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Antibacterial and wound healing analysis of gelatin/zeolite scaffolds

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ABSTRACT

In this article, gelatin/copper activated faujasites (CAF) composite scaffolds were fabricated by lyophilisation technique for promoting partial thickness wound healing. The optimised scaffold with 0.5% (w/w) of CAF, G (0.5%), demonstrated pore size in the range of 10–350 μm. Agar disc diffusion tests verified the antibacterial role of G (0.5%) and further supported that bacterial lysis was due to copper released from the core of CAF embedded in the gelatin matrix. The change in morphology of bacteria as a function of CAF content in gelatin scaffold was studied using SEM analysis. The confocal images revealed the increase in mortality rate of bacteria with increase in concentration of incorporated CAF in gelatin matrix. Proficient oxygen supply to needy cells is a continuing hurdle faced by tissue engineering scaffolds. The dissolved oxygen measurements revealed that CAF embedded in the scaffold were capable of increasing oxygen supply and thereby promote cell proliferation. Also, G (0.5%) exhibited highest cell viability on NIH 3T3 fibroblast cells which was mainly attributed to the highly porous architecture and its ability to enhance oxygen supply to cells. *In vivo* studies conducted on Sprague Dawley rats revealed the ability of G (0.5%) to promote skin regeneration in 20 days. Thus, the obtained data suggest that G (0.5%) is an ideal candidate for wound healing applications.

1. Introduction

Wound infections are caused due to invasion of injured tissues by microorganisms, that trigger body's immune system, induce inflammation, tissue damage and impede the healing process [1]. Most cases of infected wounds arise due to bacteria, originating either from the skin or external environment [2]. Skin contains normal flora of bacteria which are harmless. When it is subjected to injury, this protective barrier will be disrupted and the normal flora will then colonise the wounded site, inducing inflammation and tissue damage, thereby causing serious local and systemic complications [3]. One of the approaches for treating bacterial infected wounds is the use of biocompatible scaffolds incorporated with antibacterial agents [4]. Several polymers are used for the fabrication of such scaffolds including pectin [5], chitin [6], chitosan [7], alginate [8], collagen [9], gelatin [10], keratin [11], polyurethane [12], polycaprolactone [13], polyacrylonitrile [14], polyethylene [15] and silicon rubber [16]. Among these, gelatin is chosen as a suitable matrix due to its natural abundance, biocompatibility, biodegradability and non-immunogenicity [17]. It is a protein obtained by partial hydrolysis of collagen. It melts into liquid when heated and gets solidified when cooled [18]. Literature reports the wide use of gelatin in preparing scaffolds with antibacterial properties like electrospun gelatin fibre mats containing silver nanoparticles [19], keratin-gelatin composites [20], chitosan-gelatin/nanohydroxyapatite scaffold [21], electrospun chitosan/gelatin nanofibers containing silver nanoparticles [22], gelatin/hydroxyapatite foams [23], nanosilver/gelatin/carboxymethyl chitosan hydrogel [24], *etc.* We prepared scaffolds with antibacterial properties using gelatin as the polymer matrix.

Recently, inorganic minerals like clays and zeolites containing metals have achieved great significance compared to conventional antibacterial agents. The incorporation of metallic ions within silicate framework allowed their controlled release and prevented concentration dependent toxicity [25]. Among the different inorganic materials, copper containing minerals are prominent as

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copper ions can stimulate the proliferation of endothelial cells, promote wound healing and exhibit broad spectrum of antibacterial properties [26]. In the present study, copper exchanged faujasite (CAF) were chosen as suitable antibacterial agent. They are mineral groups of zeolite family with excellent cation exchange ability. They consist of sodalite cages connected through hexagonal prisms, forming a large central cavity of diameter ~12 Å [27]. The inner cavity is linked to 12 membered rings of diameter ~7.4 Å [28]. Seifu et al., has reported that fluorinated zeolites embedded within polymeric scaffolds can enhance oxygen delivery to cells [29]. Besides the antibacterial property, our aim was to evaluate whether CAF can provide sufficient oxygen supply to the growing skin cells at wound site.

In this work, gelatin/CAF composite scaffolds were fabricated by lyophilisation and parameters like dissolved oxygen concentration, cumulative copper release, antibacterial properties and cytocompatibility were investigated. *In vivo* studies on Sprague Dawley rats were conducted to explore the wound healing ability of prepared composite scaffold.

2. Materials and methods

2.1. Materials

Type B gelatin, glycerol (purity-99%), formaldehyde (36.5–38%), glutaraldehyde (25%) and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (France). Zeolite powder (CAF) was a kind gift from IRMA (France). Mueller Hinton agar, Luria broth (LB), sodium chloride, absolute ethanol (100%), Dulbecco's modified Eagle Medium (DMEM), MTS reagent and sterile antibiotic discs (diameter-4 mm) were acquired from Sigma–Aldrich (France). 25% (v/v) ammonia, xylene and hydrochloric acid were obtained from Merck (Damstadt, Germany). The staining reagents like Harris haematoxylin and eosin were procured from Leica Biosystems Richmund Inc. (Germany). Ketamine hydrochloride and xylazine hydrochloride were bought from Troy Laboratories, Australia. *Escherichia coli* (ATCC 25922) were obtained from microbiology department of IUT, South Brittany (Pontivy, France). NIH 3T3 fibroblast cells were procured from ATCC cell biology collection.

2.2. Preparation of gelatin/CAF composite scaffold

Gelatin/CAF porous scaffolds were synthesised by lyophilisation as reported in our previous work [30]. A 2% (w/v) of gelatin solution in water (endotoxin-free) was mixed with 5% (v/v) of glycerol (plasticiser). 2.5% (w/v) of CAF suspension was sonicated for 30 min, added to gelatin solution and immediately cross-linked using 10 μL of formaldehyde (0.38%). The solution was well mixed and poured onto petri plates. They were pre-frozen at −20 °C and lyophilised in Christ Alpha 1–2 LD Plus Freeze Dryer. G (0%) was the control scaffold synthesised without CAF. G (0.5%), G (2.5%) and G (5%) were gelatin/CAF composite scaffolds prepared with 0.5%, 2.5% and 5% (w/v) of CAF. GN (0.5%) was the composite scaffold with 0.5% (w/v) of faujasite without any copper, which was prepared to confirm whether antibacterial activity was induced by copper released from faujasite core.

2.3. SEM analysis

The morphology of scaffolds was investigated by scanning electron microscope (SEM) (JEOL-2100, Kyoto, Japan). Thin sections of scaffold were cut using razor blade. They were gold sputtered by Polaron sputtering apparatus and examined under SEM.

2.4. Dissolved oxygen measurements

Hach luminescent dissolved oxygen electrode (LDO 101-01), equipped with 1 or 3 m cables was used to measure dissolved oxygen (DO) in CAF suspension. A light emitting diode transmitted incident light which was sufficient to excite the luminophore substrate. In the presence of DO, luminescence quenching took place. The dynamic lifetime of the excited luminophore was calculated and equated to DO concentration. CAF particles were suspended in deionised water at concentrations of 0.25%, 0.5%, 2.5%, 5% (w/v), respectively and continuously stirred at 37 °C. Deionised water was used as the control. The LDO probe was calibrated before use. The probe was partially submerged in CAF suspension and the reading was recorded. Triplicates of samples were done and average of the results was plotted.

2.5. Release characteristics of copper loaded in scaffolds

0.4 g of the prepared scaffold was weighed and placed on petri plates containing saline water [0.9% (w/v) of sodium chloride]. The plates were rotated on an orbital shaker at 150 rpm for 1 h at 37 °C. Samples were washed and saline water was replaced by fresh solution. The suspensions were centrifuged at 5000 × *g* for 5 min. The supernatants were collected and the concentration of Cu^{2+} ion was measured by Varian Spectra AA 600 atomic absorption spectroscopy (AAS) (Burladingen, Germany). Cumulative copper release from samples for six days were plotted and compared. All measurements were done in triplicates.

2.6. Antibacterial studies

In vitro antibacterial activity of the prepared scaffolds was quantitatively and qualitatively evaluated using disc diffusion method and live/dead assay technique. According to the recommended standards of National Committee for Clinical Laboratory Standards (NCCLS, 2005), agar disc diffusion method was carried out against *E. coli* (ATCC 25922), a gram negative bacterial strain. The samples analysed included the optimised scaffold, G (0.5%) and control scaffold without CAF, G (0%). Along with these samples, a disc of GN (0.5%) was also tested to check whether copper present in the faujasite was actually responsible for bacterial inhibition.

The suspensions of bacteria used for inoculation were prepared by adjusting fresh cultures at Mac Farland 0.5 density (approximately 10^8 CFU/ml). The inoculum was streaked on Mueller Hinton agar medium and then air dried at room temperature. The prepared scaffolds were cut in the form of small discs of diameter 4 mm and were placed on agar medium. Afterwards, the plates were incubated at 37 °C for 24 h. All tests were carried out in triplicates. Sterile paper discs about 4 mm in diameter, impregnated with Penicillin G, Pg (10 μg/disc) and Bactopin®, Bi (μL/disc) were used as positive controls. The antibacterial activity was calculated as the mean diameter of inhibition zones (mm) developed around the samples. Photographs were taken to further support these results.

In the live/dead assay technique, the samples were cut in the form of discs, diameter 6 mm and placed in the wells of microtiter plates containing 225 μL of LB broth and 25 μL of *E. coli* culture. The plates were kept in a shaking incubator at 37 °C. After 24 h, 100 μL of the sample was eluted and seeded onto agar medium. They were stained with DMAO and EthD-III and observed under confocal microscopy (Zeiss LSM 510, Germany), to determine the number of live or dead bacteria in the well.

2.7. Susceptibility tests

E. coli was cultured in LB broth at 37 °C for 24 h and was then diluted in fresh broth to reach the required optical density. A 2.5%