. [11] and Nakano et al. [12].

Solubilized ESM

A major limitation of using native ESM for scaffold fabrication is the difficulty in controlling the shape, size, and subsequent incorporation of native ESM into scaffolds. Hence, ESM has been solubilized to obtain soluble eggshell membrane proteins (SEP) using a variety of solvents such as NaOH:ethanol mixture (3:1) [53], 3-mercaptopropionic acid and acetic acid [54], and 1,1,1,3,3,3-hexafluoro-2-propanol [55]. SEP improves mechanical properties when blended with other biocompatible polymers such as silk fibroin [56], PCL [57], PLGA [58], chitosan [59], PEO [60], PLA (poly lactic acid), and PPC (poly propylene carbonate) [55].

The biocompatibility of SEP is comparable to that of collagen type I [46, 56]. However, the total protein content of SEP is lower than that of native ESM because of the harsh solvent treatment [47]. Moreover, SEP can also be made water-insoluble using 10% acetic acid without hampering its biocompatibility [54]. Topical application and ingestion has been shown to improve skin elasticity in healthy human volunteers [61].

ESM hydrolysates

ESM hydrolysates are water-soluble derivatives of ESM, prepared by enzymatic digestion, to break the disulfide bonds that make it insoluble. ESM hydrolysate has better digestibility than native ESM in rats, with no alteration in protein utility by the body [62]. By combining enzymatic digestion with ultrasonication, stable emulsions of ESM protein hydrolysates can be prepared [63]. The anti-inflammatory properties of ESM hydrolysate is also well-established both in vitro and in vivo [26, 27].

Tissue engineering applications of ESM

ESM has been widely used as a biomaterial for 'guided tissue engineering' due to its physicochemical properties such as high tensile strength, lipid and proteinaceous content, and permeability that help mimic the human ECM.

Skin tissue engineering

The most significant contribution of ESM as a biomimetic scaffold is in skin tissue engineering. ESM has been used either in its native form or as an additive component in scaffolds. The application of native ESM in both human and rodent wounds has demonstrated rapid healing [64, 65]. Studies have shown that the surface modification of ESM with metallic nanoparticles helps increase hydrophilicity, imparts antibacterial properties, and enhances wound healing in animals through vascularization and reduction in inflammation (Fig. 5a) [66–68]. Similar results were obtained by coating ESM with antimicrobial peptides [69]. Moreover, coating of ESM with a nanofibrous layer of PCL-chitosan was found to increase the mechanical strength, provide barrier properties, and enhance healing in animals [70, 71]. The therapeutic potential of ESM for dermal injuries was enhanced by modification with the thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAAm) and drug-loaded silver nanoparticles (AgNPs) [72].

ESM powder has anti-inflammatory and growth factor-like properties that aid healing [49, 73]. Furthermore, impregnation of ESM powder into polymeric films such as chitosan improves hydrophilicity, tensile strength, water permeability, and resistance to degradation [67, 74]. ESM powder can also act as a physical crosslinker for scaffolds without affecting their physical or biological properties [9].

Solubilized forms of ESM electrospun with various natural and synthetic polymers have demonstrated promising results in skin regeneration applications [57, 75, 76]. PCL-ESM nanofibrous mats were synthesized by electrospinning to develop wound dressings with improved biocompatibility and antibacterial activity [77]. PCL combined with silk fibroin, SESM, and aloe vera gel was used to develop electrospun nanofibrous scaffolds to induce basal stem cell to keratinocyte differentiation and skin regeneration [78]. Detachable bilayered dermal patches loaded with SESM and curcumin nanoparticles exhibited better wound healing than commercial dressings [79]. SESM immobilized on culture dishes elevated the expression of MMP2, type III collagen, and decorin in HADF cells [80]. Topical application of SESM was found to increase type III collagen expression in the papillary dermis of mice and improve the elasticity of the arms while reducing facial wrinkles in humans, thereby demonstrating its anti-aging effect [81].

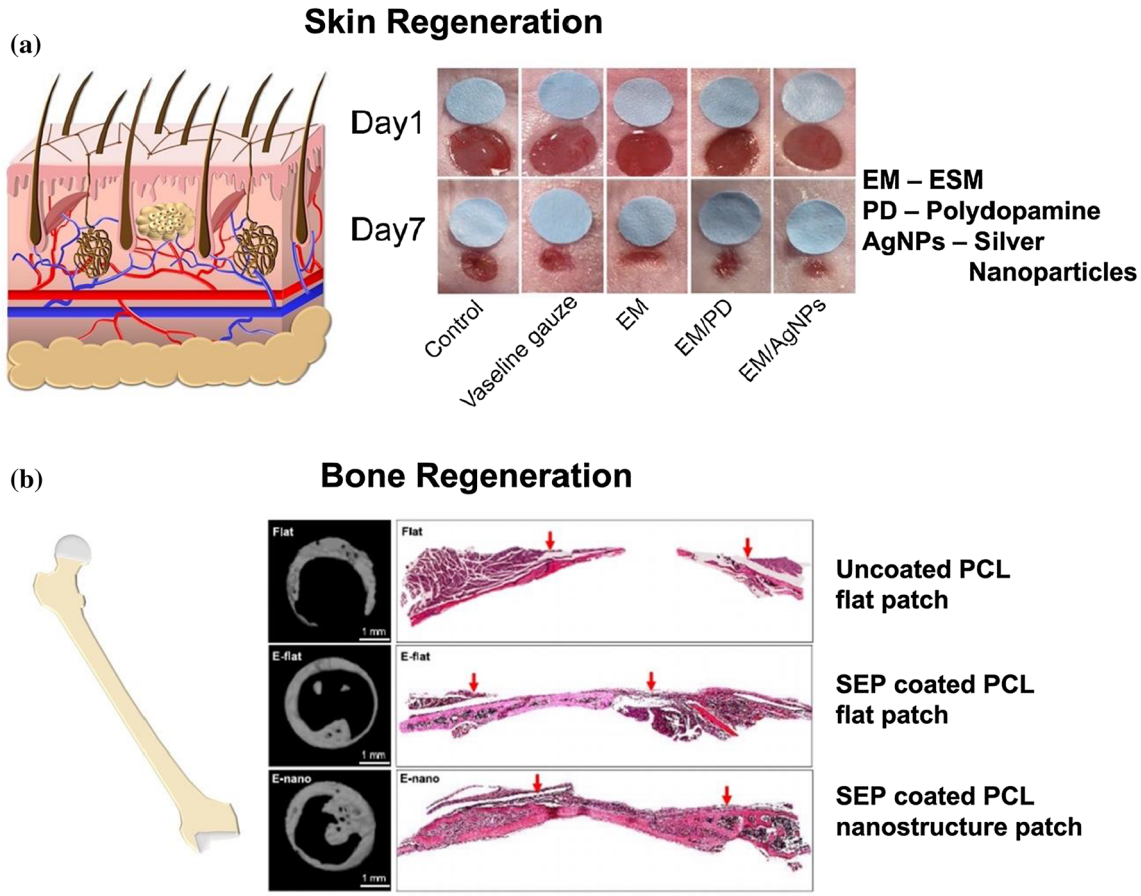


Figure 5 **a** Macroscopic appearance of full-thickness wounds in mice in control (untreated), treated with vaseline gauze, EM (ESM), EM/PD (polydopamine coated ESM), EM/AgNPs (ESM decorated with silver nanoparticles) groups. **b** Micro-CT (left) and hematoxylin and eosin stained (right) images showing the effect of uncoated, SEP-coated flat and nano-topographic PCL patches on bone regeneration in mice after 3 weeks. Reproduced with permission from [68, 82].

Bone tissue engineering

ESM serves as a natural bio-template for the mineralization of calcareous eggshells, thus making it an excellent biomaterial for bone and cartilage regeneration [15, 83]. Chen et al. utilized ESM as a biomineralization template to form an ESM-hydroxyapatite scaffold for bone tissue engineering. The hydroxyapatite improved the thermal stability and hydrophilicity of the scaffold and increased alkaline phosphatase activity expression of osteogenesis-related genes, and proteins in MC3T3-E1 cells [84]. Durmus et al. used native and powdered ESM to treat cranial defects in rabbits which showed better bone regeneration than the untreated group [85]. Furthermore, SEP combined with other polymers has been observed to induce the osteogenic differentiation of mesenchymal stem cells and promote bone formation in mice (Fig. 5b) [82, 86]. Park et al. developed scaffolds with nanotopography fiber alignment by nanoimprint lithography using ESM solution. The scaffolds enhanced osteogenic differentiation and growth factor secretion by osteoblasts, thereby demonstrating their potential for bone regeneration [87].

Cartilage tissue engineering

Pillai et al. compared RESM and AESM obtained from hen eggs as potential biomaterials for cartilage tissue engineering. They reported that autoclaving facilitated greater attachment of human meniscus cells to AESM and increased the resistance to degradation and thermal stability, implying that AESM is superior to RESM [88]. In a subsequent study, AESM was incorporated into a scaffold made of silk fibroin and polyvinyl alcohol, which exhibited mechanical properties similar to those of native human cartilage [48]. In addition, SEP in combination

with various materials such as chitosan, silk, and agarose, fabricated into three-dimensional scaffolds, provide good swelling properties, biodegradability, antimicrobial activity, and biocompatibility for successful chondrocyte regeneration [89, 90].

The tympanic membrane (TM), also known as the eardrum, is a delicate cartilaginous membrane that receives sound vibrations and transmits them to the auditory ossicles. The major constituents of this double-layered fibrous membrane are collagen types I, II and III [91]. Minor traumatic TM perforations heal on their own, but major damage requires surgical interventions [92]. ESM, being a natural collagenous matrix, has been utilized as a biomaterial for TM regeneration and has been demonstrated to facilitate faster healing in human patients with small, moderate, and large perforations of the TM (Fig. 6a) [93, 94].

Vascular tissue engineering

Several researchers have demonstrated the use of ESM for the development of vascular grafts. A bilayered, wavy-structured graft (Fig. 6b) with thermoplastic polyurethane as the external layer and heparin- and dopamine-coated ESM as the internal layer was developed, resulting in enhanced blood flow and higher human umbilical vein endothelial cell attachment and proliferation [95]. In another study, small-diameter vascular grafts using ESM extract and heparin-incorporated expanded polytetrafluoroethylene were fabricated, which showed improved hydrophilicity and cytocompatibility [96]. Li et al. synthesized polyethylene glycol diacrylate-coated ESM-based vascular grafts with mechanical properties similar to those of native human blood vessels [97].

Nerve tissue engineering

Application of ESM for nerve regeneration has demonstrated that ESM-based nerve grafts help in proliferation of primary hippocampal neurons [98] and neural precursor cells, such as PC12 cells [99, 100], under in vitro conditions. Similarly,

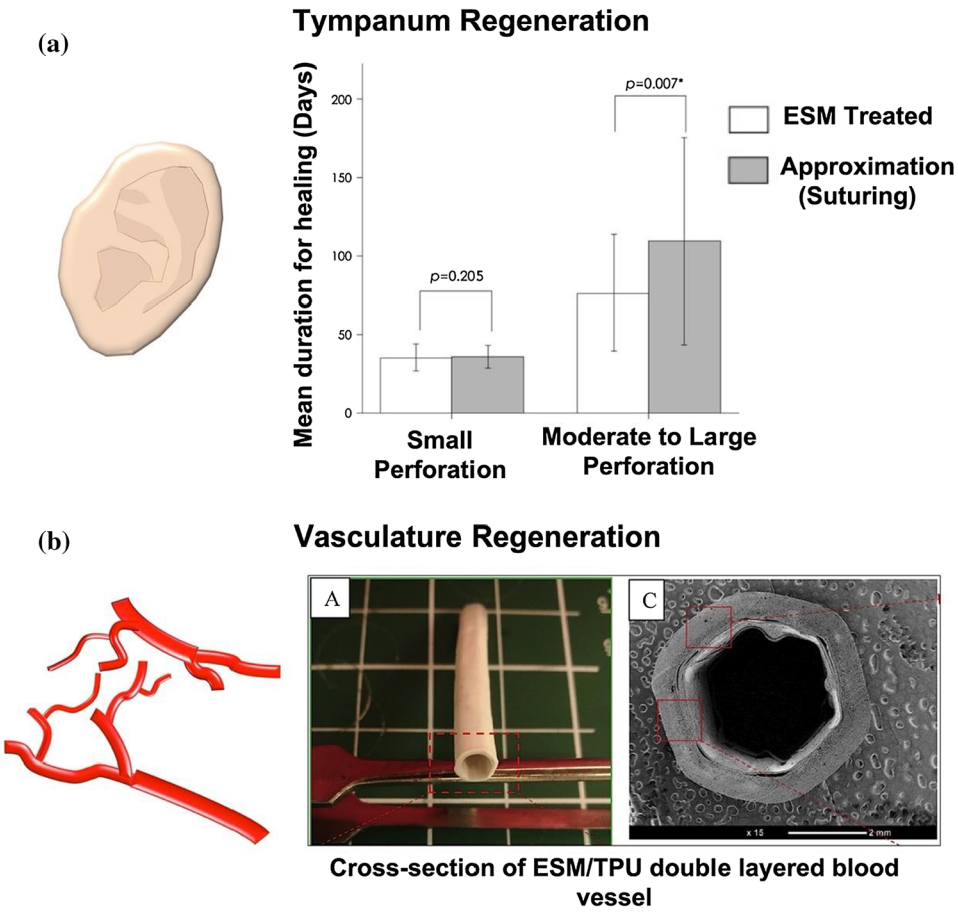


Figure 6 **a** Healing time recorded in patients with different grades of tympanic membrane perforation showing reduced time of healing with ESM than the approximation (suturing) group for moderate-to-large perforations. **b** Digital photograph and scanning electron micrograph of cross-section of double layered vascular graft made of ESM/thermoplastic urethane (TPU) (scale bar = 2 mm). Reproduced with permission from [92, 95].

experiments in rats with an incised sciatic nerve of the thigh also proved that ESM plays a vital role in nerve regeneration by improving various essential parameters (Fig. 7a) compared to treatment with autograft [101–103].

Tissue engineering in dentistry

Researchers have discovered that modification of ESM with a graphene coating can help improve the mechanical strength and hydrophilicity of the scaffold and aid the proliferation of dental pulp cells with simultaneous enhancement of mineralization and secretion of essential growth factors by the cells [104]. Powdered ESM facilitates periodontal regeneration in rats at a rate similar to commercial grafts [105]. Animal studies have demonstrated that oral administration of SESM with vitamin C can increase collagen production and accelerate orthodontic tooth movement (Fig. 7b) [106]. Gempita et al. synthesized calcia partially stabilized zirconia (Ca-PSZ) using the sol–gel method with ESM as a dental filler [107]. Moreover, hyaluronic acid extracted from ESM can promote significant regeneration of interdental papillae in guinea pigs, underlining the efficacy of the constituents of ESM [108].

Nanomedicine applications of ESM

Nanoparticles are extensively explored for enhancing drug bioavailability via targeted delivery. ESM has been used either as nanoparticles or as a platform to deliver nanoparticles for various therapeutic applications (Table 2).

Challenges in application of ESM

ESM is a promising and emerging biomaterial which has been used for diverse applications with significant application in the biomedical field. Consequently, numerous patents exist for ESM processing and related products (Table 3). Despite extensive research, only a few products have been commercialized. Examples of such products include Ovomet®, NEM®, Biovalex® (supplements to maintain healthy human joints); Dermarep™ (an affordable wound dressing that provides rapid and scarless

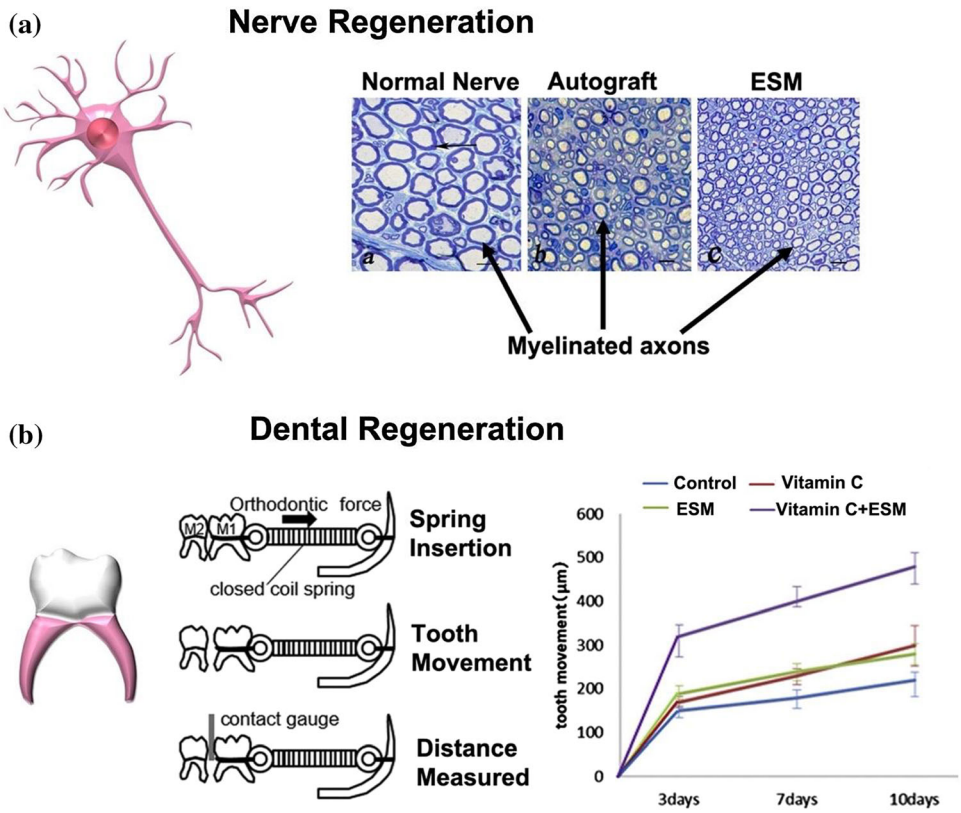


Figure 7 **a** Light microscopic images of toluidine blue stained cross-sections of regenerated nerves, 90 days after surgery, showing myelinated axons (arrow) in normal nerve, autograft and ESM treated group (scale bar = 10 μm). **b** Schematic (left) showing a spring attached to the first molar following tooth movement between the first (M1) and second molar (M2) was measured; (Right) Quantification of tooth movement. Reproduced with permission of [101, 106].

Table 2 Application of ESM in nanomedicine

No	Forms of ESM used	Study	Application	References
1	Nanoparticles (NPs)	NPs with an outer shell of powdered ESM and inner core of liposoluble vitamin-E	Vitamin E delivery	[109]
		SEP-catechin NPs	Dressings, adsorption membranes and biofilters	[110]
		SEP loaded chitosan-fucoidan nanoparticles	Mitigation of inflammation to treat inflammatory bowel disease	[111]
2	Synthesis of NPs on native ESM (bio-template)	Silver (Ag)	Tannic acid immobilization for catalysis of azo dyes	[112]
		C-ZnO/ZnS	Photocatalytic application	[113]
		Iron (Fe)	Catalytic reduction of organic contaminants	[114]
		Copper (Cu)	Adsorption of dye	[115]
		Tin oxide	Supercapacitor	[116]
		Cadmium oxide-zinc oxide nanocomposite	Antibacterial bio-membrane	[117]
		V_2O_5/ZnO	Antibacterial bio-membrane	[118]
		Synthesis of platinum or gold NPs on ESM followed by immobilization of enzymes such as glucose oxidase or horseradish peroxidase	Biosensors for glucose sensing	[119–121]
3	Native ESM	Growth of calcium carbonate crystals on ESM	Biomineralization	[122]
4	Synthesis of quantum dots from ESM ash	Microwave assisted spherical CQDs (5 nm diameter)	Fluorescent probes with an excitation and emission spectrum of 275 nm and 450 nm for labeling glutathione	[123]
		Water-soluble and fluorescent CQDs	Detection of mercury in water samples	[124]
5	Native ESM	In vitro penetration of sialic acid ointment through ESM	In vitro topical drug/irritant penetrability studies	[125]
		In vitro penetration of smokeless tobacco forms	In vitro topical drug/irritant penetrability studies	[126]
6	Native ESM	Acyclovir encapsulated ethyl cellulose-Carbopol 974P NF microspheres	In vitro platform for mucoadhesion evaluation of drugs	[127]
		Buccoadhesive tablets such as gelatin-hydroxypropyl methyl-cellulose, gelatin-sodium carboxymethylcellulose and gelatin-hydroxypropyl cellulose were compared with benzylamine and metronidazole tablets	In vitro platform for mucoadhesion evaluation of drugs	[128]

healing); BiovaBio™, Ovoderm®, Biovaderm® (skin care products).

Some obstacles need to be overcome to improve the ease of usage and scalability of ESM-based products. Quality and chemical characteristics tend to vary depending on the source of the eggs, which could impact the reproducibility of the finished product, thereby limiting its industrial scale-up. There may be a risk of microbial contamination of the product if ESM is collected from waste disposal areas and not carefully sterilized. Manual peeling of ESM is laborious and time-consuming. Thus, automation of the isolation process could make industrial scale-up feasible. Although numerous patents are available for ESM-based products, further trials are needed to commercialize such potential products. Awareness of the nutritional value of ESM must be promoted more aggressively to enhance its usage. Thus, recycling of egg-derived wastes would prove to be profitable to small-scale industries, culminating in more human employment, and channelized utilization of ESM for biomedical applications.

Table 3 Patents on biomedical applications of ESM

S. No	Patent title	Patent No	Year	Reference
1	Tissue engineering scaffolds comprising particulate egg shell membrane	US11045578B2	2016	[129]
2	Method and apparatus for the enhanced separation of calcium eggshell from organic membrane	US9873616B2	2015	[130]
3	Hepatic protection agent containing eggshell membrane and pharmaceutical composition, food additive and food using the same	US20150164962A1	2015	[131]
4	Activator of gene expression of molecular chaperone gene comprising eggshell membrane component and composition thereof	US20150196606A1	2015	[132]
5	Method and apparatus for the enhanced separation of calcium eggshell from organic membrane	US9873616B2	2015	[130]
6	Methods for treating glucose metabolic disorders	US8679551B2	2014	[133]
7	Wound care product with egg shell membrane	US10166260B2	2013	[134]
8	Eggshell film-containing micropowder, tablet, method for producing eggshell film-containing micropowder, and method for producing tablet	CN103300357A	2013	[135]
9	A cement material for renewal of damaged dental tissues	WO2014021797A2	2013	[136]
10	Dietary supplements for promotion of growth, repair, and maintenance of bone and joints	WO2012096883A1	2012	[137]
11	Pulverized eggshell membrane and chitosan as bio-cream and lotion	JP7223935	2011	[138]
12	A composition comprising powdered eggshell membrane for use in treating a pre-diabetic mammals	EP2842563A1	2011	[139]
13	Novel process for solubilizing protein from a proteinaceous material and compositions there of	20110034401	2011	[140]
14	Fiber-treating liquid, modified cloth, and process for producing the same	US20090176423A1	2009	[141]
15	Fiber comprises an eggshell membrane component useful for producing a fiber assembly, which is used as a wound dressing or a cosmetic sheet	US2009031691A1	2009	[142]
16	Hydrolysed shell membrane produced from shell membrane of hen's egg with proteinase, method for producing the same and functional material added therewith	JP2008007419A	2008	[143]
17	Fiber, fiber assembly, and fiber producing method	EP2020455A2	2008	[144]
18	Avian eggshell membrane polypeptide extraction via fermentation process	20070017447	2007	[145]
19	Therapeutic, nutraceutical and cosmetic applications for eggshell membrane and processed eggshell membrane preparations	US20080063677A1	2007	[146]
20	Protein hydrolysates and method of making	US8101377B2	2007	[147]
21	Anti-inflammatory activity of eggshell membrane and processed eggshell membrane preparations	20070178170	2007	[148]
22	Preparation of hyaluronic acid from eggshell membrane	US6946551B2	2003	[149]
23	Adhesive plaster	JP2003225298A	2002	[150]
24	Anti-peroxide external preparation for skin	US5415875A	1993	[151]
25	Process for using eggshell compositions for promoting wound healing	US3558771A	1968	[152]
26	Assisting healing of skin-denuded areas on the body with dried non-fibrous egg-shell membrane products and compositions therefore	US3194732A	1960	[153]

Conclusions and future outlook

The aim of this review is to provide a holistic view on the diverse applications of ESM. However, its application in tissue engineering has yet to be fully explored. According to the global ESM market 2019, hydrolyzed ESM outsold the unhydrolyzed form of ESM because hydrolysis increased the bioavailability of its bioactive constituents [154]. Thus, innovative technologies should be developed to obtain functional forms of ESM that contain bioactive components and produce bench-to-bedside products. More studies must be conducted, including isolation of individual biomacromolecules, to understand their role in the process of tissue regeneration.

ESM can also be used in everyday healthcare applications, such as for reducing post-chemotherapy side effects or as a supplement for enhancing muscle and bone strength in athletes. It could also serve as an alternative reinforcement material for ceramic particles. Although the utility of ESM as a biosensor, bio-template, bio-sorbent, and medicinal product has been well established, its high adsorption capacity can be further utilized to develop novel drug delivery systems and biomedical detection kits by immobilizing biomolecules and growth factors. Although the biocompatibility of ESM with stem cells has been studied, whether ESM plays a role in stem cell differentiation remains to be elucidated. 3D-bioprinting of ESM derivatives or SEP with or without further modifications is a promising field that is yet to be explored. ESM may also be used to develop bioplastics in the future. These strategies will pave the way for its wider use as a biomaterial in novel ways.

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Declarations

Conflict of interest Authors declare that there are no financial or competing interests to influence the work reported in this manuscript.

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