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REVIEW

Synthesis of Collagen Nanoparticles from Eggshell Membrane and its Applications with Special Emphasis on Wound Healing and Water Purification

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Collagen, one of the foremost copious proteins in the human physique, is a versatile natural polymer that is used in various fields of science ranging from biomedicine like tissue engineering, wound healing, and drug delivery to environmental cleansing agents including removal of oil and dyes from polluted water. This protein is very well-known because of its distinctive nature of biocompatibility, biodegradability and bioavailability owing to its composition that consists of proline, glycine and hydroxyproline. Collagen nanoparticles is one of the everlasting applications of this protein as it provides lower toxicity, increases shelf-life of the sample, improved water-binding capacity and ensures controlled and targeted release of the sample. These biologically beneficial properties increase their significance for functioning in biological systems. This review will provide insights on the various methods of collagen extraction and the major sources of this protein. The review also highlights on different types of collagen nanoparticles that can be synthesized and its diverse applications in multiple areas.

Key words: Collagen extraction, Collagen nanoparticles, Drug delivery, Eggshell membrane, wound healing/ water purification

Eggshell membrane (ESM) is primarily composed of complete protein called collagen (Ruff *et al.*, 2009a; Han *et al.*, 2023). The membrane mainly contains type I collagen, type V collagen, and type X collagen (Han *et al.*, 2023; Matsuoka *et al.*, 2019). Eggshells also contain various types of amino acids like arginine, glutamine, proline, etc (Nagamalli *et al.*, 2017; León-López *et al.*, 2019). Its ingredients are widely used in medicine, pharmaceutical, cosmetics and other industries. Soluble protein, collagen, keratin and other products are successfully extracted from eggshells currently (Chi *et al.*, 2022). Eggshells are majorly made of two important components which are the eggshell and eggshell membrane and its composition is 10-12% and 1.02% respectively (Chi *et al.*, 2022; Han *et al.*, 2023). Collagen is a biochemical product mainly found in the skin and bones of cattle and poultries. Collagen is mainly used in cornea repair, tissue replacement and skin graft. Its demand is increasing nowadays (Ponkham *et al.*, 2011), but after the outbreak of mad cow disease, bovine spongiform encephalopathy and food-and-mouth disease crises, it resulted in a hypothesis suggesting the transfer of the infectious agents from animals to humans that is not entirely confirmed due to which, there was a significant reduction in collagen trade (Dazrulhafizi *et al.*, 2023; Ponkham *et al.*, 2011). Collagen is a protein of utmost importance as it provides structural support to various tissues in the body, including skin, bones, and connective tissues (Shoulders & Raines, 2009). While traditional collagen sources often come from mammals like cows and pigs, exploring alternative sources has several potential benefits (Jafari *et al.*, 2020) . Hen eggs offer a cost-effective source of complete protein when compared to meat, poultry, and fish. As a result, eggs are known for their wide consumption and affordability globally (Ponkham *et al.*, 2011). The production of chicken eggs for human consumption approximates up to >68 million tons annually across the globe. The eggshell membrane (ESM) is abundantly available as a derivative of the food industry, associated with the eggshell (Ahmed *et al.*, 2019). The eggshell membrane contains various proteins, glycoproteins, and other bioactive molecules (Lien *et al.*, 2022; Shi *et al.*, 2021). However, due to its insoluble and stable nature, studying its formation and protein constituents can be challenging. Researchers have made efforts to characterize the eggshell membrane, but a complete understanding is still an ongoing process (Du *et al.*, 2015). The proteomic examination of the eggshell membrane indicated the presence of over a hundred proteins, with 40% of them identified as collagen and cysteine. This composition imparts outstanding biocompatibility and biodegradability to eggshell membranes (Elkhenany *et al.*, 2022).

Collagen, characterized by its triple helical structure, is a readily accessible biodegradable fibrous protein. Substantial quantities of collagen-containing waste are produced in various protein processing industries, including slaughterhouses, meat packing, leather, and related sectors (Thanikaivelan *et al.*, 2012). Collagen, a prominent biomaterial, finds extensive use in diverse clinical and industrial demands due to its remarkable biological characteristics. Collagen's key attributes—biocompatibility, biodegradability, and low antigenicity make it a highly suitable polymer for a diverse array of applications. These features contribute to its effectiveness and safety in various medical and industrial contexts, making collagen a versatile and valuable material in domains like tissue engineering, wound healing, and cosmetic formulations (Shalaby *et al.*, 2023a). Given its ability to stimulate the development of multiple cell layers in injured skin, collagen has been suggested as an optimal choice for wound dressing products. This property makes collagen well-suited for promoting effective wound healing by providing a supportive environment for cellular growth and tissue regeneration (Shalaby *et al.*, 2023b). The extracellular matrix, largely composed of collagen, serves as a structural framework in tissues, and leveraging collagen in medical dressings capitalize on its natural affinity with the body's biological processes. The exceptional biological activity and biocompatibility of collagen make it an advantageous material for medical dressings, facilitating optimal wound healing and tissue regeneration while minimizing adverse reactions (Li *et al.*, 2020a)

ESM has gained attention in innumerable biomedical implementations due to its unique composition and properties and is used in wound healing. The presence of collagen and other bioactive compounds in ESM may contribute to its wound-healing properties. It could be used in dressings or patches to promote tissue regeneration and accelerate the healing process, (Mensah, Salim, *et al.*, 2023; Ahmed *et al.*, 2019; Shalaby *et al.*, 2023b). Certainly, collagen is the primary constitutional protein found in the extracellular matrices of connective tissues (Li *et al.*, 2020a). It stands as the single most plenteous protein in the animal kingdom and is widely distributed in tissues such as the dermis (skin), tendons, and bones (Kheirabadi *et al.,* 2018) . As a crucial component of connective tissues, collagen provides strength, support, and elasticity to various structures in the body, contributing to the overall integrity and function of tissues and organs (Rath *et al.*, 2016). Non-healing skin wounds represent a significant global health concern, leading to substantial morbidity and mortality. The inability of these wounds to undergo proper healing processes can result in prolonged suffering for individuals and may contribute to severe health complications, ultimately impacting overall well-being on a global scale (Shi *et al.*, 2021). In a simplified explanation, optimal wound healing involves creating an environment that is both moist and permeable. This environment facilitates the necessary cellular processes and deposition of the extracellular matrix for effective wound repair (Mensah, Cook, *et al.*, 2023). Research indicates that wound dressings based on collagen offer numerous advantages (Zhang *et al.*, 2018). Collagen-based dressings have the potential to replicate the intricate architecture of the original tissue's extracellular matrix, including collagen and associated secondary components such as laminin, fibronectin, and glycosaminoglycans (Shalaby *et al.*, 2023b) (Huang *et al.*, 2021). Enhancing the stability of collagen scaffolds to expedite wound healing can be accomplished by incorporating bioactive molecules with therapeutic significance (You *et al.*, 2017) (Natarajan & Kiran, 2019). Silver shows promising effects in the therapy of chronic abrasions(Singh *et al.*, 2022) (Paladini & Pollini, 2019). Hence, silver in the form nanoparticles shows precise effects as it consists of antibacterial, antioxidant and anti-inflammatory properties (Rybka *et al.*, 2022). Silver, whether in the form of nanoparticles (AgNPs) or other configurations, exhibits outstanding germicidal activity against a diverse array of microorganisms, including bacteria, viruses, and fungi. Silver nanoparticles (AgNPs) have found widespread applications across various biomedical studies owing to their potent antibacterial capabilities and selective toxicity towards microorganisms (Cardoso *et al.*, 2014) . It also provided excellent tensile properties and aided in better fibril alignment almost resembling the original skin (Kwan *et al.*, 2011a). Indeed, silver nanodots (AgNPs) have attracted considerable interest in recent decades because of their exceptional antimicrobial properties. These nanoparticles display potent antibacterial, antiviral, and antifungal activities, positioning them as promising candidates in divergent fields like medicine, healthcare and beyond (Talapko *et al.*, 2020). The goal of the study is to exploit the advantageous properties of collagen nanofibers to develop a wound-healing environment that not only prevents infections through the antimicrobial activity of silver but also enhances tissue regeneration by providing a supportive scaffold for cellular processes. This integrated approach may have the potential to improve the overall effectiveness of wound care, promoting faster and more successful healing outcomes (Rath *et al.*, 2016).

Clearing oil spill:

Crude oil stands as a vital natural resource, holding immense significance in the progress of human civilization. Among the various sources of energy, oil and gas stand out as the predominant ones (Mottaghi *et al.*, 2021). The relentless growth of economies and societies renders the extensive extraction of oil and its transportation via marine routes an unavoidable necessity (Pete *et al.*, 2021; Ouyang *et al.*, 2023). The growing trend of globalization has become a significant factor leading to an excessive dependence on oil energy, resulting in continuous environmental pollution. In the last thirty years, the frequency of oil spill accidents has significantly risen, with various oil carriers being responsible for these incidents (Wolok *et al.*, 2020). One of the significant instances of marine pollution arises from oil contamination, stemming from both accidental incidents and routine ship operations (Aliyu *et al.*, 2015).

Oil spills pose a severe menace to the aquatic habitat, needlessly endangering the existence of marine life forms (Malhas & Amadi, 2023). Each barrel of oil transported globally through waterways poses a risk to the environment in terms of potential spills. While every oil spill carries the potential for disaster, the extent of long-term damage relies more on the promptness and effectiveness of cleanup rather than the volume of oil spilled (Pete *et al.*, 2021). The environment and various species face substantial consequences due to water pollution caused by synthetic dyes and oil spills (Assanvo *et al.*, 2023). In the initial decades of the 20th century, artificial dyes supplanted natural pigments derived from animals and plants. They became widely utilized in various applications, including coloring substances for fabric, dyeing processes, printing, pharmaceuticals, paper production, leather manufacturing, foodstuffs, and medicine. Consequently, a substantial volume of tainted wastewater was generated (Shalaby *et al.*, 2023a). Every year, numerous oil spills occur worldwide, causing significant environmental catastrophe to a great depth in the marine waters (Rahmati *et al.*, 2022). A successful clean up procedure is necessary for addressing a crude oil spill in seawater (Sayed *et al.*, 2021).

The primary techniques employed in treating wastewater containing petroleum pollutants include physical, biological, chemical, and physicochemical treatment. Dissolved air flotation and mechanical separation are also utilized for this purpose (Vahabisani & An, 2021). The efficiency and cost-effectiveness of a biological treatment method utilizing bacteria, fungi, and algae for the biodegradation of crude oil have recently garnered significant attention (Sayed *et al.*, 2021). Currently, materials that are derived from renewable biomass resources are taken into consideration for the separation of oil/water emulsions. Biomass materials like cotton and cellulose fibres have been successfully functionalized and considered for the segregation of oil/water emulsions (Dai *et al.*, 2019). Collagen is extensively used as a biomaterial in various clinical and industrial demands due to its biological characteristics, including tissue-compatibility, solubility, biodegradability, and low antigenic reactivity (Laasri *et al.*, 2023). These attributes distinguish itself as an eminent alternative for broad scale applications. Maturated collagen, initially recognized as a robust biopolymer adsorbent with high crosslinking and water insolubility, can be blended with metals, polymers, and/or other biopolymers to enhance efficacy and recovery (Shalaby *et al.*, 2023a). As a protein, collagen is prone to destabilization when exposed to heat or certain enzymes like collagenase. Therefore, it must be stabilized to ensure its effective use in various fields, including leather production, tissue engineering, and cosmetics (Thanikaivelan *et al.*, 2012). For most purposes, natural collagen requires modification to impart durability against denaturation and deterioration.

Sources of collagen

Three main amino acid sequence that are required for the synthesis of collagen, that is, proline-lysine-glycine. Collagen, being the super abundant protein in the human body, aids in the genesis of connective tissue, thereby imparts a supporting network to brace the organs and cells. The connective tissue also assists in delivering nutrients, preserving fats, and restoring damaged tissues. Collagen is also a crucial component of bones, skin, muscles, tendons and cartilage. Apart from these, collagen as a biomaterial is also used in cosmetics, skin regeneration templates and biodegradable matrices.

The major sources of collagen as nutriments are:

i Fish and meat: High protein-rich foods nurture collagen production as they consist of amino acids like proline, glycine and hydroxyproline that help in the synthesis of collagen. Some of the major protein-rich foods are fish, poultry and meat. It is abundantly found in the connective tissue and bones of meat. Hence, bone broth is a very good source of collagen. Marine sources also contribute to a major source of collagen. The skin and bones of fish, shellfish and sharks also contain collagen. Although eggs do not possess connective tissue, they are still a great source of collagen as the egg white contains excessive quantity of proline. The eggshell membrane contains collagen type V and type X.

ii Vegetables: Vegetables are important

precursors of collagen as they contain natural vitamin C. Primarily green leafy vegetables like spinach, lettuce, kale and other salad greens are key sources as they also contain antioxidant activities apart from boosting collagen production. Vegetables like bell peppers and tomatoes also consist of nutrients like zinc that boost collagen production. Legumes like chickpeas, kidney beans, peas and lentils are excellent sources of collagen as they naturally provide proteins, copper and vitamin C that in turn allow for the production of essential amino acids which further improves the production of collagen. Garlic contains a high amount of sulphur and it is a trace mineral that magnifies collagen production in the body.

iii Vitamins: Vitamin C is a major precursor in the building of pro-collagen. This can be obtained majorly from citrus fruits like oranges, lemons, limes and grapefruits and from tropical fruits like mango, pineapple, kiwi and guava. Guava is a great source of zinc which is another cofactor for collagen synthesis in the body. Consumption of gooseberries, raspberries and strawberries increases the synthesis of collagen thus helping to tighten the skin and therefore, delaying the process of aging.

Major sources of collagen from animal skin:

i Bovine skin: Bovine skin and tendon tissues from male and female cattle were cumulated from the local abattoir and were shifted to the laboratory on ice (Sorushanova *et al.*, 2021). Collagen, as a protein, can be obtained or derived from cow hides. Bovine collagen is abundant in essential amino acids like glycine and proline. Glycine plays a role in joint repair and muscle development. Proline helps in the regeneration of the skin and also assists in wound healing. Bovine collagen aids in ameliorating arthritis, prevents deprivation of the skeletal framework and provides a long-lasting natural skin.

ii Porcine skin: Porcine collagen is obtained from the skin and bones of pigs. Porcine collagen is considered to be similar to human collagen as they are easily digested and absorbed. Porcine collagen is also rich in proline and glycine thus, assisting in tissue repair and proper growth. Porcine collagen provides better cellular adhesive properties. The cross-linked collagen matrix demonstrated the capability of fibroblast support and maintenance over longer times by the presence of cells (De Luca *et al.*, 2016).

iii Marine skin: Marine organisms like fish, jellyfish, sponges and other invertebrates are eternal sources of collagen. Consumption of marine collagen peptides showed an increased incidence in hydroxyproline content and other essential amino acids that further showed improved skin regeneration properties, intensified dermal amplification and unvaried provision of collagen fibres in the region of the dermis. It also elevated the production of ATP in the body (De Luca *et al.*, 2016).

Major sources of collagen supplements

Supplemental collagen is the hydrolysed form of collagen as they are broken down into its simpler form which can be absorbed easily when consumed. Hydrolysed collagen is primarily available in the powdered form and also in capsulated form. These supplementary collagens are used for the same functions as that of dietary collagen in the body. It also helps in boosting gut health and ameliorates cardiovascular diseases as collagen makes arteries flexible and increases its elasticity, in turn, preventing atherosclerosis.

Types of collagen

i Collagen type I: It is also called fibril-forming collagen. It consists of alpha 1 and alpha 2 chains. Type I is existent in skin, tendon, large blood vessels, fibrocartilage, connective tissue, intestine and uterus. It is the most prevalent type of collagen in the body. Its function is to repair and replace tissues (Muthukumar *et al.*, 2018).

ii Collagen type II: It is known as fibrillar collagen and contains α1 chain. It is primarily present in cartilage, vitreous and invertebrate discs. It contributes substantially in nurturing the skeletal framework of the body and also provides biomarkers for osteoarthritis.

iii Collagen type III: Type III is again called the fibrillar collagen and is also composed of α1 chain. It is present in the dermis, intestine, bone marrow, blood vessels and the heart valve. It mainly forms a network of reticular fibres that forms the supporting tissue for the internal organs and is also involved in cardiovascular development.

iv Collagen type IV: It is also named basement membrane collagens and is made up of α1, α2, α3, α4, α5 and α6 chains. It is located in between the epithelial cells and the connective tissues that forms the general basement membrane. Type IV generally supports the matrix organization and assists in platelet adhesion and aggregate formation.

v Collagen type V: It is the fibril-forming collagen and is incorporated with chains of α1, α2, α3 and α4a polypeptides. It is found in the hair, nails, cornea and placental membranes. It is responsible for collagen fibrillogenesis and matrix assembly.

vi Collagen type VI: It is also designated as microfibrillar collagen. It consists of α1, α2, α3, α4b, α5c, α6 chains. It is present in the Descemet's membrane, tendon, muscles and in the central part of the invertebral disc. Collagen type VI aids in platelet adhesion and aggregation of vascular endothelial regions.

vii Collagen type VII: Type VII collagen is also called the anchoring fibrils. It is made of an α1 chain. The site of type VII collagen is skin, placenta, lungs and cornea. As the name suggests, its main role is to anchor fibrils at the dermal-epidermal junction.

viii Collagen type VIII: This is also termed network-forming collagens and constitutes of α1 chain. Class VIII collagen is situated in endothelial cells and Descemet's membrane. This type of collagen notably renders a duty in regulating the migration of smooth muscle cells and is also involved in the movement of endothelial cells.

ix Collagen type IX: Collagen type IX also known as fibril-associated collagen with interrupted triple helices (FACIT). They are made up of α1, α2 and α3 chains and are found particularly in the cartilage. Type IX collagen functions in forming fibres in articular cartilage.

x Collagen type X: Type X collagen is also labelled as network-forming collagens. They are mainly present in the hypertrophic cartilage and consist of α1 chains. It mainly operates in the mineralization of cartilage.

xi Collagen type XI: These are the fibril-forming collagen consisting of α1, α2 and α3 chains of collagen which are positioned in the vitreous humour, cartilage and intervertebral disc. It is involved in the structuring of the pericellular matrix.

xii Collagen type XII: Type XII collagen also known as fibril-associated collagen with interrupted helices (FACIT) consists of α1 chain and they are primarily situated in the perichondrium and the articular surface of the body. This type of collagen is responsible for controlling osteoblast differentiation and bone matrix formation.

xiii Collagen type XIII: This type of collagen is also called transmembrane collagens and it constitutes of α1 chain. They are predominantly present in foetal skin, bone and intestinal mucosa. Type XIII collagen majorly functions in cellular adhesion.

Various methods in extraction of collagen

The method for extracting collagen differs based on the raw source used, but its aim is always to remove all non-collagenous substances and isolate pure collagen as the final outcome. This involves,

- pre-treating the source tissue
- extracting collagen
- purifying it further (Matinong *et al.*, 2022)

Preliminary treatment

Preliminary treatment is mainly employed to disintegrate the covalent inter-particle attachments connecting the collagen fragments (Matinong *et al.,* 2022). As collagen in animal connective tissue is cross-linked, it breaks down at a slow pace, even when boiled. Therefore, a mild chemical treatment is necessary to break these cross-links before extraction. This involves using less concentrated acids and bases to partially hydrolyze the collagen, which helps to maintain the integrity of the collagen chains while disrupting the cross-links.

- **Acid pre-treatment:**

In acid pretreatment, the epidermal shreds are plunged into a dilute acid solution at a regulated degree of temperature. The acid penetrates into the derma, causing its expansion, hence, doubling or tripling its proportion comparatively and breaking down the cross-links through hydrolysis.

- **Alkaline pre-treatment:**

The alkaline process involves treating the original substance with a suspension containing a base. Pretreatment involves the use of mild alkalis like sodium hydroxide (NaOH) and calcium hydroxide ($Ca(OH)_2$) (Matinong *et al.*, 2022).

Alkaline pretreatment of tissues at low temperatures is a commonly used method for removing non-collagenous proteins, often serving as a preliminary step in the separation and refinement of fish collagen (Meng *et al.*, 2019)

The duration of the pretreatment depends on the stratification of the matter used. Alkalis are particularly effective for extracting collagen from large and dense substances. While using NaOH, the pretreatment process may take several days to weeks to complete.

- **Skin specific pretreatment:**

Depending on the source or characteristics of the skin, the specific pretreatment methods that may be required are soaking, fleshing, dehairing, and cutting.

Extraction

Traditional separation techniques were mostly depending on chemical hydrolysis, employing acids, alkalis, or saline solubilizers. Occasionally, these chemical extraction processes are supplemented by ultrasound or microwave assistance, as well as the use of enzymes to facilitate the extraction.

While chemical hydrolysis is prevalent in industrial applications, biological processes involving enzyme supplementation show greater promises, particularly when aiming for products that boast high nutritional value and enhanced qualities.

- **Acid hydrolysis:**

Both inorganic and organic acids can effectively break bonds in collagen, facilitating the extraction of fibrils. In acidic conditions, collagen molecules acquire a net positive charge, resulting in electrostatic repulsion that helps in molecular separation. Acid hydrolysis can be conducted using organic acids like acetic acid, citric acid, and lactic acid, as well as inorganic acids such as hydrochloric acid (Matinong *et al.*, 2022).

- **Alkaline hydrolysis:**

Alkaline hydrolysis is another method utilized for collagen extraction, typically employing aqueous solutions of sodium hydroxide (aq. NaOH) or potassium hydroxide (aq. KOH). Alkalis possess the tendency to hydrolyse collagen fibrils, potentially leading to the degradation of amino acids such as cysteine, histidine, serine, and threonine during the process. However, extractants such as calcium oxide, calcium hydroxide, and sodium carbonate may also be employed for this purpose.

Salt solubilization is less frequently utilized. Neutral brine solutions provide greater efficacy in collagen solubilization and are frequently employed in extraction processes. Various salts used include citrates, phosphates, sodium chloride, and Tris-HCl.

- **Enzyme hydrolysis:**

Enzyme hydrolysis can be combined with conventional chemical methods. This approach provides improved reaction accuracy and causes slighter damage to collagen. Consequently, it holds the potency to enhance both the yield and integrity of the extracted collagen product.

Enzyme-soluble collagen represents an innovative approach to collagen extraction, characterized by its eco-friendly nature. This method reduces reliance on organic solvents and toxic chemicals by leveraging enzymes to hasten the decomposition process, thereby producing collagen substitutes efficiently while minimizing solvent use and waste generation (Gaikwad & Kim, 2024).

Furthermore, enzymatic hydrolysis offers several advantages over chemical hydrolysis. These include enzymatic specificity, the ability to control the extent of hydrolysis, operates under moderate conditions, and a lower salt content in the resulting hydrolysate.

- **Ultrasound-assisted hydrolysis:**

Ultrasound finds extensive application in enhancing mass transfer during wet processes, particularly in tasks such as mixing, extraction and drying, which are crucial in various applications. Utilizing ultrasound waves with a frequency of 20 kHz or higher in the course of collagen isolation enhances the gain of the material and shortens the time required for the process. The collagen extraction process is impacted by both the ultrasound's amplitude and treatment duration. Research indicates that higher amplitudes can reduce extraction time and improve yield. Ultrasound waves comprise alternating compression and rarefaction cycles that can transmit through solid, liquid, or gas mediums, causing molecules to detach from their original positions to shift (Kumar *et al.*, 2020). Ultrasound-assisted extraction (UAE) involves the use of mechanical energy from ultrasound waves to the samples. Sonication leads to cavitation, the creation of tiny vacuum bubbles or voids within the liquid. These bubbles collapse at the solid sample, generating confined elevated temperatures (around 45°C) and pressures (around 50 MPa). Ultrasound treatment is typically simple, diminishes reliance on corrosive chemicals, and offers an economically viable and safe extraction method.

- Supercritical fluid extraction (SFE):

SFE has become a prominent green extraction method, offering several benefits over traditional techniques. It is highly effective for extracting natural chemical components such as flavonoids, ethereal oils, seed oils, carotenes, and fatty acids from plant materials. SFE acts as a renewable substitute to conventional methods of extraction. Key factors driving the substantial growth of SFE include its increased selectivity, higher extraction procurement, superior fractionation capabilities, and lower ecological impact. Notably, SFE is gaining traction in heavy metal recovery and drug delivery sectors, demonstrating increasing reliability (Uwineza & Waśkiewicz, 2020).

- Deep eutectic solvent:

The utilization of deep eutectic solvent (DES) presents an environmentally sustainable method for extracting collagen from various sources including plants, animals, and marine organisms. This method involves the establishment of hydrogen bond interactions, characterized by at least one hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD) (Jafari *et al.*, 2020b).

Post extraction purification

In addition to collagen, a raw collagen extract generally includes neutral salts and non-collagen proteins. To isolate collagen fractions with different molecular weights, the extract must be purified through various steps, including filtration and centrifugation. One common purification technique is "salting out," where a high concentration of salt is added to the extract to precipitate either the target proteins or the unwanted proteins from the solution (Matinong *et al.*, 2022).

Collagen nanoparticles

Collagen nanoparticles are a highly favourable biomaterial because of their various beneficial advantages such as increased bioavailability, high biocompatibility, enhanced biodegradability, and high tensile strength. These properties are majorly available in natural polymeric nanoparticles. Other efficient natural nanoparticles are keratin, soy, silk, and elastin nanoparticles.

Collagen is an abundant protein present in the body. It is extensively used in drug delivery, in the production of hydrogels, cancer therapy, and collagen shields for eye disease.

Collagen nanoparticles are more beneficial than other naturally occurring nanoparticles because they provide a high contact surface, low toxicity, minimum amount of antigenicity, and increased cationic-charge density potential due to the presence of abundant amino acids in the protein. These collagen nanoparticles also increase cell retention, maintain their shape under heat, and reduce the danger of by-products created during its metabolism (El-Sawah *et al.*, 2024).

- Collagen-silver nanoparticle composites:

For centuries, silver has been harnessed in its ionic or nanocrystalline state for its antimicrobial properties, effectively combating severe bacterial infections, including those caused by antibiotic-resistant strains. Currently, silver nanoparticles (Ag NPs) hold significant utility across various domains like medicine and biosensing. Their smaller size and larger surface area confer robust chemical, magnetic, and plasmonic attributes, rendering them indispensable in diverse applications (Grigore *et al.*, 2017) .

Recent advancements in silver-infused dressings have expanded their applicability to various wound types that exhibit colonization or infection. Numerous studies have been conducted to integrate silver nanoparticles into collagen hydrogels for applications in tissue engineering.

- Collagen-copper nanoparticle composites:

Copper nanoparticles (Cu NPs) have garnered significant attention in wound healing research owing to their potent antibacterial characteristics, effectively inhibiting a broad range of bacterial strains. These nanoparticles release Cu2+ ions, which induce disruption in cell walls and membranes by modifying protein structure or interfering with enzyme function (Kushwaha *et al.*, 2022).

The collagen-Copper Oxide matrix loaded with Copper Oxide nanoparticles (CuO NPs), along with the incorporation of various herbs such as *Andrographis paniculata*, *Senna auriculata*, and *Mimosa pudica*, exhibited remarkable efficacy against bacterial strains, particularly *S. aureus* and *E. coli* (Kushwaha *et al.*, 2022).

- Collagen-curcumin nanoparticles:

Intrinsic plant-based ingredients like curcumin (CUR) are recognized for their antimicrobial and anti-inflammatory properties. When CUR is combined with collagen scaffolds, it causes a decline in laceration, absolute re-epithelialization, and the genesis of granulated tissue. This suggests that CUR-infused nano collagen scaffolds serve as an effective template for epidermal revival.

- Collagen- Titanium dioxide (TiO_2) nanoparticles:

Over the past few decades, there has been increasing engrossment in hybrid materials incorporating nanoparticles, particularly due to their diverse range of applications. TiO_2 nanoparticles, for instance, are widely utilized in cosmetics and sunscreen products. Studies have documented the antibacterial properties of TiO_2 nanoparticles against various types of bacteria, including both gram-positive and gram-negative strains (Kalirajan *et al.*, 2019).

The oil removal process relies on a straightforward adsorption mechanism, and it does not display unmediated interactivity between the oil and collagen molecules within the hybrid scaffolds. Another significant contributor to the oil sorption capacity of collagen materials is the highly porous arrangement created during the freeze-drying process. The oil adsorption is likely facilitated by capillary action, which promotes the scattering of oil into the empty spaces within the macropores of the hybrid scaffold. This phenomenon results in pores with relatively hydrophobic surfaces, promoting high oil adsorption. Incorporating TiO_2 nanoparticles into the hybrid collagen scaffold may enhance surface severity at the nanoscale and increase hydrophobicity, thereby facilitating rapid oil adsorption.

- Collagen-magnetic nanoparticles:

Combining nanoparticles with non-toxic polymers can create nanobiocomposites, which hold substantial promise for various biomedical and environmental applications. Magnetic iron oxide has shown minimal cytotoxicity even at high concentrations, making it an excellent magnetic material for bio-diagnostics and visualizing purposes. The interaction between helical collagen fibres and spherical superparamagnetic iron oxide nanoparticles (SPIONs) has been confirmed through colorimetric, microscopic, and spectroscopic techniques. This nanocomposite exhibits specific oil absorption and magnetic tracking abilities, making it ideal for oil removal applications (Thanikaivelan *et al.*, 2012).

- Collagen-hydroxyapatite (HA):

Collagen is widely acknowledged as a highly beneficial natural biomaterial. In biomedical applications, it can be processed into scaffolding material to improve cell migration, aid in recovery of injuries, and support tissue rejuvenation.

Hydroxyapatite (HA) is widely used as an artificial bone replacement material and has gained substantial attentiveness for rigid tissue implementations because of its biological activity, bioresorbable, and compostable properties. Nevertheless, HA's inherent brittleness, low fracture toughness, and vulnerability to fatigue failure pose challenges. To address these issues, composite materials combining HA with polymers such as collagen are highly recommended for osseous remodelling. Currently, a loosely structured and porous HA/Collagen composite has been evolved, offering sponge-like resilience and excellent handling qualities (Xie *et al.*, 2023) .

- Collagen- chitosan nanoparticles:

Chitosan is a linear polycarbohydrate derived from the deacetylation of chitin; a native polymer found in the exoskeletons of crustaceans. It is a homopolymer comprising of D-glucosamine units (deacetylated) and N-acetyl-D-glucosamine units (acetylated), connected by β-(1,4) glycosidic bonds. Chitosan is extensively utilized in cosmetics and skincare formulations due to its antibacterial, antioxidant, and skin-regenerating properties.

Enzymatic, acidic, or alkaline hydrolysis of collagen can be employed to obtain collagen peptides, also known as collagen hydrolysates, which is the primary structural protein found in skin, bones, and connective tissues. The decreased molecular weight of collagen peptides showed enhanced bioavailability compared to intact proteins.

The use of collagen peptides in food supplements, cosmetics, and pharmaceutical products is experiencing rapid growth. Pickering emulsions stabilized with polysaccharide/protein complex particles have gained increasing research recognition as they demonstrate high stability and long shelf life. Emulsions stabilized by solid particles, known as Pickering emulsions adsorb at the interface between oil and water. They are recognized as environmentally favourable options to traditional emulsion systems because they do not require added emulsifiers(Cheng *et al.*, 2020)

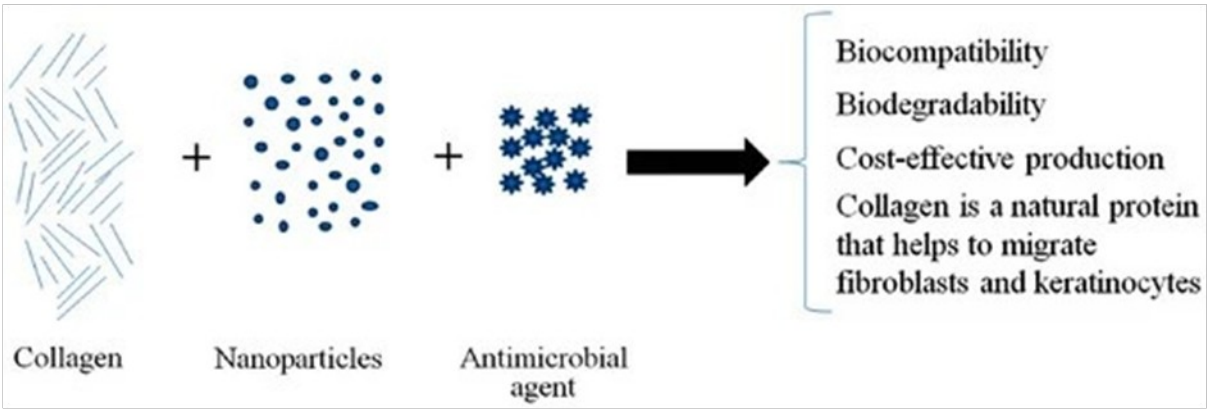

Figure 1. The advantages of collagen nanoparticles. Courtesy: (Grigore *et al.*, 2017)

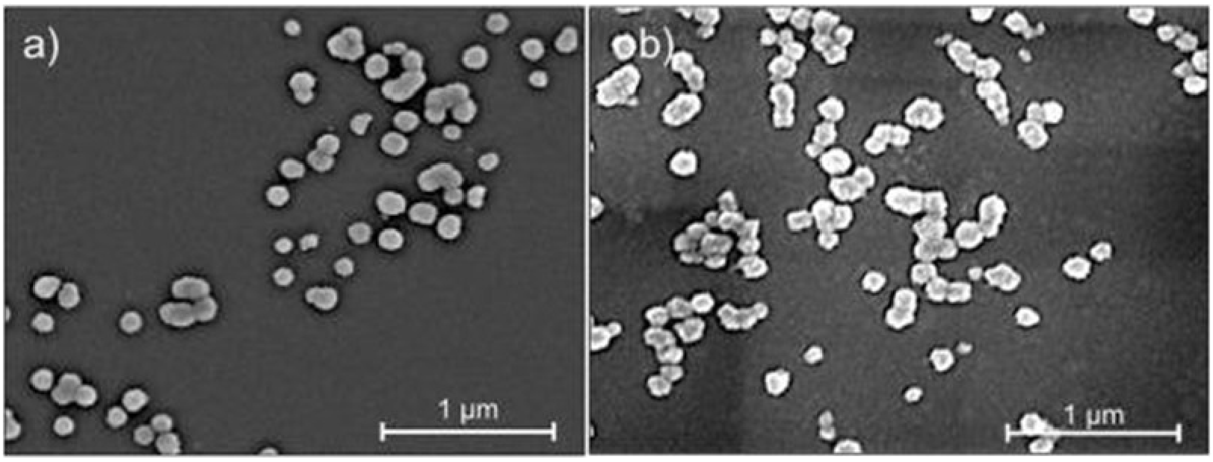

Figure 2. SEM images showing stable collagen-peptide functionalized chitosan nanoparticles after the drug discharge process. **a**) pH 1.5, and **b**) pH 7.4. Courtesy: (Anandhakumar *et al.*, 2017)

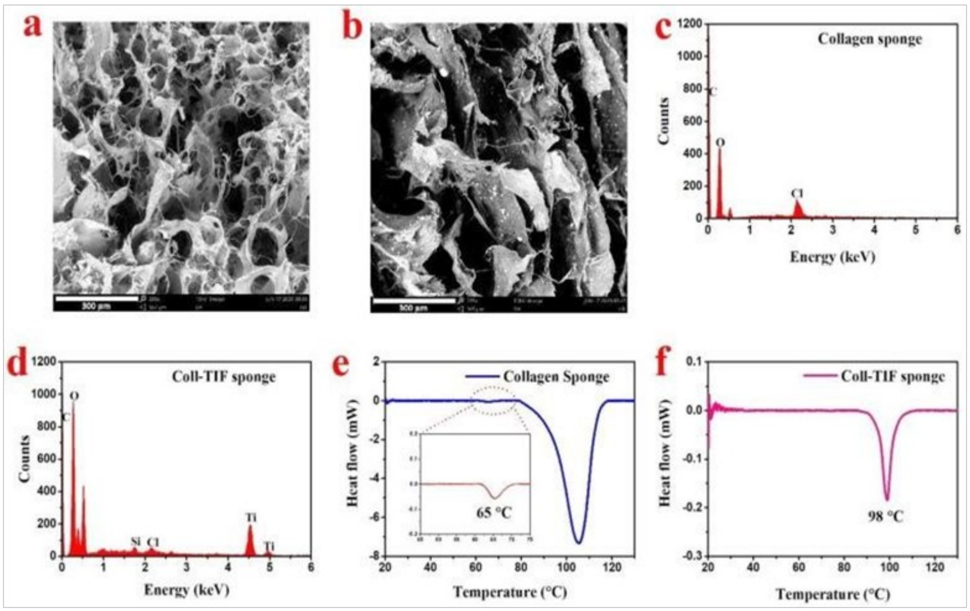

Figure 3. SEM images of (a) native collagen sponge and (b) Coll-TIF sponge. EDAX spectra of (c) native collagen sponge and (d) Coll-TIF sponge. DSC curves of (e) native collagen sponge and (f) Coll-TIF sponge. Courtesy: (Nagaraj *et al.*, 2021)

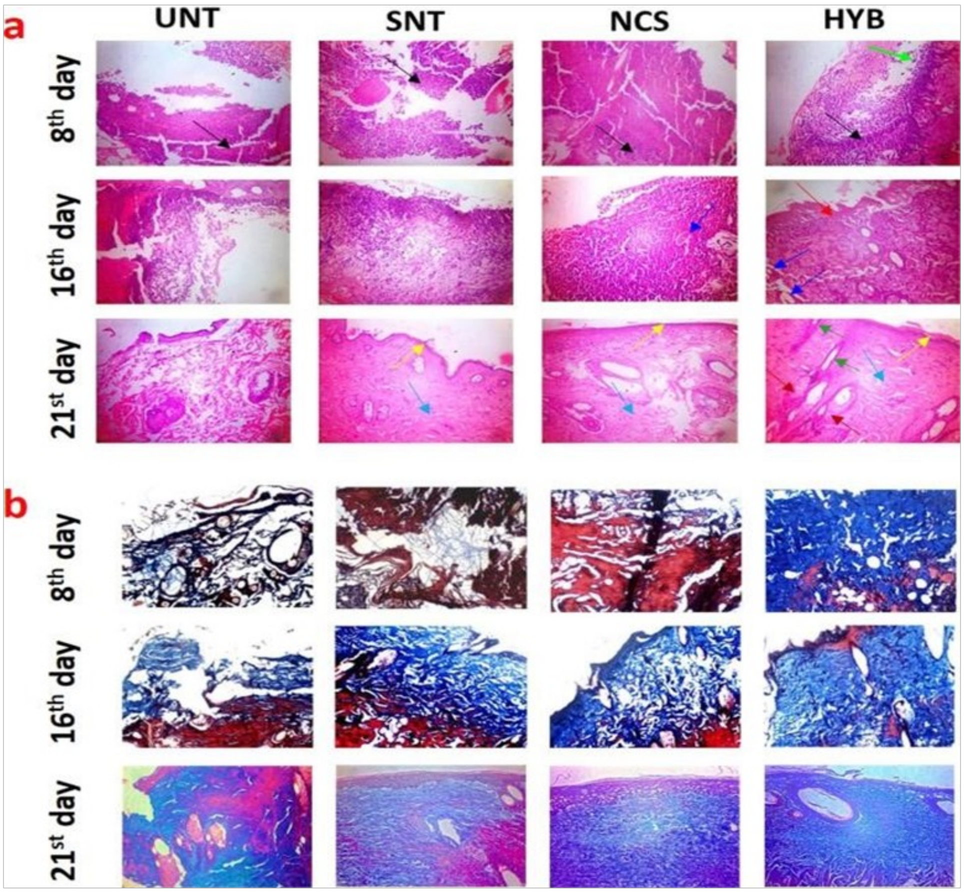

Figure 4. Tissular analysis. (a) H&E staining of granulation tissue collected from all categories at various days, black colour arrows demonstrating the inflammatory cells, light green colour arrow showing the new fibroblasts, red colour arrows showing the new epithelium, yellow colour arrows revealing the epidermis, sky blue colour arrows showing the dermis layer, navy blue colour arrows illustrating the blood vessels, dark green colour arrows showing the new hair follicles and brown colour arrows elucidating the sebaceous gland. (b) Masson trichrome staining of granulation tissue collected from all groups at different days showing the collagen deposition. The blue colour region in the images shows the deposition of collagen. Courtesy: (Kalirajan *et al.*, 2019)

Applications of collagen

Drug delivery:

Collagen-peptide functionalized chitosan nanoparticles were found to be an effectual carrier system for encapsulation and it was an evident biomaterial for the release of doxorubicin which is a drug that is used to treat cancer. In-vitro drug release was experimentally performed using buffers like phosphate-buffered saline, acetate-buffered saline, and hydrochloric acid buffer as release medium utilizing the dialysis method. The collagen nanoparticle containing the appropriate buffer was placed in a dialysis membrane tubing in a standard flask kept in an incubator shaker. The aggregate of doxorubicin that was released was assessed through the UV-visible spectroscopy by estimating the increase in absorbance value.

These in-vitro drug liberation profiles revealed that the drug release emerges in two types of stages which are the burst release stage up to 20 hours and the sustained release stage from 20 hours to 1 week. At disparate pH values, it was noticed that the release of drugs was increased when the pH was decreased from an alkaline (pH 7.4) value to an acidic (pH 1.5) value. The concentration of the drug released was increased for a week because the drug doxorubicin contains an amino group with a pka of 8.6 and phenol groups with a pka of 9.5. The electrostatic interaction between the amino group of the drug and the carboxyl group of the collagen peptide chitosan nanoparticle is primarily involved in the encapsulation of the drug and the drug-nanoparticle interaction is stronger at higher pH values which then lead to lower drug release. This proves that when the pH is decreased to an acidic level, the amount of drug released is of a higher concentration (Anandhakumar *et al.*, 2017).

Collagen nanobiosponge:

The leather and tanning industry is known to produce plenty of collagen waste. This biowaste can be used to produce collagen nano bio-sponges. The textile industry also releases excess dyes into water bodies which in small proportions can be toxic for both humans and marine organisms. This collagen bio-sponge can be used for the eradication of dye from water bodies hence assisting in water remediation.

Titanium dioxide nanoparticles were surface functionalized using ammonia and water. The surface functionalized titanium dioxide nanoparticles were crosslinked with collagen fibres by Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) coupling. Then, the synthesised collagen-titanium dioxide functionalized fibres (coll-TIF) were dissolved in glacial acetic acid, following which it was transferred to Petri plates and kept for freezing. The coll-TIF obtained was then lyophilized to procure the collagen-titanium dioxide functionalized nano bio-sponge.

The FTIR spectroscopy revealed the mechanical stability of the degradable collagen nano bio-sponge and this bio-sponge also showed great potency in degrading rhodamine B under visible light irradiation which is a dye component that contaminates water. Hence, this cost-effective collagen-titanium dioxide functionalized nano biosponge is a credible approach for water bioremediation (Nagaraj *et al.*, 2021).

Cosmetics:

Collagen is a great source to boost the properties of an eternal and perpetual skin. Formulating collagen into beauty products has shown benefits in enhancing skin health, moisture and elasticity and, together with daily usage, will reduce signs of aging (Lo and Fauzi, 2021). Thus, collagen is one of the natural components used in various cosmetic products. Supplementation of marine collagen peptides to assess the clinical parameters of the dermis of the face. Collagen from fish skin was derived and formulated into gelatinous capsules along with grape skin extract, coenzyme Q, luteolin, and selenium which were developed with the commercial name CELERGEN.

This marine collagen peptide was subjected to assessment for various parameters of the skin like epidermal and dermal thickness, aging, elasticity, and sebum content through ultrasound examinations. It was shown that the CELERGEN enhanced skin elasticity and sebum production. The photoprotective effect of dietary marine collagen peptides was also tested on mice after chronic UV irradiation. This test indicated that dietary collagen supplementation did improve the immunity of the skin causing decreased loss of water from the skin helping in hydrated skin, restoring cutaneous collagen and elastin levels and it also maintained type I and type III collagen ratio. Hence, dietary marine collagen peptides as a supplement showed a significant potency in preventing the degeneration of the skin and thus assisting in skin replenishment (De Luca *et al.*, 2016).

Wound healing:

Collagen scaffolds that were prepared from cowhide also showed a significant influence on wound healing when it was tested on Albino Wistar rats. The Wistar rats were subjected to skin burn and then treated with collagen scaffolds. The results were observed after 21 days. It was observed that the collagen scaffolds influenced regeneration and thorough re-epithelialization of the degraded part of the skin within 21 days of ministration. Histological analysis was done by Hematoxylin and Eosin (H&E) staining and Masson's trichrome staining. On the 0^{th} day, H&E staining of the seared wound tissue showed the deprivation of junctures between the dermis and epidermis, and there was complete damage of the skin appendages which can be classified as a gaping second-degree burn. The collagen scaffold accelerated this wound healing by the 8^{th} day and, by the 16^{th} day, the wounded tissue showed less inflammation. At the end of tissue treatment on the 21^{st} day, the histological analysis showed completely developed cells and tissues with good skin formation, hair papilla, sebaceous glands, dermal, and epidermal layers with newly formed blood vessels. Masson's trichrome staining also revealed a high expression of collagen fibres by the 16^{th} day of treatment which positively increased by the 21^{st} day of treatment. It also showed the tightly packed collagen fibres, hence proving that collagen scaffolds are indeed extraordinary proteins that aim to manifest a promising approach in tissue regeneration and wound healing which in turn, can be utilized for tissue engineering (Kalirajan *et al.*, 2019).

Water purification:

Water contamination caused by synthetic dyes and oil spills has a profound effect on the environment and various species (Sayed *et al.*, 2021). In the early 20th century, synthetic dyes were replaced by natural dyes derived from animals and plants across various applications, including fabric dyeing, printing, pharmaceuticals, paper production, leather processing, and food production. This shift led to a substantial increase in coloured wastewater generation, with the textile industry emerging as the largest consumer of dyes, accounting for 60% of the total usage (Shalaby *et al.*, 2023a). Recently, there has been considerable interest in composites made from biopolymers sourced from natural or biological origins. By combining nanoparticles with biodegradable polymers, nanobiocomposites can be created, offering promising applications in both biomedical and environmental fields (Thanikaivelan *et al.*, 2012). Matured collagen, known for its high crosslinking and water insolubility has been recognized as an effective biopolymer adsorbent material. It can be enhanced by blending with metals, polymers, and/or other biopolymers to enhance efficiency and recovery (Shalaby *et al.*, 2023a). Iron oxide nanoparticles, specifically magnetic iron oxide have demonstrated minimal cytotoxicity even at significantly high concentrations. This characteristic makes iron oxide nanoparticles exceptionally suitable as magnetic materials for bio-diagnostics and imaging applications (Thanikaivelan *et al.*, 2012). The integration of magnetic nanoparticles with collagen polymer presents a novel approach to leverage unique properties in collagen biopolymer-magnetic nanoparticle interactions. This innovative combination effectively stabilizes collagen waste fibers. Specifically, the magnetic iron oxide nanoparticles are strategically employed to enhance the removal of dyes from wastewater through magnetic tracking. However, there has been limited exploration in utilizing reusable biological materials derived from waste. The integration of polymers derived from biological sources with diverse materials into a single composite combining nanoparticles is highly desirable to enhance its properties for specific application (Shalaby *et al.*, 2023a).

CONCLUSION

In contemporary times, nanomaterials offer promising avenues for combating microbial diseases and disorders. Chronic wounds, particularly those plagued by drug-resistant microbes, present a significant challenge to the healthcare system. Recent research has shown that composite hydrogels containing antibiotic-loaded nanoparticles and collagen are effective for promoting wound healing and preventing the formation of microbial biofilms in chronic wounds. While collagen has been used in biomedical research for many years, recent studies focus on silver nanoparticles stabilized with collagen, exploring their biocompatibility and antibacterial properties. Silver and silver-collagen nanoparticles possess dual capabilities: they can effectively eradicate microorganisms while also promoting skin regeneration and the distinctive properties of silver nanoparticles suggest they can effectively prevent wound infections and enhance the healing of damaged tissues. Collagen exhibits excellent properties such as biocompatibility, biodegradability, thickening, and water-binding capacity. Moreover, utilizing non-mammalian sources or extracellular matrix components for collagen production enhances cost effectiveness. Thus, collagen provides an optimal structure for cellular ingrowth to facilitate effective wound healing.

Water pollution caused by synthetic dyes and oil spills greatly affects the environment and living organisms. In response, a low-cost, eco-friendly, and easily biodegradable magnetic hybrid bio-sponge nanocomposite using renewable resources like collagen can be used. It was experimentally demonstrated that collagen and magnetic collagen nanocomposites can adsorb dye, thereby reducing the cytotoxicity of polluted water, and can also accumulate under the influence of a magnetic field.

CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

List of abbreviations

ESM- Egg Shell Membrane

UAE- Ultrasound Assisted Extraction

SFE- Supercritical Fluid Extraction

DES- Deep Eutectic Solvent

HBA- Hydrogen Bond Acceptor

HBD- Hydrogen Bond Donor

$Ca(OH)_2$- Calcium Hydroxide

Aq. NaOH- Aqueous Sodium Hydroxide

Aq. KOH- Aqueous Potassium Hydroxide

Ag NPs- Silver Nanoparticles

Cu NPs- Copper Nanoparticles

CuO NPs- Copper Oxide Nanoparticles

CUR- Curcumin

TiO_2 – Titanium Dioxide

SPIONs- Superparamagnetic Iron Oxide Nanoparticles

HA- Hydroxyapatite

EDC/NHS- Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N- hydroxysuccinimide

Coll-TIF- collagen-titanium dioxide functionalized fibres

H&E staining- Hematoxylin and Eosin staining

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