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In vitro corrosion, cytotoxicity and hemocompatibility of bulk nanocrystalline pure iron

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Abstract
Bulk nanocrystalline pure iron rods were fabricated by the equal channel angular pressure (ECAP) technique up to eight passes. The microstructure and grain size distribution, natural immersion and electrochemical corrosion in simulated body fluid, cellular responses and hemocompatibility were investigated in this study. The results indicate that nanocrystalline pure iron after severe plastic deformation (SPD) would sustain durable span duration and exhibit much stronger corrosion resistance than that of the microcrystalline pure iron. The interaction of different cell lines reveals that the nanocrystalline pure iron stimulates better proliferation of fibroblast cells and preferable promotion of endothelialization, while inhibits effectively the viability of vascular smooth muscle cells (VSMCs). The burst of red cells and adhesion of the platelets were also substantially suppressed on contact with the nanocrystalline pure iron in blood circulation. A clear size-dependent behavior from the grain nature deduced by the gradual refinement microstructures was given and well-behaved in vitro biocompatibility of nanocrystalline pure iron was concluded.

(Some figures in this article are in colour only in the electronic version)

1. Introduction
As a body-friendly and essential micronutrient [1], iron is not only mostly distributed in hemoglobin, reticuloendothelium system and organs such as liver and spleen [2], but also widely involved in oxygen fixation and reaction to metabolization [3]. Recently, pure iron has been developed as a potentially biodegradable material for use in such devices as cardiovascular stents [4–7]. The in vitro corrosion behavior and cytotoxicity evaluation of the commercially pure iron were reported previously [5]. Even more, long-term [6] and short-term [7] in vivo animal tests with porcine, dogs or rabbits validated that pure iron implants had neither obvious localized adverse effects nor systemic allergy. However, the main barrier to pure iron being employed as a biodegradable stent material lies in the endothelialization fulfillment [8], mechanical failure [4] or restenosis due to the thickness of the struts [7]. Various techniques have been used recently on pure iron, such as plasma ion implantation and deposition technology [9, 10], surface nanocrystallization [11] or metal alloying [12] to enhance its physical and biological properties.
Bulk nanocrystalline/nanostructured materials by severe plastic deformation were developed and spotlighted in wide fields [13]. Under the inspiration of both ultra-high practical strength and superior biological performance of nanocrystalline titanium [14] by equal channel angular pressure (ECAP), we are motivated to prepare three-dimensional bulk nanocrystalline pure iron. On the one hand, the three-dimensional bulk nanostructure can eliminate the interface mismatch problem between the nanocrystalline surface layer and the microcrystalline substrate of the above-mentioned surface nanocrystallized pure iron, on the other hand the strengthening through severe plastic deformation might be beneficial to its application as stent for pure iron, since promoting radial strength by the material itself means a thinner strut/low profile of stent during design, and thus may reduce the risk of thrombosis. In this study, we for the first time investigate the in vitro corrosion and biocompatibility of three-dimensional bulk nanocrystalline pure iron, and study also the effect of the severe deformation degree on the in vitro biological performance of the pure iron.

2. Experimental details

2.1. Sample preparation

Nanocrystalline pure iron (initially from commercial pure iron (>99.8 wt%)) was fabricated via ECAP by way of Bc route [13] up to eight passes. Four sample groups of zeroth, second, fourth and eighth pass ECAPed Fe (Φ8 mm × 1.5 mm) were chosen. They were mechanically polished up to 2000 grit, ultrasonically cleaned in acetone, absolute ethanol and distilled water, and then quickly dried in the open air. For the extraction preparation in the cell test, all the samples were sterilized in the autoclave at 121 °C for 30 min.

2.2. Materials characterization

An x-ray diffractometer (XRD, Rigaku DMAX 2400) using Cu Kα radiation was employed for the identification of the constituent phases. Transmission electron microscopy (TEM, JEM 200CX) operated at 120 kV was performed to validate the grain size and microstructural morphology of the experimental sample foils obtained by twin-jetting with 5 vol.% perchloric acid and 95 vol.% ethanol as electrolyte at 50 kV and 30 mA after standard grinding, polishing and dimpling procedures. Optical microscopy (OM, Olympus BX 51M) was used for microstructural observation of a coarse-grained sample after 5 vol.% nitric acid and 95 vol.% ethanol etching in a standard way. The tensile tests were carried out at a strain rate of 4 × 10⁻⁴ s⁻¹ with an Instron 3366 universal test machine, whereas the micro-hardness was measured using an HMV-2T (Shimadzu, Japan) micro-hardness tester.

2.3. Electrochemical and immersion testing of corrosion

The platinum-made counter-electrode and the reference electrode by a saturated calomel electrode (SCE) were utilized in the three-electrode cell for electrochemical measurements. The area of the working electrode exposed to the solution was 0.1256 cm². All the measurements were carried out on an electrochemical workstation (CHI660C, China) at a temperature of 37 °C in Hank’s solution. The polarization was scanned from −1.0 to 1.0 V (vs SCE) with a rate of 20 mV s⁻¹. The surface morphology of the samples after corrosion was characterized by environmental scanning electron microscopy (ESEM, AMRAY-1910 FEI).

The natural immersion test was carried out in Hank’s solution, 20 ml for each sample according to ASTM-G31-72 [15] at 37 °C in a water bath. After 1, 3, 7 and 14 days, the samples were removed from the soaking solution, gently rinsed with distilled water and quickly dried in the case of oxidation. The change of the surface chemical composition of experimental samples was analyzed by XPS (AXIS Ultra, Kratos). Inductively coupled plasma atomic emission spectrometry (Leeman, Profile ICP-AES) was employed to measure the concentration of Fe ions dissolved from the samples into the solution. An average of three measurements was taken for each group.

2.4. Cellular responses

Murine fibroblast cells (L-929), vascular smooth muscle cells (VSMCs) and human umbilical vein endothelia cells (ECV304) were adopted to evaluate the cytotoxicity of zeroth, second, fourth and eighth pass ECAPed Fe by indirect assay and direct seeding on the surface of the samples. Extracts were prepared using Dulbecco’s modified Eagle’s medium (DMEM) serum-free medium as the extraction medium with the surface area of the extraction medium ratio being 3 cm² ml⁻¹ [16] in a humidified incubator with 5% CO₂ at 37 °C for 72 h. DMEM here consists of 10% fetal bovine serum (FBS), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin. Negative controls of DMEM-cultured cells and positive controls of DMSO-added cells were involved. The optical density (OD) values by MTT assay was measured by a microplate reader (Bio-RAD680) at 570 nm with a reference wavelength of 630 nm. A cell density of 5 × 10⁴ ml⁻¹ was seeded directly on the substrate of different samples in the 24-well plate. Cellular attachment, spread and detailed morphologies were studied by ESEM after Au sputtering.

2.5. Hemocompatibility

Diluted blood was prepared with normal saline (4:5 ratio by volume) by healthy human blood containing sodium citrate (3.8 wt%) in the ratio of 9:1. Experimental samples were immersed in a 10 ml saline tube pre-incubated at 37 °C for 30 min, following 60 min incubation after addition of 0.2 ml diluted blood into this tube. The saline solution was used as a negative control and deionized water as a positive control. Then, all the tubes were centrifuged for 5 min at 3000 rpm and the supernatant was carefully taken out to the cuvette for spectrophotometric analysis at 545 nm. The hemolysis was calculated by the following formula and averaged by three replicates:

\[
\text{Hemolysis} = \frac{\text{OD(test)} - \text{OD(negative control)}}{\text{OD(positive control)} - \text{OD(negative control)}} \times 100\%
\]
Figure 1. Photographs of experimental pure iron samples after zeroth, second, fourth and eighth pass ECAP.

Platelet-rich plasma was prepared by centrifuging whole blood for 10 min at a rate of 1000 rpm min\(^{-1}\). Then it was dripped onto the sample surface and incubated for 1 h at 37 °C. Thereafter PBS was used to remove the non-adherent platelets. The adhered platelets were fixed in 2.5% glutaraldehyde solution for 1 h at room temperature followed by dehydration in a gradient ethanol/distilled water mixture (from 50% to 100%) for 10 min each. The morphologies of platelet-attached experimental samples were observed by ESEM. The platelet numbers at random fields of view for each material group were counted.

2.6. Statistics analysis

All experiments were performed at least three times, data averaged and expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and significance was considered at \(P \leq 0.05\).

3. Results

3.1. Materials characterization

Figure 1 shows the macroscopic pictures of pure iron rod samples which were just ECAPed after zeroth, second, fourth and eighth pass. Microscopically speaking, a mean grain size of about 50 \(\mu m\) can be found for the initial zeroth pass ECAPed Fe, as illustrated by the metallographic observation under OM (figure 2(a)). After two ECAP passes and the follow-up annealing process, the average grain size of experimental pure iron decreases to about 40 \(\mu m\), as shown in figure 2(b). The representative nanostructure of eighth pass ECAPed Fe was investigated by a bright field observation mode under TEM, which indicates a distinct-oriented polycrystalline structure of very fine crystal grains ranging in size from 80 to 200 nm, as given in figure 2(c). These microstructural observation results coincided well with the peak positions and the grain size calculated by the half-width of peaks in XRD patterns, as illustrated by figure 3.

Figure 4 demonstrates the micro-hardness value of pure iron ECAPed after zeroth, second, fourth and eighth ECAP pass (114 ± 16, 351 ± 22, 397 ± 27 and 444 ± 31 kgf mm\(^{-2}\)) and the tensile strength variation for zeroth, second, fourth and eighth pass ECAPed Fe samples (262 ± 13, 313 ± 18, 381 ± 24 and 470 ± 29 MPa), reflecting the mechanical performance as a function of grain size.

Figure 2. Optical microscopic images of (a) zeroth pass ECAPed Fe; (b) second pass ECAPed Fe and the TEM image of (c) eighth pass ECAPed Fe, with the electron diffraction pattern inset showing the nanocrystalline diffraction ring feature.
Figure 3. XRD patterns of zeroth, second, fourth and eighth pass ECAPed Fe samples.

3.2. Electrochemical corrosion and immersion tests

Figure 5(a) reveals the electrochemical curves of open circuit potential (OCP) mode, from which it can be seen that all curves of the experimental samples go down sharply at the beginning and become gradually stable after a certain period. The ending OCP values of fourth and eighth pass ECAPed Fe stay high above those of zeroth and second pass ECAPed Fe.

Figure 5(b) shows the potentiodynamic polarization curves of zeroth, second, fourth and eighth pass ECAPed Fe, from which no pitting corrosion but a typical passivation duration in the anodic polarization zone can be revealed. In spite of minor divergence, two individual groups can be divided. One is of ultrafine-grained eighth and fourth pass ECAPed Fe (group I) with lower current density and higher potential ([1.66 ± 0.09] × 10^{-6} A cm^{-2} and [2.32 ± 0.12] × 10^{-6} A cm^{-2}; −0.600 ± 0.008 V and −0.616 ± 0.006 V for eighth and fourth pass ECAPed Fe, respectively), and the other is coarse-grained second and zeroth pass ECAPed Fe (group II; [7.11 ± 0.10] × 10^{-6} A cm^{-2} and [7.79 ± 0.13] × 10^{-6} A cm^{-2}; −0.671 ± 0.005 V and −0.642 ± 0.003 V for second and zeroth pass ECAPed Fe, respectively) with obvious attacks at grain boundaries and deeper grooves mostly distributed on their surface (see figure 6).

A standard immersion test under ASTM directive was designed to make clear the fundamental natural corrosion behavior of the pure iron in simulated physiological fluid. Figure 5(c) reveals the release amount of Fe ion in a short-term immersion in Hank’s solution for the experimental pure iron plate samples, with the extension of the immersion time. It can be seen that on the 14th day of natural immersion corrosion, the released dosages of the fourth and eighth pass ECAPed Fe (group I) samples were about half of those released from zeroth and second pass ECAPed Fe (group II).
samples. It means that the Fe ion leakage decreases sharply with the decrement of the grain size of pure iron samples at the same time point since day 4. Meanwhile, the quantity of Fe ions gradually increases with the time increment and shows a significant ($P < 0.05$) difference among different material groups except for the case on day 1. According to the plot, the mean amount of Fe ion released from the experimental samples is 10.83, 9.7, 4.66 and 2.77 $\mu$g ml$^{-1}$ till the 14th day immersion, and the corrosion rate is 18.0 ± 4.3, 13.7 ± 1.4, 9.9 ± 3.8 and 7.9 ± 4.3 $\mu$g cm$^{-2}$ h$^{-1}$ for zeroth, second, fourth and eighth pass ECAPed Fe samples, respectively.

Figure 6 shows typical surface images of the zeroth, second, fourth and eighth pass ECAPed Fe samples observed by SEM after the electrochemical corrosion. For the zeroth pass ECAPed Fe, a multitude of bigger and deeper holes appeared all over the working area (figure 6(a)), while on the surface of the second and fourth pass ECAPed Fe (figures 6(b) and (c)), the isolated group of rough potholes was uncovered with more original intact area. As for the nanocrystalline sample of eighth pass ECAPed Fe, only small dimples were present on the superficial surface after the electrochemical corrosion in Hank’s solution (figure 6(d)).

Figure 7 displays the SEM images of the zeroth, second, fourth and eighth pass ECAPed Fe samples after static immersion in Hank’s solution for 14 days. Cracks of the covered thick corrosion products on the surface of microcrystalline samples of zeroth and second pass ECAPed Fe can be evidently seen, whilst the surface of ultrafine-grained fourth and eighth pass ECAPed Fe demonstrates only shallow pits and limited attachment of the inorganic salts, with the original scratch during sample preparation still observable.

Figure 8 shows the XPS characterization on the surface of the experimental examples. As revealed by the high sensitivity (survey) scan and high resolution spectrum of Fe 2p in figures 8(a)–(d), Fe$_2$O$_3$ and Fe$_3$O$_4$ mixtures are found to be the main corrosion products covering the surface of experimental pure iron samples after 14 days’ immersion in Hank’s solution, although their contents were sample dependent. The inset high-resolution spectra further reveal that elements Ca and P were present on the surface of the fourth pass ECAPed Fe sample but not on the surface of zeroth pass ECAPed Fe.

### 3.3. Cellular response and hemocompatibility evaluation

Figure 9 shows the cell viability cultured in zeroth, second, fourth and eighth pass ECAPed Fe extraction medium for 1, 2 and 4 days, with three cell line models. The number of fibroblast cells (L-929) of the zeroth and eighth pass ECAPed Fe groups was much higher than that with the groups of second and fourth pass ECAPed Fe, as shown in figure 9(a). As for the VSMC line, the viability of cells co-cultured with the nanostructured eighth pass ECAPed Fe extract group was significantly lower than that with the groups of second and fourth pass ECAPed Fe, as shown in figure 9(b). Conversely, the proliferation of ECs co-cultured with the extract of the nanostructured eighth pass ECAPed Fe extract group was much higher than that co-cultured with the zeroth pass ECAPed Fe extract group (figure 9(c)).

Figure 10 demonstrates the SEM images of the cell morphologies after direct culture on top of the zeroth, second, fourth and eighth pass ECAPed Fe plate samples. It can
be easily seen that many cells severely shrank or wrinkled due to the surrounding corrosion products resulting from the chemical reaction of Fe substrate and aqueous extractions. Relatively speaking, it can be found that the cell lines of L-929 and ECs grew and attached well on the Fe substrate layer by layer, especially on the surface of the eighth pass ECAPed Fe sample, while VSMCs show less activity and single thin cell spread on the samples, without significant proliferation. Good correspondence could be found between the direct observation and indirect cell viability evaluation.

Figure 7. SEM images of sample surface morphology on (a) zeroth pass ECAPed Fe; (b) second pass ECAPed Fe; (c) fourth pass ECAPed Fe and (d) eighth pass ECAPed Fe samples after 14 days’ immersion in Hanks’ solution.

Figure 8. High intensity XPS on the surface of (a) zeroth pass ECAPed Fe; (b) second pass ECAPed Fe; (c) fourth pass ECAPed Fe and (d) eighth pass ECAPed Fe samples after 14 days’ immersion in Hank’s solution.
Figure 9. Cell proliferation of ECAPed pure iron samples cultured with (a) L-929; (b) VSMC and (c) ECV304. *Significant against cell proliferation on control group at $P \leq 0.05$.

Figure 10. SEM observation showing the morphologies of cellular attachment for different cell lines ((a)–(d) for L-929, (e)–(h) for VSMCs and (k)–(n) for ECs) on different samples ((a), (e), (k) for zeroth pass ECAPed Fe; (b), (f), (l) for second pass ECAPed Fe; (c), (g), (m) for fourth pass ECAPed Fe; (d), (h), (n) for eighth pass ECAPed Fe).

Figure 11(a) reveals the hemolysis rate for zeroth, fourth and eighth pass ECAPed Fe samples, which were all lower than 5%, reflecting their good hemocompatibility. In order to further understand the nature of blood interaction with different Fe substrates, a direct study of platelet adhesion was carried out. For coarse-grained substrates of initial zeroth pass ECAPed Fe, a pattern of shaggy and loose platelet balls was found on the sample surface (figure 11(b)). The crowded and aggregated platelet clusters were positively scattered on the surface of the fourth pass ECAPed Fe, as shown in figure 11(c). Adhered platelets with a conglobated shape and smooth surface, having the lowest surface energy and free energy and minimizing the activity interfaced with the substrate, were found on the surface of the nanostructured eighth pass ECAPed Fe, as given in figure 11(d).

4. Discussion

It is clearly known that rigidity and body strength of ECAP-induced nanocrystalline pure Fe are highly improved over those of the traditional microcrystalline pure iron samples, far beyond satisfying the demand of commercial stents (withstanding up to 1000 mm Hg crush pressure [17]). About
Figure 11. (a) Hemolysis rate of zeroth, fourth and eighth pass ECAPed Fe and the SEM image showing the platelet adhesion morphology on (b) zeroth pass ECAPed Fe, (c) fourth pass ECAPed Fe and (d) eighth pass ECAPed Fe samples.

twofold or fourfold difference in tensile strength is clearly shown when the grain size of pure iron decreases from microcrystalline to nanocrystalline, which brings it well into correspondence with the classic Hall–Petch rule [18, 19] and our previous research [20].

Cell-induced electrochemical corrosion and active cellular destruction of surface mechanisms were established to explain the reason and pointed the way forward to the feasibility of utilizing the corrosion of metals [21]. Ever since, in vitro and in vivo research on the corrosion and other specific investigations of metallic stents has been carried out, most of which were made up of iron or magnesium alloys. Table 1 summarizes the data of the corrosion rate for various pure iron materials/devices we can find from the literature. Disregarding the size of samples and measurement methods under different circumstances, our present result for the corrosion rate for the case of zeroth pass ECAPed Fe (18.0 μg (cm² h)⁻¹) is in good consistency with those (equivalently based on commercially pure iron) from other researchers’ reports (from 18.3 to 20.4 μg (cm² h)⁻¹) [5, 22]. Talking about the nanocrystalline eighth pass ECAPed Fe, the nanocrystalline nature can effectively slow down the severe corrosive procedure of pure iron (7.9 μg (cm² h)⁻¹).

Meanwhile, the enhanced tensile/yield strength of the nanocrystalline eighth pass ECAPed Fe makes it possible that stents can be designed and realized with much thinner struts and lighter mass, yet without the loss of sufficient mechanical support. In addition, iron stents with a major bulk within early corrosion would maintain the radial/longitudinal strength to support the blood pressure in a vessel and prohibit the occurrence of recoil [23–25] in an adorable duration. Referred to the maximum value of 7 μg ml⁻¹ for complete degradation of the iron stents [5], the corrosion rate and durable lifespan of the nanocrystalline eighth pass ECAPed Fe sample in this investigation fit well as a biodegradable stent material.

Concerning the cytotoxicity of Fe ion, Huang et al [5] pointed out the critical concentration of Fe ion collected from various iron-containing salt solutions to cause cytotoxicity of ECs is 50 μg ml⁻¹, and much lower iron concentration (<10 μg ml⁻¹) may even produce a favorable effect on the metabolic activity of ECs. Our results regarding the cell proliferation of ECV304 are in good agreement with the statement above for the dissolved ion concentration after immersion in DMEM for extraction of zeroth, second, fourth and eighth pass ECAPed Fe in the present study is about 2.6, 2.45, 2.16 and 1.9 μg ml⁻¹, respectively, far below.
the critical concentration of intoxication induced by Fe ions [26]. A similar situation exists for the L929 cell line, that is, no cytotoxicity is demonstrated when L929 cells were incubated with extracts from all the experimental samples for 4 days. However, for VSMCs, a depressed proliferation and migration of VSMCs of eighth pass ECAPed Fe groups can be found from the present results. Together with the cell attachment and spread results, larger number even multilayers in the cell lines of L-929 cells and ECs are clearly shown on the fourth and eighth pass ECAPed pure iron over those on the zeroth and second pass ECAPed pure iron, while an apparently decreased number of VSMCs grow on all the substrates. No pseudopods from cells can be observed on the eighth pass ECAPed pure iron sample. All the direct assays above exhibit good agreement with the indirect cytocompatible evaluation. According to the mechanisms of Fe(II)-mediated cellular responses by gene expression [27], the neutoinima proliferation would also suppress the thrombogenicity and restenosis, with the nanocrystalline eighth pass ECAPed pure iron was significantly illustrated, implying a reduced risk of neointimal hyperplasia, thrombogenicity and restenosis.

5. Conclusions

In summary, the bulk nanocrystalline eighth pass ECAPed pure iron in our study exhibited an excellent combination of both surface and integrated bulk properties, which is essential for biosafety and biostability of successful stent implant materials. Specifically:

1. An elevation of doubled tensile strength and four-time folded micro-hardness of the nanocrystalline eighth pass ECAPed pure iron over the microcrystalline counterpart is clearly concluded after the ECAP-oriented nanocrystallization, which may help providing early-stage enhanced mechanical behavior and sufficient strength in the follow-up duration.

2. Superior corrosion resistance and a slower degradation rate (7.9 μg (cm² h)⁻¹ versus 18.0 μg (cm² h)⁻¹) of the nanocrystalline eighth pass ECAPed pure iron over that of the zeroth pass ECAPed pure iron are deduced from the electrochemical measurements and static ion immersion test in the simulated body fluid. Nevertheless, a tinier corrosion product with slimmer structure due to the powerful physical parameters above can guarantee a desired duration for the nanocrystalline eighth pass ECAPed pure iron after a durable corrodbile procedure in balance.

3. All the samples, no matter whether microcrystalline or nanocrystalline structures, show well-behaved in vitro hemocompatibility, with a credible hemolysis rate less than 5%. Smaller quantity and free activation of adherent platelets can be detected on the surface of the nanocrystalline eighth pass ECAPed pure iron.

4. Preferential cellular response was found. Cell growth of VSMCs is clearly inhibited for the cases of the nanocrystalline eighth pass ECAPed Fe (less than 60% up to 4 days culture), while the promotion on ECs growth and L-929 cytocompatibility (both more than 80%) are greatly improved.

More importantly, the above results depict a clear size-dependent evolution from the nature of microcrystalline grain to nanoscale microstructures. As a result, bulk nanocrystalline eighth pass ECAPed pure iron, a new member of this bulk nanometal family with both well-behaved compatible and hemocompatible performances, would be a novel promising alternative to the future body implants.
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