



# Investigation of a cyanine dye assay for the evaluation of the biocompatibility of magnesium alloys by direct and indirect methods



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## ABSTRACT

Magnesium and its alloys are promising candidates for a new generation of biodegradable metals in orthopaedic applications due to their excellent biocompatibility, biodegradability, and mechanical properties that are similar to natural bone. However, direct *in vitro* assessment of these materials in the presence of cells is complicated by degradation products from the alloy that lead to a false positive for the most commonly used cell adhesion and cell proliferation assays. In this paper, a cyanine dye was used to quantitatively evaluate the *in vitro* biocompatibility of a Mg AZ31 alloy by both direct and indirect methods. The cytotoxicity of the corrosion products was evaluated via an indirect method; a 25% decrease in cell viability compared to control samples was observed. Moreover, direct assessment of cell adhesion and proliferation showed a statistically significant increase in cell number at the surface after 72 h. In addition, the degradation rate and surface characteristics of the Mg AZ31 alloy were evaluated for both direct and indirect tests. The degradation rate was unaffected by the presence of cells while evidence of an increase in calcium phosphate deposition on the magnesium alloy surface in the presence of cells was observed. This study demonstrates that a cyanine dye based assay provides a more accurate assessment of the overall *in vitro* biocompatibility of biodegradable metals than the more commonly used assays reported in the literature to date.

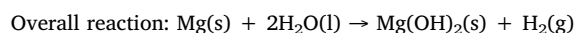
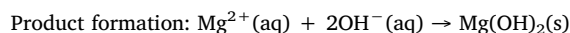
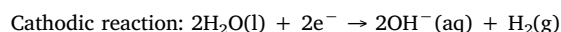
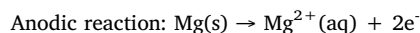
## 1. Introduction

Biomaterials are used in the replacement and treatment of diseased or injured tissue in different parts of the body; some examples include cardiovascular, dental and orthopaedic implants [1]. The development of biomaterials for orthopaedic applications has been a significant challenge to biomaterials scientists. The optimum biomaterials for orthopaedic implants should be non-toxic and biocompatible with the human body [2]. Furthermore, they should have excellent mechanical properties for the intended application and for applications such as fracture fixation, these biomaterials should be biodegradable to prevent the need for additional surgery to remove the implant after healing has occurred [3].

Magnesium and its alloys are a promising alternative for biodegradable orthopaedic implants that decrease stress shielding and enhance new bone growth due to their biodegradability, biocompatibility, and mechanical properties that are similar to natural bone. The biodegradability of magnesium provides a good advantage for short term implants that can dissolve after healing without the need for a second surgery to remove the implant.

Although magnesium itself is biocompatible [4,5], the degradation process in physiological environments that are rich in chloride ions can cause several complications for the surrounding tissues due to the rapid pH change and hydrogen gas production. The electrochemical reaction that occurs is shown below. Magnesium metal is oxidized to produce magnesium ions while water is reduced to form hydroxide ions and hydrogen gas [6].

### General degradation mechanism of magnesium



Magnesium alloys contain small amounts of additional elements such as aluminum, zinc, manganese and rare earth elements that enhance the mechanical strength and corrosion resistance of these materials. In addition, alloying elements that play an important role in biological activity such as calcium and strontium may also be present.

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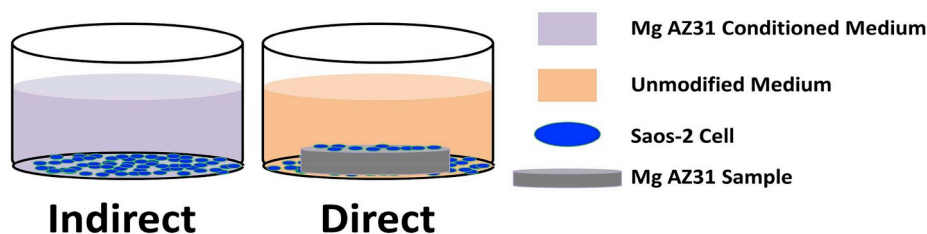


Fig. 1. Schematic diagram of indirect and direct methods for characterization of the biocompatibility of magnesium alloy materials.

The release of magnesium ions, hydroxide ions, hydrogen gas, and other alloying elements during the biodegradation process affects the overall biocompatibility of magnesium alloys. Therefore, it is essential to develop accurate testing methods to determine the biocompatibility of magnesium and its alloys to ensure that they are safe for use in medical applications [7].

According to the International Organization for Standardization (ISO), various methods and conditions are suggested to evaluate the *in vitro* biocompatibility of recently developed biomaterials including direct and indirect techniques that quantify the cytotoxicity of biomaterials and cell adhesion/proliferation on biomaterials respectively. These two methods are illustrated schematically in Fig. 1 [8,9].

The direct method involves direct contact between the material and the cultured cells while the indirect method involves exposing healthy, growing cells to an extract created by immersing the material in cell culture medium for a specified period of time. It should be noted that, the current ISO standards for the biological evaluation of medical devices were not specifically developed for biodegradable metallic materials.

For magnesium and its alloys, an indirect method is the most common way to evaluate their biocompatibility. This indirect method involves studying the effects of cell culture media that has been pre-conditioned through exposure to the magnesium material on already growing cells [7]. This indirect test evaluates the effect of the soluble degradation products on the cell viability. Many studies use this indirect method of evaluation for magnesium because a false positive is commonly observed when the assays are conducted in the presence of the magnesium material [10]. For example, the MTT assay, which is commonly used to directly quantify cell proliferation at the surface of biomaterials, involves the conversion of a yellow tetrazolium salt into a purple formazan dye by chemical reduction. Using this assay in the presence of biodegradable metals leads to a false positive result as the MTT dye is reduced by the electrons released during metal oxidation [10]. In addition, it has also been shown that the MTT assay can lead to false positive results at higher pH values [10]. As biodegradable metals corrode, the pH rises due to reduction of water which produces hydroxide ( $\text{OH}^-$ ) ions. Furthermore, high levels of  $\text{Mg}^{2+}(\text{aq})$  ions have been shown to inhibit the reduction of the tetrazolium dye leading to false negative values [10].

Although evaluating the cytotoxicity of the degradation products for biodegradable materials is one indicator of their biocompatibility, cell adhesion and cell proliferation at the surface of these materials are also important factors to consider. In addition, this false positive has caused some materials scientists to avoid these *in vitro* tests altogether and proceed directly to *in vivo* testing. While *in vivo* testing gives a more complete evaluation of the biocompatibility of an implant material, *in vitro* testing is still an essential screening tool to choose the most likely candidate materials and thus minimize expense and the number of animals that must be sacrificed. Therefore, it is very important to establish an alternative *in vitro* assay that can be used in a direct method to more accurately mimic the expected *in vivo* conditions. In this study, a non-common assay with a cyanine dye that strongly fluoresces only when bound to cellular nucleic acids was evaluated for its ability to determine the biocompatibility of a magnesium alloy by both direct and indirect methods.

The determination of cellular nucleic acid content provides a reasonable measure of cell numbers. These types of assays do not rely on a colorimetric change due to chemical reduction but rather take advantage of the interaction of a fluorescent dye with cellular DNA and thus should not exhibit the false positive result discussed above. This makes them suitable for quantifying cell adhesion and proliferation directly in the presence of a biodegradable metal. The cyanine dyes bind to double helical DNA by either intercalation between the base pairs of DNA or insertion into the minor groove of the double helical DNA resulting in high fluorescence enhancement [11–14].

The CyQUANT assay that was used in this study is an example of an assay that is based on the binding of a cyanine dye to nucleic acids. It is a quick and reliable method that can detect as few as 10–50 cells and as high as 25,000–50,000 cells depending on cell type. The linear range of this assay can be further extended to cell numbers as high as 100,000–250,000 cells by increasing the cyanine dye concentration [11].

In contrast to indirect techniques that evaluate cell viability in the presence of pre-conditioned cell media, direct methods allow for evaluation of the biocompatibility of magnesium alloys in the presence of the biocorrosion process. The effect of magnesium ions that dissolve into cell culture media, the rapid pH change, the hydrogen gas produced and the deposition of corrosion products during biocorrosion all have an influence on biocompatibility. However, direct evaluation methods have been avoided by many researchers for biodegradable magnesium materials due to false positive results from the most commonly used assays [7,10].

Fig. 2 shows the results of our literature survey of the methods used to evaluate the biocompatibility of magnesium materials from 2014 to 2018. Of the 150 journal articles surveyed, 70% reported using an indirect method to evaluate biocompatibility. As shown in Fig. 2, the most common method used to evaluate the biocompatibility of magnesium was the indirect method with the MTT assay. Other assays used for the indirect method include the MTS, WST-1, WST-8 and Alamar Blue assays. All of these assays are based on a colorimetric change when a dye is chemically reduced in response to cellular metabolism and are therefore unsuitable for quantifying cell adhesion/proliferation in the presence of magnesium due to the expected false positive result. Despite the known false positive, the MTT assay was also employed in a direct biocompatibility test in 17% of the articles surveyed. Moreover, about 8% of the articles evaluated the biocompatibility of magnesium by a direct method using other assays that also undergo a colorimetric

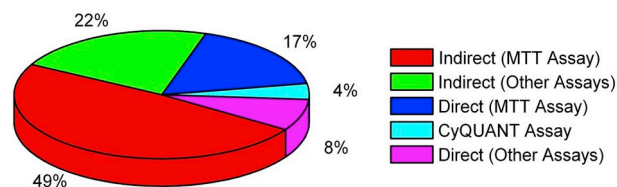


Fig. 2. Survey of Methods Used to Evaluate the Biocompatibility of Magnesium and Magnesium Alloys in Publications from 2014–2018. (A complete list of the 150 references used to prepare this figure can be found in the supplemental information).

change via chemical reduction. Overall only 4% of the articles reviewed evaluated biocompatibility by a direct method that did not employ an assay that would be expected to give false positive results. This literature survey highlights the need for a reliable method to quantify cell adhesion/proliferation for cells in direct contact with magnesium biomaterials. Cyanine dye based assays, used in only 6 of the 150 articles surveyed, should not result in a false positive and can be used to quantify cell numbers by both direct and indirect methods.

The objective of this research was to determine the applicability of a cyanine dye assay, the CyQUANT assay, for the *in vitro* evaluation of the biocompatibility of biodegradable magnesium materials by both direct and indirect methods.

## 2. Materials and methods

### 2.1. Materials

Mg AZ31 foil (0.81 mm thickness) was purchased from Alfa Aesar (US). Acetone (reagent grade) was purchased from Caledon Laboratory Chemicals (Canada). Bovine Calf Serum (BCS) and Trypan Blue were purchased from Fisher Scientific (Canada). McCoy's 5a culture medium and Trypsin/EDTA (1X) were purchased from Corning (Canada). Phosphate Buffered Saline (PBS) (1X) and Penicillin-Streptomycin Solution were purchased from HyClone (Canada). The CyQUANT Cell Proliferation Assay kit was purchased from Life technologies (Canada). Ethyl alcohol (95%) and methanol were purchased from Commercial Alcohols (Canada). All chemicals were used as received without further purification. The Saos-2 cell line was purchased from American Type Culture Collection (ATCC, Bethesda, MD USA).

### 2.2. Preparation of Mg AZ31 alloys

Mg AZ31 alloy sheets were cut into circular discs with a diameter of 1.27 cm. These discs were polished on both sides with a 320 grit P400 grinding paper and oil-based lubricant (Buehler). In order to remove any excess polishing oil from the surface of the alloys, the samples were sonicated in acetone for 20 min and then rinsed in deionized water for 30 s. Finally, the samples were air dried.

### 2.3. Cell culture

Human osteosarcoma Saos-2 cells were cultured in McCoy's 5a cell culture medium containing 15% bovine calf serum and 1% penicillin/streptomycin at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. The cell culture medium was changed every two days. Once the cells grew to 70–80% confluence, they were washed with warm (37 °C), sterile phosphate buffered saline (PBS) and detached from the flask with Trypsin/EDTA and then centrifuged at 2500 rpm for 5 min. The supernatant was discarded, and the cells were re-suspended in fresh McCoy's medium. The number of cells in the suspension was determined using the Trypan Blue dye with a Neubauer hemocytometer.

### 2.4. Direct method procedure

The Mg alloy samples were sterilized in 70% ethanol for 20 min and then rinsed with warm PBS. The samples were placed in the wells of a 24-well plate, 25,000 cells in 2 mL of McCoy's medium were added to each well and the plate was incubated for 24, 48, and 72 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. After the desired incubation period, a 25 µL aliquot was taken from the cell culture medium and diluted to 25 mL with 2% HNO<sub>3</sub> in a volumetric flask to quantify the magnesium ions dissolved in the cell culture medium. Subsequently, the Mg alloys were gently rinsed with warm PBS in order to remove non-adherent cells, then the plate was frozen at –20 °C for at least 24 h for the cells to lyse. For cell number determination, the plate was allowed to thaw and was analyzed using the CyQUANT assay, according to the supplier's

instructions. Briefly, 200 µL of CyQUANT solution was added to each sample and incubated for 5 min. At the end of the incubation period, 100 µL of the CyQUANT solution was pipetted from each well to a black fluorescent 96-well plate (Costar) and the fluorescence intensity of each solution was measured with a Fluostar Optima spectrofluorometer. The cell number was then determined using a calibration curve of cell number vs. fluorescence intensity. In addition, cells grown on the bare well surface (in the absence of magnesium discs) was used as a positive control group for cell adhesion while the blank consisted of Mg AZ31 disks incubated in culture medium in the absence of cells. The entire experiment was repeated two times on different days to ensure the reliability of the results. Each individual experiment was an analysis of 3 samples at each incubation time.

The percentage of Saos-2 cells adhered directly to the Mg AZ31 surface as a function of time was determined using this direct method. The quantitative data is reported as a percentage of the number of cells attached to the surface in comparison to the initial number of cells seeded (25,000 cells). The cell adhesion percentage was calculated by the following equation:

$$\text{Cell Adhesion\%} = \left( \frac{\text{number of adhered cells}}{25,000} \right) * 100$$

The lack of false positive for the CyQUANT assay was confirmed through a control experiment that measured the fluorescence of the dye in the presence of Mg AZ31 but in the absence of cells.

### 2.5. Indirect method procedure

#### 2.5.1. Preparation of magnesium conditioned media (magnesium extract)

Prior to the indirect experiment, all magnesium alloy samples were sterilized in 70% ethanol for 20 min followed by washing with warm PBS. The Mg alloy samples were placed in individual wells of a 24-well plate that contained only 2 mL of McCoy's medium (media volume/surface area = 1.3 mL/cm<sup>2</sup>) and then incubated for 24, 48, and 72 h at 37 °C in a 5% CO<sub>2</sub>. After the incubation periods, a 25 µL aliquot of the cell culture medium was taken from each well and diluted to 25 mL using 2% HNO<sub>3</sub> in a volumetric flask. The remaining conditioned medium was stored at –80 °C until needed. The magnesium alloy samples were then rinsed in water and set aside for further analysis. Cell culture medium was used as a control to compare the concentrations of magnesium ions in the presence and absence of the magnesium alloy samples.

#### 2.5.2. Evaluation of cell viability

To determine the effect of magnesium on the viability of cells, 15,000 cells were seeded in triplicate a 96-well plate. The cells were incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere for 24 h to allow cell attachment to the plate surface. After 24 h of incubation, the cell culture medium was removed and 200 µL of the magnesium extract (Mg-conditioned medium) was added to the 96-well plate with the already growing cells. The plate was then incubated for 24 h at 37 °C in a 5% CO<sub>2</sub> atmosphere. After 24 h, the magnesium conditioned medium was removed, and the cells in the 96-well plates were rinsed with warm PBS in order to remove non-adherent cells, then the plate was frozen at –20 °C overnight for the cells to lyse. The cell numbers were then determined using the CyQUANT assay as described in section 2.4. Cells grown in the absence of magnesium extract were used as a control group to determine the cell viability. The entire experiment was repeated two times on different days to ensure the reliability of the results. Each individual experiment tested 3 samples of each type (Cells in unmodified growth medium and cells exposed to Mg AZ31 conditioned growth medium (24 h extract, 48 h extract and 72 h extract)).

The percentage of viable Saos-2 cells in the presence of Mg AZ31 conditioned growth medium was determined using this indirect method. The quantitative data are reported as a percentage of the

number of cells in the presence of Mg AZ31 conditioned growth medium in comparison to the number of cells in the control group. The cell viability percentage was calculated using the following equation:

$$\text{Cell viability\%} = \left( \frac{\# \text{ of cells after exposure to Mg extract}}{\# \text{ of cells in control sample}} \right) * 100$$

## 2.6. Instrumental analysis

### 2.6.1. Attenuated total reflectance-Fourier transform infrared microscopy (ATR-FTIR)

Attenuated total reflection Fourier transform infrared microscopy (ATR- FTIR) was used to analyze the surface of the Mg AZ31 alloy discs after exposure to the cells and the cell culture medium in the direct method and to the cell culture medium alone in the indirect method. A Bruker Optics Hyperion infrared microscope with an attenuated total reflectance objective and a germanium crystal were employed. The main goal of this analysis was to determine the chemical functional groups present on the surface of the magnesium alloys after the direct and indirect method. Spectra were collected in three spots for each sample and were corrected with the atmospheric compensation function of the OPUS software.

### 2.6.2. Flame atomic absorption spectroscopy (FAAS)

A Perkin Elmer AA analyst 400 flame atomic absorption spectrometer was used to determine the degradation rate of the magnesium alloys after exposure to the cells and the growth medium in the direct method and to the growth medium alone in the indirect method. This technique was used to measure the concentration of magnesium ions that were released into the cell culture medium in the presence and absence of cells. The amount of magnesium released from the samples was quantified using a calibration curve for standards of known concentrations of magnesium (0.05, 0.1, 0.2, 0.5 mg/L). Unmodified cell culture medium was used as the control sample. The results are reported as the average  $\pm$  standard deviation for three different samples of each type.

### 2.6.3. Scanning electron Microscopy-Energy Dispersive X-ray spectroscopy (SEM-EDS)

Scanning electron microscopy was used to evaluate the surface topography of the samples after the direct and indirect cell tests. SEM images were obtained using a digital scanning electron microscope (JEOL 6400) operated at 20 kV with a beam current of 1 nA. Energy Dispersive X-ray Spectroscopy (EDS) was used to determine the elemental composition of the degradation products on the surface. Samples to be analyzed were coated with a thin film of carbon to render the samples conductive.

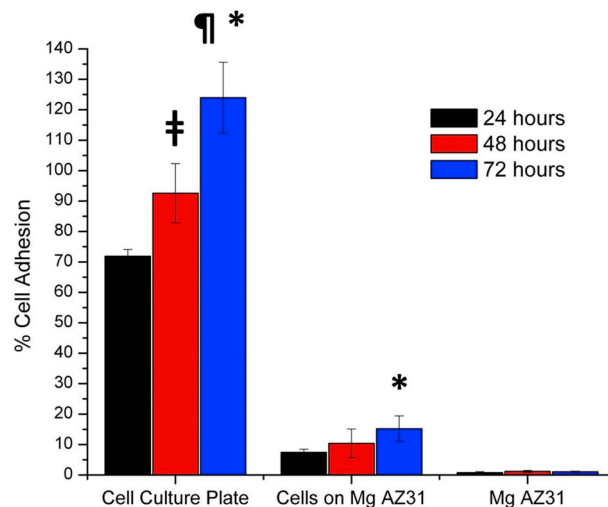
## 2.7. Statistical analysis

Quantitative results are reported as an average  $\pm$  standard deviation of multiple sample trials. The number of samples used in each data is indicated in the figure captions. Statistical significance of the observed differences in the % cell adhesion and % cell viability data was determined with a paired student t-test. A value of  $p < 0.05$  was assumed to indicate statistically significant differences.

## 3. Results and discussion

### 3.1. Evaluation of the biocompatibility of Mg AZ31 alloy using the CyQUANT assay

The main objective of this study was to demonstrate that a cyanine dye based assay can be used to evaluate cell adhesion/proliferation in direct contact with magnesium and its alloys with no false positive



**Fig. 3. The Percentage of Saos-2 Cells Adhered to the Mg AZ31 Surface as a Function of Time Evaluated by the Direct Method.** Results are reported as a percentage of the number of cells attached to the surface in comparison to the initial number of cells seeded. “Cell Culture Plate” is the data for the control group, “Cells on Mg AZ31” is the data for cells adhered to the Mg AZ31 surface and “Mg AZ31” is the negative control showing that there is no false positive in the absence of cells. Data are the average of 6 Samples (3 Replicates of 2 Independent Experiments). \* Indicates  $p < 0.05$  between 24 h and 72 h, † Indicates  $p < 0.05$  between 48 h and 72 h, and ‡ Indicates  $p < 0.05$  between 24 h and 48 h.

result and to evaluate the CyQUANT assay as a tool to quantify the *in vitro* biocompatibility of magnesium alloys by both direct and indirect methods.

#### 3.1.1. Direct method

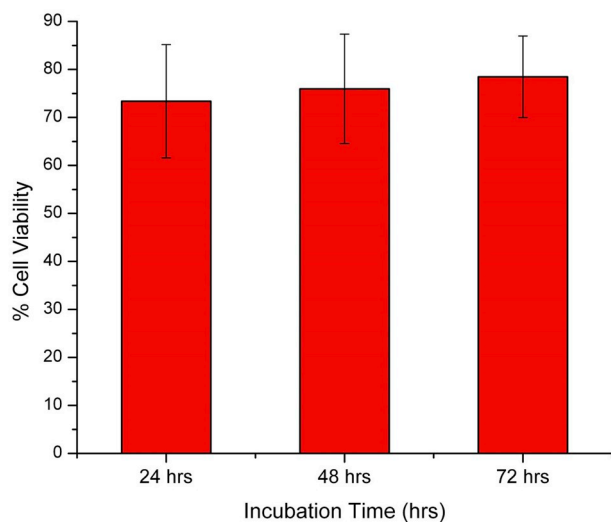
In the direct method, cells were grown directly on the surface of the Mg AZ31 discs for a selected time interval (24 h, 48 h, and 72 h) as shown in Fig. 1. After the incubation time, the magnesium disc was removed, and the non-adherent cells were rinsed from the surface in order to quantify only the cells adhered to the Mg alloy surfaces.

Fig. 3 shows the percentage of cells adhered to the Mg AZ31 samples compared to the initial number of cells seeded after incubation periods of 24, 48, and 72 h. The results show that the percentage of cells on the positive control (cell culture plate) increased as a function of time indicating cell proliferation in the well. In addition, the percentage of cells on the Mg AZ31 alloy surface increased significantly over time but with overall lower cell numbers in comparison to the control samples. The lower percentage of cells on the Mg AZ31 surface may be attributed to the corrosion of the magnesium alloy surface in aqueous environments resulting in the production of hydrogen gas bubbles and an increase in pH of the growth medium. However, the results show a statistically significant difference ( $p < 0.05$ ) in cell number on the magnesium alloy samples after 72 h. In fact, after 72 h the cell number increases by 105% on the magnesium alloy surfaces in comparison to an increase of only 70% for the control samples. This indicates that although the initial adhesion of cells to the magnesium samples is low, the adhered cells were able to proliferate significantly over 72 h. Thus, using the CyQUANT assay, we were able to show that Mg AZ31 has the ability to promote cell adhesion and cell proliferation over time.

#### 3.1.2. Indirect method

In the indirect method, already growing cells on the surface of a plastic 96-well plate were exposed to cell culture medium that was pre-conditioned in the presence of Mg AZ31 samples for 24, 48 and 72 h as shown in Fig. 1. After incubation of the cells in the presence of the conditioned medium for 24 h, the magnesium conditioned cell culture





**Fig. 4.** The Percentage of Saos-2 Cell Viability Using the Indirect Method in the Presence of Mg Alloy Conditioned Extracts that had been Pre-conditioned for 24, 48 and 72 h. The quantitative data are reported as a percentage of the number of viable cells in the presence of Mg AZ31 conditioned extract compared to the number of viable cells in unmodified cell culture medium (Control). Data are the Average of 6 Samples (3 Replicates of 2 Independent Experiments).

medium was removed, the wells rinsed with PBS to remove non-adherent cells and the number of viable cells determined with the CyQUANT assay. Cells grown in the cell culture well (in the absence of magnesium extract) were applied as a control group in this study to determine the cell viability for each sample group.

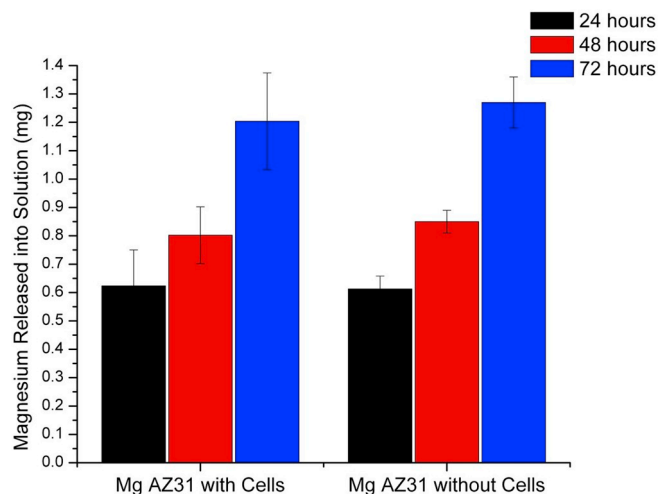
Due to the biodegradation of the Mg alloy samples, magnesium ions, hydroxide ions and ions of alloying elements are released into the cell culture medium. The rise in pH and the presence of potentially toxic ions may negatively affect the cell viability. Fig. 4 shows the viability of Saos-2 cells in the presence of the Mg-conditioned medium after 24, 48 and 72 h. It was observed that the cells exposed to the conditioned Mg AZ31 extract had similar cell viability regardless of conditioning time. According to ISO, if the cell viability is reduced by more than 30%, the material is considered to be cytotoxic [9]. However, these results show that cell growth in the Mg alloy conditioned cell culture medium for 24, 48 and 72 h all decreased the cell viability by only 25% on average. No statistically significant difference in the percentage of cell viability was noted for the different conditioned media ( $p > 0.05$ ).

### 3.2. Determination of the biodegradation rate of Mg alloy AZ31 in the cell culture medium in the presence and absence of cells

In order to evaluate the influence of cells on the biodegradation rate of Mg AZ31, the amount of magnesium dissolved in cell culture medium as a function of time was determined by flame atomic absorption spectroscopy (FAAS). Magnesium ions are released into cell culture medium as the magnesium metal is oxidized to  $Mg^{2+}$  in the aqueous solution.

Fig. 5 shows a graph of the amount of magnesium dissolved into the cell culture medium as a function of time over a 72-h period in the presence (direct method) and absence (indirect method) of cells. The amount of Mg ions released into the cell culture medium was the same in both cases. Therefore, the cells did not inhibit or enhance the corrosion of Mg alloys AZ31 up to 72 h.

However, Agha et al. reported that the influence of cells on the degradation of pure Mg and Mg alloys was more apparent after 14 days of immersion than 4 days of immersion in cell culture medium in their study on the degradation of magnesium in the presence and absence of osteoblast cells. A decrease in the degradation rate of pure Mg and



**Fig. 5.** The Total Amount of Mg Dissolved as a Function of Time in the Cell Culture Medium in the Presence and Absence of Cells. Each data point represents an average of 3 samples; the error bars are the sample to sample standard deviation.

Mg-10Gd was observed in the presence of cells compared with the samples in the absence of cells. These results were attributed to the formation of more hydroxyapatite in the presence of osteoblast cells which increased over time [15].

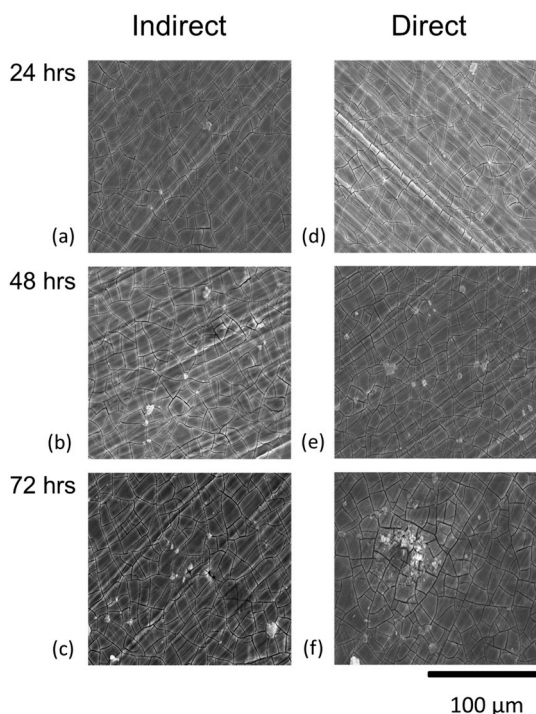
In addition, although the concentration of magnesium in the cell culture medium at 72 h was close to two times the amount measured at 24 h there was no increase in the observed cell cytotoxicity measured by the indirect method. For the direct method, cell proliferation was observed despite the increase in magnesium ions in the cell culture medium. These results are consistent with the literature as it has been previously reported that an increase in magnesium ion levels can stimulate new bone growth [16,17]. Numerous studies have demonstrated enhancement of bone growth around the corroded magnesium implant *in vivo* [18–20]. As has been shown in the literature, the presence of magnesium on orthopaedic implants may enhance osteoblastic cell adhesion *in vitro*. Pietak et al. has shown that magnesium-based substrates can promote bone cell attachment on the implant surface in an *in vitro* experiment [21]. Furthermore, Li et al. have shown that Mg-1Ca alloy did not induce toxicity to cells and had high activity of osteoblast and osteocytes around the alloy in an *in vivo* experiment [20].

### 3.3. Surface characterization of the Mg AZ31 alloy samples after direct and indirect methods

After each incubation time, the samples were analyzed by SEM-EDS in order to evaluate the morphology and surface elemental composition of the magnesium alloy after exposure to the mixture of the cells and the cell culture medium in the direct method and to the unmodified cell culture medium in the indirect method. The samples were also analyzed by ATR-FTIR in order to identify the precipitation products on the surface. One of the expected degradation products on the surface of magnesium alloys after exposure to the cell culture medium solution are calcium phosphates [22]. This is due to their low solubility in aqueous solution, particularly at elevated pH. As the magnesium alloy degrades, the pH rises, resulting in precipitation of calcium phosphate species such as hydroxyapatite as shown in the precipitation reaction below:



Furthermore, other precipitation products are possible on magnesium alloy surfaces after exposure to cell culture media such as other calcium phosphates, magnesium phosphate, magnesium hydroxide and



**Fig. 6. Representative SEM Images of the Magnesium Alloy AZ31 Surfaces after the Direct and Indirect Methods.** (a, b, c) Samples after 24, 48, 72 h by the direct method and (d, e, f) Samples after 24, 48, 72 h by the indirect method.

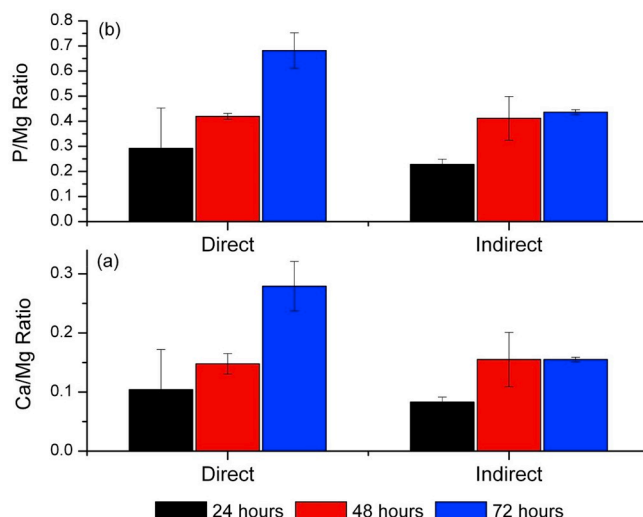
magnesium carbonate [23].

Fig. 6 shows representative SEM images of the surface of the Mg AZ31 alloy samples after incubation in the cell culture medium in the presence (a, b, c) and absence (d, e, f) of Saos-2 cells. The SEM image of a polished bare magnesium surface before testing showed that the polished Mg AZ31 surface was flat with lines due to polishing (image is not shown). The SEM images of all of the samples after either the direct or indirect test are completely covered with a layer of corrosion product. The cracking of the layer is due to dehydration of the corrosion product as it dries. This layer was readily observed on the magnesium substrate after 24 h of exposure to the cell culture medium both in the presence and absence of cells. Thus, the presence of cells in the culture medium does not change the surface morphology of the layer deposited on the magnesium alloy surface. The presence of both calcium and phosphorus on these surfaces was confirmed by EDS indicating that the deposited layer contains calcium phosphate as expected.

All of the samples were analyzed by EDS in order to determine the elemental composition of the degradation products that had been formed on the surface of the magnesium alloy after both the direct and indirect cell tests.

Fig. 7 shows the Ca/Mg ratio and the P/Mg ratio as a function of time for samples exposed to cell culture medium alone (indirect test) and cell culture medium and cells (direct test). The EDS results indicate that the Ca/Mg and P/Mg ratio on the surface of Mg alloys for both the direct and indirect methods was slightly increased with increased incubation time from 24 to 48 h. After 72 h, both the ratio of Ca/Mg and P/Mg that was deposited on the surface of the Mg alloys after the direct method increased significantly indicating an increase in the amount of deposited calcium and phosphorus in the presence of cells with increasing incubation time. In comparison, the ratios of Ca/Mg and P/Mg on the surface of Mg AZ31 in the absence of cells (indirect method) after 72 h were not increased. Thus, although the morphologies are similar as seen in Fig. 6, the amount of the calcium phosphate deposited appears to be influenced by the presence of cells.

These results can be attributed to the ability of the Saos-2 osteoblast cells to produce calcium phosphate as has been previously reported



**Fig. 7. The (a) Ca/Mg and (b) P/Mg Ratio on the Surface of Mg AZ31 after the Direct and Indirect Methods.** Each data point represents an average of 3 samples and 9 spots on each sample; the error bars are the sample to sample and spot to spot standard deviation.

[24–26]. Human osteoblast cells play an important role in the formation of bone by inducing and regulating the mineralization of the extracellular matrix. Saos-2 cells can also more closely mimic the *in vivo* environment than other commonly used osteoblast cell lines [15,24–26]. Agha et al. found more hydroxyapatite on a magnesium surface in the presence of cells in comparison to the absence of cells. Specifically, their study reported that they found higher amounts of Ca and P near the cells than underneath the cells [15].

In addition, Strzelecka-Kiliszek et al. compared the formation of minerals on two selected osteoblastic cell models: osteosarcoma Saos-2 cells and human foetal hFOB 1.19 cells and it was observed that Saos-2 cells had higher amount and composition of minerals compared to hFOB 1.19 cells and that Saos-2 cells mineralized better than hFOB 1.19 cells since the ratio of calcium to phosphate was closer to hydroxyapatite. Therefore, the choice of osteoblast cell line is an important consideration for the direct method since each cell line shows different characteristics in terms of the extracellular matrix and mineralization production [27].

The magnesium alloy samples were also analyzed by ATR-FTIR in order to further identify the precipitation products on the surface. Fig. 8 shows infrared spectra of MgAZ31 surfaces before and after exposure to the cells. The IR spectrum for a polished and cleaned magnesium alloy has a small peak at  $3700\text{ cm}^{-1}$  (O–H stretch) which indicates the presence of Mg–OH bonds at the surface. A broad peak at  $3000\text{ cm}^{-1}$  (O–H stretch) was also observed coupled with a peak at  $1640\text{ cm}^{-1}$  (O–H bend) due to surface adsorbed water. Carbonate ( $\text{CO}_3^{2-}$ ) was also observed at ( $1450\text{ cm}^{-1}$ ) due to the reaction of the magnesium substrate with carbon dioxide in the atmosphere. Furthermore, peaks at  $2850\text{--}2930\text{ cm}^{-1}$  ( $\text{CH}_2/\text{CH}_3$  stretch) were observed indicating a small amount of organic contamination on the surface. As shown in Fig. 8, all the sample surfaces exhibited a strong phosphate peak at  $1050\text{ cm}^{-1}$  confirming that the phosphorus peaks observed in the EDS spectra are due to the presence of phosphate,  $\text{PO}_4^{3-}$ , at the magnesium alloy surface. Furthermore, a carbonate peak at ( $1450\text{ cm}^{-1}$ ) was still observed after both the direct and indirect test; this is attributed to the presence of carbonate within the deposited surface layer. The deposition of calcium phosphates and other phosphate species on the surface of the Mg alloy may lead to enhanced bone-implant contact and induce early bone formation [19,20]. Therefore, the presence of cells in direct contact with this layer is a very important factor for the evaluation of the biocompatibility of magnesium biomaterials.

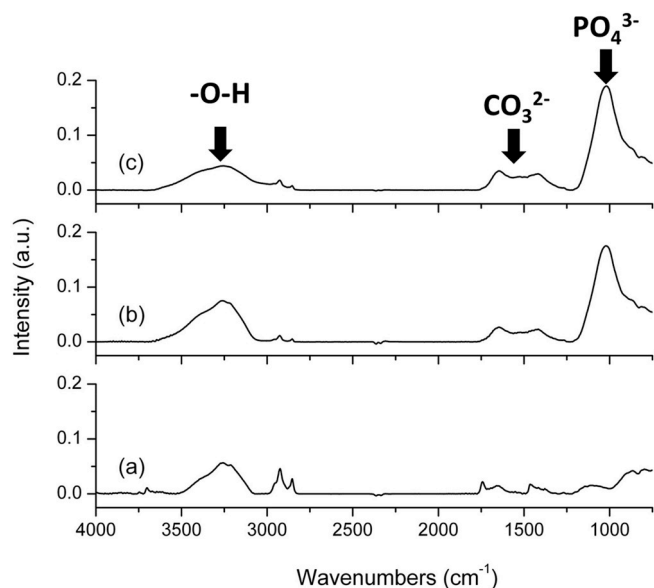


Fig. 8. IR Spectra of MgAZ31 after the Direct and Indirect Methods (72 h Incubation Time). (a) Sample before test, (b) Sample after direct method and (c) sample after indirect method.

#### 3.4. Comparison of the direct and indirect methods using the CyQUANT assay

Materials developed for medical applications must be biocompatible, which means they can not have toxic or carcinogenic effects and must also be tolerated by the human body without inducing inflammation or injury to the surrounding tissue [28]. *In vitro* studies of the biocompatibility of a material are an important preliminary step to investigate the potential safety of using these materials for *in vivo* studies [29]. The *in vitro* study of the biocompatibility of biodegradable magnesium alloys is mainly carried out using cell culture [29]. There are two primary methods that have been adapted from the international standards organization for evaluating the biocompatibility of biomedical devices [8,9]. The first method involves direct contact between the material and the cultured cells (direct method). The second method is to evaluate the compatibility of the materials with cells using an indirect method in which growing cells are exposed to an extract created by immersing the material in cell culture medium for a period of time.

The most commonly used assays for both of these methods are colorimetric assays that depend on a chemical reduction reaction. Using these assays for magnesium materials in a direct method leads to a false positive result as the dye is reduced by the electrons released during the oxidation of the magnesium substrate itself [29]. Consequently, the indirect method is the most popular method for analyzing the biocompatibility of Mg based biomaterials. Although some authors have reported analyzing samples exposed to only magnesium (the blank) and then subtracting the signal obtained from the samples in the presence of cells, this is still problematic because the observed intensity of the magnesium “blank” depends on the corrosion rate of the sample [30]. This can be influenced by the mechanism of corrosion of the alloy especially in the case of pitting corrosion where high sample to sample variability may be observed. The CyQUANT assay used in this study is an excellent alternative as it is an assay based on nucleic acid binding and can therefore be used in both indirect and direct biocompatibility tests.

The cell viability measured by the CyQUANT assay in this study was approximately 80%. This is consistent with reported values from the literature that used the MTT assay to determine the *in vitro* cytotoxicity of Mg AZ31. For example Li et al. reported a high (approx. 90%) cell

viability on both Mg AZ31 and  $N^+$  implanted Mg AZ31 [31]. Sunil et al. also found similar results for the cytotoxicity of a fine grained Mg AZ31 alloy [32]. In addition, Lopes et al. have reported that Mg AZ31 is not cytotoxic with a > 80% metabolic activity for Saos-2 cells as measured by the MTT assay [33].

In this paper, the results obtained from the direct method using the CyQUANT assay indicated the ability of Mg AZ31 to promote cell adhesion and cell proliferation over time. The initial cell adhesion numbers are low, likely a result of the surface roughness. It has been shown that rougher surfaces lead to higher corrosion rates [34]. This would have a direct impact on cell attachment due to increased levels of hydrogen gas production. However, it is clear from our results that the adherent cells are healthy because they are able to proliferate over time; in fact the cell number more than doubles from 24 to 72 h. Furthermore, the EDS results show that the calcium and phosphorus levels on the surface increase from 24 to 72 h in the presence of cells. This result indicates increased surface mineralization due to cell activity. This result is consistent with other studies that have shown the presence of healthy cells on Mg AZ31 surfaces after 24–72 h [31–33]. Moreover, the extremely low fluorescence measurements observed for Mg AZ31 alloys without cells indicates that the CyQUANT assay does not yield a false positive result in the presence of magnesium. Therefore, this assay provides a better assessment of the overall behaviour of cells with Mg based biomaterials because the cells are growing in a direct contact with the biomaterial and are therefore exposed to all environmental changes in the cell culture medium, including Mg ion release, an increase in pH, hydrogen gas production and the deposition of biodegradation products.

The Mg AZ31 alloy was used as a model material in this research. This alloy has been shown to have minimal cytotoxicity in this and other studies. In addition, an *in vivo* test in rabbits has demonstrated no adverse health effects to the animals after 60 days post-implantation [33]. Although these results are promising, further *in vivo* testing to determine the long term effects of aluminum released from this alloy during degradation needs to be conducted. In particular, we must ensure that no neurotoxicity and/or bone disease develop in the host animal.

#### 4. Conclusion

This research demonstrates that the cyanine dye (CyQUANT assay) can be used to evaluate the *in vitro* biocompatibility of biodegradable magnesium alloys by both direct and indirect methods. A particular advantage of this assay is the ability to quantitatively measure cell numbers directly on the surface of magnesium biomaterials allowing for a more complete evaluation of the behaviour of cells in the presence of all of the degradation products that are produced. In addition, the CyQuant assay is commercially available, simple to use and can be implemented in any laboratory that has access to a fluorescence microplate reader making it an excellent alternative to the conventionally used MTT assay. The combined results from the direct and indirect biocompatibility tests performed in this study indicate that cell proliferation readily occurs on Mg AZ31 surfaces and that the soluble products of biodegradation are only slightly cytotoxic.

#### Author contribution

Afrah Al Hegy: Funding acquisition, Formal analysis, Writing – original draft.

Ryan Smith: Formal Analysis

Eric Gauthier: Conceptualization, Supervision, Formal analysis, Writing -review & editing

Joy Gray-Munro: Conceptualization, Supervision, Formal analysis, Writing – review & editing.

## Declaration of competing interest

There is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioactmat.2019.12.002>.

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