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## Biodegradable porous FeMn( $-x$ Ag) alloys: assessment of cytocompatibility, mechanical, magnetic and antibiofilm properties†

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In this work, porous FeMn( $-x$ Ag) alloys are fabricated through powder metallurgy methods. The effects of porosity and Ag addition on the microstructure, biodegradability, magnetic and mechanical properties of the alloys are investigated. Studies on the cytocompatibility, inflammatory cytokine response and anti-bacterial effect are also conducted. The fabricated alloys exhibit a macro- and nanoporous structure, with uniformly distributed silver particles. The biodegradability tests reveal that the release of Mn to the Hank's solution is higher than that of Fe, without significant differences between the alloys. The degradation products consist mainly of Fe, Mn, O and compounds enriched in Ca, P and Cl. As-sintered alloys show a low saturation magnetization value (below  $1 \text{ emu g}^{-1}$ ), which does not increase significantly with immersion time. The results on biocompatibility indicate that all tested alloys are non-cytotoxic, but the addition of Ag might interfere with cell proliferation. However, the ions released by the FeMn( $-x$ Ag) alloys do not induce an inflammatory response in macrophages. The obtained results on microbiological interactions reveal that although no significant bactericidal effect is observed at 4 h between FeMn control and FeMn-5Ag, a significant reduction in the total biofilm biomass of both live and dead bacteria is observed after 24 h in Ag containing FeMn-5Ag surfaces.

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## 1 Introduction

In recent years, the application of biodegradable metals (BMs) and polymers as potential candidates for temporary medical implants has been of growing interest.<sup>1–6</sup> This class of materials is expected to corrode gradually *in vivo* once they have fulfilled their role.<sup>2,7</sup> Their main advantage is the possibility of avoiding the implant removal surgery, hence limiting the cost of medical treatment and decreasing the risk of infections, which is

increased during the revision surgery.<sup>8</sup> It has been shown that biodegradable materials are in general less prone to biomaterial-associated infections than non-degradable materials, most probably due to the degradation processes that reduce the area available for bacterial colonization and, therefore, the formation of biofilm.<sup>9</sup> Among various tested alloys, Mg-based and Fe-based alloys are especially attractive due to their very good biocompatibility.<sup>2,7</sup> Mg-based alloys show very low toxicity and their Young's modulus is close to that of the human bone. However, their degradation rate is too rapid for some applications, resulting in H<sub>2</sub> gas evolution and bubbling upon immersion. In addition, they are hard to process, and their strength and ductility are not satisfactory.

Over the past few years, increasing attention has been paid to Fe-based alloys as they show good processability, mechanical strength and low toxicity,<sup>10–13</sup> however, the degradation rate is too slow to be applied as biodegradable implants.<sup>7,14</sup> Moreover, Fe-based alloys tend to be ferromagnetic, which is undesirable for magnetic resonance imaging (MRI) purposes. Thus, several approaches to improve the degradation rate of Fe-based alloys are being explored, such as alloying with different elements (*e.g.*, Mn),<sup>11,13,15</sup> using new fabrication methods to tune the microstructure such as selective laser melting (SLM),<sup>16,17</sup>

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introducing porosity,<sup>17–21</sup> adding small amounts of noble elements,<sup>22–25</sup> or adding C to promote twinning-induced plasticity (TWIP),<sup>7,26–28</sup> amongst others. The addition of elements such as Cu and Ag, which are more noble than Fe or Mn, is being investigated as a way of not only improving corrosion rates (through galvanic pairs) but also because they bring antimicrobial properties to the resulting alloys.<sup>22,25</sup> Powder metallurgy is especially appealing to introduce insoluble elements like Ag to the Fe–Mn matrix in a homogenous manner. However, the effect of adding Ag in terms of its role in the degradation rate remains still controversial: while most authors agree that Ag causes increased degradation rates of Fe-based alloys,<sup>22–24,29</sup> some studies report that Ag additions promote galvanic coupling only in the first stages of degradation and, after formation of the degradation layer, the Ag role is not being fulfilled anymore.<sup>27</sup> Similarly, one can find dissimilar conclusions on the effect of silver on antibacterial activity. In the report by Liu<sup>30</sup> no antimicrobial properties were confirmed in Fe70.3–Mn28.9–Ag0.8 alloy, while in the research published by Sotoudehbagha<sup>22</sup> antibacterial activity in the alloys containing 1 and 3 wt% of Ag was observed.

The main aim of this study is to fabricate and characterize porous Fe–Mn alloys with different additions of Ag in terms of their microstructure, mechanical and magnetic properties, degradation, biocompatibility and antibacterial activity. For this purpose, four different compositions have been investigated: equiatomic FeMn, and FeMn with 1, 3 and 5 wt% of silver. The relatively high content of Mn is proposed to ensure the non-magnetic character of the investigated alloys. Up to date, neither a long-term biodegradability test nor a study of the evolution of magnetic properties upon immersion has been performed in terms of ion release and degradation surface analysis for porous, Ag-containing equiatomic Fe–Mn alloys.

## 2 Materials and methods

### 2.1 Fabrication of alloys

Commercial powders of iron (97% purity, Merck, Germany), manganese ( $\geq 99\%$  purity, Merck, Germany) and silver ( $\geq 99.9\%$  purity, Merck, Germany) were used to prepare four types of samples: FeMn, FeMn–1Ag, FeMn–3Ag and FeMn–5Ag, where the ratio of Fe : Mn was adjusted to 50 : 50 (at%) and the additions of silver are given in wt% (1, 3 and 5 wt%, respectively). Mixtures of powders were alloyed using a planetary ball milling device (Pulverisette 5, Fritsch, Germany) under an Ar atmosphere at a rotation speed of 300 rpm, for 10 h and with a 10 : 1 ball-to-powder weight ratio. The procedure of weighing powders and sealing the container was conducted in a glove box under Ar atmosphere ( $\text{H}_2\text{O} < 0.1$  ppm,  $\text{O}_2 < 0.1$  ppm) to prevent oxidation and atmospheric contamination. The particle size of as-milled powders was measured using the ImageJ software (National Institutes of Health, USA) from scanning electron microscopy (SEM) images. After ball-milling, the powders were further refined in an agate mortar, to avoid leaving large agglomerates. Subsequently, as-prepared powders were uniaxially cold-pressed at 100 MPa to form

cylindrical green pellets (discs) of 10 mm diameter and 2–3 mm in thickness. The green pellets were then loaded in a vacuum furnace and sintered at 900 °C at  $5 \times 10^{-5}$  mbar for 4 h at a heating rate of 1 °C min<sup>-1</sup>. As Mn has a high vapor pressure, higher sintering temperatures and longer dwelling times might lead to significant losses of Mn content due to sublimation,<sup>18,31</sup> therefore the temperature was kept at 900 °C. Moreover, it has been shown that the losses of Mn in ball-milled powders are lower than in powders prepared using other methods.<sup>31</sup>

### 2.2 Structural characterization

The structural and compositional characterization of the fabricated materials was conducted using an SEM microscope (Zeiss Merlin, Zeiss, Germany) operated at 15 kV equipped with an energy-dispersive X-ray spectroscopy (EDS) detector. The phase compositions of ball-milled powders, sintered alloys and alloys after immersion in Hank's balanced salt solution (HBSS) were examined by X-ray diffraction (XRD, Panalytical X'Pert powder diffractometer, Malvern Panalytical, UK) using Cu K $\alpha$  radiation. The measurements were conducted in an angular range from 20° to 100° with a step size of 0.026° in the Bragg-Brentano geometry. The XRD results were analyzed using Rietveld refinement (MAUD software),<sup>32</sup> from which crystallite size and lattice parameters were determined. ImageJ software was used to assess the macroporosity of sintered alloys by analyzing the SEM images. Archimedes' method was used to determine the density of the specimens. Then, the porosity values were estimated by comparing the theoretical (*i.e.*, for the fully dense materials) and experimental densities, according to the formula (eqn (1)):

$$\% \text{ porosity} = \left( 1 - \left( \frac{\text{density}_{\text{experimental}}}{\text{density}_{\text{theoretical}}} \right) \right) \quad (1)$$

### 2.3 Biodegradability tests

Biodegradability tests were conducted by immersing samples of as-fabricated alloy pellets for various periods (7, 14, 28, 56, and 84 days) in HBSS at  $37.5 \pm 1$  °C. Each sample, with a surface area of approximately 100 mm<sup>2</sup>, was immersed in a container containing approximately 30 mL HBSS. The volume of the solution adhered to the ASTM G31-72(2004) standard, using a volume/surface area ratio of 0.3 mL mm<sup>-2</sup>. At least three replicas were prepared at each time point. The HBSS (H8264, Merck, Germany) is a popular physiological solution used to reproduce the conditions in the human body, which contains CaCl<sub>2</sub>, MgSO<sub>4</sub>, KCl, KH<sub>2</sub>PO<sub>4</sub>, NaHCO<sub>3</sub>, NaCl, Na<sub>2</sub>HPO<sub>4</sub> and glucose. After each period, 2 mL of solution from at least three replicas was taken for the ion release concentration analysis, which was conducted using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7900, Agilent, USA). Before the ICP-MS analysis, the aliquots were diluted with HNO<sub>3</sub>. Samples immersed for 14, 28, 56, and 84 days were withdrawn from the containers for structural analysis of corrosion products formed both on the surface and the cross-section of the pellets. XRD analysis was performed to identify the corrosion products formed on the surface of FeMn and FeMn–5Ag alloys after 7 and 28 days of immersion.



## 2.4 Characterization of mechanical and magnetic properties

The mechanical properties were studied using nanoindentation (Anton Paar NHT<sup>3</sup> nanoindenter, Anton Paar, Austria). Before indentation, the discs were polished to a mirror-like surface, with the final polishing step using 1  $\mu\text{m}$  diamond particles paste. Maximum applied loads of 100 and 500 mN were used to assess the behaviour of the material during deformation. A series of at least 50 measurements were performed on each sample to obtain statistically significant data. The load-displacement curves were used to calculate the hardness ( $H_B$ ) and reduced Young's modulus ( $E_r$ ), using the method of Oliver and Pharr.<sup>33</sup> Apart from that, the elastic ( $W_{\text{elast}}$ ) and plastic ( $W_{\text{plast}}$ ) energies of deformation were obtained from the load-displacement curves by assessing the areas between the unloading curve and displacement axis ( $W_{\text{elast}}$ ) and between the loading and unloading curves ( $W_{\text{plast}}$ ). Finally, the elastic recovery was calculated as the ratio between elastic ( $W_{\text{elast}}$ ) and total ( $W_{\text{elast}} + W_{\text{plast}}$ ) energies during nanoindentation.

A vibrating sample magnetometer (VSM, MicroSense, USA) was used to analyze the magnetic properties of the samples before (as-fabricated) and after incubation in HBSS, at room temperature. The maximum applied magnetic field was 20 kOe. The results were normalized over sample weight to obtain the value of saturation magnetization ( $M_s$ ).

## 2.5 Cell proliferation assays and cytotoxicity tests

For cell proliferation and cytotoxicity experiments, human osteosarcoma Saos-2 cells (ATCC HTB-85) were used. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), under standard conditions (37 °C, 5% CO<sub>2</sub>).

Experiments were conducted in a medium conditioned in the presence of the different FeMn(-xAg) alloys. To prepare the conditioned media, the samples were sterilized with UV light for 3 min and incubated in 15 mL of supplemented DMEM under standard conditions for 7, 14, 28, and 56 days. Then, the alloys were removed, and the conditioned media were kept at 4 °C protected from light until used. In parallel, control media were also prepared following the same incubation times and conditions but without the presence of any alloy (aged media). Osteoblasts (Saos-2 cells) proliferation was determined using Alamar Blue cell viability Reagent (Thermo Fisher Scientific) before (day 0) and after (day 3) cells' exposure to the conditioned media. Briefly,  $5 \times 10^4$  cells were seeded into each well of a 24-well plate with fresh medium. Cells were cultured for 24 h to allow cell attachment, and then the medium was replaced with fresh medium with 10% Alamar Blue, and cells were incubated for 4 h in standard conditions in the dark. Then, the supernatant was collected (day 0), and the fluorescence was measured at 585 nm wavelength after excitation at 560 nm on a Spark multimode microplate reader (Tecan, Männedorf, Switzerland). After supernatant collection, FeMn(-xAg) conditioned media for different incubation times were added to each well. Cells were cultured for 3 more days in a conditioned medium and the

Alamar Blue assay was repeated according to the previously described protocol (day 3). Aged and fresh media were used as controls. Experiments were performed in triplicate.

Immediately after proliferation assays, cytotoxicity was assessed in cells incubated with media conditioned for 7 and 56 days using the live/dead viability/cytotoxicity kit for mammalian cells (Invitrogen), according to the manufacturer's protocol. Images from different regions of the well were captured using an Olympus IX71 inverted microscope equipped with epifluorescence.

## 2.6 Quantification of inflammatory cytokines secretion

THP-1 monocyte cells were used to analyze the potential immunological response induced by the FeMn(-xAg) alloys. Monocytes were grown in RPMI 1640 medium (Gibco) supplemented with 25% FBS (Gibco) under standard conditions. To differentiate monocytes into macrophages,  $4 \times 10^5$  THP-1 cells were seeded into 24-well plates and treated with 0.16  $\mu\text{M}$  phorbol-12-myristate-13-acetate (Sigma-Aldrich, Saint Louis, MO, USA) for 72 h. Then, cells were washed and incubated in a fresh medium for 24 h before carrying out the experiments. The fresh medium was replaced by a conditioned RPMI medium (prepared as explained above) and macrophages were incubated for 5 h and 24 h to allow the secretion of inflammatory cytokines. As a positive control, 1  $\mu\text{g mL}^{-1}$  of lipopolysaccharide (LPS) (Sigma-Aldrich) was added to the macrophages culture. As a negative control, macrophages were cultured in a fresh medium. After 5 h and 24 h, supernatants were collected and used to quantify cytokine secretion. Inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were evaluated by flow cytometry using cytometric bead array (CBA) (Becton-Dickinson, East Rutherford, NJ, USA). Cytokine concentrations in the supernatant were analyzed according to the manufacturer's protocol. Negative control was considered as basal secretion level and used for statistical comparisons. Experiments were performed in triplicate.

## 2.7 Assessment of *S. aureus* adhesion and viability

From a frozen stock, *S. aureus* ATCC 25923 was streaked onto a 5% horse blood agar (HBA) plate (Media Department, Clinical Microbiology Laboratory, Sahlgrenska University Hospital, Sweden) and incubated at 37 °C overnight. After incubation, colonies were taken and inoculated into 4 mL tryptic soy broth (TSB) (Scharlau, Barcelona, Spain) to achieve an OD<sub>546</sub> of 0.13, equivalent to  $10^8$  CFU mL<sup>-1</sup>, which was diluted 1:1000 into 40 mL of TSB to achieve a suspension of  $10^5$  CFU mL<sup>-1</sup>. One mL of this suspension was then added to each well of a 48-well plate (Nunclon Delta-surface; Thermo Fisher Scientific, Waltham, USA) containing 9 mm diameter discs with the test surface (FeMn-5Ag) and controls of FeMn or Ti (Christers Finmekaniska AB, Skövde, Sweden). The discs were then incubated statically at 37 °C for 4 or 24 h to assess bacterial adhesion and biofilm formation as well as bacterial viability.

After each incubation period, each disc was rinsed three times in sterile 0.9% saline to remove non-adhered bacteria. Discs were then stained using LIVE/DEAD™ BacLight™ Bacterial



Viability Kit, for microscopy & quantitative assays (Invitrogen, Waltham, USA) following the manufacturer's instructions. The discs were rinsed three times to remove any unbound stain and fluorescence was assessed in a plate reader (FluostarOmega, BMG LABTECH, Ortenberg, Germany) and confocal laser-scanning microscope (Nikon C2+, Nikon, Tokyo, Japan) with a 100 $\times$  water dipping objective (CFI Plan 100XC W). Five z-stacks were taken at randomly chosen fields of view on each sample and images were taken at 3  $\mu$ m intervals through the biofilm layers. Biofilm thickness and biomass were analyzed in BiofilmQ.<sup>34</sup> From the plate reader, the average fluorescence intensity of each well scan was taken as the representative live/dead distribution for each disc. Experiments were performed in triplicate with duplicate material samples.

## 2.8 Statistical analysis

All quantitative data were analyzed with GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA) and presented as the mean  $\pm$  standard error of the mean. Statistical differences were assessed by one-way analysis of variance (ANOVA) with a Bonferroni correction for cytocompatibility and inflammatory response analyses and with Fisher's least significant difference post-test for microbiological analyses. A value of  $p < 0.05$  was considered significant.

# 3 Results and discussion

## 3.1 Microstructure and compositional analysis

**3.1.1 Morphology and phase composition of as-sintered alloys.** The SEM images of the initial and ball-milled powders are presented in Fig. S1 (ESI<sup>†</sup>). The initial particle size was less than 50  $\mu$ m for the initial Fe, Mn, and Ag powders. The sizes of the ball-milled powders were in the range of 1–40  $\mu$ m. Fig. 1 shows the XRD patterns of the powders ball-milled for 10 h (Fig. 1a) and the as-sintered FeMn, FeMn–1Ag, FeMn–3Ag and FeMn–5Ag alloys (Fig. 1b). The ball-milled powders have an austenitic structure, which is in accordance with the Fe–Mn phase diagram for this Mn content.<sup>35</sup> XRD patterns of ball-milled powders show wide peaks and a crystallite size which

decreases with increasing the Ag content. Crystallite sizes are 14, 14, 13 and 10 nm for the FeMn, FeMn–1Ag, FeMn–3Ag, FeMn–5Ag, respectively. The lattice constant increases upon the addition of Ag, showing values of 3.625 Å for FeMn, 3.630 Å for FeMn–1Ag, 3.633 Å for FeMn–3Ag and 3.637 Å for FeMn–5Ag.

The FeMn and FeMn–1Ag sintered alloys maintain the austenitic structure, while FeMn–3Ag and FeMn–5Ag alloys consist of a duplex structure, comprising  $\gamma$ -austenite (fcc structure) and  $\epsilon$ -martensite phase (hcp structure). The occurrence of the martensite phase was not expected as alloys containing above 27.3 at% of Mn are known to be mainly austenitic.<sup>13</sup> This phase transition could be caused by the occurrence of discrete inhomogeneities in the element distribution, forming regions of lower Mn content and thus promoting the martensitic transformation. This phenomenon was already observed before by Zhang,<sup>21</sup> but the explanation of martensitic transformation under furnace cooling conditions was not given. In this case, the martensitic transformation could be further enhanced by the presence of Ag. Nevertheless, both  $\gamma$ -austenite and  $\epsilon$ -martensite phases are antiferromagnetic, therefore the occurrence of the martensitic phase should not interfere with MRI. A shift in the diffraction peaks towards higher  $2\theta$  angles is observed in the sintered alloys with higher Ag contents. The crystallite size of the alloys subjected to sintering increases when compared to as-milled powders, and values of 38, 39, 18 and 13 nm for FeMn, FeMn–1Ag, FeMn–3Ag and FeMn–5Ag, respectively, are obtained. Therefore, as in the case of powders, the addition of silver is leading to a decrease in the crystallite size. A peak of low intensity can be observed at  $2\theta = 35^\circ$ , which was attributed to Fe<sub>2</sub>O<sub>3</sub>. The occurrence of the Fe<sub>2</sub>O<sub>3</sub> phase was observed in other studies<sup>35</sup> and it originates from the sintering method, which is always connected with some level of oxidation. Nevertheless, Fe<sub>2</sub>O<sub>3</sub> is also biocompatible,<sup>37,38</sup> therefore its presence should not compromise the biocompatibility of the tested alloys.

Fig. 2 shows SEM micrographs of the morphology of sintered (a–c) FeMn, (d–f) FeMn–1Ag, (g–i) FeMn–3Ag and (j–l) FeMn–5Ag alloys. The SEM images under various magnifications were analyzed using ImageJ software and the % area of macroporosity and the size of pores were calculated. Manganese is foreseen to diffuse into the iron matrix during sintering and



Fig. 1 XRD patterns of (a) ball-milled powders and (b) sintered FeMn(–xAg) alloys.





Fig. 2 SEM micrographs of sintered and surface-polished samples of FeMn (a–c), FeMn–1Ag (d–f), FeMn–3Ag (g–i) and FeMn–5Ag (j–l) samples. The % area macroporosity was determined using ImageJ by analyzing SEM images of the regions of macroporosity. The unlabeled red arrows provide reference for the Ag particles.

create a solid solution, while silver may form non-soluble precipitates in the FeMn matrix, as the solubility of Ag in Fe is very low.<sup>39,40</sup> The morphologies of the green pellets, *i.e.* pressed powders before sintering, are shown in Fig. S2 (ESI<sup>†</sup>). All tested alloys show a similar initial porosity in the range of 19–26%, as calculated using ImageJ based on SEM images. The process of sintering typically creates some additional level of porosity resulting from the rearranging of Fe and Mn, *i.e.*, voids are left behind by the metals upon diffusion. Another factor causing porosity is the change in the crystalline structure from bcc-Fe and bcc-Mn to fcc- $\gamma$  (FeMn) and hcp- $\epsilon$  (FeMn). This porosity is expected to have a beneficial impact on the proliferation of cells within the pores and, what is more, it can also improve the degradation rate due to an increased surface area exposed to the bodily environment.<sup>18,41</sup> In the tested materials, two different levels of porosity can be distinguished – namely macroporosity with an average pore size of around 10  $\mu\text{m}$  – and nanoporosity with a pore size of around 100 nm, as presented in Fig. 2(c, f, i and l). The calculated levels of total porosity are presented in Table 1, which shows that porosity increases with Ag content. While the addition of only 1 wt% of Ag does not cause a significant decrease in the density, the addition of 3 and 5 wt% of Ag leads to a higher total porosity (above 30%). This phenomenon is attributed mainly to the

decreased compressibility due to the presence of non-alloyed silver particles in the ball-milled powder.<sup>42</sup>

Silver particles are uniformly distributed within the Fe–Mn matrix, as indicated by red arrows in Fig. 2. To further analyze the distribution of Ag precipitates in the Fe–Mn matrix, EDS compositional maps, presented in Fig. S3 and S4 (ESI<sup>†</sup>), show that Ag precipitates exhibit a polydisperse distribution within a size range of 0.5–3  $\mu\text{m}$ . Larger agglomerates of Ag were observed, particularly in the FeMn–5Ag sintered alloy, as shown in Fig. S4b (ESI<sup>†</sup>). The presence of Ag-rich precipitates could in principle enhance the micro galvanic corrosion, hence inducing faster degradation rate of tested alloys.<sup>22–24</sup>

EDS compositional analysis of the samples furnished the weight percentage of individual elements, as listed in Table 1. The amounts of Ag are close to the nominal values (1, 3 and 5 wt%), while the weight percentages of Fe and Mn are slightly different from the nominal equiatomic composition (50.5 wt% Fe and 49.6 wt% Mn), with a lower amount of Mn than the initial value. Manganese is an element very prone to sublimation under high temperatures;<sup>39</sup> therefore it may be assumed that partial sublimation of Mn occurred, lowering its final amount.

**3.1.2 Surface morphology and chemical composition upon immersion.** The changes in surface morphology of FeMn and

Table 1 Actual chemical composition, macroporosity, density, and total porosity of sintered alloys. Total porosity accounts for both macro- and nanopores. Macroporosity was estimated from ImageJ software processing of SEM images, whereas the total porosity was determined by the Archimedes method

Sample	wt% Fe	wt% Mn	wt% Ag	Macro-porosity (% area)	Density ( $\text{g cm}^{-3}$ )	Total porosity (%)
FeMn	54.3	45.7	0	18.39 $\pm$ 3.00	5.50 $\pm$ 0.16	28.2 $\pm$ 2.1
FeMn–1Ag	56.7	42.0	1.3	17.80 $\pm$ 1.54	5.38 $\pm$ 0.11	30.1 $\pm$ 1.4
FeMn–3Ag	54.9	42.5	2.6	20.61 $\pm$ 1.34	4.94 $\pm$ 0.04	36.3 $\pm$ 0.5
FeMn–5Ag	54.5	41.1	4.4	22.02 $\pm$ 0.30	4.87 $\pm$ 0.12	37.7 $\pm$ 0.6



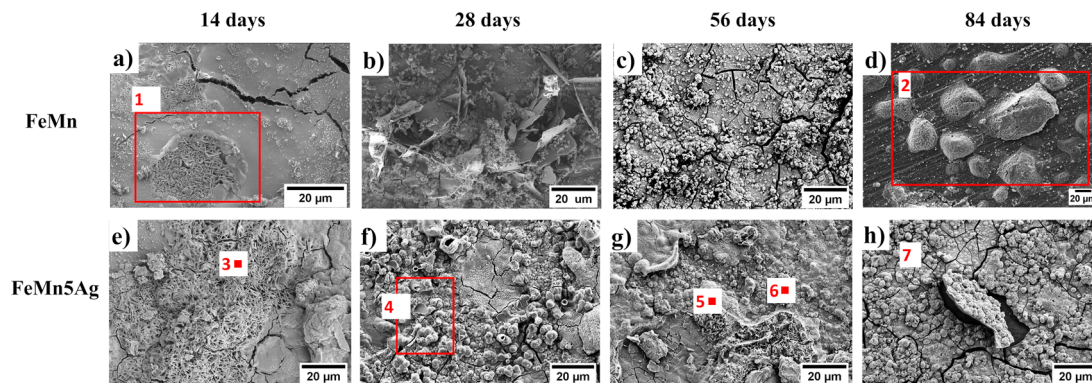


Fig. 3 Top-view SEM images of the degraded surface of FeMn (a–d) and FeMn–5Ag (e–h) incubated for 14 (a and e), 28 (b and f), 56 (c and g) and 84 (d and h) days in HBSS. (EDS analysis on the highlighted areas and locations is presented in Table 2).

FeMn–5Ag alloy upon immersion are presented in Fig. 3. The degradation process of the alloys led to the formation of a degradation layer. The EDS analyses, performed on the areas highlighted in Fig. 3 and summarized in Table 2, reveal that the degradation layer consists not only of Fe, Mn, and O, but also degradation products enriched in Cl, P and Ca, which are elements present in the HBSS, show up. Silver was not detected on the degradation surface, which suggests that it is not prone to be released during incubation in HBSS. In the first stages of degradation, namely after 14 days of immersion the corrosion products formed on the surface are enriched in Ca–P clusters, forming a flaky structure that could be identified as calcium phosphate-based apatite. These clusters tend to disappear at longer immersion periods, being replaced by different corrosion products.

The globular precipitates, shown in Fig. 3f and h mainly consist of O, Mn, Ca and P. Increasing immersion period leads to the formation of a relatively thick oxidation layer, as shown in Fig. 3c and h. Even though the layer has the appearance of compact and homogenous, more detailed analysis (Fig. 3c) reveals a cracked structure caused most probably by dehydration due to air-drying after removal from HBSS.<sup>44,45</sup> The cracked oxide layer is prone to detachment, as shown in Fig. 3h. After detachment, the exposed area allowed HBSS to penetrate further into the pores. As shown in Table 2, the degradation layer of samples incubated for 84 days (Fig. 3d and h) contains high levels of Cl<sup>−</sup>, namely 12.89 and 15.4 wt% for FeMn and FeMn–5Ag, respectively. The Cl<sup>−</sup> ions are aggressive to the surface of

the alloy and they can break the passive oxidation layer, therefore leading to the formation of the pits.<sup>2,23,46</sup> As can be seen in Fig. 3d, Cl-rich precipitates have formed on the exposed surface of FeMn alloys, most probably after Cl<sup>−</sup> ions have broken the oxide layer.

To gain further insight into the degradation products formed on the surfaces upon immersion, XRD analysis was performed on the FeMn and FeMn–5Ag samples after 7 and 28 days of immersion, as presented in Fig. S5 (ESI<sup>†</sup>). The XRD patterns obtained after 7 days did not demonstrate any additional features compared with the as-prepared samples (Fig. 1b). This can be explained by the thin nature of the oxide layer as well as the less abundant presence of other corrosion products. However, after 28 days of immersion, an increase in the intensity of some of the peaks was observed. Corrosion products such as MnPO<sub>4</sub>, MnCO<sub>3</sub>, Fe<sub>2</sub>O<sub>3</sub> and MnO were identified. It is worth noting that some of the oxide peaks can overlap, therefore we might expect more corrosion products on the surface.

To unveil the degradation mechanism, cross-sections of all tested alloys immersed for 14, 28, 56 and 84 days in HBSS were examined (Fig. 4). One can observe the formation of a degradation layer at the utmost sample surface, which is particularly visible in the samples immersed for 14 and 28 days, with a thickness of around 30 μm. This layer was presumably cracked due to dehydration upon air drying or during the curing of the mounting resin, as mentioned before.<sup>11,27</sup> Remarkably, this layer becomes thinner or even disappear for longer immersion periods.

At the same time, an increase of the incubation period favors higher penetration of the HBSS towards the bulk of the material since the HBSS is able to permeate through the open-pore structure of the pellets. As a result, corrosion products formed inside the pores, mainly consisting of iron and manganese oxides, as shown in Fig. S6 (ESI<sup>†</sup>). Brighter contrast (due to charging) is associated with low-conducting corrosion products made of O, Ca, P and Cl. When comparing individual alloys, the degradation layer of Ag-containing alloys seems to be thinner after 56 and 84 days of immersion than that of FeMn. Nevertheless, at the same time, the corrosion products consist mainly of metal hydroxides and carbonates, accompanied by calcium, phosphorus

Table 2 Chemical composition of areas and points indicated in Fig. 3 as measured by EDS

Area/point		Elements (wt%)					
		Fe	Mn	O	Cl	P	Ca
FeMn	1	34.6	22.5	38.3	0.5	3.1	1.0
	2	19.3	20.1	30.9	12.9	8.4	8.4
FeMn–5Ag	3	23.4	—	68.9	0.5	3.8	3.4
	4	12.5	22.3	38.3	—	8.8	18.2
	5	40.5	27.1	31.1	1.4	—	—
	6	27.9	21.1	42.2	0.8	5.8	2.2
	7	6.3	42.9	25.1	15.4	—	10.4





Fig. 4 Cross-section SEM images of FeMn, FeMn-1Ag, FeMn-3Ag and FeMn-5Ag specimens after 14, 28, 56 and 84 days of immersion in HBSS. 'S' stands for sample and 'DL' to the degradation layer formed on top of the sample.

and chlorine elements, as mentioned before.<sup>11,20,47</sup> The amount of Cl is notably higher in the Ag-containing alloys which can be attributed to localized pitting. Since porosity increases with Ag content, the Cl-rich corrosion products are accumulated inside the pores to a larger extent in samples with higher Ag wt%.

The EDS compositional maps of the cross-sections of the alloys immersed for 84 days, presented in Fig. S6 (ESI<sup>†</sup>), show the distribution of Fe and Mn alloying elements as well as of foreign elements that have accumulated inside the pores. Fe and Mn are uniformly distributed in the matrix, but the upper degradation layer shows a lower concentration of those elements than the bulk. The abundant presence of oxygen was observed, both in the degradation layer and the sample, mostly inside the pores. Other elements, such as Ca, Cl and P, were also present, mostly in the degradation layer. The EDS analyses performed in the degradation layer show that its Mn content is lower than that of Fe. For example, in the case of FeMn-5Ag, the content of Fe was around 4–5 times higher than the content of Mn (33.3 wt% of Fe and 8.5 wt% of Mn after 14 days of immersion, and 51.7 wt% of Fe and 11.1 wt% of Mn after 84 days of immersion). This suggests that the release of Mn ions to the HBSS occurs easier than the release of Fe ions.

**3.1.3 Released ion concentration.** It has been shown before that addition of noble elements, such as Pt and Pd,<sup>15,28,42</sup> to the

Fe–Mn matrix can lead to the formation of micro galvanic cells that can cause an accelerated corrosion rate. Additions of Ag particles into the Fe-based alloys have also been tested, but so far mixed results have been reported in the literature. Most of the studies have shown an enhanced corrosion rate<sup>22–24</sup> but a neutral effect has also been observed,<sup>26,27</sup> as well as a decrease in corrosion rate in Fe–Ag alloys.<sup>48</sup> Silver has a more positive standard corrosion potential compared to Fe and Mn, hence it can be assumed that during corrosion the Fe–Mn matrix acts as an anode, and it is therefore oxidized, while the Ag particles act as a cathode and thus accept electrons.<sup>23,30</sup> Another possible mechanism to explain the role of Ag in the acceleration of the corrosion rate is that during corrosion of the Fe–Mn matrix, the Ag particles will form a separate phase, which has the ability to break the Fe oxide layers.<sup>23,43</sup>

The concentration of Fe and Mn ions released from the materials into the HBSS after 7, 14, 28, 56 and 84 days of immersion is presented in Fig. 5a and b. It can be observed that Mn release is higher than Fe release for all tested alloys and that the ion concentration increases with the immersion time, which is in agreement with the previously shown EDS results. Mn is more prone to be released due to a lower standard corrosion potential when compared with Fe (*i.e.*, Mn<sup>2+</sup> ions are more susceptible to be released to the HBSS than Fe<sup>2+</sup> or Fe<sup>3+</sup>).





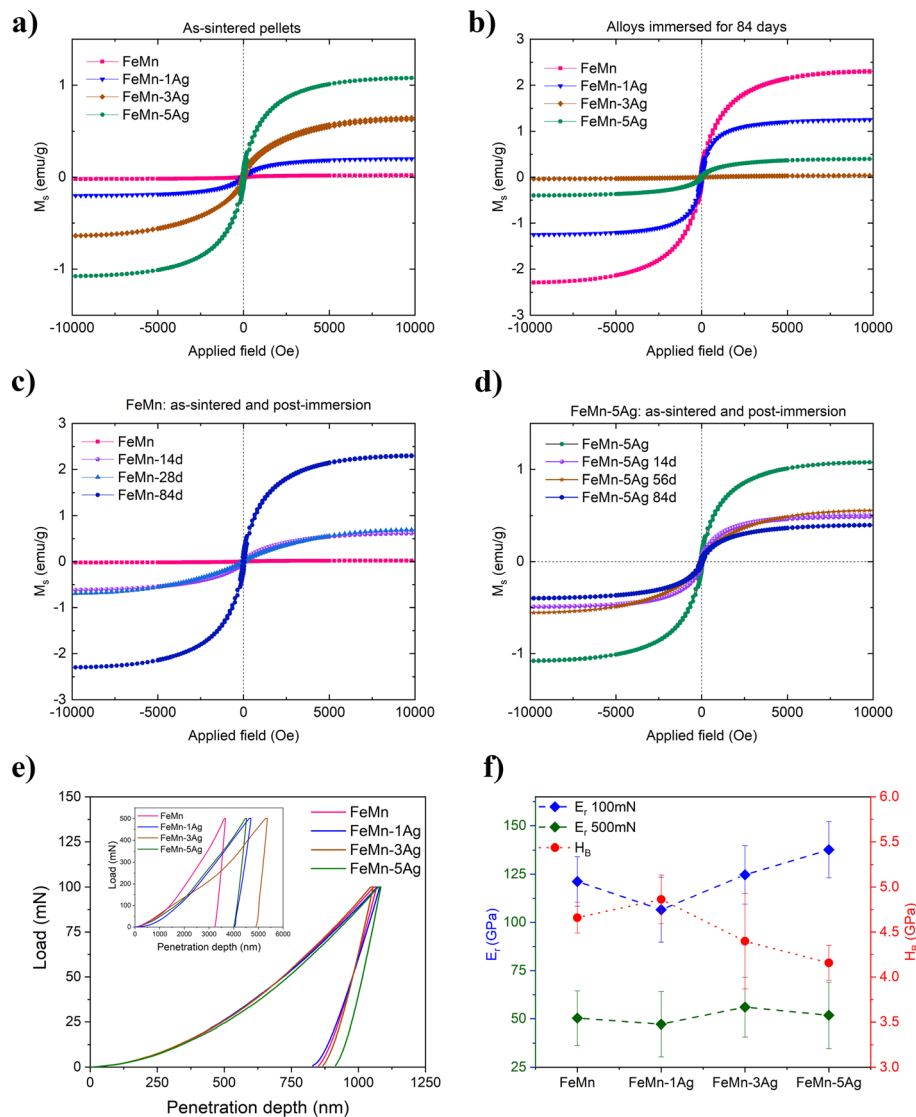


Fig. 6 Magnetization curves (a–d) for (a) as-sintered samples, (b) alloys after immersion in HBSS for 84 days, and detailed changes of magnetization upon immersion for different periods for (c) FeMn and (d) FeMn–5Ag. Load-displacement curves (e) of the FeMn(–xAg) alloys under 100 and 500 mN (insert) maximum applied loads, and (f) corresponding reduced Young's modulus ( $E_r$ ) under 100 mN (blue) and 500 mN (green) of maximum applied loads, together with hardness ( $H_B$ ) (red). Please note the different Y-axis scales between the panels.

influence of both levels of porosity (macro and nano) on the reduced Young's modulus ( $E_r$ ) and hardness ( $H_B$ ). Representative load-displacement curves of tested alloys, together with calculated values of  $E_r$  and  $H_B$  are presented in Fig. 6e, f and Table 3, respectively. The load-displacement curves (Fig. 6e) show higher variability under higher load, which is mainly due to different levels of macroporosity among the tested alloys. On the other

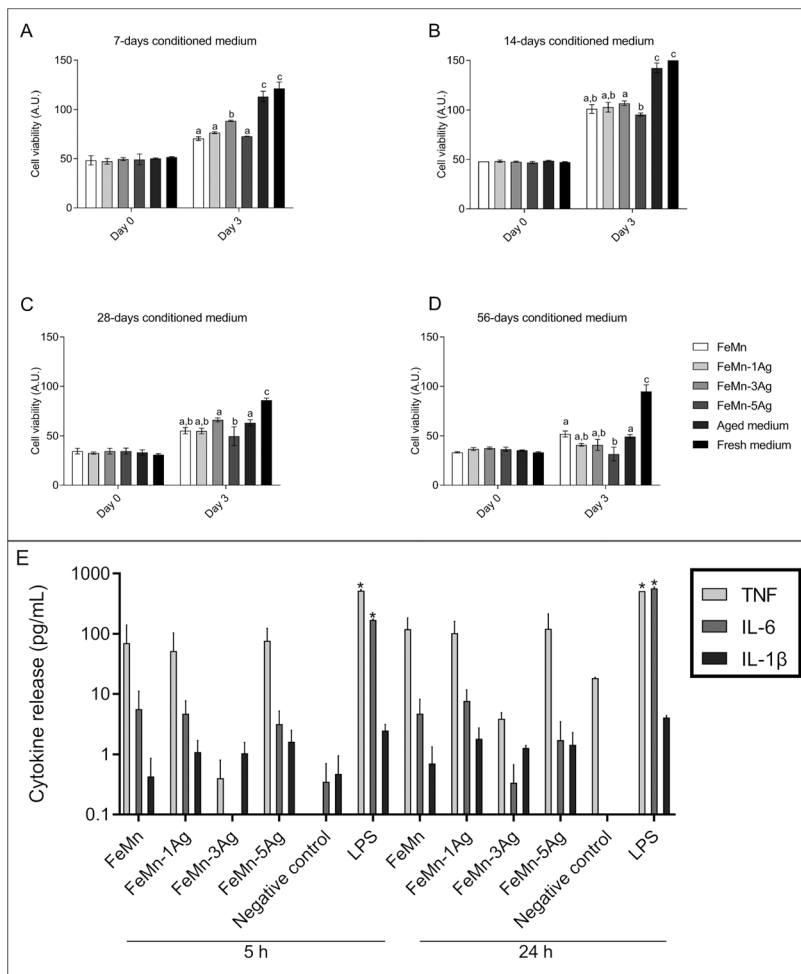
hand, the curves obtained under 100 mN of maximum load almost overlap and the resulting values of  $E_r$  are more similar among the various samples, as shown in Fig. 6f. The nanoindentation tests conducted under a maximum applied load of 500 mN show that the alloys have a comparable value of  $E_r$ , around 50 GPa, which is relatively close to that of a human bone (cortical bone has Young's modulus value of around 20 GPa).<sup>55</sup> The

Table 3 Values of Young's modulus ( $E_r$ ), hardness ( $H_B$ ),  $H_B/E_r$  ratio and energies of deformation ( $W_{\text{elast}}/W_{\text{total}}$  and  $W_{\text{plast}}/W_{\text{total}}$ ) for FeMn, FeMn–1Ag, FeMn–3Ag and FeMn–5Ag alloys

Sample	$E_r$ (500 mN) GPa	$E_r$ (100 mN) GPa	$H_B$ (GPa)	$H_B/E_r$ (wear resistance)	$W_{\text{elast}}/W_{\text{total}}$	$W_{\text{plast}}/W_{\text{total}}$
FeMn	50.39 ± 14.16	121.24 ± 12.84	4.66 ± 0.17	0.038 ± 0.003	0.22 ± 0.03	0.78 ± 0.03
FeMn–1Ag	47.24 ± 16.86	106.60 ± 16.82	4.86 ± 0.27	0.046 ± 0.008	0.26 ± 0.04	0.74 ± 0.03
FeMn–3Ag	56.09 ± 15.36	124.65 ± 15.13	4.40 ± 0.53	0.035 ± 0.006	0.20 ± 0.03	0.80 ± 0.10
FeMn–5Ag	51.81 ± 17.13	137.67 ± 14.58	4.16 ± 0.19	0.030 ± 0.003	0.18 ± 0.02	0.82 ± 0.04







**Fig. 7** Cell proliferation of Saos-2 cells before (day 0) and after (day 3) culture with media conditioned for 7 (A), 14 (B), 28 (C) and 56 (D) days with FeMn, FeMn-1Ag, FeMn-3Ag and FeMn-5Ag alloys, measured by Alamar Blue fluorescence. Aged medium, consisting of DMEM medium incubated for the same incubation times and conditions but without any alloy, and fresh medium were used as controls. Bars with different alphabetical superscripts are significantly different from each other ( $p < 0.05$ ), whereas bars with the same alphabetical superscripts are not significantly different from each other. (E) Logarithmic representation of cytokine release by macrophages exposed to 56 days FeMn(-Ag) conditioned media. Secretion was analyzed by the CBA test at 5 and 24 h of culture. LPS: Lipopolysaccharide, positive control. Asterisks above the columns indicate significant differences compared with the negative control.

was higher than 90% in all cases. These results are in agreement with Alamar Blue results, and suggest that, while FeMn(-xAg) materials might interfere with cell proliferation, they are not cytotoxic.

The cytotoxicity of biodegradable metallic alloys is mainly attributed to the released degradation particles<sup>58</sup> and metallic ions<sup>13</sup> which can promote or inhibit both cell proliferation and metabolic activities. In addition, the corrosion of porous alloys can produce a general change in pH and osmolarity that can contribute to a decrease in the cell viability. The results obtained showed that media conditioned with the FeMn-5Ag alloy reduced cell proliferation compared with the other alloys containing less amount of Ag, indicating that certain amount of silver can alter cell proliferation. In fact, the FeMn-5Ag alloy was the one with the highest Ag release after 84 days in Hank's solution ( $6.35 \mu\text{g L}^{-1}$ ).

The effect of Ag on cell viability is controversial and depends on the state of the Ag, *i.e.* whether Ag is present in the culture as

a salt, as nanoparticles, or as part of the alloy. In our case, Ag was incorporated within the FeMn matrix in the form of particles during the fabrication step.

It has been shown that the metal salt  $\text{AgNO}_3$  produces a high cytotoxic effect in mammalian cell cultures,<sup>59,60</sup> with an  $\text{IC}_{50}$  of  $\text{AgNO}_3$  in the range of  $2\text{--}11 \mu\text{M}$  ( $300\text{--}1900 \mu\text{g L}^{-1}$ ) depending on the cell type.<sup>61</sup> On the other hand, studies using nanoparticles concluded that  $6 \mu\text{M}$  silver nanoparticles do not have a significant impact on cytotoxicity of epithelial cells.<sup>62</sup> Similar results were observed specifically using osteoblasts, as the presence of silver nanoparticles coating-alloys did not induce any cytotoxic effect on MG63 osteoblasts.<sup>63,64</sup>

Our results showed values at the  $0.88\text{--}6.35 \mu\text{g L}^{-1}$  range of Ag released from the alloys. The highest values observed for FeMn-5Ag after 84 days were far from the concentrations considered cytotoxic. In this regard, our cytotoxic results are in agreement with those of previous studies (viability higher



than 90%). However, the cell proliferation rate was reduced when osteoblasts were cultured in the presence of the alloy-conditioned media. After culture with 7 and 14 days conditioned medium, a decrease in cell proliferation was observed regardless of the alloy, which indicates that not only Ag can interfere with the cell cycle. The presence of Fe and Mn ions, or the relatively high corrosion rate could be responsible for the reduced proliferation rate. The high corrosion rate could increase the pH and osmolality, as previously observed.<sup>65</sup> The release of Ag from the FeMn-5Ag after 28 and 56 of media conditioning could also contribute to the lowest cell proliferation observed.

### 3.5 Inflammatory cytokine response

To find out whether the ions released by alloys activate the secretion of inflammatory cytokines, 56 days conditioned medium was added to macrophage cultures. We analyzed the pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF, which are commonly produced by macrophages in response to bacterial endotoxin or LPS.<sup>66</sup> According to Chanput *et al.*, stimulating THP-1 macrophages with LPS results in a dramatic increase in the secretion of IL-1 $\beta$ , IL-6, and TNF, which reaches a plateau after 18 h of stimulation.<sup>67</sup>

Results of the CBA showed that ions released by FeMn(-xAg) alloys did not activate the secretion of IL-1 $\beta$ , IL-6 or TNF in macrophages when compared with a non-stimulated control. However, when macrophages were exposed to LPS (positive control), an increase in the concentration of the three cytokines was detected in the culture medium, even though IL-1 $\beta$  increase was not statistically significant (Fig. 7e). Ubanako *et al.* also obtained the same results when using low amounts of LPS to stimulate THP-1 derived macrophages, that is, little or

no secretion of IL-1 $\beta$ , IL-6 and TNF.<sup>68</sup> It has been reported that the presence of metal debris and even low concentrations of metal ions can induce an inflammatory response.<sup>69</sup> In this regard, it has been observed that the release of Fe, Mn and Ag could induce the secretion of inflammatory cytokines; Fe and Mn are considered non-allergenic elements, although Ag is.<sup>70</sup> However, the results obtained for cytokine release indicated that concentrations lower than 0.5 mg L<sup>-1</sup> of Fe, 2.3 mg L<sup>-1</sup> of Mn and 0.9  $\mu$ g L<sup>-1</sup> of Ag did not induce the inflammatory response in macrophages.

### 3.6 Microbial interactions with FeMn(-xAg)

In this investigation, the effect of FeMn and FeMn-5Ag alloys on *S. aureus* viability and biofilm formation was tested. The addition of silver was tested due to its widely known antimicrobial capacity.<sup>71,72</sup> Biofilm biomass and thickness were assessed using confocal laser-scanning microscopy (CLSM) (Fig. 8A–D) and fluorescence plate reading (Fig. 8E–H) after 4 and 24 h of growth. Moreover, representative images of the biofilm formed on FeMn and FeMn-5Ag, obtained using CLSM are shown (Fig. 8I and J).

When comparing the FeMn-5Ag to the FeMn material, whilst no significant difference in biofilm viability was observed at 4 h, a significant reduction in the total live and dead biomass was observed at the 24 h time point (Fig. 8A and B). Importantly, the total biofilm thickness on FeMn-5Ag was directly comparable to that of the titanium control (Fig. 8C and D), known for its biocompatibility in bone-implant applications, showing analogous performance of the FeMn-5Ag materials.

According to the fluorescence plate reading data, at 4 h the total biofilm viability was unaffected by the alloying of 5% silver compared to FeMn (Fig. 8E and F). A reduction in biofilm



**Fig. 8** *Staphylococcus aureus* ATCC 25923 biofilm morphology on FeMn-5Ag compared to FeMn and machined titanium (Ti) controls after 4 and 24 h incubation, analysed using quantitative confocal laser-scanning microscopy (CLSM) to assess biofilm biomass (A and B) and thickness (C and D). Fluorescence intensity of total live and dead cells in biofilms grown for 4 h (E and F) and 24 h (G and H) on FeMn-5Ag, FeMn and Ti using fluorescence plate reading. Bars with different alphabetical superscripts are significantly different from each other ( $p < 0.05$ ), whereas values with the same alphabetical superscripts are not significantly different from each other. CLSM images of the biofilm formed on the surface of FeMn (I) and FeMn-5Ag (J) alloys (NIS-Elements Viewer, Nikon). Green: Live *S. aureus* cells; Red: Dead *S. aureus* cells.







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