Toxicity Assessment of Fe-Mn-Al Ternary Alloys Using a Probit Dose-Response Model and an Augmented Simplex Design

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This study establishes a toxicity assessment of the mixture of Fe^{3+} , Mn^{2+} , and Al^{3+} ions by employing a Probit dose-response model and an augmented simplex design. The toxicity of individual Fe^{3+} , Mn^{2+} , and Al^{3+} metal ions with respect to *Escherichia coli* DH5 α is in the order of $\text{Mn}^{2+} > \text{Fe}^{3+} > \text{Al}^{3+}$. The mixture of Fe^{3+} , Mn^{2+} , and Al^{3+} ions shows a hump region and a saddle region in the IC_{50} values contour plot. Fe-Mn-Al ternary alloys may possess a lower toxic risk when the ratio of released metal ions approaches the hump region of the contour plot. Fe-Mn-Al ternary alloys may present an increased toxic risk when the ratio of released metal ions gets close to the saddle regions of the contour plot. [doi:10.2320/matertrans.M2014108]

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1. Introduction

Fe-Cr-Ni stainless steels, Co-Cr based alloys, pure Ti metal, and Ti-6Al-4V alloys are traditionally used as biocompatible metallic materials in biomedical applications such as replacing hard tissue.¹⁾ Among them, stainless steels are typically used as implant materials for normal medical and dental applications, joint replacement, fixation of bone fractures, and for orthopedic purposes because of their low cost, good mechanical properties and excellent corrosion resistance.²⁻⁴⁾ However, interactions between stainless steel and living tissues usually lead to corrosion of the chromium oxide layer and release nickel ions, which may disturb the proliferation and differentiation relationship in osteoblastic human alveolar bone cell cultures and induce undesirable allergies or cancer.⁵⁻⁷⁾ Recently, shape memory alloys (SMAs) have been extensively developed in advanced medical applications because of their unique shape memory effect and superelasticity.⁸⁾ Compared to other SMAs, nickel-titanium (TiNi) SMAs are the most widely investigated because they could offer good biofunctionality for particular biomedical applications, such as in laparoscopic surgery, intracoronary stents, ligament replacement, shape memory microvalves for the control of drug delivery, and for bone stamp and osteosynthesis devices.⁹⁻¹¹⁾ Unfortunately, the degradation of TiNi SMAs implants also involves the release of nickel ions, severely restricting their practical biomedical applications.¹²⁾

The biocompatibility characteristics of biomaterials should be carefully considered when they are used for implant materials. Shettlmore and Bundy¹³⁾ reported that the toxicity of aqueous metal solutions is representative of the ionic degradation products of orthopedically implanted 316L stainless steel by a Microtox[®] bacterial bioluminescence assay. Their study developed a mathematical model to predict the toxicity of mixtures of Ni^{2+} , Cr^{3+} , and Fe^{3+} ions based on the proportions of the metal ions determined through selective leaching of 316L stainless steel. They then proposed

that the ions present in this ratio exhibit antagonistic interactions, resulting in a decrease in the toxicity of degradation products from 316L stainless steel. The advantage of this method is that the toxicity of the implant materials can be rapidly and economically predicted and analyzed using bacteria prior to toxicology studies under *in vivo* conditions using animals. However, this method can only determine the toxicity of three-component Fe-Ni-Cr ionic systems at a specific concentration ratio. Consequently, it is extremely costly and time-consuming to determine the toxicities of other biomedical materials also composed of Fe, Cr, and Ni ions but at different proportions. In order to overcome this shortcoming, Chang *et al.*¹⁴⁾ established a toxicity assessment of the mixture of Fe^{3+} , Cr^{3+} , and Ni^{2+} ions using an augmented simplex design to verify the toxicity of releasing Fe^{3+} , Cr^{3+} , and Ni^{2+} at a various combination of concentrations.

Fe-Mn-Al alloys have attracted research attention for many years because of their specific properties, such as high strength, high ductility and good corrosion resistance; these alloys are economical and can be used to replace conventional Fe-Cr-Ni stainless steels.¹⁵⁻¹⁸⁾ Recently, Omori *et al.* reported that Fe-Mn-Al alloys with specific chemical compositions or with the addition of a small amount of Ni into polycrystalline Fe-Mn-Al, can exhibit shape memory effect and superelasticity.^{19,20)} Consequently, Fe-Mn-Al alloys are promising candidates to serve biomaterials because they possess the advantages of both Fe-Cr-Ni stainless steels and TiNi SMAs, but contain a much lower nickel concentration. The main purpose of this study is therefore to investigate the toxicity effects of Fe^{3+} , Mn^{2+} and Al^{3+} metal ions on bacteria (*Escherichia coli* DH5 α) by Probit dose-response analysis and augmented simplex design to simulate with an assess the toxicity of the mixture of Fe^{3+} , Mn^{2+} , and Al^{3+} ions at different concentration ratios.

2. Experimental Procedures

2.1 Materials and methods

The Fe, Mn, and Al metal ions used in this study were high-purity atomic absorption spectroscopy (AAS) standard

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solution (1,000 mg/L) purchased from Merck. Each AAS solution was diluted to various concentrations, and mixed with sodium citrate buffer solution, $0.5 \times \text{LB}$, and deionized water to formulate metal ion solutions ranging between 0 and 1,000 mg/L. The toxic levels of the selected ion concentrations for the *Escherichia coli* DH5 α were appropriate in the context of typical mammalian cells research reported before, in which the metallic ion concentrations were approximately 100 ppm.^{21–23} The *Escherichia coli* DH5 α used in this study was provided by Professor Bor-Yann Chen, NIU, Taiwan. *Escherichia coli* DH5 α was pre-cultured in Luria–Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C, 125 rpm for 12 h. Cultured *Escherichia coli* DH5 α solutions were then added to each metal ion solution at a volume ratio of 1 : 100. The concentration of *Escherichia coli* DH5 α was determined by measuring the cell solution optical density (OD) value every 30 min for the initial 5 h, and then every 1 h up until 24 h. The cell solution OD value was measured at 600 nm (OD_{600}) in a spectrophotometer (GENESYS 20, Thermo Scientific) using cultured broth as the blank cuvette.

2.2 Dose-response analysis

The dose-response curves of Fe, Mn, and Al metal ions were analyzed using a Probit dose-response model.²⁴ The Probit dose-response model suggests that the tolerance capacity of bacteria in response to toxic material in a given population exhibits a log-normal distribution. The mid-point inhibitive concentration (IC_{50}) on the dose-response curve is typically selected for metal ion toxicity comparisons because it is simpler to interpolate the mid-point IC_{50} accurately than it is to make extrapolated estimates of IC_0 and IC_{100} . The semi-logarithmic plot of metal ion concentration as a function of the obtained response is assumed to behave as a linear relationship. Thus, the Probit dose-response model converts a sigmoidal-shaped dose-response curve into a linear normal equivalent deviation (NED) scale. For example, 50% and 84.1% responses correspond to NED scale values of 0 and 1, respectively. Additionally, a Probit unit (P) in the model equals a NED scale value plus 5. The concentration formulae are shown as follows:^{24–27}

$$Y = A + B \log Z \quad (1)$$

$$P = \frac{1}{2} \left[1 + \operatorname{erf} \left(\frac{Y - 5}{\sqrt{2}} \right) \right] \quad (2)$$

$$\operatorname{erf}(x) \equiv \frac{2}{\sqrt{\pi}} \int_0^x e^{-\xi^2} d\xi \quad (3)$$

where A and B are the intercept and Hill slope of the dose-response relationship; Z and Y are the metal concentration (mol/L) and Probit unit, respectively; P is the response (%) corresponding to the administered metal; $\operatorname{erf}(x)$ is an error function. Note that the response variable is normalized so as to be located between 0 and 1. The conversion relationship for provoked response is given by Chen *et al.*^{25,26} For example, 55% and 85% responses correspond to 5.13 and 6.04 Probit units, respectively. The relative growth rate can be determined from μ/μ_0 , where μ denotes the maximum specific growth rate and the reference parameters μ_0 are determined from growth curves of zero concentration of a metal-free culture.²⁷ The kinetic parameter μ is specifically

Fig. 1 The $\ln OD_{600}$ values of the *Escherichia coli* DH5 α solutions cultured in medium with various pH values as a function of time.

determined from the growth curves of the designed experimental cultures. Microbes in a metal-laden environment may exhibit a significant decrease in the growth rate of bacterial cultures. All inocula used in this study were selected from pre-cultures in metal-free media.

3. Results and Discussion

3.1 Effect of pH values on the growth of *Escherichia coli* DH5 α

Figure 1 presents the $\ln OD_{600}$ values of *Escherichia coli* DH5 α solutions cultured in medium with various pH values from 3.45 to 6.78 as a function of time. Figure 1 shows that the $\ln OD_{600}$ values for the curves of pH = 4.47, 4.81, 5.14, and 6.78 all generally increase with culture time, indicating that *Escherichia coli* DH5 α grow prosperously when the pH values of the culture media are between 4.47 and 6.78. Figure 1 also shows that the $\ln OD_{600}$ values for the curve of pH = 4.12 only slightly increase with the increase of culture time, suggesting that the growth of *Escherichia coli* DH5 α is retarded. Figure 1 also shows that the $\ln OD_{600}$ values for the curves of pH = 3.54 and 3.74 do not show appreciable difference throughout the culture time. This indicates that the growth of *Escherichia coli* DH5 α is totally inhibited when the pH values of the culture media are 3.54 and 3.74. Accordingly, in this study, we controlled the pH value of the culture medium at pH = 4.8 after mixing the metal ions solutions by adding appropriate volume of sodium citrate buffer solution to ensure the growth of *Escherichia coli* DH5 α will not be affected by the pH value of the culture medium.

3.2 Tolerance capacity of bacteria in response to single metal ion

Figure 2(a) plots the $\ln OD_{600}$ values of *Escherichia coli* DH5 α solutions added with Al^{3+} ion solutions of different concentrations as a function of time. The $\ln OD_{600}$ value of *Escherichia coli* DH5 α without adding any Al^{3+} ion (control group) is also plotted in Fig. 2(a) for comparison. As shown in Fig. 2(a), the $\ln OD_{600}$ values for each curve are extremely small in the first hour. The deferment of the *Escherichia coli*

Fig. 2 (a) The $\ln OD_{600}$ values of the *Escherichia coli* DH5 α solutions added with Al^{3+} metal ion with different concentrations as a function of time. (b) The $\ln OD_{600}$ value as a function of time measured in the exponential growth stage for each curve in (a).

Fig. 3 (a) The relation between Y value and concentration Z and (b) relative growth rate curves of Al^{3+} metal ion predicted by the Probit model.

DH5 α growth at this stage is termed as the lag time. Thereafter, the $\ln OD_{600}$ values for each curve increase significantly until 5 h, which indicates that *Escherichia coli* DH5 α exhibit an exponential growth at this stage. After 5 h, on the other hand, the $\ln OD_{600}$ values for each curve gradually approach a static state until 24 h. Figure 2(a) also shows that the $\ln OD_{600}$ values decrease with increasing Al^{3+} ion concentration when determined at the same time. This feature reveals that the addition of Al^{3+} ion into solutions significantly inhibits the growth of *Escherichia coli* DH5 α . Figure 2(b) plots the $\ln OD_{600}$ values as a function of time for the exponential growth stage of each curve shown in Fig. 2(a). Figure 2(b) shows that each curve exhibits a linear relationship during exponential growth. Therefore, the specific growth rate, μ , for *Escherichia coli* DH5 α with various concentrations of Al^{3+} ion can be determined from the slope of each fitting line. Meanwhile, the maximum specific growth rate μ_0 can be determined from the slope of the fitting line of the control group. Accordingly, the response value can be calculated from the equation $(\mu_0 - \mu)/\mu_0$ from Fig. 2(b), and then changed into Probit value Y and plotted in Fig. 3(a). Consequently, as shown in Fig. 3(a), the relation between the value of Y and concentration Z can be calculated and plotted by means of the Probit model. From Fig. 3(a), the concentration formula for the Al^{3+} ion can be obtained from the fitting line as $Y = -8.35 + 2.33 \log Z$ ($R^2 = 0.994$). From

this equation, the dose-response curve can be plotted from the relation between relative growth rate (μ/μ_0), and concentration Z , as illustrated in Fig. 3(b). From Fig. 3(b), the IC_{50} value for the Al^{3+} ion can be determined to be 19232.8 mol/L. In the same manner, the concentration formulae for Fe^{3+} and Mn^{2+} ions are determined to be $Y = -9.97 + 2.60 \log Z$ ($R^2 = 0.996$) and $Y = -8.30 + 2.38 \log Z$ ($R^2 = 0.993$), respectively. Figure 4 plots all the dose-response curves for single Fe^{3+} , Mn^{2+} , and Al^{3+} ions. From Fig. 4, the IC_{50} values for Fe^{3+} , Mn^{2+} , and Al^{3+} ions are 15692.9, 6891.4, and 19232.8 mol/L, respectively. This indicates that the toxicity of these metal ions with respect to *Escherichia coli* DH5 α is in an order of $Mn^{2+} > Fe^{3+} > Al^{3+}$.

3.3 Tolerance capacity of bacteria in response to solutions containing two or three kinds of metal ions

Figures 5(a) and 5(b) plot the dose-response curves for solutions containing two or three kinds of metal ions, respectively, with different specific concentration ratios. From Fig. 5(a), the IC_{50} value for $Fe^{3+} : Mn^{2+} = 1 : 1$, $Fe^{3+} : Al^{3+} = 1 : 1$, and $Mn^{2+} : Al^{3+} = 1 : 1$ can be determined to be 14590.4, 20379.8, and 32886.5 mol/L, respectively. This indicates that the toxicities of the solutions containing two kinds of metal ions in the ratios of $Fe^{3+} : Al^{3+} = 1 : 1$ and $Fe^{3+} : Mn^{2+} = 1 : 1$ are comparable and are slightly higher than that in the ratio of $Mn^{2+} :$

Fig. 4 Relative growth rate curves of Fe^{3+} , Mn^{2+} and Al^{3+} metal ions predicted by the Probit model.

Fig. 6 A schematic diagram of triangle ternary graph for the augmented mixture design of Fe^{3+} , Mn^{2+} and Al^{3+} ions.

Fig. 5 Relative growth rate curves of Fe-Mn-Al solutions containing (a) two and (b) three of Fe^{3+} , Mn^{2+} and Al^{3+} ions predicted by the Probit model.

$\text{Al}^{3+} = 1 : 1$. Since Mn^{2+} ion possesses the highest toxicity compared to Al^{3+} and Fe^{3+} ions, this unexpected result corresponds to the fact that the interactions between these metal ions may cause synergism, antagonism, or additivity effects in the solutions containing two kinds of metal ions. From Fig. 5(b), the IC_{50} values for $\text{Fe}^{3+} : \text{Mn}^{2+} : \text{Al}^{3+} = 1 : 1 : 1$, $\text{Fe}^{3+} : \text{Mn}^{2+} : \text{Al}^{3+} = 1 : 1 : 4$, $\text{Fe}^{3+} : \text{Mn}^{2+} : \text{Al}^{3+} = 1 : 4 : 1$, and $\text{Fe}^{3+} : \text{Mn}^{2+} : \text{Al}^{3+} = 4 : 1 : 1$ can be determined to be 20693.9, 21958.3, 14332.8, and 17168.3 mol/L, respectively. The fact that the toxicity of $\text{Fe}^{3+} : \text{Mn}^{2+} : \text{Al}^{3+} = 1 : 4 : 1$ is slightly higher than other concentration ratios can be assigned to the higher toxicity of the Mn^{2+} ion. However, this effect is not significant because the complex toxicity interactions between these three metal ions are difficult to determine.

3.4 Toxicity assessment of a mixture of Fe^{3+} , Mn^{2+} , and Al^{3+} ions

The toxicity of the mixture of Fe^{3+} , Mn^{2+} , and Al^{3+} ions on *Escherichia coli* DH5 α can be further assessed by an augmented simplex design, which is the most common type of design for experiments involving mixtures. As shown in Fig. 6, the factorial space constituted by all possible concentration ratios of Fe^{3+} , Mn^{2+} , and Al^{3+} ions is a

triangle, of which the vertices correspond to Fe^{3+} (point A), Mn^{2+} (point B), and Al^{3+} (point C) ions. In addition to the vertices, the triangle's central point (point G), the medium points along the lines connecting the vertices (points D–F), and the medium points between the central point and the vertices (points H–J) are also considered in the augmented simplex design.²⁸⁾ All the responses of points A to J are also determined as the IC_{50} values described in Sections 3.2 and 3.3. These response values can be formulated by a typical quadratic model as

$$\text{IC}_{50} = 17094.97x + 7340.87y + 18218.45z - 4222.14xy - 8674.81xz + 57049.74yz \quad (4)$$

where x , y and z represent the concentration ratios of Fe^{3+} , Mn^{2+} , and Al^{3+} , respectively. Figures 7(a) and 7(b) show the three-dimensional plot and the two-dimensional contour plot, respectively, of the formulated quadratic model. As shown in Fig. 7, the IC_{50} values show a saddle region close to pure Mn (denoted as region I) and a hump region around $\text{Mn}^{2+} : \text{Al}^{3+} = 1 : 1$ (denoted as region II). Accordingly, we can predict that the implant material may increase the toxicity risk when the ratio of metal ions released from it in human tissue is close to region I. On the other hand, implant material may possess a lower toxic risk for prostheses applications

Fig. 7 (a) Three-dimensional and (b) two-dimensional contour plot of the IC_{50} value for the mixture of Fe^{3+} , Mn^{2+} and Al^{3+} ions.

when the ratio of released metal ions approaches region II. However, further dose-response experiments are still required to determine the toxicity of metal ions released from implant materials into surrounding tissues.

4. Conclusions

The toxicity of Fe^{3+} , Mn^{2+} , and Al^{3+} metal ions with respect to *Escherichia coli* DH5 α is in an order of $Mn^{2+} > Fe^{3+} > Al^{3+}$. The IC_{50} values for solutions containing two or three kinds of metal ions exhibit deviations from the expected additive toxicity because the interactions between the metal ions cause synergistic or antagonistic effects. The IC_{50} values contour plot for the mixture of Fe^{3+} , Mn^{2+} , and Al^{3+} ions shows a saddle region close to Mn^{2+} and a hump region around $Mn^{2+} : Al^{3+} = 1 : 1$. Fe-Mn-Al ternary alloys may risk increased toxicity when the ratio of metal ions released from it in human tissue is close to the saddle region in the augmented simplex model contour plot. Fe-Mn-Al ternary alloys may risk a lower toxicity for prostheses applications when the ratio of released metal ions approaches the hump region in the contour plot. The toxicity tests using bacteria in this study can be useful and applicable to estimate the toxicity of metal ions in aqueous environment.

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