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Preparation of copper-containing bioactive glass/eggshell membrane nanocomposites for improving angiogenesis, antibacterial activity and wound healing

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ABSTRACT

Effectively stimulating angiogenesis and avoiding wound infection are great challenges in wound care management. Designing new healing dressings with requisite angiogenic capacity and antibacterial performance is of particular significance. In order to achieve this aim, we prepared a copper (Cu)-containing bioactive glass nanocoating (40–50 nm) with uniform nanostructure on natural eggshell membrane (Cu-BG/ESM) by the pulsed laser deposition (PLD) technique. The surface physicochemical properties including hydrophilicity and hardness of ESM were significantly improved after depositing Cu-BG nanocoatings. Meanwhile, 5Cu-BG/ESM films containing 5 mol% Cu stimulated proangiogenesis by improving vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF)-1α protein secretion as well as angiogenesis-related gene expression (VEGF, HIF-1α, VEGF receptor 2 (KDR) and endothelial nitric oxide (eNos)) of human umbilical vein endothelial cells (HUVECs). When used to treat full-thickness skin defects in mice, 5Cu-BG/ESM films enhanced the healing quality as confirmed by the significantly improved angiogenesis (as indicated by CD31 expression) and formation of continuous and uniform epidermis layer *in vivo*. Furthermore, 5Cu-BG/ESM films could maintain a sustained release of Cu^{2+} ions and distinctly inhibited the viability of bacteria (*Escherichia coli*). The results indicate that Cu^{2+} ions released from Cu-BG/ESM nanocomposite films play an important role for improving both angiogenesis and antibacterial activity and the prepared nanocomposite films combined Cu-containing BG nanocoatings with ESM are a promising biomaterial for wound healing application.

Statement of Significance

Designing new healing dressings with requisite angiogenic capacity and antibacterial performance is of particular significance in wound care management. In our study, we successfully prepared copper-containing bioactive glass/eggshell membrane (Cu-BG/ESM) nanocomposites with uniform bioactive glass nanocoatings by using pulsed laser deposition (PLD) technology. Due to the deposited Cu-BG nanocoatings on the surface of ESM, Cu-BG/ESM nanocomposites possessed significantly improved physicochemical and biological properties, including surface hydrophilicity, hardness, antibacterial ability, angiogenesis rate *in vitro* and wound healing quality *in vivo* as compared to pure ESM and BG/ESM films. Our study showed that prepared nanocoatings on Cu-BG/ESM nanocomposites offer a beneficial carrier for sustained release of Cu^{2+} ions which played a key role for improving both angiogenesis and antibacterial activity. The prepared nanocomposites combined Cu-containing BG nanocoatings with ESM are a promising biomaterial for wound healing application.

1. Introduction

The repair of wounds is a complex biological process in which various intracellular and intercellular pathways should be activated and coordinated in order to reduce the risk of infection and

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further enhance the healing quality [1,2]. Skin generally needs to be protected with a dressing immediately once an injury occurs. An ideal dressing biomaterial for skin wound should possess the ability to effectively stimulate angiogenesis and protect the wound from infection during healing process. In addition, it should have suitable physical and mechanical properties, such as porous structure and toughness [3,4]. It remains significant challenging to achieve such kind of dressing biomaterials for effective wound healing of skin tissues. Two of major challenges regarding the development of wound dressings are how to enhance angiogenesis and prevent bacterial infections during the process of wound healing. Angiogenesis plays multifunctional roles in wound healing since new blood vessels are responsible for transport of nutrients and growth factors and removal of waste products from the new tissue formation site [5,6]. In addition, the formation of new blood vessels is of great significance to sustain newly formed granulation tissue [7]. Avoiding wound infection caused by bacterial intrusions is the other important issue during healing process. Once the wound site is infected by microorganisms, the healing process will be significantly impeded [8].

Recently, there has been a growing tendency to incorporate inorganic ions into biomaterials for stimulating angiogenesis differentiation of stem cells for bone regeneration [9,10]. Among bioactive ions, copper (Cu^{2+}) ions can promote angiogenesis by stabilizing the expression of hypoxia-inducible factor (HIF-1α) and secretion of vascular endothelial growth factor (VEGF), therefore artificially mimicking hypoxia, which plays a critical role in the recruitment and differentiation of cells and in blood vessel formation [11]. In addition, Cu is one of the well-studied antimicrobial agents against *Escherichia coli O157*, *methicillin-resistant Staphylococcus aureus* and *Clostridium difficile* [12,13]. Considering the advantages, it is interesting to incorporate Cu^{2+} ions into wound healing biomaterials for skin regeneration.

Bioactive glasses (BGs) have been extensively applied in hard tissue engineering because of their high bioactivity, osteoconduction and osteostimulation [14,15]. Recently, an increasing number of studies focus on application of BGs in contact with soft tissues and wound healing, including vascularization and cardiac, lung, nerve, gastrointestinal and laryngeal tissue repair [16–18]. Considering the numerous advantages of Cu^{2+} ions and BGs mentioned above, preparation of Cu-containing bioactive glass may stand for a promising strategy to accelerate wound healing. However, one of shortcoming for bioactive glass particles used as wound healing materials is that they have no mechanical supporting and not easy for treatment [19]. In addition, since bioactive glass particles have sharp morphology, which may result in potential inflammation reaction when directly exposed to tissue cells [20].

Eggshell membrane (ESM) is a thin (thickness: 60–80 μm), highly-collagenized fibrous connective tissue comprised of both inner and outer layers [21]. ESM is a cheap material with high biosafety, which can be easily obtained. As a natural material, ESM has a biopolymeric fibrous network with porous structure and the fibers are composed mainly of proteins (80–85%), of which ~10% are collagens (types I, V and X). The fascinating porous fibrous structure provides ESM with high surface area, reasonable adhesion and gaseous exchange ability [22]. In addition, the antibacterial activity of ESM, which is critical for wound healing, has been verified in previous works [23]. The unique properties of ESM have inspired many scientists, who have used them as biotemplate, biosensor or medicine [22,24].

Inspired by the advantages of Cu^{2+} ions, bioactive glass (BG) and ESM, we are setting out to prepare a porous Cu-BG/ESM nanocomposite film for wound healing of skin tissue. Pulsed laser deposition (PLD) has been established as a versatile technique for preparing nanosized inorganic material film on a variety of substrates [25,26]. By using this method, it is convenient to prepare uniformly nanosized films which completely keep compositional consistency with the target by adjusting the laser energy density or the pulse repetition rate [27]. Furthermore, PLD is particularly suitable for room temperature growth of films especially when the substrate is biomaterials [28]. Therefore, the aim of this study is to prepare and characterize Cu-containing BG (Cu-BG) nanocoatings on the surface of ESM film and investigate the effect of nanocomposite film on the angiogenesis of endothelial cells *in vitro* and antibacterial activity as well as the wound healing ability *in vivo*.

2. Materials and methods

2.1. Preparation and characterization of copper-containing bioactive glass-ceramics bulks

Copper (Cu)-containing bioactive silicate glass-ceramic powders with different Cu contents (0, 2 and 5 mol%) were synthesized by a sol–gel method using tetraethyl orthosilicate (TEOS, 98%), triethyl phosphate (TEP, 99.8%), $Ca(NO_3)_2{\cdot}4H_2O$ and $Cu(NO_3)_2{\cdot}3H_2O$ as primary materials (analytical grade, Sigma–Aldrich). The corresponding chemical formulation was $(Cu_{0.0x})(Ca_{0.25-0.0x})P_{0.05}Si_{0.75}$, x = 0, 2, 5. Briefly, to prepare silicate bioactive glass-ceramics containing 5 mol% Cu (designated 5Cu-BG), 25 mL of 2 M HNO_3 and 157 mL of TEOS were added into 100 mL of deionized water and hydrolyzed at room temperature for 30 min. Then 47.2 g of $Ca(NO_3)_2{\cdot}4H_2O$, 8.5 mL TEP and 12 g of $Cu(NO_3)_2{\cdot}3H_2O$ were orderly added into the as-hydrolyzed solution and stirred for 5 h until the sol became uniform and transparent. The sealed sol was aged for 48 h at 60 °C and dried at 120 °C for 24 h, followed by the ball milling treatment. The ground dry gel powders were then being calcined at 800 °C in air for 3 h at a heating rate of 2 °C min^{-1}. The calcined particles were ground and sieved to 400 meshes to obtain 5Cu-BG powders. Silicate bioactive glass without Cu (designated 0Cu-BG), with 2 mol% Cu (designated 2Cu-BG) were prepared by the same procedure by adding corresponding Cu source.

To prepare the Cu-containing glass-ceramic discs, the synthesized xCu-BG (0, 2 and 5Cu-BG) powders were uniaxially pressed by using a steel mould (Ø20 mm) at 20 MPa, and polyvinyl alcohol (6 wt.%) water solution was used as the binder. The green compacts were subsequently sintered in air at 1250 °C for 3 h, with a heating rate of 2 °C min^{-1}.

The crystal phase of the sintered samples was characterized by X-ray diffraction (XRD; Rigaku D/Max-2550V, Geigerflex, Japan). XRD was performed using CuKα radiation (λ = 1.5418 Å) at a scanning rate of 10° per min in the 2θ range 10–80°.

2.2. Preparation and characterization of Cu-BG/ESM nanocomposite films

For extracting eggshell membrane (ESM), the eggshell was firstly cleaned to remove the albumen and yolk, and then the ESM was carefully peeled off from the eggshell. The ESM was then carefully cleaned with deionized water to completely remove the residual albumen. The clean ESM was cut into small pieces (15 × 15 mm) and dried in the room temperature.

The as-sintered Cu-containing glass-ceramic discs were used for the target materials to coat ESM films. The ESM films were fixed on the sample stage in the chamber of pulsed laser deposition (PLD) instrument using adhesive tape. The Cu-containing glass-ceramic discs were ablated using focused laser fluence of 160 MJ with a pulsed repetition rate of 5 Hz at room temperature. The O_2 ambient was set with a pressure of 20 MPa and treating time of 40 min. The fabricated composite films were named as xCu-BG/ESM (0, 2 and 5Cu-BG/ESM).