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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 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.^{1–6} This class of materials is expected to corrode gradually *in vivo* once they have fulfilled their role.^{2,7} 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.⁸ 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.⁹ Among various tested alloys, Mg-based and Fe-based alloys are especially attractive due to their very good biocompatibility.^{2,7} 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 H₂ 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,^{10–13} however, the degradation rate is too slow to be applied as biodegradable implants.^{7,14} 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),^{11,13,15} using new fabrication methods to tune the microstructure such as selective laser melting (SLM),^{16,17}

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introducing porosity,^{17–21} adding small amounts of noble elements,^{22–25} or adding C to promote twinning-induced plasticity (TWIP),^{7,26–28} 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.^{22,25} 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,^{22–24,29} 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.²⁷ Similarly, one can find dissimilar conclusions on the effect of silver on antibacterial activity. In the report by Liu³⁰ no antimicrobial properties were confirmed in Fe70.3–Mn28.9–Ag0.8 alloy, while in the research published by Sotoudehbagha²² 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 5×10^{-5} mbar for 4 h at a heating rate of 1 °C min⁻¹. 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,^{18,31} 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.³¹

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 α 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),³² 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 ± 1 °C. Each sample, with a surface area of approximately 100 mm², 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⁻². 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 CaCl₂, MgSO₄, KCl, KH₂PO₄, NaHCO₃, NaCl, Na₂HPO₄ 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 HNO₃. 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.





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.^{39,40} The morphologies of the green pellets, *i.e.* pressed powders before sintering, are shown in Fig. S2 (ESI[†]). 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- γ (FeMn) and hcp- ϵ (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.^{18,41} In the tested materials, two different levels of porosity can be distinguished – namely macroporosity with an average pore size of around 10 μm – 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.⁴²

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[†]), show that Ag precipitates exhibit a polydisperse distribution within a size range of 0.5–3 μm . Larger agglomerates of Ag were observed, particularly in the FeMn–5Ag sintered alloy, as shown in Fig. S4b (ESI[†]). The presence of Ag-rich precipitates could in principle enhance the micro galvanic corrosion, hence inducing faster degradation rate of tested alloys.^{22–24}

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;³⁹ 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 (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.^{44,45} 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[−], namely 12.89 and 15.4 wt% for FeMn and FeMn–5Ag, respectively. The Cl[−] 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.^{2,23,46} As can be seen in Fig. 3d, Cl-rich precipitates have formed on the exposed surface of FeMn alloys, most probably after Cl[−] 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[†]). 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 MnPO₄, MnCO₃, Fe₂O₃ 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.^{11,27} 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[†]). 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.^{11,20,47} 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[†]), 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,^{15,28,42} 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^{22–24} but a neutral effect has also been observed,^{26,27} as well as a decrease in corrosion rate in Fe–Ag alloys.⁴⁸ 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.^{23,30} 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.^{23,43}

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.*, Mn²⁺ ions are more susceptible to be released to the HBSS than Fe²⁺ or Fe³⁺).



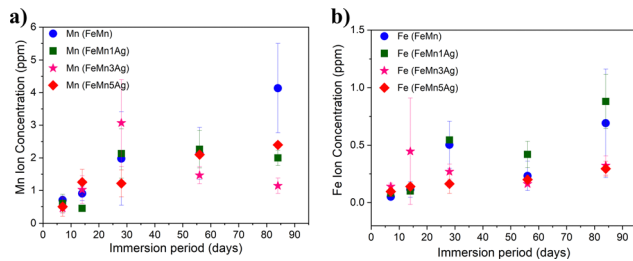


Fig. 5 Concentration of (a) Mn and (b) Fe ions released into the HBSS at different immersion periods as measured with ICP-MS. Please note the different Y-axis scale in panels of Mn (a) and Fe (b) release.

In the first stage, up to 28 days of immersion, a sharp increase in the release is observed, especially in the case of Mn for all tested specimens and it tends to slow down upon further immersion. Nevertheless, an increase in both Mn and Fe release after 84 days of immersion is observed in the case of FeMn and FeMn-5Ag, as well as an increase of Fe release of the FeMn-1Ag sample.

However, no significant differences in the amounts of released ions were observed between the tested alloys. The silver-containing alloys did not show an increase in the ion release of either Fe or Mn when compared to the alloy without Ag. Similar behavior has already been observed in literature with Fe-Mn-C-xAg alloy.^{26,27} Apparently, Ag had a negligible influence on the biodegradability of the material. Although Ag could in principle induce microgalvanic corrosion in contact with the less noble FeMn environment, the amounts of leached Fe and Mn ions in FeMn and FeMn-5Ag, as determined by ICP, were similar. This suggests that other effects, likely the porosity, play a major role.

The trend in the curve's shape of the ion concentration indicated a linear dependence up to 28 days and then a more stable increase during a longer immersion period. It can be mainly attributed to the degradation products formed on the surface that are partially blocking the dissolution of Fe and Mn at longer immersion periods. Due to the same reason, the corrosion rate does not increase with the immersion time for the Ag-containing alloys, as the release of silver is being blocked by corrosion products formed on the surface and inside the pores. Nevertheless, the release of silver was also measured, and it was 3 orders-of-magnitude lower than the release of Fe and Mn. Namely, the highest Ag release was observed for the FeMn-5Ag and was $1.23 \pm 0.21 \mu\text{g L}^{-1}$ after 14 days of immersion, then it decreased to $0.88 \pm 0.22 \mu\text{g L}^{-1}$ after 56 days of immersion and again increased up to $6.35 \pm 3.65 \mu\text{g L}^{-1}$ after 84 days in HBSS. In the FeMn-1Ag and FeMn-3Ag, the highest concentration of Ag was also observed after 84 days of immersion and was $1.65 \pm 0.55 \mu\text{g L}^{-1}$ and $1.3 \pm 0.34 \mu\text{g L}^{-1}$, respectively. Thus, as could be suspected, the release of Ag ions increases with the initial Ag content. The increased amount of Ag released after 84 days of immersion, when compared with the amount released after 56 days, can be connected with the increased amounts of Cl-rich precipitates on the degradation surface that can contribute to breaking the degradation layer and, hence, enabling further degradation.

A previous study reported that Fe-Mn alloys (Fe35Mn and Fe25Mn), obtained through powder sintering and cold rolling

and resintering cycles, released around 1.5 ppm and 2 ppm for Mn and Fe ions after 84 days of immersion, respectively.¹¹ In another report, FeMnSiPd alloys obtained through arc melting,¹⁵ released around 1.5 ppm for both elements up to 120 days of immersion. The release of ions in our tested alloys was higher, presumably due to the higher level of porosity, which enhances the degradation.

3.2 Magnetic properties

The magnetic behavior of as-sintered and immersed alloys is presented in Fig. 6a-d. The as-sintered alloys (Fig. 6a) show very low saturation magnetization, with values of 0.019, 0.199, 0.626 and 1.077 emu g^{-1} for FeMn, FeMn-1Ag, FeMn-3Ag and FeMn-5Ag, respectively. For comparison, the value of saturation magnetization (M_S) of the 304 austenitic stainless steel SS304, commonly used as implant material is 1.25 emu g^{-1} .^{36,49} On the other hand, the M_S value of pure iron is as high as 221.7 emu g^{-1} .⁵⁰ This means that the FeMn-5Ag alloy, which has the highest value of M_S among the tested alloys, has only 0.49% the magnetization of Fe. In fact, the Fe-based alloys with manganese content above 30 wt% have been reported to be antiferromagnetic as they consist mainly of antiferromagnetic γ -austenite and ϵ -martensite phases.^{51,52} therefore enhancing the materials' compatibility with MRI imaging. In our case, the M_S value increased with higher Ag content, which might be attributed to slightly higher porosity of those alloys (which in turn could lead to higher amount of Fe_2O_3 after sintering).^{53,54}

Following immersion in HBSS, the magnetization of FeMn progressively increased with the immersion time, as shown in Fig. 6c. The alloys incubated for 84 days (Fig. 6b) showed a M_S value of 2.301, 1.249, 0.0367 and 0.396 emu g^{-1} for FeMn, FeMn-1Ag, FeMn-3Ag and FeMn-5Ag, respectively. When comparing the gradual differences in the magnetic properties upon increasing immersion time, a similar trend was noticed in the FeMn alloy – the lowest value of magnetization is shown by the as-sintered material and it increased gradually – after 14 and 28 days the value was similar, around 0.690 emu g^{-1} , with an increase up to 2.290 emu g^{-1} after 3 months of immersion. This observation can be ascribed to a higher release of Mn than Fe ions, which leads to a higher level of Fe-enriched regions in the material than before immersion. On the contrary, in the case of the FeMn-5Ag, an increase of the incubation time led to a small decrease in the M_S values, starting from 1.077 emu g^{-1} for as-sintered alloy and then decreasing, firstly to around 0.550 emu g^{-1} after 14 days of immersion and then to a minimum value of 0.393 emu g^{-1} after 3 months of immersion. In any case, even though the M_S values change upon degradation, the level of M_S is sufficiently low to assure good compatibility of the alloys with MRI.

3.3 Mechanical properties

Nanoindentation was used to analyze the micro/nano-mechanical properties of the tested alloys. The tests were conducted under two different maximum loads, namely 100 and 500 mN. The lower load was used to extract information from the nanoporous regions of the material, while the higher loads may be able to assess the





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).⁵⁵ The

Table 3 Values of Young's modulus (E_r), hardness (H_B), H_B/E_r ratio and energies of deformation (W_{elast}/W_{total} and W_{plast}/W_{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_{elast}/W_{total}	W_{plast}/W_{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 particles⁵⁸ and metallic ions¹³ 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 AgNO_3 produces a high cytotoxic effect in mammalian cell cultures,^{59,60} with an IC_{50} of AgNO_3 in the range of 2–11 μM ($300\text{--}1900 \mu\text{g L}^{-1}$) depending on the cell type.⁶¹ On the other hand, studies using nanoparticles concluded that 6 μM silver nanoparticles do not have a significant impact on cytotoxicity of epithelial cells.⁶² Similar results were observed specifically using osteoblasts, as the presence of silver nanoparticles coating-alloys did not induce any cytotoxic effect on MG63 osteoblasts.^{63,64}

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.⁶⁵ 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 β , IL-6 and TNF, which are commonly produced by macrophages in response to bacterial endotoxin or LPS.⁶⁶ According to Chanput *et al.*, stimulating THP-1 macrophages with LPS results in a dramatic increase in the secretion of IL-1 β , IL-6, and TNF, which reaches a plateau after 18 h of stimulation.⁶⁷

Results of the CBA showed that ions released by FeMn(-xAg) alloys did not activate the secretion of IL-1 β , 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 β 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 β , IL-6 and TNF.⁶⁸ It has been reported that the presence of metal debris and even low concentrations of metal ions can induce an inflammatory response.⁶⁹ 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.⁷⁰ However, the results obtained for cytokine release indicated that concentrations lower than 0.5 mg L⁻¹ of Fe, 2.3 mg L⁻¹ of Mn and 0.9 μ g L⁻¹ 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.^{71,72} 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

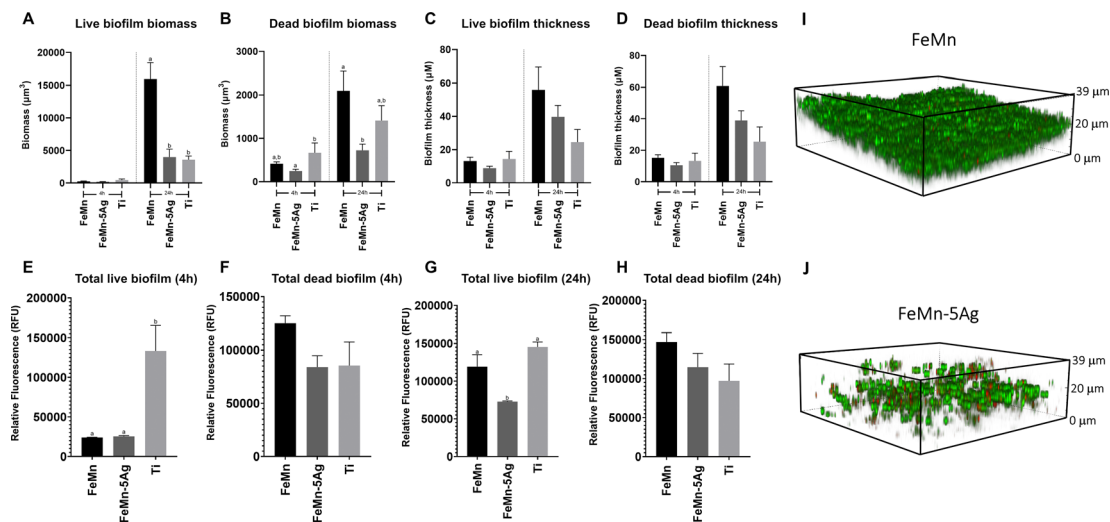


Fig. 8 *Staphylococcus aureus* ATCC 25923 biofilm morphology on FeMn-5Ag compared to FeMn and machined titanium (Ti) controls after 4 and 24 h incubation, analysed using quantitative confocal laser-scanning microscopy (CLSM) to assess biofilm biomass (A and B) and thickness (C and D). Fluorescence intensity 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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