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Nonvolatile buffer coating of titanium to prevent its biological aging and for drug delivery

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ABSTRACT

The osseointegration capability of titanium decreases over time. This phenomenon, defined as biological aging of titanium, is associated with the disappearance of hydrophilicity and the progressive accumulation of hydrocarbons on titanium surfaces. The objective of this study was to examine whether coating of titanium surfaces with 4-(2-Hydroxylethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, a nonvolatile zwitterionic chemical buffering agent, could prevent the time-dependent degradation of the bioactivity of titanium. Commercially pure titanium samples, prepared as disks and cylinders, were acid-etched with H_2SO_4. A third of the samples were used for experiments immediately after processing (new surfaces), while another third were stored under dark ambient conditions for 3 months (3-month-old surfaces). The remaining third were coated with HEPES after acid-etching and were stored for 3 months (HEPES-coated 3-month-old surfaces). The 3-month-old surfaces were hydrophobic, while new and HEPES-coated 3-month-old surfaces were superhydrophilic. Protein adsorption and the number of osteoblasts attached during an initial culture period were substantially lower for 3-month-old surfaces than for new and HEPES-coated 3-month-old surfaces. Alkaline phosphatase activity and calcium deposition in osteoblast cultures were reduced by more than 50% on 3-month-old surfaces compared to new surfaces, whereas such degradation was not found on HEPES-coated 3-month-old surfaces. The strength of *in vivo* bone-implant integration for 3-month-old implants, evaluated by the push-in test, was 60% lower than that for new implants. The push-in value of HEPES-coated 3-month-old implants was equivalent to that of new implants. Coating titanium surfaces with HEPES containing an antioxidant amino acid derivative, *N*-acetyl cysteine (NAC), further enhanced osteoblast attachment to the surfaces, along with the increase level of intracellular glutathione reserves as a result of cellular uptake of NAC. These results suggest that HEPES coating of titanium surfaces maintained their superhydrophilicity for at least 3 months and resulted in a continuous retention of bioactivity and osteoconductivity similar to freshly prepared surfaces. This coating technology may be useful for preventing biological aging of titanium and delivering biological molecules for synergistic enhancement of bone-titanium integration.

1. Introduction

Recent reports on time-dependent degradation of the bioactivity of titanium, defined as biological aging of titanium, led to the first discovery of time-related changes in biological potential of biomaterials, and provided significant clinical impacts in the field of osseous implant therapy [1,2]. *In vitro* bioactivity, such as those resulting in the attraction and proliferation of osteogenic cells, is reduced by 40–80% on 4-week-old titanium surfaces compared to newly prepared surfaces [1,2]. In terms of *in vivo* bone integration capability, at an early healing stage in an animal model, the strength of implant fixation and the percentage of bone coverage around implants for 4-week-old surfaces were reduced to less than 50% of those for new surfaces [1]. It should be noted that the biomechanical and histological indices of these aged implants showed lower values not only in the early stage of healing but also in the late stage. This indicated that the aging of titanium implants resulted in not only delayed but also a compromised level of bone-implant integration. In fact, in the late stage of healing, bone-implant contact remained less than 60% for 4-week-old implants,

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while it reached over 90% for new implants [1]. Commercially available orthopedic and dental implants are sold as storable medical devices. These products invariably age during their inventory and distribution, as well as storage before use. It is unlikely that these products are used within 4 weeks after manufacturing. Therefore, the pivotal implication is that the biological capabilities of commercially available implants may have been unpredictably and unavoidably compromised.

In addition, the scientific significance of biological aging of titanium is crucial for exploring future advancements in this field. Titanium forms a passive, protective oxide layer on its surface once exposed to the atmosphere. Because of the chemical stability of this oxidized surface (primarily TiO_2), titanium had been categorized into a group of bioinert materials, which means that they do not interact with host cells/tissues. Accordingly, the biological capability of titanium surfaces had been accepted as innate and invariable over time. The necessity for correcting these assumptions was firmly suggested by the discovery of biological aging of titanium. A series of reports have indicated that freshly prepared titanium surfaces were bioactive, whereas the commonly available titanium surfaces, including commercial products, that have been exposed to the atmosphere long enough are bioinert [1–3]. These new findings were expected to alter the long-held assumption of titanium that titanium is a bioinert material. Titanium should be defined as bioactive under the condition that the surface is new. Moreover, the demonstration of substantial differences in bioactivity of titanium surface at different stages of their aging implied that standardizing the age of titanium samples is required in future research designs in the filed or otherwise a careful interpretation is required for the results. Biological aging is seen to occur in all surface topographies of titanium that were tested, ranging from microtopographical features (e.g., acid-etched surfaces) to a relatively smooth morphology (e.g., machined surfaces) [1–3]. Currently, there are no effective measures to prevent this phenomenon.

Although the exact mechanisms underlying the biological aging of titanium have not been fully elucidated, this phenomenon is associated with disappearance of hydrophilicity and the progressive contamination of hydrocarbons on titanium surfaces over time [1,2]. For example, naturally induced superhydrophilicity of freshly prepared acid-etched titanium surfaces only lasts for a week [1]. The atomic ratio of carbon, which is less than 20% on a fresh titanium surface, increases to approximately 60% after 4 weeks of exposure to the atmosphere [1,2]. We hypothesized that coating a newly prepared titanium surface with a nonvolatile liquid could prevent or reduce the time-dependent degradation in its bioactivity by preserving its hydrophilicity and avoiding excessive chemical contamination from the atmosphere. HEPES s is a nonvolatile zwitterionic buffering agent with a molecular formula of $C_8H_{18}N_2O_4S$. It is a stable nonreactive agent that is widely used for cell culture reagents and for storing biological molecules and cells [4–7].

An issue here is that HEPES is a highly viscous liquid and can not be thinly coated onto material surfaces under normal conditions. Successful bone-titanium integration requires an intimate juxtaposition of titanium surfaces and newly formed bone. A significantly thick intervention of a nonbiological layer, which might prevent osteogenic cells from reaching the titanium surfaces, was deemed to be unsuitable. Our experimental strategy was to utilize the superhydrophilic nature of newly prepared titanium surfaces. Here, we demonstrate that HEPES buffer can spread and form a thin layer exclusively on freshly prepared titanium surfaces, but not on old titanium surfaces.

The objective of this study was to determine if a thin layer of coating with HEPES buffer on freshly prepared titanium surfaces could prevent the biological aging of titanium. The bioactivity and osseointegration capability of titanium samples after 3 months of storage (3-month-old titanium surfaces), with and without HEPES coating, as well as those of freshly processed titanium samples (new titanium surface) were compared. We also tested the potential of HEPES coating as a carrier vehicle for biological molecules to enhance the osteogenic environment around titanium surfaces. *N*-acetyl cysteine (NAC) is an amino acid derivative and known as an strong antioxidant [8]. NAC is easily deacetylated into cysteine, which is an important precursor of glutathione [8]. The glutathione-mediated redox cycle is the most important removal system for exogenous and endogenous reactive oxygen species (ROS) [9,10]. Therefore, cellular uptake of NAC results in the increased level of antioxidant defense capacity [11]. Also, the NAC-mediated direct scavenging effect for polymerization-induced reactive oxygen species has been demonstrated in various biomaterials [12–14]. In particular, a recent study demonstrated that NAC-incorporated poly(methyl methacrylate) (PMMA) resin-based bone cement showed significant improvement in its osteoconductivity [11]. Besides these exogenous oxidants, cells are subjected to substantial degrees of oxidative stress by mechanical and chemical stimuli during invasive surgery *in vivo* and even during various steps in cell culture procedures. To test the feasibility of titanium surface-mediated drug delivery, we examined the effect of HEPES/NAC coating to further enhance the osteogenic environment around titanium surfaces.

2. Materials and methods

2.1. Preparation of HEPES-coated titanium samples

Commercially pure titanium samples prepared as disks (20 mm in diameter, 1 mm in thickness) and cylinders (1 mm in diameter, 2 mm in length) were acid-etched with H_2SO_4 using a previously established protocol [15–17]. The surface morphology of the acid-etched surface was examined by scanning electron microscopy (SEM) (XL30, Philips, Eindhoven, Netherlands). A third of the samples were used for experiments immediately after processing (new surface), while another third was stored under dark ambient conditions for 3 months (3-month-old surface). The remaining third were coated with HEPES buffer immediately after acid etching, and were stored for 3 months (HEPES-coated 3-month-old surface). HEPES buffer was prepared as a filter-sterilized 1 M stock solution at pH 7.2. Ten μl and 0.3 μl of HEPES were used to coat titanium disks and cylinders, respectively. To indirectly measure the thickness of HEPES coated on titanium surfaces, 3 μl of HEPES was dropped onto new titanium disks. The thickness of HEPES was calculated by dividing the volume of HEPES drop (3 μl) by the area of spread. Hydrophilicity of titanium surfaces was assessed by measuring the contact angle of a 10 μl ddH_2O drop. To test the potential use of HEPES coating as a carrier vehicle, titanium disks coated with NAC were also prepared. NAC (Sigma-Aldrich, St. Louis, MO) was prepared as a 0.5 mol/L stock solution in HEPES buffer whose pH was adjusted to 7.2. Ten μl of HEPES/NAC was used to coat titanium disks, and the disks were stored for 3 months (HEPES/NAC-coated 3-month-old surface). Electrostatic potential of HEPES-coated titanium surfaces was measured using a coulomb meter (NK-1001, Kasuga Denki, Inc. Tokyo, Japan).

2.2. Protein adsorption

Bovine serum albumin, fraction V (Pierce Biotechnology, Inc., Rockford, IL) was used as a model protein. A previously established bicinchoninic acid (BCA)-based colorimetric detection was used [3,15]. The BCA reagent is used to detect Cu^{+1}, which is formed when Cu^{+2} is reduced by proteins in an alkaline environment [18–20]. Three hundred μl of a protein solution (1 mg/ml protein/saline) was spread over a titanium disk using a pipette. After 3 or 24 h of incubation under sterile humidified conditions at 37 °C, the solution containing nonadherent proteins was removed and mixed with microbicinchoninic acid (Pierce Biotechnology) at 37 °C for 60 min. The amount of the removed protein was quantified using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT) at 562 nm. A color response curve that produced a consistent standard of proof (correlation coefficient = 1.00) was used for protein estimation. The total amount of protein initially applied was also quantified with this method. The proportion of protein adsorption was calculated as the percentage of proteins adsorbed to titanium surfaces relative to the total amount of proteins initially applied.