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Improving the osteointegration of Ti6Al4V by zeolite MFI coating

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ABSTRACT

Osteointegration is crucial for success in orthopedic implantation. In recent decades, there have been numerous studies aiming to modify titanium alloys, which are the most widely used materials in orthopedics. Zeolites are solid aluminosilicates whose application in the biomedical field has recently been explored. To this end, MFI zeolites have been developed as titanium alloy coatings and tested *in vitro*. Nevertheless, the effect of the MFI coating of biomaterials *in vivo* has not yet been addressed. The aim of the present work is to evaluate the effects of MFI-coated Ti6Al4V implants *in vitro* and *in vivo*. After surface modification, the surface was investigated using field emission scanning electron microscopy (FE-SEM) and energy dispersive spectroscopy (EDS). No difference was observed regarding the proliferation of MC3T3-E1 cells on the Ti6Al4V (Ti) and MFI-coated Ti6Al4V (M–Ti) ($p > 0.05$). However, the attachment of MC3T3-E1 cells was found to be better in the M–Ti group. Additionally, ALP staining and activity assays and quantitative real-time RT-PCR indicated that MC3T3-E1 cells grown on the M–Ti displayed high levels of osteogenic differentiation markers. Moreover, Van-Gieson staining of histological sections demonstrated that the MFI coating on Ti6Al4V scaffolds significantly enhanced osteointegration and promoted bone regeneration after implantation in rabbit femoral condylar defects at 4 and 12 weeks. Therefore, this study provides a method for modifying Ti6Al4V to achieve improved osteointegration and osteogenesis.

1. Introduction

Titanium alloys are the most prevalent material used in orthopedic clinical practice because of their good biocompatibility and mechanical properties. However, there is still concern about the integration between titanium alloys and bone tissue (osteointegration) considering the inert nature of titanium alloys. Osteointegration is a crucial factor that affects the success of orthopedic implants. A typical example is the early loosening in hip or knee prostheses, which is a serious complication in total joint replacement [1]. If the osteointegration of an implant is unstable, the implant will experience micromotions that will activate the osteoclasts, leading to further implant loosening and finally implant failure [2]. To overcome this problem, titanium alloy surface modifications that focus on the surface morphology [3–6] and surface chemistry [7–10] are continuously being explored with the aim of achieving a stable integration between the bone and implant.

Zeolites are solid aluminosilicates with a uniform microporous structure and have been generally applied to catalysis, adsorption and ion exchange for commercial applications. Researchers have also widely investigated other potential applications for zeolites [11], which have shown great potential for biomedical applications [12]. Zeolites have been used as MRI contrast agents [13], antimicrobial coatings [14,15], drug delivery systems [16–19], and tissue engineering agents [20]. Recently, Seifu et al. demonstrated that fluorinated zeolites as novel oxygen vectors embedded in three-dimensional polyurethane scaffolds are capable of providing a sufficient oxygen supply to cells for tissue engineering [21]. Pellegrino et al. confirmed that zeolites can limit the negative effects caused by oxidative stress by adsorbing the reactive oxygen species [22]. Ninan et al. demonstrated that gelatin/hyaluronic acid (HA)/faujasite porous scaffolds can enhance wound healing by accelerating re-epithelization and collagen deposition [23].

Abbreviations: MFI, three letter code for zeolite nomenclature given by the International Zeolite Association; FE-SEM, field emission scanning electron microscopy; EDS, energy dispersive spectroscopy; Ti, Ti6Al4V; M-Ti, MFI coated Ti6Al4V.

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Among the different zeolites, Bedi et al. reported that the MFI zeolite can be synthesized on a titanium alloy surface as a coating to improve biocompatibility, corrosion resistance, osteoconductivity and osteoinductivity *in vitro* [24–26]. These studies showed that the MFI coating is a promising surface modification for titanium alloys. To the best of our knowledge, however, there have been no studies on the osteointegration of MFI coatings *in vivo*, which is important for orthopedic implants.

In this study, we investigated whether MFI-coated Ti6Al4V (M–Ti) exhibits greater biocompatibility and osteointegration compared with bare Ti6Al4V (Ti) substrates *in vitro* and *in vivo*. For this purpose, we created a MFI zeolite coating on a Ti6Al4V surface. The surface morphology, apatite formation ability, cell proliferation, surface cell morphology and cell osteogenic differentiation were investigated *in vitro*. We established a rabbit femur implant model to determine whether the MFI zeolite coating could improve the osteointegration and osteogenesis peri-implant.

2. Materials and methods

2.1. Substrate and MFI coating preparation

Medical Ti6Al4V (Shenyang National Laboratory for Materials Science) was used as the substrate. Disk-shaped samples (Ø12 mm × H2 mm) were used for *in vitro* assays, and cylindrical samples (Ø3 mm × L10 mm) were used for *in vivo* experiments. All of the samples were abraded with 2000-grit SiC paper and then cleaned with acetone (15 min), ethanol (15 min) and deionized water (15 min) in an ultrasonic bath.

The zeolite precursor solution was prepared by mixing tetrapropylammonium oxide (TPAOH, 50%), tetraethylorthosilicate (TEOS), and deionized water with a molar composition of TEOS: 0.2TPAOH:19.2H_2O, which was then heated for 4 h in an autoclave at 398 K. The samples were dipped into the obtained precursor solution for 2 h followed by air blowing for a few seconds and were subsequently dried in air for 12 h at 313 K.

A low-concentration synthesis solution was prepared by mixing TEOS, TPAOH and deionized water with a molar composition of TEOS: TPAOH: H2O = 1: 0.023: 178. In most cases, the samples were added to the above solution. The mixture was then transferred to a Teflon-lined stainless steel autoclave with a capacity of 500 ml. The autoclave was sealed and heated at 433 K for 0–48 h. After the hydrothermal treatments, the samples were washed thoroughly with hot water, dried at 373 K in air, and subsequently calcined at 823 K for 6 h to remove the organics from the zeolite framework.

2.2. Surface characterization

The morphology of the samples (Ti and M–Ti) was inspected by field emission scanning electron microscopy (FE-SEM, S-4800, HITACHI, Japan). An energy dispersive spectroscopic (EDS) analysis was performed to identify the chemical composition of the samples using EMAX. Prior to detection, the samples were sputtered with a thin layer of platinum (Pt) using a typical sputtering instrument to improve the surface conductivity.

2.3. Simulated body fluid (SBF) immersion experiments

The simulated body fluid (SBF) solution was prepared according to a previously described protocol [27], and the composition is presented in Table 1. The specimens were immersed in 15 ml of fresh SBF and incubated at 37 °C.The samples were removed from the water bath after 2 and 4 days, washed with deionized water twice and dried overnight in an air oven at 37 °C. FE-SEM (S-4800, HITACHI, Japan) was used to observe the apatite formation on the surface of the samples. The samples were sputtered with a thin layer of Pt to improve the surface conductivity.

Table 1
The ion concentration of SBF.

Ion	Ion concentrations (mM)
Na^+	142.0
K^+	5.0
Mg^{2+}	1.5
Ca^{2+}	2.5
Cl^-	147.8
HCO_3^-	4.2
HPO_4^{2-}	1.0
SO_4^{2-}	0.5
pH	7.40

2.4. Cell culture

Mouse preosteoblast cells (MC3T3-E1) were cultured in α-MEM medium supplemental with 10% fetal bovine serum (FBS, Gibco), 100U ml^{-1} penicillin and 100ug ml^{-1} in a humidified incubator at 37 °C with 5% CO_2. The medium was changed every 2 days.

2.5. Cell proliferation and morphology

MC3T3-E1 cells were seeded on the samples (Ti and M–Ti) at a density of 2 × 10^4/well in 24-well culture plates and cultured for 1, 4 and 7 days to evaluate the cell proliferation using Cell Count Kit-8 (CCK-8, Dojindo, Japan). Briefly, at each time point, the samples were transferred to new 24-well culture plates. Then, CCK-8 solution with a 10% volume of the medium was added to the wells, and the samples were incubated at 37 °C for 1 h. Next, 100 μl of the reaction solution was transferred into a new 96-well plate, and the optical density was measured at 450 nm using a microplate reader.

After incubation for 2 days, the samples were washed with PBS and fixed in 2.5% v/v glutaraldehyde at 4 °C overnight. The samples were then dehydrated through an ethanol series, critical-point dried and sputtered with Pt. The samples were observed using FE-SEM (S-4800, HITACHI, Japan).

2.6. Alkaline phosphatase (ALP) staining and activity assay

MC3T3-E1 cells were seeded on Ti and M–Ti samples at a density of 2 × 10^4/well in 24-well culture plates. After cell adherence, the cells were cultured using osteogenic medium (complete medium supplemented with 50 mg/L ascorbic acid, 10^{-8} M dexamethasone and 10 mM β-glycerol phosphate). The medium was changed every 2 days. After osteogenic medium treatment for 4 days and 7 days, the samples were stained with a BCIP/NBT alkaline phosphatase color development kit (Beyotime) according to the manufacturer's instructions. Images were captured with a zoom-stereo microscope. To determine the alkaline phosphatase (ALP) activity, the samples were washed twice with PBS and disrupted by freezing at −80 °C for 20 min with PBS containing 0.1% Triton X-100. Enzyme activity was detected using a commercially available kit (Beyotime), according to the manufacturer's instructions. The protein content was determined with a BCA kit (Thermo Scientific), and the alkaline phosphatase activity was expressed in arbitrary units per microgram of protein content.

2.7. Quantitative real-time RT-PCR

After osteogenic medium treatment for 7 and 14 days, the expressions of runt-related transcription factor-2 (Runx2), osteocalcin

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