Bacterial Biofilm Formation on Resorbing Magnesium Implants

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Abstract

Background: Implant-associated infections are a result of bacterial adhesion to an implant surface and subsequent biofilm formation at the implantation site. This study compares different magnesium materials based on their ability to resist bacterial adhesion as well as further biofilm formation. Material and Methods: The surfaces of four magnesium-based materials (Mg2Ag, Mg10Gd, WE43 and 99.99% pure Mg) were characterized using atomic force microscope. In addition, the samples were tested for their ability to resist biofilm formation. Planktonic bacteria of either S. epidermidis or E. faecalis were allowed to adhere to the magnesium surfaces for two hour followed by rinsing and, for S. epidermidis, further incubation of 24, 72 and 168 h was carried out. Results: E. faecalis had a significantly stronger adhesion to all magnesium surfaces compared to S. epidermidis (p = 0.001). Biofilm growth of S. epidermidis was different on various magnesium materials: the amount of bacteria increased up to 72 h but interestingly a significant decrease was seen at 168 h on Mg2Ag and WE43 surfaces. For pure Mg and Mg10Gd the biofilm formation reached plateau at 72 h. Surface characteristics of resorbable magnesium materials were changing over time, and the surface was generally less rough at 168 h compared to earlier time points. No correlation was found between the surface topology and the amount of adherent bacteria. Conclusion: In early stages of biofilm adhesion, no differences between magnesium materials were observed. However, after 72 h Mg2Ag and WE43 had the best ability to suppress S. epidermidis' biofilm formation. Also, bacterial adhesion to magnesium materials was not dependent on samples' surface topology.

Keywords

Bacterial Biofilm, Magnesium, Surface Topology

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1. Introduction

Surgical site infection is one of the most commonly reported infection and accounts for 14% - 16% of all nosocomial infections among hospital patients [1] [2]. It is a major problem in orthopedics leading to implant failure and in severe cases may result in amputation and mortality [3]. Implant infections in dental and maxillofacial fields can lead to implant failure [4].

Implant-associated infections are the result of bacteria adhesion to an implant surface and subsequent biofilm formation at the implantation site [3]. Sources of infectious bacteria include the environment of the operating room, surgical equipment, clothing worn by medical and paramedical staff, resident bacteria on the patient’s skin and bacteria already residing in the patient’s body [3] [5]. Thus, secondary operations involving implant removal highly increases the risk of nosocomial infections.

Designing metallic implants that would resorb at the same rate as that of bone healing would minimize the risk of postoperative infections, decrease high costs associated with repeated surgeries, minimize recovery times, and thus promote higher quality of life to each individual patient. Magnesium has attracted much attention for its potential use in trauma and orthopedics fields due to its mechanical properties [6], biocompatibility [7], biodegradability and ability to stimulate new bone formation [8] [9]. It was shown that magnesium has higher antibacterial activity than titanium, and that this activity is further strengthened in presence of silver [10]. It is thus desirable to compare different magnesium alloys based on their bacteriostatic and bactericidal abilities.

It seems that bacterial adhesion to surface is highly dependent on surface roughness (Sa) and developed surface area ratio (Sdr) [4] [11]. The level of bacterial adherence to moderately rough titanium surfaces (Sdr 58%) was five times greater than to smooth titanium surfaces (Sdr 2.8%) [4]. Magnesium resorbs mainly by pitting corrosion [12] which results in surface changes and might promote bacterial adhesion. Thus, it is important to compare whether any correlation between biofilm adhesion and surface changes which occur during resorption exist. Parameters describing spatial properties, like Sds, as well as hybrid properties, like Sdr, might further differentiate surfaces with similar Sa characteristics [13].

Enterococci, specifically Enterococcus faecalis, is the third most common cause of nosocomial infection, and most infections in hospitalized patients are associated with the use of indwelling medical devices [14]. E. faecalis, a Gram-positive constituent of the human intestinal microbiome, has become a prominent pathogen of health care-associated infections over the past 3 decades [15]. Between 1980 and 2008, the frequency of nosocomial infections caused by Enterococcus faecium, the other frequently encountered enterococcal pathogen, increased by 8.8% [16]. E. faecalis and E. faecium infections together accounted for 16.0% of central line-associated bloodstream infections, 14.9% of catheter-associated urinary tract infections, and 11.2% of surgical site infections as reported by the United States National Healthcare Safety Network between 2006 and 2007 [17]. E. faecalis is also the primary causative agent of enterococcal endocarditis [18] [19] and is the most frequently isolated pathogen in secondary endodontic infections [20].

Staphylococcus epidermidis and Staphylococcus aureus represent, in absolute, the main causative agents of infection in orthopedics [21]. S. epidermidis is the most frequently isolated member of the group of coagulase-negative staphylococci from implant-associated infections and they are associated with nosocomial infections [3]. S. epidermidis, a Gram-positive, non-spore forming facultative anaerobe that grows by aerobic respiration or fermentation, with diameters ranging from 0.5 - 1.5 mm, belong to the normal microbiota of the human skin. They are characterized by individual cocci, which divide in more than one plane to form grape-like clusters [3].

The aim of this study was to compare magnesium materials on their ability to resist bacterial adhesion as well as further biofilm formation. The surface changes were measured in order to find the possible correlation between biovolume and surface characteristics.

2. Materials and Methods

2.1. Sample Production

The following materials were used to produce alloys for this study: magnesium (99.99%, Xinxiang Jiuli Magnesium Co. Ltd., China), yttrium (99.95%, Grirem Advanced Materials Co. Ltd., China), gadolinium (99.95%, Grirem Advanced Materials Co., Ltd., China), rare earth mixture (Grirem Advanced Materials Co. Ltd., China), and silver (99.99%, ESG Edelmetall-Handel GmbH & Co. KG, Germany).

Three magnesium-based materials were produced: Mg2Ag (1.89% Ag, the rest was Mg), Mg10Gd (8.4% Gd,
the rest was Mg), and WE43 (3.45% Y, 2.03% Nd, 0.84% Ce, the rest was Mg). Pure magnesium (99.97% Mg) was used as a control. The concentrations of magnesium Mg, Y, Nd and Ce were determined by spark emission spectrometer (Spectrolab M, Spectro, Germany) and the concentrations of Ag and Gd were determined by X-ray fluorescence spectrometer (Bruker AXS S4 Explorer, Bruker AXS GmbH., Germany). The materials were cast at HZG-MagIC.

The three magnesium alloys (Mg2Ag, Mg10Gd, WE43) were produced by permanent mould gravity casting. After melting the pure Mg, the melt was held at 720°C and the preheated alloying elements were added with continuous stirring for 15 minutes. The melt was then poured into a preheated (550°C) permanent steel mould treated with boron nitride. During the casting process cover gas was used (SF₆ and Ar mixture). The alloys were homogenized with a T4 heat treatment prior to extrusion in Ar atmosphere at 550°C (Mg10Gd and WE43) and at 420°C (Mg2Ag) for 6 h. Afterwards the alloys were extruded indirectly with an extrusion ratio of 4:25. The chamber of the extrusion machine was set to 370°C and the billets (d = 30 mm) were preheated for one hour at 370°C (Mg2Ag), at 390°C (WE43) and at 430°C (Mg10Gd). The extrusion speed was between 3 and 4.5 mm/sec. Pure Mg was cast by permanent mould direct chill casting. The cast billet (d = 110 mm) was extruded indirectly with an extrusion ratio of 1:84. The billet temperature was maintained at 340°C and the speed of the extrusion was 0.7 mm/sec. Discs (10 mm diameter and 1.5 mm thickness) were machined from the extruded bars.

2.2. Sample Sterilization

The samples were sonicated for 20 min in dry isopropanol, dried and gamma-sterilized at the BBF Sterilisation-service GmbH facility (Kernen, Germany) with a total dosage of 29 kGy.

2.3. Bacterial Strains and Culture

The strains used for biofilm assays were _E. faecalis_ ATCC 29212 and _S. epidermidis_ C121, isolated from the external side of peritoneal dialysis catheter [22]. All strains were routinely maintained on blood agar or in Todd-Hewitt broth (30 g·l⁻¹; Difco Laboratories, Becton Dickinson & Co, Sparks, MD) at 37°C in 5% CO₂.

2.4. Biofilm Formation Assays

The magnesium discs were pre-coated in human serum for 18 h and then washed twice in 2 ml potassium phosphate buffer (PBS) for 10 min at 37°C. Overnight broth cultures of _S. epidermidis_ or _E. faecalis_ was inoculated (1:10 dilution) into fresh, pre-warmed Todd-Hewitt broth and incubated at 37°C in 5% CO₂ to the mid-exponential growth phase (optical density at 600 nm ≈ 0.6). The bacterial suspension was centrifuged at 3000 rpm for 10 min at 5°C washed once in PBS and re-suspended in 10% human serum to a final concentration of approximately 1 × 10⁸ cells ml⁻¹. The bacterial suspension was added to a microtiter plate with the magnesium discs and the bacteria were allowed to adhere for 2 h at 37°C on a rocking platform at 300 cycles per hour. Following incubation for 2 h, the surfaces were rinsed twice in 2 ml PBS with pH 7.5 to remove loosely bound cells. _S. epidermidis_ was then further incubated for 24, 72 and 168 h respectively. Adhered cells were stained using the Live/Dead Bac Light staining kit (Molecular Probes) and then visualized using a fluorescent microscope (Aristoplan, Leitz). Ten images per surface were captured with a digital camera and the number of bacteria on each image was counted by hand in a field area of 15,600 µm². All experiments were carried out in triplicates for each surface.

2.5. Surface Roughness Characterization

Magnesium discs which were pre-coated in human serum for 18 h and then washed twice in 2 ml potassium phosphate buffer (PBS) for 10 min at 37°C. The micro titer plates with these magnesium discs in human serum but without bacteria were incubated at 37°C on a rotary shaker at 300 cycles per hour in 5% CO₂ for 2, 24, 72 and 168 h. The human serum was changed every second day. At different time points the discs were removed and allowed to dry at room temperature. Surface characterization was performed by atomic force microscope, AFM (XE-100, Park Systems Corp, Suwon, Korea). Measurement areas of 10 × 10 µm in three random positions were selected for each disc. The measurements were performed at a scan rate of 0.50 Hz. The images acquired from AFM were subjected to leveling and Gaussian filtering with a cut-off of 2.5 µm was applied using the software MontainsMap® Universal 6.2 (Digital Surf, Besancon, France) and 3-D parameters such as Sₘ, Sₚ,
Sds were analyzed.

2.6. Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, v18, SPSS Inc, Chicago, USA). The significance level was set at 5%. Standard analyses comparing different groups were conducted via one-way repeated measures analysis of variance (ANOVA). One-way repeated measures ANOVA was performed with the Dunn or Holm-Sidak post-hoc test. Since the data was non-normally distributed, Kruskal-Wallis test was performed to compare parameters between the groups. All graphs were plotted using SPSS.

3. Results

3.1. Adhesion of S. epidermidis and E. faecalis to Magnesium Surfaces

E. faecalis had significantly stronger adhesion than S. epidermidis with respect to the same samples (p = 0.001). Comparison between adhesion of S. epidermidis and E. faecalis to magnesium materials after 2 h of incubation is presented in Figure 1. This was true for all tested materials. The viability of the adhered cells of both E. faecalis and S. epidermidis was high (>95% for both strains) and not statistically different from each other. No significant differences were found between materials within the same bacterial adhesion assay. In general, all magnesium samples were densely covered with E. faecalis, whereas S. epidermidis colonies were scarcely spread over the surface. Figure 2 shows this trend for Mg2Ag.

Figure 1. Adhesion of S. epidermidis and E. faecalis to magnesium materials after 2 h of incubation. (A) Live S. epidermidis; (B) Live E. faecalis; (C) Dead S. epidermidis; (D) Dead E. faecalis. *Field area represents 15,600 µm².
3.2. S. epidermidis Growth over Time

Biofilm growth of *S. epidermidis* on magnesium materials over time is presented in Figure 3(a). In general, Mg2Ag and WE43 had similar pattern of the biofilm growth, whereas pure Mg was comparable to Mg10Gd. The number of adherent bacteria increased up to 72 h of incubation for all tested samples. Between 72 h and 168 h, the biofilm reached its plateau for pure Mg and Mg10Gd. Whereas, for Mg2Ag and WE43, the amount of adhered *S. epidermidis* decreased between 72 and 168 h.

The viability of the cells remained high (>95%) at all time-points as shown by the low number of dead cells. There was no significant difference between the groups in the amount of dead *S. epidermidis* at the different time points. Even inside the group, no statistically significant differences were observed (Figure 3(b)). The pattern of *S. epidermidis* growth on pure Mg over time is shown in Figure 4.

3.3. Characterisation of Surface Roughness

Surface topology was quantified starting from 0 h when bacteria were seeded onto the samples. It was observed that $S_a$ values for pure Mg significantly increased from 0 to 2 h, and then slightly decreased at 168 h (Figure 5). The surface of Mg2Ag stayed stable over time with no significant changes in $S_a$. WE43 was comparable to Mg2Ag but with a significant $S_a$ decrease between 24 and 168 h. Significant decrease in $S_a$ was observed for Mg10Gd at 0 and 168 h.
Figure 4. Adherence of *S. epidermidis* to pure Mg at (A) 2 h; (B) 24 h; (C) 72 h; (D) 168 h. Scale bar represents 30 µm.

Figure 5. *S*ₐ, average surface roughness, of magnesium alloys over time.
The pattern of $S_{dr}$ change over time was very similar to $S_a$ and is presented in Figure 6. $S_{dr}$ behavior was similar for all tested materials (Figure 7). In general, $S_{dr}$ decreased from 0 h to 2 h but then stayed quite stable over time. No correlation between the surface topology and amount of adherent bacteria were found in this study.

4. Discussion

Implant-associated infections are the result of bacteria adhesion to an implant surface and subsequent biofilm formation at the implantation site. This study compared magnesium materials on their ability to resist bacterial adhesion as well as further biofilm formation. The surface changes were measured in order to find the possible correlation between biovolume and surface characteristics.

Adhesion of *E. faecalis* was significantly better than adhesion of *S. epidermidis* to the same samples. This means that different bacterial strains have different ability to bind to magnesium surfaces under the same conditions which can be supported by previous studies on microbiology [23]. In this study, no correlation was found between the surface topology and the ability of the bacteria to adhere to the magnesium surface, although this correlation was observed in previous research [4]. Resorption of magnesium is a very complex chemical process. Surface topology is one of many factors that might influence cell and bacteria adherence and there are many more aspects that should be taken into account when analyzing magnesium’s performance *in vitro*.

It has been shown in previous studies that magnesium changes the surrounding environment; it makes pH more basic [24] [25] which in turn stimulates precipitation of Ca$^{2+}$ ions from the solution [24]-[26] thereby changing the osmolality [26] and thus reacts with the medium to form H$_2$ gas [27]. The surrounding environment also has an effect on magnesium since it promotes formation of the protective layer on its surface [27]. Protective layer slows down further degradation but is brittle and cleaves off from the surface quite easily [28]. This means that the surface topology of magnesium materials is constantly changing. In this study, it was observed that in the first hours $S_a$ increased which can be explained by removal of surface irregularities and particles that are present on the surface. Later, the summits start to resorb and their tops break off from the surface.
The higher pH inhibits enzyme activities that are essential to bacterial life, i.e., metabolism, growth, and cellular division [29].

It was shown in previous studies that *S. epidermidis* grows best in slightly more acidic pH close to around 6.35 but demonstrates the ability to grow in a range of pHs [30] [31]. In this study *S. epidermidis* seemed to be resistant to alkali pH due to the effects of magnesium, as revealed by the increase in the bacterial number up to 72 h for all samples tested. After 72 h Mg2Ag and WE43 suppressed further bacterial growth on their surfaces. This might be due to the release of Ag2+ ions which are known for their antibacterial properties [10]. In case of WE43, the decreased number of adherent bacteria after 72 h might be due to high resorption rate of this alloy [32] and consequent formation of H2 gas which acts as a physical barrier for further biofilm growth. Pure Mg and Mg10Gd both had bacteriostatic effect after 72 h which might be either due to the change in the surrounding environment which became less favorable for *S. epidermidis*, or due to the high biovolume which suppresses further biofilm growth.

It was shown in previous studies that unlike *S. epidermidis, E. faecalis* can survive in highly alkaline pH, and some clinical isolates require 72 h at pH 12.5 to be killed [33]. Mild alkaline mediums (pH 7 - 9) had no effect on *E. faecalis* vitality and high alkaline condition (pH > 10) led to a significant decline in the survival rate of bacteria in one study [34]. Also the biofilm cells of *E. faecalis* were more alkaline tolerant than corresponding planktonic cells [34]. This can explain why there were more *E. faecalis* adhered to magnesium samples compared to *S. epidermidis* which are less resistant to alkali pH. Based on the previous research results it can be hypothesised that *E. faecalis* would have continued its biofilm growth if the experimental time would have been prolonged.
Dead *E. faecalis* and *S. epidermidis* were few in this study. The surface was predominantly occupied by live bacteria. Thus, magnesium seems to have no bactericidal effect. With time no increase in dead *S. epidermidis* was observed, their number was stable over the observation period.

Magnesium alloys seemed to suppress the adhesion of *S. epidermidis* in the first hours of experiment. Unlike *S. epidermidis*, *E. faecalis* binded to magnesium more readily at 2 h. One of the limitations of this study is that it does not compare magnesium to titanium surfaces and it is thus not possible to see whether magnesium is better in its ability to prevent biofilm adhesion than titanium. Resorption of magnesium alloys is a complex process which involves alterations of material’s surface and changes in the surrounding environment. Thus the task of the future research in this area would be determination of factors that suppresses bacterial vitality.

5. Conclusion

Various bacterial strains had different adherence capacity to magnesium-based materials. There were no differences between different magnesium materials based on their ability to withstand biofilm formation at early stages up to 72 h. However, after 72 h Mg2Ag and WE43 had more favorable properties than pure Mg and Mg10Gd in their ability to suppress bacterial growth. These changes in biofilm growth and adherence could not be attributed to the changes in surface topology.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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