Cytotoxicity and corrosion behaviour of magnesium and magnesium alloys

in Hank´s solution after processing by high-pressure torsion

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Abstract

It is well known that processing by severe plastic deformation using high-pressure torsion (HPT) promotes grain refinement and increases the strength of magnesium and its alloys. The present research was conducted to evaluate the effect of such processing on cytotoxicity and corrosion behaviour in Hank´s solution by using samples of commercial purity magnesium and AZ31, AZ91 and ZK60 magnesium alloys. All samples were subjected to electrochemical testing and hydrogen evolution testing before and after processing by HPT and the results show that this processing improves the corrosion resistance of pure magnesium, has no significant effect on the AZ31 and AZ91 alloys but reduces the corrosion resistance of the ZK60 alloy. The observations support the conclusion that grain refinement improves the corrosion resistance of metals with a tendency for passivation but impedes the resistance of metals without passivation. In addition, *in vitro* cytotoxicity tests were performed on the processed materials and show cell viability in all samples. The results demonstrate that HPT processing may be used to improve the performance of magnesium and its alloys as biodegradable implants.

*Keywords*: corrosion; cytotoxicity; high-pressure torsion; magnesium; severe plastic deformation

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**1. Introduction**

Magnesium and its alloys have low density and low elastic modulus compared to other structural metallic materials and they also have a higher fracture toughness than ceramics. These metals are also widely present in the human body and excess levels are readily expelled in the urine [[1](#_ENREF_1)].Thus, magnesium has attracted significant attention as a biomaterial and particularly for orthopedic implants. The important constraints associated with these materials are their low strength and their rapid corrosion in a physiological environment. In practice, magnesium is highly reactive and a double layer of MgO and Mg(OH)2 is formed on its surface in an aqueous media[[2](#_ENREF_2)]. This layer does not lead to complete passivation and, as a consequence, magnesium degrades at rates that prevent their use in long term implants but nevertheless enable their use as temporary implants.

A recent review on metallic implant biomaterials placed magnesium alloys in the third generation of biomaterials with the biodegradation playing a role in bioactivity and targeting tissue regeneration [[3](#_ENREF_3)]. For example, pure magnesium was used to produce a bone fixation device that allowed the fracture to heal and led to the formation of new bone tissue around it during the degradation [[4](#_ENREF_4)]. Magnesium scaffolds also showed good osteogenesis during degradation [[5](#_ENREF_5)] and biodegradable stents were produced from a WE43 magnesium alloy [[6](#_ENREF_6)]. Despite the good biological response of magnesium, the mechanical strength of the highly pure metal is too low for any significant load-bearing applications and therefore alloying and/or metal processing routes must be undertaken. The rate of corrosion also should be controlled in order to extend the total lifespan of the implant and thereby to reduce and/or prevent the formation of hydrogen gas bubbles which are a by product of any corrosion. Finally, the effect of the release of alloying elements during degradation needs significant clarification.

Multiple approaches have been considered in order to reduce the rate of corrosion of magnesium. The development of surface protective layers is effective for an initial protection but care must be taken since any break in this protective layer may lead to severe localized corrosion. The use of alloying elements usually reduces the corrosion resistance compared to the highly pure magnesium [[7](#_ENREF_7)] but these elements may trigger the formation of second phase precipitates which effectively compromise the corrosion resistance due to galvanic corrosion. Thermo-mechanical and thermal treatments are often used to control the amount and distribution of any second phase precipitates and to refine the grain structure which, in some situations, leads to an improvement in the corrosion resistance. For example, hot rolling and processing by equal-channel angular pressing (ECAP) reduced the rate of degradation of an AZ31 alloy [[8](#_ENREF_8)] and a decrease in the corrosion current density with decreasing grain size was reported in pure magnesium [[9](#_ENREF_9)].

It is now well established that processing through the application of severe plastic deformation (SPD) can effectively refine the grain structure and improve the mechanical strength of metallic materials [[10](#_ENREF_10), [11](#_ENREF_11)] but the use of these SPD techniques is not easy for processing magnesium and its alloys due to their limited formability at room temperature. As a consequence, the SPD processing is usually conducted at high temperatures so that the grain refinement is then less effective. However, high-pressure torsion (HPT) is an SPD procedure that may be used to process these materials at low temperatures due to the imposition of high hydrostatic stresses that restrict the development of any cracking [[12](#_ENREF_12)]. This technique has been used for the processing of pure magnesium and numerous magnesium alloys and generally it produces grain sizes that are smaller than 1 µm. The mechanical strength is improved significantly in all alloys at room temperature and there is an exceptional increase in ductility in pure magnesium [[13](#_ENREF_13)]. Additionally, there are reports of improved corrosion resistance in pure magnesium processed by HPT [14,15].

An earlier report documented the effect of HPT processing on the structure, hardness and corrosion in 3.5 m/v % NaCl of commercial purity magnesium (CP-Mg) and AZ31, AZ91 and ZK60 magnesium alloys [[14](#_ENREF_14)]. The results showed that ultrafine grains (<1 µm) were produced in all materials and hardness values of >100 kgf.mm-2 were observed in the alloys. Polarization and electrochemical impedance spectroscopy (EIS) tests revealed no significant changes in behaviour in these metals after processing by HPT. Based on these earlier data, the present research was undertaken to examine and compare the corrosion behaviour of the same four materials (CP-Mg, AZ31, AZ91 and ZK60) in Hank´s solution before and after HPT processing and also to provide some information on cytotoxicity.

**2. Experimental materials and procedures**

Commercial purity magnesium (CP-Mg) and the AZ91 alloy were received as cast slabs and the AZ31 and ZK60 alloys were received as extruded rods. The AZ91 alloy was solution treated at 693 K for 24 h and water quenched. For all materials, discs of 10 mm diameter and 0.85 mm thickness were prepared and processed by HPT at room temperature using a quasi-constrained facility [[15](#_ENREF_15), [16](#_ENREF_16)] with a nominal pressure of 6.0 GPa, a rotation rate of 2 rpm and with negligible deformation heating. The earlier experiments [[14](#_ENREF_14)] showed that the average initial grain size, *d*, was very coarse in the as-cast CP-Mg (~1000 µm) and in the solution-treated AZ91 alloy (~110 µm) but it was reasonably refined in the extruded AZ31 (~16 µm) and the ZK60 (~2.9 µm) alloys. Processing by HPT gave significant grain refinement with average grain sizes of ~0.51 µm in CP-Mg, ~0.13 µm in AZ31 and ~0.10 µm in AZ91 after 10 turns of HPT and ~0.16 µm in ZK60 after 5 turns of HPT.

Samples were prepared for corrosion testing from materials before and after HPT processing by grinding the disc surfaces on #4000 abrasive paper and conducting the tests in Hank´s solution using the composition given in Table 1. A potentiostat (Autolab PGSTAT 100N) with 3 electrodes (Ag/AgCl as reference, platinum as counter electrode and metal sample as the working electrode) was used for the electrochemical tests. The specimens were immersed in solution for at least 600 s before starting each test in order to stabilize the potential. The scan rate in the polarization tests was 1 mV s-1 and the amplitude in the impedance spectroscopy test was 10 mV in relation to the open circuit potential. Hydrogen evolution tests were used to estimate the mass-loss rate. The samples were immersed in Hank´s solution and held in suspension by a cotton thread. A funnel directed the hydrogen gas bubbles which were collected using an inverted burette to facilitate tracking the gas volume evolution. The samples were photographed using a digital camera and a stereo-microscope after 7, 14, 21 and 28 days of immersion in order to evaluate the macroscopic corrosion features. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) were used to examine microscopic features on the corroded surfaces after polarization and impedance testing.

The cytotoxicity of the different alloys was estimated using a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) test and LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR, USA); the experimental procedure was described in detail in an earlier report [[17](#_ENREF_17)]. Briefly, a human osteosarcoma cell line (SAOS-2) from the Rio de Janeiro Cell Bank (BCRJ, Brazil) was used in the experiments. The cells were maintained in a controlled 5% CO295% humidity incubator at 37 °C with a culture medium constituted by basal Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic. The cells were kept with a culture medium for a negative control groupand cells were treated with 1% triton-X 100 in a culture medium for a positive control group.For the MTT test, 3×104 cells mL-1 were seeded in a 24-well plate with supplemented DMEM and the well was maintained under controlled conditions for 24 h and then exposed to the materials for 24 h. After exposure, the material was removed, 210 µL of fresh medium and 170 µL of MTT solution (5 mg mL-1) were added and the cells were incubated for 4 h. Finally, 170 µL of SDS solution/4% HCl was placed and incubated for 12 h and the absorbance at 595 nm was quantified. Three measurements were carried out for each sample and the results were then expressed as a fraction of the cell function. Measurements of the negative control group (functional cells) were considered 100%.For the LIVE / DEAD assay, 6 × 104 cells were seeded on glass coverslipswith 2 mL with supplemented DMEM. The cells and media were maintained under controlled conditions for 24 h and then exposed to the materials for 24 h. Thereafter, the cells were rinsed twice with phosphate-buffered saline solution and examined using a fluorescent confocal microscope.

**3. Experimental results**

3.1. Cytotoxicity

*In vitro* experiments were carried out on the HPT-processed samples. The analysis using MTT and LIVE/DEAD assays showed that pure magnesium and the AZ31, AZ91 and ZK60 alloys do not generate a cytotoxic environment after HPT processing. As indicated in Fig. 1, the results obtained from the MTT test revealed no significant differences in the mitochondrial activity of cells exposed to the different alloys for 24 h when compared to the negative control group (p<0.05). The cell metabolic activity was over 80%, implying a high cell viability.In Fig. 1 the metabolic activity of a positive control group (Triton) is also presented for comparison. As shown in Fig. 2, the results of the LIVE/DEAD assay demonstrated that most of the cells exposed to different alloys for 24 h preserved a vital status. Live cells were stained with green fluorescence whereas dead cells were stained with red fluorescence. The live cells assumed a normal morphology characterized by a fusiform shape and these results support the MTT findings giving evidence of a satisfactory cell viability.

3.2. Polarization tests

The polarization curves for the different alloys before and after HPT are shown in Fig. 3. The CP-Mg has the lowest corrosion potential followed by AZ91, AZ31 and with the ZK60 alloy having the highest potential. The potential for CP-Mg is in agreement with another report [[18](#_ENREF_18)] but the present results show a lower potential for the AZ91 which is attributed to the preliminary solution treatment. The CP-Mg, AZ31 and AZ91 alloys display a region of passivation-like behaviour in which there is a reduced variation in current with increasing potential. The CP-Mg, AZ31 and AZ91 alloys also showed a transpassivation region where the current densities increased abruptly at high potentials. This passivation-like behaviour was reported earlier for pure magnesium[[19](#_ENREF_19), [20](#_ENREF_20)] and the AZ31 [[21](#_ENREF_21), [22](#_ENREF_22)] and AZ91[[20](#_ENREF_20), [21](#_ENREF_21)] alloys. By contrast, the ZK60 alloy exhibits a continuous increase of anodic current density as the potential rises and there is no significant change in the shape of the curve after HPT processing, although HPT processing shifts the corrosion potential to higher values. Table 2 provides a summary of data from the polarization tests for the different samples.

The surfaces of the samples after the polarization tests were observed by SEM and the areas of localized corrosion are illustrated in Fig. 4 together with the distributions of magnesium, oxygen, aluminium and zinc. It is readily apparent that the areas of localized corrosion in CP-Mg and the AZ31 and ZK60 alloys are covered by magnesium and oxygen rich products. Second phase particles rich in aluminium and manganese were observed within the area of localized corrosion in the AZ31 alloy. Al-Mn precipitates are common in this alloy and they were also reported in areas of localized corrosion in AZ31 after 30 min of immersion in a phosphate-buffer solution [[23](#_ENREF_23)] and after 2 h in 3.5% NaCl[[24](#_ENREF_24)]. A zinc and zirconium rich precipitate is observed in Fig. 4 on the left side of the area of localized corrosion in the ZK60 alloy processed by HPT. Localized corrosion in the AZ91 takes place around an area that is rich in Mg and Al due to the β phase which has a high aluminium content. There is evidence that this phase accelerates corrosion in AZ91 [[25](#_ENREF_25)].

3.3. EIS

The curves for EIS for the different materials are shown in Fig. 5 where, due to the very significant differences in the scale of the curves, a second illustration is also presented. The AZ91 alloy displays the largest capacitive arc, ZK60 is the smallest and HPT processing has no effect on the curves for these alloys. However, a significant increase in the diameter of the capacitive arc is observed in CP-Mg and there is a ten-fold increase in the AZ31 alloy after HPT. Figure 6 shows the equivalent circuits used to model the EIS curves. Two types of circuits were considered: in (a) there is a serial connection for the CP-Mg (as-cast and after HPT), AZ31 (after HPT) and AZ91 (solution treated and after HPT) and in (b) there is an inductive loop for the AZ31 (extruded) and ZK60 (extruded and after HPT). The three relevant time constants may be related to an external corrosion product such as magnesium hydroxide (R2/CPE1), an intermediate corrosion product layer (R3/CPE2) and to the metal/corrosion product interface (R4/CPE3). The constant phase element (CPE) was used due to the heterogeneous surface of the working electrode [[26](#_ENREF_26)]. The inductive element in circuits (Fig. 6 b) can be attributed to adsorbed species on the electrode surface [[27](#_ENREF_27)]. The circuit parameters for each material are summarised in Table 3. It is known that the capacitive arc, which is proportional to the polarization resistance (RP), tends in EIS tests to increase with immersion time [[23](#_ENREF_23), [25](#_ENREF_25), [28](#_ENREF_28)] but the present results show there is a larger arc in CP-Mg processed by HPT than in the as-cast Mg even after 15 days of immersion in Hank´s solution [[29](#_ENREF_29)]. The capacitive arc of the HPT-processed AZ31 is also larger than the maximum reported after long periods of immersion in similar alloys [[22](#_ENREF_22), [30](#_ENREF_30)] and the AZ91 alloy displays a larger arc than reported for long periods of immersion in Hank´s solution [[30](#_ENREF_30)]. By contrast, the HPT-processed ZK60 alloy exhibits comparable behaviour to that reported for a similar alloy tested using a phosphate buffer saline solution [[21](#_ENREF_21)].

The appearances of the surfaces of samples after the EIS tests are shown in Fig. 7 together with higher magnifications of the corrosion features. The extruded AZ31 and the HPT-processed ZK60 alloys exhibit a significant area covered by corrosion products whereas the other samples only exhibit small and well-distributed points of corrosion. Isolated areas with thick Mg and O rich layers are observed in the CP-Mg and ZK60 alloy and AZ91 displays localized corrosion near Si and Al rich phases. The HPT-processed AZ31 alloy shows no evidence of localized corrosion after the EIS test.

3.4 Hydrogen evolution tests

The hydrogen volume and rate of degradation are plotted as a function of time in Fig. 8 for the four different materials. The total volume of hydrogen is larger in both the as-cast CP-Mg and the HPT-processed ZK60 alloy indicating a more severe degradation in these samples. There is a general trend of decreasing rate of degradation during long exposure periods. There was no significant change in the hydrogen volume after 2 days of immersion of HPT-processed CP-Mg, AZ31 and AZ91 and the solution treated AZ91 alloy. The penetration rates in these samples were less than 0.1 mm/year after 2 days of immersion.

The samples are shown in Fig. 9at different periods of immersion in Hank´s solution and areas of localized corrosion after 28 days of immersion are presented in Fig. 10. Severe localized corrosion takes place in the as-cast CP-Mg within 7 days leading to the formation of a hole in the disc after 14 days whilst areas away from the hole exhibit no significant corrosion. The HPT-processed CP-Mg displays a more uniform corrosion throughout the sample surface although localized corrosion was observed at the edge of the disc after 14 days of immersion. This localized corrosion at the border failed to propagate significantly in the next 14 days suggesting that HPT processing reduces the tendency for localized corrosion in this material.

The extruded AZ31 and the solution-treated AZ91 alloys reveal signs of localized corrosion which are not as severe as in the as-cast CP-Mg. These areas of localized corrosion are filled with a corrosion product that appears to slow down its propagation. By contrast, the HPT-processed AZ31 and AZ91 alloys display a more widespread corrosion within the first days of immersion although areas of localized corrosion are also observed.

The ZK60 alloy shows evidence of significant degradation but nevertheless the corrosion appears to be generally uniform. This is in agreement with the polarization test for the ZK60 alloy which suggested homogeneous corrosion behaviour for this material. It is apparent that HPT processing is detrimental for this alloy since the rate of degradation appears to increase after SPD processing.

**4. Discussion**

4.1. Cytotoxicity

It was shown earlier that HPT significantly refines the grain size and increases the strength of pure magnesium and the AZ31, AZ91 and ZK60 alloys[[14](#_ENREF_14)]. Improving the mechanical properties is important for load-bearing orthopaedic implants but, in addition, the biocompatibility and corrosion behaviour must be fully examined.

It is well established that pure magnesium is biocompatible. The effect of texture, concentration and the pH of extruded pure magnesium samples on a mouse osteoblast cell viability were investigated recently and the results showed that different textures and concentrations up to 4 x 103μg mL-1had no effect on the cells whereas the cell viability was lower than the control for pH 10.0 [[31](#_ENREF_31)]. Grain refinement of cast pure magnesium by small (0.25% and 1%) additions of Zr enhanced the osteoblast cell activity and proliferation in experiments performed *in vitro*[[32](#_ENREF_32)]. The present results demonstrate that the pronounced grain refinement produced by HPT does not compromise the *in vitro*biocompatibility of pure magnesium.

The cytotoxicity of different magnesium alloys was analyzed on human osteosarcoma cells (MG63) and there was acceptable toxicity (grade I) in extruded and ECAP-processed AZ31 except for one ECAP condition that led to a grade II toxicity [[33](#_ENREF_33)]. It was also shown that non-cytotoxic coatings may be used in this alloy. An AZ31 alloy coated with polycaprolactone showed a higher rat skeletal muscle cell viability than annealed samples[[34](#_ENREF_34)]. *In vitro* experiments demonstrated that a composite magnesium phytic acid/apatite coating, synthesized on an AZ31 substrate, enhanced the cell viability of osteoblasts[[35](#_ENREF_35)]. Mouse osteoblastic cells (MC3TE) exposed to magnesium alloy extracts showed suitable cytocompatibility for AZ31 and AZ91 (untreated, anodized) but a reduced cell viability was reported in ZK60 [[21](#_ENREF_21)].Similarly, murine fibroblast cells (L-929) and human osteosarcoma cells (MG63) were significantly affected by 100% extracts of as-cast and extruded ZK60 alloy, indicating that these materials generated cytotoxicity [[36](#_ENREF_36)]. Despite these results, it was shown that a Mg-Zn-Zr alloy with lower zinc content, ZK30, displays a low degradation rate and good biocompatibility in rabbit bone marrow stromal cells[[37](#_ENREF_37)].

Overall, the results reported in the present study show appropriate cytocompatibility of AZ31, AZ91 and ZK60 after processing by HPT and this demonstrates that HPT processing does not compromise the *in vitro* biocompatibility of these magnesium alloys.

4.2. Corrosion

In order to discuss the results of the corrosion tests in this investigation it is important to initially clarify their limitations. It has been shown that corrosion rates estimated from Tafel extrapolation of polarization tests are not reliable [[38](#_ENREF_38)]. Furthermore, polarization tests and EIS tests display significant variations depending on the sample immersion time [[22](#_ENREF_22), [23](#_ENREF_23), [28](#_ENREF_28), [30](#_ENREF_30), [38](#_ENREF_38)]. Hydrogen evolution tests may underestimate the corrosion rate for slower rates due to hydrogen diffusion to the sample [[7](#_ENREF_7)] and solubilisation in the solution. Finally, some corrosion tests of magnesium alloys in simulated body fluids were carried out at 37°C to provide corrosion conditions similar to the human body. As a consequence, the available data display significant differences in electrochemical tests for similar alloys. Thus, care was taken in the present experiments to maintain similar immersion conditions and times for all samples before the electrochemical tests to permit direct comparisons between them.

The current results suggest a stronger influence from the alloy composition than from the HPT processing on the corrosion behaviour of magnesium. The zinc-rich ZK60 alloy displays generalised corrosion at fast rates whereas CP-Mg, AZ31 and AZ91 exhibit localized corrosion. The hydrogen evolution tests and the appearance of the samples after 28 days of immersion in Hank´s solution reveal that the aluminium-rich alloys, AZ31 and AZ91, corrode at a slower rate and are less prone to severe corrosion localization than the as-cast CP-Mg. This is not consistent with the faster corrosion reported for AZ91 than high-purity Mg in Hank´s solution at 37 °C[[18](#_ENREF_18)] and in 3% NaCl solution at room temperature [[39](#_ENREF_39)]. It is also not consistent with the similar corrosion reported in these materials in Hank´s solution at room temperature [[39](#_ENREF_39)]. This difference in behaviour is attributed to the solid solution treatment that was given to the AZ91 alloy in the present experiments. Thus, it was shown earlier that this thermal treatment significantly reduces the fraction of the β phase and the HPT processing at room temperature does not promote significant precipitation [[14](#_ENREF_14)]. Also, the thermal treatment increases the amount of aluminium in solid solution and these effects enhance the corrosion resistance of this magnesium alloy. Furthermore, in support of the results obtained in the current study, EIS tests showed an improved corrosion resistance in the solution treated AZ91 alloy compared to the CP-Mg in a 3.5% NaCl solution before and after HPT processing [[14](#_ENREF_14)].

It is concluded from the present experiments that HPT processing may either increase or decrease the corrosion resistance of magnesium and its alloys. This apparent dichotomy is explained based on the tendency of the material to develop a protective layer of corrosion products. It appears that grain refinement may either increase or decrease the corrosion resistance of metals depending upon the stability and effectiveness of the oxide film. For example, experiments show that an increase in corrosion resistance with grain refinement is expected in metals with low corrosion rates and a decrease is expected in conditions of high dissolution rates [[40](#_ENREF_40)]. This is in agreement with the present results since grain refinement by HPT processing of the ZK60 alloy, which exhibits the faster corrosion rate and no trend to passivation, promoted an increase in the corrosion rate. On the other hand, CP-Mg and the AZ31 and AZ91 alloys display passivation-like behaviour in the polarization test and HPT processing appears to improve their corrosion resistance. It is important to note also that an increase in corrosion resistance due to grain refinement to the ultrafine range was reported in a Mg-Y-RE alloy after friction stir processing [[41](#_ENREF_41)].

Finally, it should be noted that the Al-rich AZ31 and the AZ91 alloys exhibit small second phase particles and the SEM-EDS analysis shows they tend to trigger localized corrosion. Despite the presence of these particles, these alloys do not undergo severe localized corrosion after up to 28 days of immersion in Hank´s solution which suggests that the aluminium in solid solution improves the quality of the protective surface layer. The long term consequences of the release of aluminium in the human body will require a future comprehensive investigation but nevertheless the present results indicate that this is a significant alloying element for enhancing the corrosion resistance of magnesium.

**5. Summary and conclusions**

1. CP-Mg and AZ31, AZ91 and ZK60 alloys were processed by HPT and subjected to biocompatibility tests. Electro-chemical tests and hydrogen evolution tests were used to evaluate the corrosion behaviour in Hank´s solution at room temperature and the surface features after corrosion were analysed using SEM-EDS.

2. HPT processing does not compromise the biocompatibility of the different magnesium alloys. MTT tests showed metabolic activity over 80% for cells subjected to 24 hours of contact with all materials. A LIVE / DEAD assay showed that the great majority of the cells are live and with good morphology after contact.

3. CP-Mg and the AZ31 and AZ91 alloys exhibit a passivation-like behaviour in polarization tests whereas the ZK60 alloy exhibits generalised corrosion behaviour. However, localized corrosion takes place in the CP-Mg and the AZ31 and AZ91 alloys. Severe propagation of localized corrosion is observed only in the as-cast CP-Mg but this is prevented by HPT processing.

4. The AZ91 alloy displays the largest polarization resistance due to an initial solid solution treatment that reduced the amount of second phase particles and increased the aluminium content in the magnesium matrix. The ZK60 alloy exhibits the smallest resistance. Processing by HPT significantly increases the polarization resistance of the AZ31 alloy and CP-Mg.

5. Processing by HPT improves the corrosion resistance of samples with a tendency for passivation (CP-Mg, AZ31 and AZ91) and decreases the resistance of the ZK60 alloy which exhibits generalised corrosion.

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