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Influence of mixed organosilane coatings with variable RGD surface densities on the adhesion and proliferation of human osteosarcoma Saos-2 cells to magnesium alloy AZ31

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ABSTRACT

In the last decade, the use of magnesium and its alloys as biodegradable implant materials has become increasingly accepted. However, surface modification of these materials to control the degradation rate in the early stages of healing and improve their biocompatibility is crucial to the successful implementation of magnesium alloy implants in medicine. Cell adhesion and proliferation at the implant surface is a vital factor for successful integration of a biomaterial within the body. Cells accomplish this task by binding to ligands such as the arginine-glycine-aspartic acid peptide sequence (RGD) commonly found on adhesive proteins present in the extracellular matrix. In this paper, we report a biomimetic surface modification strategy involving deposition of a mixed organosilane layer on Mg AZ31 followed by covalent immobilization of RGD peptides through a heterobifunctional cross-linker molecule. Our results indicate that with optimized deposition conditions uniform organosilane coatings were successfully deposited on the Mg AZ31 substrate. Furthermore, we have demonstrated that the surface density of immobilized RGD can be varied by depositing organosilane layers from solutions containing two different organosilanes in specified ratios. Increases in cell adhesion and cell proliferation were observed on the surface modified substrates.

1. Introduction

In the last decade, the use of magnesium and its alloys as a biodegradable orthopedic implant material has become increasingly accepted. They are considered an ideal candidate for this purpose due to their desirable mechanical properties [1–3] and the important role of magnesium in the body [4–7]. Several recent studies on the *in vitro* and *in vivo* performance of these materials indicate that in addition to being non-toxic, magnesium based implants exhibit good biocompatibility, osteoconductivity and osseointegrative properties [8–14]. However, the major drawback to the use of magnesium materials as orthopedic implants is their poor corrosion resistance in chloride containing environments leading to fast degradation along with the production of large volumes of hydrogen gas [10]. This process has a negative impact on the mechanical integrity of the implant leading to loss of implant stability before the impaired bone tissue has sufficiently healed and poor osteointegration of the magnesium implant [9,10,13]. In order to control the corrosion rate and extend the functional lifetime of magnesium implants in the human body, appropriate coatings with excellent adhesion as well as the ability to enhance the biocompatibility and slow the corrosion rate are crucial [15].

Organosilane coatings provide a unique opportunity to provide both the necessary corrosion resistance and the ability to biofunctionalize the magnesium implant surface. In particular, trialkoxysilanes have been commonly used as coupling agents to produce covalent bonds between organic and inorganic materials [16–19]. The general structure of a trialkoxysilane is $RSi(OR')_3$, where R′ is typically an alkyl group such as $-CH_3$ or $-CH_2CH_3$ and R is an organofunctional substituent that typically has a hydrocarbon bridge, $(-CH_2-)_n$, linked to the central silicon atom through an Si–C bond and terminated with a specific functional group [16]. An appropriate choice of functional group allows covalent immobilization of biomolecules of interest [16,17,19,20]. Under appropriate reaction conditions, a series of hydrolysis and condensation

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reactions leads to a stable multilayer coating of cross-linked polysiloxane molecules that are also covalently bonded to the metal surface. Based on previous research, a thiol terminated organosilane, (3-Mercaptopropyl)trimethoxysilane (MPTS) can act as a water barrier coating to improve corrosion resistance [16,17,21,22]. In addition, the thiol functional group of MPTS provides reactive sites for the covalent immobilization of functional biomolecules [23].

For orthopedic implants, it is essential to establish a surface that is osteoconductive and osteoinductive and does not lead to fibrous tissue formation [24]. For most types of adherent cells, cell adhesion generally depends on the interaction between ligands in the extracellular matrix (ECM) and the corresponding integrin receptors on the cell surface; this process is essential for cell survival of non-malignant cells [25,26]. The arginylglycylaspartic acid (RGD) amino acid sequence has been found in a number of extracellular molecules, and this specific tri-peptide has been shown to play a key role in cell attachment [27–29]. Thus an appropriate surface modification that immobilizes RGD containing peptides to a substrate should improve cell-surface adhesion onto biomaterials [30–34]. Furthermore, Hu et al. also demonstrated that the presence of the RGD sequence can enhance the differentiation of osteogenic cells [35]. Although immobilizing the RGD-containing sequence to a substrate surface can efficiently mimic the natural ECM environment and significantly enhance cell adhesion to substrate surfaces there are still some differences in comparison to the natural ECM. For example, the RGD surface density, orientation and distribution have been shown to have an effect on cell adhesion, cell spreading and cell proliferation [36–39].

The main objective of this study was to develop a surface modification strategy for Mg AZ31 that gives controlled degradation rates and improved cell/surface interactions. Organosilane coatings with different thiol surface densities were prepared by varying the ratio of two different organosilanes, MPTS and tetraethoxysilane (TEOS), in the coating solution. A heterobifunctional crosslinker was used to covalently immobilize the RGD peptide to the thiol functional groups, resulting in coatings with variable RGD surface densities. The proposed surface modification procedure is illustrated in Fig. 1. The influence of MPTS/TEOS ratio on the surface chemistry, corrosion resistance and biocompatibility of the surface modified Mg AZ31 was evaluated.

2. Experimental details

2.1. Materials

Mg AZ31 foil (1 mm thickness) and 3-Maleimidopropionic acid N-hydroxysuccinimide ester (SMP) were purchased from Alfa Aesar (US). 3-Mercaptopropyltrimethoxysilane (MPTS), Tetraethoxysilane (TEOS), N,N-Dimethylformamide (DMF), Sodium Hydroxide, Arginylglycylaspartic acid (RGD) and gold nanoparticle suspension were purchased from Sigma-Aldrich (Canada). Sulfuric acid and Trypan Blue were purchased from Fisher Scientific (Canada). Phosphate Buffered Saline (PBS) (1x), McCoy's 5a, Trypsin EDTA (1X) and Fetal Bovine Serum (FBS) were purchased from Corning (Canada). Penicillin-Streptomycin Solution was purchased from HyClone (Canada). CyQUANT Cell Proliferation Assay was purchased from Life technologies (Canada). Acetone (reagent grade) was purchased from Caledon Laboratory Chemicals (Canada). Ethyl alcohol (95%) and methanol were purchased from Commercial Alcohols (Canada). All chemicals were used as received without further purification. The Saos-2 cell line was purchased from American Type Culture Collection (US) and cultured for further experiments.

2.2. Preparation of silanized Mg AZ31

Organosilane solutions were prepared with 3 different volume ratios of MPTS to TEOS including 1:1, 3:1 and pure MPTS while keeping the total volume of organosilane in the coating solution constant as described in Table 1. The pH of all silane solutions was adjusted to 4.20 by dropwise addition of 0.1 M H_2SO_4 immediately after mixing of the organosilane/water/methanol mixture. All solutions were aged with stirring for 7 h to ensure complete hydrolysis and optimum conditions for condensation of the silanol groups with hydroxyl groups on the substrate surface.

Mg AZ31 foil was machined into 1 cm diameter circular samples. The samples were polished to a 1 μm mirror finish with a diamond polishing suspension; AutoMet lapping oil (Buehler, Canada) was used as the lubricant. The polished AZ31 coupons were sonicated for 15 min in acetone and then rinsed well with deionized water for 5 min. The polished, degreased coupons were immersed in 0.05 M NaOH solution at 50 °C for 1 h, rinsed with copious amounts of deionized water and immediately air-dried. Each coupon was immersed in 25 ml of organosilane solution at 50 °C without stirring for 20 h. The coated coupons were removed from solution, immediately air-dried and then cured in an oven at 100 °C for 1 h.

2.3. Study of distribution of thiol functional groups

It is well documented that gold nanoparticles (AuNP) can specifically bond to thiol functional groups [40,41]. Therefore, each of the three coating types was exposed to 400 μl of a 10 nm diameter citrate stabilized AuNP suspension to evaluate the distribution of the thiol functional groups at the coating surface. After 1 h, the samples were removed from the AuNP suspension, rinsed with deionized water three times and immediately air-dried. A control group was also prepared by exposing the three coatings to a sodium citrate solution under the same conditions. The surface topographies of the coatings before and after treatment were evaluated by atomic force microscopy (AFM). This experiment was repeated 3 times.

2.4. Magnesium ion release rate – immersion test in 3.5% NaCl

The magnesium ion release rate of the coated samples compared to uncoated Mg AZ31 was evaluated by monitoring the concentration of magnesium in solution as a function of time upon immersion in 0.9% (w/v) NaCl solution at room temperature. The samples were mounted in epoxy with only the coated surface exposed to ensure a constant surface area from sample to sample. Each sample was immersed in 50 ml of NaCl solution. After 1, 3, 5, 7 and 14 days, the solutions were mixed well, a 100 μl aliquot was removed and diluted to 10 ml in a 2% nitric acid solution. The concentration of magnesium in the diluted solutions was determined using a Perkin Elmer AAnalyst flame atomic absorbance spectrometer (285.21 nm, air/acetylene flame).

2.5. Immobilization of the RGD peptide

The RGD peptide was covalently bonded through the surface thiol functional groups on the coated Mg AZ31 substrates using the heterobifunctional cross-linker SMP as previously described in the literature [23, 42]. Briefly, the SMP solution was prepared at a concentration of 5 mg/ml in dimethylformamide (DMF) and then diluted with pure ethanol to a final concentration of 1.7 mg/ml. Each silanized Mg AZ31 sample was exposed to 500 μl of freshly prepared SMP and allowed to react at room temperature for 1 h. After reaction with the crosslinker, the samples were rinsed three times with pure ethanol and once with deionized water to remove