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Development of polyelectrolyte chitosan-gelatin hydrogels for skin bioprinting

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Abstract

Bioprinting is an emerging technology that offers the unique ability to deposit and pattern different cells and matrix materials to fabricate three-dimensional (3-D) tissue constructs. Markedly, bioprinting demonstrated great potential for skin tissue engineering ranging from the development of 3-D skin tissue models to *in-situ* bioprinting of skin directly over the wound site. Nevertheless, one of the major challenges that impede the progress in this field is the limited choices of printable biomaterials. In this paper, we report the development of printable polyelectrolyte chitosan-gelatin hydrogel for potential applications in tissue engineering of skin. The oppositely charged functional groups from chitosan and gelatin respectively first interacted at a specific pH range to form a polyelectrolyte complex, followed by further pH-dependent crosslinking. The pH-crosslinked polyelectrolyte chitosan-gelatin hydrogel was then evaluated in terms of its rheological behavior, biocompatibility, printability and lastly material stability under physiological conditions. The polyelectrolyte chitosan-gelatin hydrogel remained in a robust gel-state over the temperature range of 20-40 ^{o}C and facilitated cellular attachment and proliferation. Furthermore, it demonstrated good printability and the multi-layered hydrogel construct was mechanically stable after subjecting it to physiological conditions for 7 days.

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Keywords: Additive manufacturing; Bioprinting; Polyelectrolyte chitosan-gelatin; Hydrogels; Skin tissue engineering

1. Introduction

Fascinating advancements have been made in the field of tissue engineering over the past three decades; it has emerged as a multi-disciplinary field that engages scientists, engineers and clinicians to strive towards the goal of creating anatomically relevant tissue constructs for replacement of malfunctioning tissues/organs [1]. Nevertheless, one of the major hurdles faced was the inability to replicate the sophisticated compartmentalization of different cell types within native tissues by simply seeding cells over the pre-formed polymeric scaffolds. Bioprinting, which can be defined as "the use of 3-D printing technology that incorporate viable living cells with biomaterials to create tissues", has promising prospects for fabrication of complex 3-D multicellular tissue constructs. The bioprinting technology not only enables the simultaneous deposition of different biomaterials and multiple cell types, it also provides flexibility in the design and fabrication of customizable patient-specific tissue-engineered constructs [2].

Despite being in its stage of infancy, bioprinting has already demonstrated great potential for fabrication of multi-layered skin [3-5], cartilage [6, 7] and liver constructs [8]. It was envisioned that fabrication of simple tissues such as skin would be a reality in the near future [9] and some recent works on bioprinting of skin constructs include fabrication of hydrogel constructs comprising different skin cells (keratinocytes and fibroblasts) [3, 4] and *in-situ* printing of skin cells and biomaterials directly over the wound site [10]. Despite the initial zeal, progress in bioprinting is severely impeded due to limited choices of printable biomaterials.Among the different biomaterials that have been

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used for skin tissue engineering applications, chitosan demonstrates promising results for wound healing applications [11-13]. Chitosan is a linear copolymer of D-glucosamine and *N*-acetyl-D-glucosamine, which is prepared by the *N*-deacetylation of insoluble chitin in the presence of alkaline solution [14]. It is one of the most abundant natural polysaccharides which can be found in crab and shrimp shells. It was shown that chitosan degrades *in vivo* via enzymatic hydrolysis by lysozymes [15] to form the by-product, glucosamine, which does not pose any antigenicity. Chitosan is a widely utilized biomaterial as it triggers hemostasis and expedites tissue regeneration. This can be attributed to its ability to induce migration of inflammatory cells and activation of fibroblasts to produce multiple cytokines that expedite wound healing [16]. Furthermore, chitosan-based biomaterials are shown to exhibit antimicrobial properties which is beneficial for skin tissue engineering applications [17]. Chitosan powders are soluble at low pH and the amine groups in chitosan are protonated at pH lower than 6 to confer the poly-cationic behavior to chitosan. As the pH increases above 6, the amine groups become deprotonated and chitosan becomes insoluble. This soluble-insoluble transition occurs at its pKa value around pH 6 – 6.5, which is dependent on degree of *N*-deacetylation and molecular weight [18]. Previous works on printing of chitosan hydrogel include a dual-approach dispensing method [19] and direct deposition of pre-crosslinked chitosan [20]. In the first approach, acellular chitosan scaffolds were fabricated by simultaneous deposition of coagulation medium (NaOH) directly above the chitosan filaments to initiate the crosslinking process. In the latter approach, it was reported that pre-crosslinked chitosan was too viscous to be used in the bioprinter. Hence, further modifications are required to increase the printability of chitosan-based hydrogels.

One of the strategies that could be implemented is to induce the formation of a polyelectrolyte complex between positively charged chitosan and negatively-charged polymer to improve its printability. Gelatin exhibits negative charges when the pH of medium is above its isoelectric point (pH_{iso} = 4.7) [21] and it is commonly used for biomedical applications due to its significantly low cost, simple processability, good biocompatibility and biodegradability under physiological conditions. The positively charged ammonium ions from chitosan react with carboxylate groups from the ampholytic gelatin to form a polyelectrolyte complex. Notably, the use of polyelectrolyte chitosan-gelatin scaffolds/films [22-25] demonstrated great potential for skin tissue engineering applications. The preparation of polyelectrolyte chitosan-gelatin scaffolds is usually done via freeze-drying [22, 24, 25] or solvent-casting approaches [22, 23] and to the best of our knowledge, bioprinting of polyelectrolyte chitosan-gelatin hydrogel has yet to be reported.

In this paper, the development of polyelectrolyte chitosan-gelatin (PCG) hydrogel for potential bioprinting of tissue-engineered skin construct is presented. The crosslinking mechanism was first discussed, followed by characterization of PCG hydrogels in terms of rheological behaviour at varying shear rates and temperatures and biocompatibility, lastly we evaluated its printability at different printing pressures and material stability under physiological conditions. These outcomes will provide valuable insights into development of printable hydrogels for bioprinting of 3-D tissue constructs.

2. Materials and methods

2.1. Materials

Gelatin (porcine skin, Type A) and chitosan (low molecular weight) powders were obtained from (Sigma Aldrich, Singapore). Other reagents like acetic acid, sodium hydroxide (NaOH) and phosphate buffered saline (PBS) solution (pH 7.4 at 0.01 M) were sterile-filtered before use.

2.2. Cells

Neonatal human foreskin fibroblasts (HFF-1 from ATCC® SCRC-1041™) were used in this study. The cell line was cultured in a HERAcell 150i cell incubator (Thermo Scientific) at 37 °C in 5% CO_2 using ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% fetal bovine serum (HyClone™ from GE Healthcare). Culture media was changed every 3 days and the cells were routinely passaged in tissue culture flasks (Passage 5-6) to ensure that the cells do not reach senescence stage. The adherent HFF-1 cells were harvested using 0.25% trypsin/ ethylenediaminetetraacetic acid (EDTA) (Invitrogen) at 90% confluency and they were maintained as cell suspensions at desired cell density before use.

2.3. Preparation and crosslinking of polyelectrolyte chitosan-gelatin (PCG) hydrogels

The polyelectrolyte chitosan-gelatin hydrogel (PCG) was prepared as illustrated in Fig. 1.

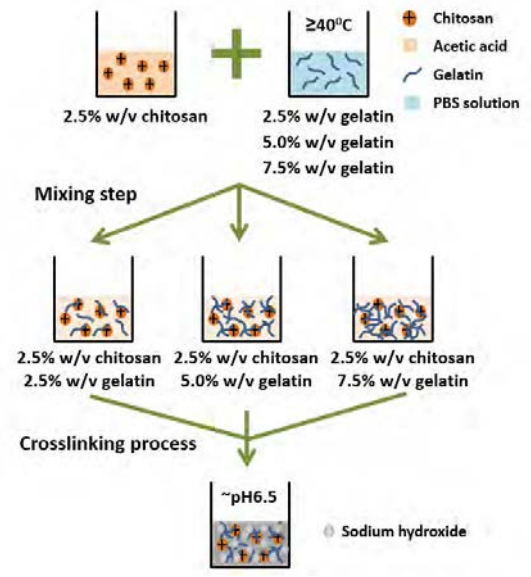

Fig. 1. Preparation and crosslinking of polyelectrolyte chitosan-gelatin hydrogels