Good Biocompatibility and Sintering Properties of Zirconia Nanoparticles Synthesized via Vapor-phase Hydrolysis

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ZrO₂ nanoparticles were synthesized by a vapor-phase hydrolysis process, and characterized in terms of crystalline structures, hardness and microstructures by X-ray diffraction, Vickers hardness test method, and atomic force microscopy (AFM) measurements. Moreover, in vitro cytotoxicity evaluation and hemolysis assay showed that the nanoparticles possessed good biocompatibility. Hardness investigations and AFM measurements indicated that both the sintering temperature and compression force played an important role in determining the physical behaviors (hardness, roughness and density) of flakes of the ZrO₂ nanoparticles. When ZrO₂ nanoparticles synthesized at 500 °C were pressed into flakes under 6 MPa and sintered at 1400 °C, the resulting flakes exhibited an optimal combination of hardness (534.58 gf·mm⁻²), roughness (0.07 μm) and density (4.41 g·cm⁻³). As the Vickers hardness value of human bones is of 315~535 gf·mm⁻² and the density of adult femur is about 1.3~1.7 g·cm⁻³, the experimental results showed that the ZrO₂ flakes were comparable to human bones with a higher density. As a result, the synthesized ZrO₂ NPs may be useful for biomedical applications, especially for bone repair and replacement in future.

The field of biomaterial technology has rapidly progressed over the last few decades with the advent of advanced medical devices and implants developed from metals and ceramics. It is known that implantation of biomaterials also causes a cascade of reactions in the biological environment. For instance, the insertion of some implants may lead to bacterial infections along the bone/material interface due to poor biocompatibility of the implants despite total disinfection prior to the surgeries. After the bacterial proliferation period, the biomaterial-associated infections can hardly be cured by traditional systemic antibiotic therapy. Hence, there is a pressing need for the development of safe biocompatible implants. The success of rapid osseointegration of orthopedic implanted materials is dependent on the formation capability between the implants and bones, when the implants are embedded in a living body. Metal alloys (e.g., Ti, Sr, Co, Cr, etc.) are generally expected to be bioinert with biological systems in the human body. Consequently, implants made of metal alloys used in bone reconstruction typically lack desired osseointegration properties, which limits their biological fixation with bone tissues and consequently long-term in vivo stability. Moreover, metal ions can be released from alloy implants due to its frequent interactions with the surrounding physiological environment, which may lead to detrimental effects on the long-term health of the patients.

Zirconia (ZrO₂), as one of the most important oxide materials, exhibits excellent properties including low thermal conductivity, high thermal expansion, good thermal stability, fine mechanical strength, good fracture toughness and high thermal shock resistance. Thus, ZrO₂ has been used in a broad range of applications as catalysts/catalyst supports, oxygen sensors, fuel cells, biological materials, automobile parts and thermal barrier coatings on metal components. ZrO₂ nanomaterials have also been employed in medical and orthopedic applications, mainly for repair and replacement of diseased and damaged parts of human skeleton.

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bones, teeth and joints due to their good biocompatibility, osseointegration, and bioinertness\textsuperscript{20}. In fact, because of nontoxicity to the surrounding tissues, implants based on ZrO\textsubscript{2} nanoparticles (NPs) have been utilized for clinical total hip replacements, and as a prevalent biomaterial in prosthetic dentistry and dental implantology\textsuperscript{5,13,16,21,22}. This is largely ascribed to the good physical performance of sintered ZrO\textsubscript{2} devices, in terms of hardness, density, roughness and stability.

Currently, the synthetic methods of nano-sized ZrO\textsubscript{2} mainly include gas-phase methods (e.g., gas-phase chemical synthesis\textsuperscript{23–26} and chemical vapor deposition\textsuperscript{27–29}), and liquid-phase methods (fast precipitation\textsuperscript{30–33}, sol–gel\textsuperscript{34–36}, solvent evaporation, and hydrothermal treatment). However, up to now, reports remain scarce involving a comprehensive and detailed investigation of the sintering properties of ZrO\textsubscript{2} NPs under different conditions.

In this paper, ZrO\textsubscript{2} NPs were synthesized by using a simple vapor-phase hydrolysis process at controlled temperatures, and utilized to prepare nanoflakes by compression and sintering. By a systematic variation of the compression force and sintering temperature, the hardness and density of the resulting ZrO\textsubscript{2} nanoflakes were maximized whereas the roughness was minimized. The physical properties such as hardness, roughness as well as density of the sintered ZrO\textsubscript{2} nanoflakes were better than those of the human bone. Furthermore, \textit{in vitro} cytotoxicity and hemolysis evaluation showed that the ZrO\textsubscript{2} NPs possessed good biocompatibility. These findings suggest great potential of the ZrO\textsubscript{2} NPs as a biomcompatible material for medical implants for bone tissue engineering because they meet the demand of high physical properties of artificial hard tissues.

### Results

#### Structures of ZrO\textsubscript{2} NPs.

The structures of the obtained ZrO\textsubscript{2} NPs were first characterized by X-ray diffraction (XRD) measurements. Figure 1 shows the XRD patterns of ZrO\textsubscript{2} NPs synthesized at different temperatures (400, 500 and 600 °C). A series of well-defined peaks can be identified at 2\textdegree; = 30.2, 35.0, 50.4, 60.0 and 62.7\textdegree; which were ascribed to the diffractions of the (101), (110), (200), (211), and (202) crystalline planes of cubic phase ZrO\textsubscript{2} (JCPDS card no. 49-1642)\textsuperscript{21}, respectively. In addition, the asymmetric line shape of the peaks at 35.0, 50.4 and 60.0\textdegree; suggested the formation of a tetragonal phase. The shoulder at 2\textdegree; = 34.5\textdegree; was the diffraction of the (002) crystalline plane of tetragonal phase ZrO\textsubscript{2} (JCPDS card no. 42-1164), and those at 2\textdegree; = 50.2\textdegree; and 59.1\textdegree; corresponding to the diffractions of the (112) and (103) crystalline planes\textsuperscript{19}. Furthermore, from Fig. 1, it can be seen that the crystallinity of the ZrO\textsubscript{2} NPs increased with increasing synthesis temperature from 400 °C to 600 °C.

#### SEM Analysis.

Further structural insights were obtained in SEM measurements. From Supporting Figure S1, it can be seen that the ZrO\textsubscript{2} NPs are mostly in the range of 15 to 65 nm in diameter. Statistical analysis based on more than 50 particles showed that the average diameter of the nanoparticles decreased with increasing synthesis temperature, 40 nm at 400 °C, 35 nm at 500 °C, and 30 nm at 600 °C, as manifested in the core size histograms (Supporting Figure S2).

#### In Vitro Cytotoxicity.

Interestingly, the resulting ZrO\textsubscript{2} NPs were found to exhibit low cytotoxicity, as manifested in \textit{in vitro} studies with human umbilical vein endothelial cells lines (HUVEC). Experimentally, ZrO\textsubscript{2} NPs were dispersed under sonication at varied concentration (up to 1 mg·mL\textsuperscript{−1}) into dulbecco's modified eagle medium (DMEM) and added to the HUVEC cell culture. The \textit{in vitro} cytotoxicity of the ZrO\textsubscript{2} NPs in HUVEC cells was evaluated by CCK-8 assay. Control experiments were also carried out by dispersing the ZrO\textsubscript{2} NPs in deionized water, and phosphate buffer saline (PBS) (Fig. 2). From Fig. 3, it can be seen that the ZrO\textsubscript{2} NPs (up to 500 μg·mL\textsuperscript{−1}) exerted virtually no effect on cell viability after co-incubation for 24 h. For example, the HUVEC cells retained 92% of viability even at the concentration of 500 μg·mL\textsuperscript{−1} of ZrO\textsubscript{2} NPs synthesized at 400, 500, or 600 °C.

Hemolysis of the ZrO\textsubscript{2} NPs was also evaluated by incubating the NPs with red blood cells (RBCs) for 4 h. It can be seen that the hemolytic percentages of RBCs were lower than 3.6% for the NPs synthesized at 400, 500, and 600 °C even at the concentration as high as 800 μg·mL\textsuperscript{−1}, implying that these NPs had a negligible hemolytic activity (Fig. 4a–c). Therefore, it can be concluded that the ZrO\textsubscript{2} NPs exhibit good biocompatibility and thus can act as a promising bio-ceramic materials for prosthetic dentistry and dental implantology\textsuperscript{37,38}. 

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**Figure 1.** XRD patterns of ZrO\textsubscript{2} NPs synthesized at different temperatures.
Physical Hardness. With such remarkable cytocompatibility and hemocompatibility, ZrO₂ NPs-based materials may be viable candidates for biomedical applications. Thus, the ZrO₂ NPs were pressed into nanoflakes and subjected to sintering at elevated temperatures. Significantly, the obtained nanoflakes exhibited remarkable Physical characteristics. First, Vickers hardness tests were performed to examine the materials hardness, which was quantified by the peak load ($P_{\text{max}}$) and projected contact area ($A$), $H = \frac{P_{\text{max}}}{A}$.

The results were listed in Table 1. It can be seen that for ZrO₂ NPs prepared at 400 °C, at the same sintering temperature, there is a maximum hardness that varies with the compression force (Supporting Figure S3); and at the same compression force, there is also a maximum hardness that varies with the sintering temperature. The maximum hardness (603.25 gf·mm⁻²) could be found at the compression force of 3 MPa and sintering temperature of 1200 °C. Similarly, for the ZrO₂ NPs prepared at 500 and 600 °C, the maximum hardness can be identified at 3 MPa and 1200 °C, and 3 MPa and 1400 °C, respectively.

As is known, when the ZrO₂ NPs are pressed into flakes, at low compression forces the flakes are likely crack-free such that the hardness increases with increasing compression force; however, at too high a compression force, (micro) cracks would start to form in the flakes, leading to reduced hardness. Likewise, at relatively
Table 1. Physical hardness (gf·mm\(^{-2}\)) of ZrO\(_2\) flakes. The ZrO\(_2\) flakes prepared by compression of ZrO\(_2\) NPs synthesized at 400, 500 and 600 °C under 2, 3, 6, 10, 14 and 18 MPa and sintering at 800, 1000, 1200 and 1400 °C (C. Force: compression forces, S. Temp: sintering temperature, Syn. Temp: Synthesis temperature).

| S. Temp | C. Force | 2 MPa | 3 MPa | 6 MPa | 10 MPa | 14 MPa | 18 MPa |
|---------|----------|-------|-------|-------|--------|--------|--------|
| 800 °C  | 800 °C   | 150.28| 411.64| 200.55| 232.89 | 324.56 |
| 1000 °C | 1000 °C  | 188.00| 460.30| 311.34| 299.65 | 431.36 | 420.47 |
| 1200 °C | 1200 °C  | 543.85| 603.25| 440.13| 467.21 | 460.85 | 454.70 |
| 1400 °C | 1400 °C  | 450.51| 477.59| 462.29| 452.65 | 419.63 | 405.69 |
| 500 °C  | 800 °C   | 99.71 | 284.30| 233.39| 248.84 | 266.57 | 262.87 |
| 1000 °C | 1000 °C  | 103.38| 331.60| 387.94| 375.85 | 357.65 | 356.67 |
| 1200 °C | 1200 °C  | 548.40| 562.00| 476.36| 473.04 | 468.07 | 458.26 |
| 1400 °C | 1400 °C  | 383.63| 542.62| 534.58| 477.21 | 468.65 | 460.44 |
| 600 °C  | 800 °C   | 195.33| 343.27| 133.21| 263.46 | 251.32 | 246.58 |
| 1000 °C | 1000 °C  | 104.90| 252.67| 232.78| 371.88 | 446.96 | 425.60 |
| 1200 °C | 1200 °C  | 575.50| 621.30| 492.09| 502.75 | 530.99 | 408.04 |
| 1400 °C | 1400 °C  | 503.22| 765.98| 488.67| 463.27 | 417.01 | 412.79 |

low sintering temperatures, thermal stress in the particle cores was minimal and the flake hardness increased with increasing sintering temperature; in contrast, thermal stress became increasingly significant during high-temperature calcinations which led to the formation of cracks and hence reduced hardness\(^{39,40}\). Taken together, these results suggest that both the compression force and sintering temperature play an important role in the determination of the hardness of the flakes, and the maximum hardness may be manipulated by sintering temperature and compression force.

**Surface Roughness.** The surface morphologies of the ZrO\(_2\) nanoflakes were then analyzed by AFM measurements. From the AFM topographs in Fig. 5, the root mean square (rms) roughness of the ZrO\(_2\) nanoflakes was evaluated as a function of sintering temperature and compression force\(^{41}\). Experimentally, the rms roughness was quantified by taking an average of five data points measured over an area of 4\(\mu\)m \(\times\) 4\(\mu\)m (Table 2). It can be seen that under the same compression force, the surface roughness of the flakes diminished with increasing sintering temperature (Supporting Figure S4).

From Table 2, it can also be seen that at the same sintering temperature, the roughness of the flakes remained almost invariant with the compression force. Yet, at a constant compression force, the roughness of the flakes decreased with increasing sintering temperature, consistent with the results in Fig. 5. Interestingly, at the same sintering temperature, the ZrO\(_2\) surface roughness remained almost constant (Fig. 6), independent of the compression force. With increasing sintering temperature, the ZrO\(_2\) NPs tended to agglomerate and the surface roughness decreased accordingly. It has been reported that the surface properties play a critical role in the stability and function of bone-rebuilding materials\(^{39}\). Thus, changing the sintering temperature to reduce flakes roughness may enhance the biomedical applications of the ZrO\(_2\) flakes.

**Flake Density.** Density is another important parameter in the assessment of materials for biomedical implants. The density of the ZrO\(_2\) nanoflakes was quantitatively evaluated by using a precision electronic hydrometer\(^{42,43}\), and the results are summarized in Table 3.

One can see that the density of the flakes varied with the sintering temperature and compression force, ranging from 2 to 7 g·cm\(^{-3}\), much higher than that of adult femurs (1.3–1.7 g·cm\(^{-3}\))\(^{44,45}\). At this optimal point, the flakes hardness is comparable to that of the human skeleton while the density is far larger. Thus, the ZrO\(_2\) flakes may be used as biological materials for hip replacements, rosthetic dentistry and dental implantology. This is being pursued in ongoing studies.

**Discussion**

ZrO\(_2\) NPs were synthesized by a simple vapor-phase hydrolysis process\(^{47}\). Vickers hardness investigation indicated that both the sintering temperature and compression force played an important role in the determination of the hardness of the ZrO\(_2\) flakes. AFM studies showed that the surface roughness of the ZrO\(_2\) flakes gradually decreased with increasing sintering temperature. In addition, the density of the ZrO\(_2\) flakes was also determined within the context of sintering temperature and compression force. With a systematic variation of these two parameters, hardness and density of the ZrO\(_2\) nanoflakes were maximized and roughness was minimized simultaneously\(^{46,48}\). When ZrO\(_2\) NPs synthesized at 500 °C were pressed into flakes under the compression force of 6 MPa
and sintering at 1400 °C, the resulting flakes exhibited an optimal combination of hardness (534.58 gf·mm⁻²), roughness (0.07 μm) and density (4.41 g·cm⁻³).

In conclusion, the experimental results show that both the sintering temperature and compression force played an important role in determining the physical behaviors (hardness, roughness, and density) of ZrO₂ flakes. Thus, by changing compression force and sintering temperature, the hardness parameters of the ZrO₂ flakes can be adjusted and comparable to those of human bones, along with a higher density. More importantly, the in vitro cytotoxicity and hemolysis evaluation shows that the ZrO₂ NPs have good biocompatibility. Therefore, it is believed the ZrO₂ NPs have promising application for bone tissue engineering and regenerative medicine.

Table 2. Surface roughness (μm) of ZrO₂ flakes. The ZrO₂ flakes prepared by compression of ZrO₂ NPs synthesized at 400, 500 and 600 °C under 2, 3, 6, 10, 14 and 18 MPa and sintering at 800, 1000, 1200 and 1400 °C.
Methods

Material Preparation. ZrO₂ NPs were synthesized by a vapor-phase hydrolysis procedure and the experimental apparatus had been reported in a previous study. In brief, the reactor was made of two glass tubes that were externally heated in a vertical furnace. ZrCl₄ was sublimated at 350 °C and carried by a N₂ gas (99.9%) into the reaction chamber via the nozzle. Water vapor was introduced into the reaction chamber from around the nozzle by dry air. These two gas streams were mixed rapidly, reacted, and formed ZrO₂ NPs at atmospheric pressure. Then, the aerosol was cooled by a water jacket tube and filtered at the exit of the reactor for analysis, followed by washing. Three samples of ZrO₂ NPs were synthesized at different temperatures (400, 500 and 600 °C) and pressed into flakes at different compression force (2, 3, 6, 10, 14 and 18 MPa), which were then subjected to sintering for 6 h at 800, 1000, 1200 and 1400 °C, respectively.

Cell Culture and Cytotoxicity Evaluation. Human umbilical vein endothelial cells lines (HUVEC) were used to evaluate the cytotoxicity of ZrO₂ NPs. The cells were cultured in a complete medium Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovineserum (FBS, GIBCO) and streptomycin/penicillin

Table 3. Density (g·cm⁻³) of ZrO₂ flakes. The ZrO₂ flakes prepared by compression of ZrO₂ NPs synthesized at 400, 500 and 600 °C under 2, 3, 6, 10, 14 and 18 MPa and sintering at 800, 1000, 1200 and 1400 °C.

| C. Force (MPa) | Density (g·cm⁻³) | Syn. Temp 2MPa | 3MPa | 6MPa | 10MPa | 14MPa | 18MPa |
|---------------|-----------------|----------------|------|------|-------|-------|-------|
| 400°C         |                 | 4.38           | 3.09 | 2.59 | 3.87  | 5.69  | 5.86  |
| 500°C         |                 | 3.50           | 3.85 | 5.57 | 5.94  | 6.05  | 5.60  |
| 600°C         |                 | 3.76           | 4.04 | 4.63 | 5.66  | 5.55  | 6.33  |
| 800°C         |                 | 3.76           | 4.04 | 4.63 | 5.66  | 5.55  | 6.33  |
| 1000°C        |                 | 3.76           | 4.04 | 4.63 | 5.66  | 5.55  | 6.33  |
| 1200°C        |                 | 3.76           | 4.04 | 4.63 | 5.66  | 5.55  | 6.33  |
| 1400°C        |                 | 3.76           | 4.04 | 4.63 | 5.66  | 5.55  | 6.33  |

Figure 6. Surface roughness of ZrO₂ flakes. The ZrO₂ flakes prepared by sintering at 1400 °C under different compression forces of ZrO₂ NPs synthesized at different temperatures (a) 400 °C, (b) 500 °C, and (c) 600 °C.
(100 μg mL⁻¹ Hyclone) at 37°C in amosmotized 5% CO₂ incubator. The viability of the treated cells was measured by the Cell Counting Kit-8 (CCK-8) assay. Firstly, HUVEC cells were seeded into a 96-well cell culture plates at the densities of 2 × 10⁴ cells/ well, in 100 μL of a complete culture medium at 37°C for 24 h. Afterward, the ZrO₂ NPs dispersions were diluted with a fresh medium to the desired concentrations and then added to each well to replace the original culture medium. After another 24 h, the culture medium was removed and replaced by 10 μL of CCK-8 in serum-free media. After incubation for 2 h at 37°C, the optical density of each well was read at 450 nm on a microplate reader (Spectra Max M2MDC, USA)³⁷,⁵⁰–⁵⁴.

Hemolysis Assay of ZrO₂ NPs were carried out as follows. Kunming mice (5 weeks old) were purchased from Vital River (Beijing, China) and the whole blood was obtained from the mice. All the animals experiments were performed in accordance with the guideline and regulation for the care and use of laboratory animals of Ministry of Science and Technology of People’s Republic of China’s requirements. The Animal Study Committee of the Ministry of Science and Technology of the People’s Republic of China has approved the experiments. Briefly, mice blood were centrifuged and diluted 10 times with PBS to obtain red blood cells (RBCs). Then, 0.2 mL of diluted RBCs were added to 0.8 mL of PBS containing ZrO₂ NPs at various concentrations (15, 30, 60, 120, 250, 500, and 800 μg mL⁻¹), 0.8 mL of distilled water (positive control), and 0.8 mL of PBS (negative control), respectively. After that, the mixtures were kept at room temperature for 4 h, before they were centrifuged at 12000 rpm for 3 min. Absorbance of the supernatants was measured by a UV-vis spectrophotometer and hemolysis percentage of RBCs was calculated based on the absorbance at 541 nm using equation (1):

\[
\text{Hemolysis(%) } = \frac{A_{\text{sample}} - A_{0\%}}{A_{100\%} - A_{0\%}} \times 100\%
\]

where \(A_{\text{sample}}\), \(A_{0\%}\), and \(A_{100\%}\) are the absorbance of the supernatant of the samples, the negative control, and positive control, respectively.

Materials Characterizations. The composition, morphologies of the nanoparticles prepared above were characterized by X-ray powder diffraction (XRD, Bruker D8, Cu Kα radiation, \(\lambda = 1.54 \AA\)). The size distribution and morphology of the nanoparticles were examined by scanning electron microscopy (SEM, HITACHI, S4800, 15 kV) studies. The hardness of the flakes was evaluated by using a Vickers hardness instrument (UHL Microhardness Testers VMHT. VMH-001) equipped with a cube-corner diamond tip, where the sintered flakes were cleaned by a stream of high-purity nitrogen. To minimize substrate contributions, the indentation experiments were performed in load control, with the load of 50 gf. The loading and unloading speed was 5 × 10⁻⁶ m s⁻¹, and the time under the peak load was 15 s⁴⁸,⁴⁹,⁵⁵–⁵⁸. Ten random tests were performed for each sample to evaluate the material hardness. The surface morphology was analyzed using AFM and the rms roughness was obtained for the ZrO₂ flakes. The rms roughness data were obtained over an area of 4 μm × 4 μm at five different points of the sample and averaged⁴⁰,⁴⁹,⁶⁰. The density of the flakes was characterized with a precision electronic hydrometer (DH-120M) by equation (2),

\[
\rho = \frac{I.M.}{I.M. - M.M.} \times \rho_{H_2O}
\]

where \(\rho\) is the density of the flakes, I.M. is inherent mass, M.M. is measured mass of the flakes and \(\rho_{H_2O}\) is the density of water at 20°C.

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Acknowledgements
This work was supported by the Natural Science Foundation of China (No. 21471103, 51002180, and 11275218), National Basic Research Programs of China (973 program, No. 2015CB932104), the Project of Excellent Talents of Beijing (No. 203135407707), Beijing Natural Science Foundation (No. 2162046), and the Scientific Research Base Development Program of the Beijing Municipal Commission of Education.

Author Contributions
J.W. and W.Y. conducted the experiment, J.W. and W.Y. wrote the manuscript. Q.W., S.C., X.H. and M.G. discussed and reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Wang, J. et al. Good Biocompatibility and Sintering Properties of Zirconia Nanoparticles Synthesized via Vapor-phase Hydrolysis. Sci. Rep. 6, 35020; doi: 10.1038/srep35020 (2016).

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