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ZnO quantum dots modified bioactive glass nanoparticles with pH-sensitive release of Zn ions, fluorescence, antibacterial and osteogenic properties†

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Zinc (Zn)-containing materials have osteogenic and antibacterial activities while bioactive glass nanoparticles (BGN) show bone-bonding ability, as well as osteoconductive and osteoinductive properties. Zn-containing BGN are therefore considered to be promising materials for various biomedical applications, particularly in bone regeneration. In this study, we report a convenient method to prepare Zn-containing BGN by coating ZnO quantum dots (QDs) on BGN via electrostatic interactions. The synthesized ZnO–BGN nanocomposite particles are spherical and highly dispersed, and exhibit a unique fluorescence behavior under UV excitation, emitting three wavelengths in the violet, blue and green range. ZnO–BGN showed apatite-forming ability upon immersion in simulated body fluid, but their apatite formation was delayed compared to BGN. Interestingly, ZnO–BGN showed a rapid release of Zn ions at pH 4 but a far slower release at pH 7.4. ZnO–BGN also exhibited antibacterial effects on both Gram-positive and Gram-negative bacteria at the concentrations of 1, 0.1, and 0.01 mg mL^{−1}. Higher concentrations could lead to stronger antibacterial effects. The LDH and live/dead assays indicated that ZnO–BGN had no significant cytotoxicity towards human mesenchymal stem cells (hMSC) at concentration of 0.1 and 0.01 mg mL^{−1}, but ZnO–BGN inhibited the relative proliferation of hMSC compared to BGN and the control according to the MTT assay. Notably ZnO–BGN improved the osteogenic differentiation of hMSC as indicated by the determination of the alkaline phosphatase activity. In conclusion, coating quantum dots on BGN is a promising strategy to produce Zn-containing BGN. The synthesized ZnO–BGN are potential materials for bone regeneration, considering their apatite-forming ability, unique ion-release behavior, effective antibacterial activity, non-cytotoxicity, and osteogenic potential.

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1. Introduction

Bioactive glass nanoparticles (BGN) are promising materials for biomedical applications, particularly in hard tissue regeneration,

due to their bone-bonding capability, biocompatibility, osteoconduction and osteoinduction properties.^{1,2} In comparison with conventional micron-sized bioactive glass particles, BGN have more uniform shapes, a larger specific surface area, and consequently higher bioactivity and protein adsorption capability.^{1,2} These characteristics make BGN suitable materials as building blocks for nanocomposites or as injectable biomaterials in various applications.^{2,3} BGN can also act as carriers for delivering biomolecules (e.g. growth factors and anticancer drugs), owing to the small particle size, for specific intended purposes.^{4,5}

The incorporation of biomolecules in bioceramics or bioactive glasses (BG) can enhance the therapeutic effects of the materials in bone regeneration⁶ or anticancer treatments.⁵ However, the relatively short shelf-life, the sensitivity to outer conditions, and the high cost of most biomolecules (e.g. bone morphogenetic protein) constrain the widespread adoption of biomolecules. Alternatively, materials containing therapeutic ions are drawing attention regarding effective use in bone

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regeneration, as these materials can release therapeutic ions and thus lead to positive biological responses depending on the type and amount of released ions.^{7,8} Among a large number of ions with therapeutic effects, zinc (Zn), being an important element of alkaline phosphatase (ALP) and a key mediator of bone matrix mineralization,⁹ plays a significant role in bone formation.^{10,11} Additionally, Zn ions show antibacterial effects, being therefore beneficial for the inhibition of infection.¹² Zinc-containing BG have shown enhanced calcified matrix deposition and ALP activity compared with BG without zinc.¹³ Moreover, Zn-containing BG scaffolds were shown to exhibit antibacterial activity while retaining biocompatibility with human osteoblast-like cells (HOS).¹⁴ Zinc-containing BGN are therefore attractive materials for bone regeneration, as BGN can interact with cells at the nanoscale, thus delivering Zn ions more effectively and at more appropriate rates than conventional (μm sized) particles.

Unfortunately, the synthesis of highly dispersed BGN containing multifold metallic ions is still challenging through conventional sol-gel based methods, as metallic precursors can deteriorate the stability of nanoparticles during synthesis and consequently cause aggregation and irregularity in the resulting BGN.^{15,16} Zn-containing BGN showing high bioactivity have been synthesized *via* a quick alkali-mediated sol-gel method,^{17,18} but the dispersion and uniformity of the synthesized BGN should be improved when they are considered for use in nanocomposites. As an alternative, metal or metal oxide nanoparticles can be coated on nanoparticles to introduce metallic ions, while at the same time the high dispersion of the nanoparticles is maintained.^{19,20}

In addition to metal or metal oxide nanoparticles, quantum dots (QDs) can also be used to introduce metallic ions into other nanoparticles. QDs are nanocrystals in the size range of 2–10 nm, being promising in energy applications due to their unique photoelectric properties.²¹ QDs are also used in bio-imaging (*e.g.* for real-time monitoring of intracellular processes and *in vivo* molecular imaging).^{22,23} Notably, the luminescent properties of QDs could be retained after their combination with other materials (*e.g.* bioactive glass nanoparticles).²⁴ ZnO QDs are relatively weakly toxic towards cells and environmentally friendly, compared to other types of QDs (*e.g.* Cd-related QDs).²⁵ ZnO QDs are also effective antibacterial agents, due to their either killing or growth suppression effects on bacteria.²⁶ Furthermore, owing to their unique degradation behavior (rapid degradation under acidic conditions but slow degradation under neutral or basic conditions), ZnO QDs could be used as nanolids to cap nanopores on drug carriers^{27,28} to allow loaded drugs to be released at low pH (a low pH is associated with inflammation, bacterial infection or cancer).^{7,29}

Based on the above rationale, we hypothesized that highly dispersed Zn-containing BGN could be prepared by coating ZnO QDs on BGN. To this end, monodisperse BGN were first synthesized *via* a modified Stöber method as described in previous studies.^{30,31} ZnO QDs were then coated on BGN exploiting electrostatic interactions that are non-destructive to both interacting materials. This coating method avoided the possible aggregation and irregular particle size caused by the addition of Zn precursors. The as-synthesized ZnO QDs coated BGN (ZnO-BGN) were

observed by scanning transmission electron microscopy (STEM) and scanning electron microscopy (SEM), and the morphological results confirmed their spherical shape and high dispersion. Furthermore, the fluorescence property, apatite-forming ability, antibacterial activity, and the ion release behavior of ZnO-BGN were investigated. Their cytotoxicity towards cancerous HepG2 and human bone mesenchymal stem cells (hMSC) was evaluated by using the LDH (lactate dehydrogenase) leakage, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and live/dead assays. Finally, the stimulation of osteogenic differentiation of hMSC by ZnO-BGN was investigated.

2. Materials and methods

2.1. Synthesis of BGN

In a typical synthesis, one solution containing 2.25 mL of tetraethyl orthosilicate (TEOS, 98%) and 25 mL of ethanol was mixed with another solution containing 4.5 mL of ammonium hydroxide (28%), 10 mL of ethanol and 15 mL of deionized water under continuous stirring. The mixture was allowed to react for 30 min before $\text{Ca}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$ (1.45 g) was added. The mixture was stirred for 90 min and then the suspension was centrifuged at a rate of 7830 rpm (Centrifuge 5430R, Eppendorf, Germany) to obtain whitish deposits. The deposits were washed twice with water and once with ethanol before being calcined at 700 °C for 2 h at a heating rate of 2 °C min⁻¹, and subsequently cooled down overnight in a furnace. All the used chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany) without further purification.

2.2. Coating of ZnO QDs on BGN

Briefly, 0.2 g of BGN was added to a solution containing 10 mL each of ethanol and deionized water, and then the mixtures were ultrasonically dispersed for 15 min. Consequently, 1 mL of ZnO QDs stock solution (2.5 wt% in isopropanol; N-10, Nanograde AG, Switzerland) was added to the suspension under stirring. The pH of mixtures was adjusted to 7.4 with diluted ammonium hydroxide, and the mixture was then allowed to react for another 6 h before being centrifuged and washed three times with ethanol. During the whole process, the pH value of the suspension was monitored and kept at 7.4. The collected ZnO-BGN were then dried at 60 °C for 6 h and calcined at 600 °C for 1 h at a heating rate of 2 °C min⁻¹, and were then cooled down overnight.

2.3. Characterization of ZnO-BGN

The surface morphology of ZnO-BGN was observed using SEM (Auriga and Merlin Gemini II of Zeiss, Germany) and (S)TEM (Tecnai G2 20 TWIN and Titan³ G2 60-300, both FEI, USA) with bright-field (BF) imaging. ZnO-BGN were dispersed in ethanol, and then dropped onto aluminum specimen stubs and copper grids for SEM and TEM observation, respectively. All samples were observed without being sputter-coated. Energy-dispersive X-ray spectroscopy (EDS) mapping was conducted using a Quantax 800 (Bruker, USA) microanalysis system during the

SEM observation in order to investigate the distribution of ZnO QDs in ZnO–BGN, while the concentration of ZnO QDs in ZnO–BGN was detected by using EDS (X-MaxN Oxford Instruments, UK) on ZnO–BGN pellets. The particle size and distribution were determined according to SEM images using ImageJ (NIH, USA), and the number of counted particles was more than 300.

The surface charge and dispersity of ZnO–BGN were measured using a Zetasizer Nano ZS (Malvern Instruments, UK) instrument with a 4 mW HeNe laser (633 nm) and a light scattering detector positioned at 90°. BGN and ZnO–BGN were measured in phosphate-buffered saline (PBS, pH 7.4) at concentration of 0.1 g L⁻¹.

Fourier transform infrared spectroscopy (FTIR) was performed on a Nicolet 6700 FTIR spectrophotometer (Thermo Scientific, USA) in a transmission mode under ambient conditions. Samples and KBr were mixed and made into pellets for FTIR at a ratio of 1:100 by weight. Spectra were collected in the region from 400 and 3600 cm⁻¹ with a resolution of 4 cm⁻¹.

X-ray diffraction (XRD) was performed using a D8 ADVANCE X-ray diffractometer (Bruker, USA) in the 2θ range of 20–80° with Cu Kα radiation. All samples were dispersed in ethanol and then dropped onto low-background silicon wafers (Bruker AXS, USA). A step size of 0.014° with a dwell time of 1 s per step was used.

The fluorescence properties of ZnO–BGN were investigated by recording the photoluminescence spectra (PL) (emission spectra) using a spectrophotometer (F-4500, Hitachi, Japan). All samples were tested under UV excitation (320 nm) in pure ethanol at concentration of 2 mg mL⁻¹ under ambient conditions. In order to evaluate the fluorescence stability of ZnO–BGN in physiological fluid, the PL of ZnO–BGN soaked in stimulated body fluid (SBF) and PBS for 21 days was also measured.

2.4. *In vitro* mineralization

The *in vitro* mineralization of ZnO–BGN was investigated by soaking the samples in SBF according to the protocol of Kokubo *et al.*³² Briefly, ZnO–BGN were soaked in SBF at concentration of 1 mg mL⁻¹ in an incubator (KS 4000i control, IKA, Staufen, Germany) at 37 °C and with an agitation speed of 120 rpm for different periods of time. The SBF was changed twice per week to keep the concentration of ions in SBF stable. At each pre-determined time point, the samples were removed from the SBF and rinsed gently with deionized water before being dried at 60 °C for 24 h. The dried samples were subsequently characterized using FTIR, XRD and SEM. The *in vitro* mineralization of BGN without ZnO was also evaluated as a control.

2.5. Ion release from ZnO–BGN

The assessment of ion release from ZnO–BGN was performed in PBS (pH 7.4) and citric acid buffer (pH 4). In brief, ZnO–BGN were soaked in each buffer at concentration of 1 mg mL⁻¹ at 37 °C. At each pre-determined time point, half of the supernatant was collected and replenished with fresh PBS or citric acid buffer solution. The release of Ca and Zn ions was measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES, SPECTRO CIROS-CCP spectrometer).

2.6. Antibacterial activity

To assess the antibacterial activity of ZnO–BGN, Gram-negative *E. coli* and Gram-positive *S. aureus* were used as model bacteria. Both strains were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany) and cultured on nutrient broth (Roth, Karlsruhe, Germany). According to the manufacturers' instructions, the strains were inoculated in fresh media and incubated at 37 °C overnight under shaking conditions to reach a density of 1–5 × 10⁷ cfu per mL for further antibacterial assay. The antibacterial activity of ZnO–BGN was assessed quantitatively based on the absence of light caused by the turbidity of bacterial suspension.³³ Briefly, the samples were added in PBS at concentration of 1, 0.1, and 0.01 mg mL⁻¹. Then a sterilized 96-well plate was inoculated with 50 μL of particle/PBS suspension and 50 μL of diluted bacterial suspension (1–5 × 10⁵ cfu per mL). Bacterial growth was evaluated after culture for 24 h at 37 °C and their optical density (OD) at 600 nm was then recorded using a Microplate reader (7530, Cambridge Technology, Inc., Karlstad, Sweden). Considering the possible interference from the samples, the OD value of the samples at corresponding concentration before culture was removed as the background. The results were shown as relative bacterial viability being calculated as below:

$$\text{Relative bacterial viability (\%)} = \frac{\text{Sample OD}}{\text{Blank control OD}} \times 100\%$$

The well containing 50 μL of pure PBS and 50 μL of bacterial suspension was used as blank control. The experiment was performed in triplicate.

2.7. Cell source

Human mesenchymal stem cells were purchased from Lonza (Basel, Switzerland) and HepG2 cells were purchased from ATCC (Manassas, USA). Both cell lines were maintained in phenol red free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) batch tested fetal bovine serum (FBS), 50 U mL⁻¹ of penicillin and 50 mg mL⁻¹ of streptomycin, 1% (v/v) L-glutamine and 4.5 g of glucose. DMEM was purchased from Invitrogen (UK), and the cell culture was performed in a humidified atmosphere of 5% CO₂, 95% air at 37 °C.

2.8. MTT assay

Both of hMSCs and HepG2 proliferation over time after exposure to BGN and ZnO–BGN were evaluated using the MTT assay. Briefly, around 4000 cells were seeded in each well of 96-well plates and were allowed to grow for 24 h. Afterwards, the cells were exposed to BGN and ZnO–BGN at concentration of 0.1 or 0.01 μg mL⁻¹, for 24 and 72 h. After the treatment, the medium was removed from each well, and the cells were rinsed three times with PBS. Then 100 μL of new medium with 10 μL of MTT solution (Amresco, Solon, USA) at concentration of 5 mg mL⁻¹ was added to each well. After incubation for another 4 h at 37 °C, MTT formazan was measured at 560 nm using an ELX Ultra Microplate Reader (Biotek, USA). The results were

converted to relative cell viability according to the following equation:

$$\text{Relative cell viability (\%)} = \text{Sample (OD)} / \text{Control (OD)} \times 100\%$$

2.9. LDH assay

The cytotoxicity of BGN and ZnO-BGN towards hMSCs was assessed by monitoring lactate dehydrogenase (LDH) leakage into the culture medium using a cytotoxicity detection kit (Roche, Germany). In brief, 4000 hMSC cells were seeded in 96-well plates. After culture for 24 h, the medium was replaced by fresh medium containing BGN and ZnO-BGN at concentration of 0.1 or 0.01 mg mL⁻¹, respectively. After culture for 24 h and 72 h, the supernatants were collected and the cells were removed from the medium through centrifugation. The LDH assay was performed for each sample in triplicate according to the manufacturer's protocol. Absorbance was measured at 500 nm with an ELX Ultra Microplate Reader. The results were shown as relative LDH leakage according to the following equation:

$$\text{Relative LDH leakage (\%)} = \text{Sample (OD)} / \text{Control (OD)} \times 100\%$$

2.10. Live/dead assay

The live/dead assay was performed to assess hMSCs viability after their exposure to BGN and ZnO-BGN by using a cell viability imaging kit (Life Technologies, Carlsbad, USA). This detection employed two fluorescent dyes: NucBlue[®] Live reagent for live cells producing blue fluorescence (ex/em 360/460 nm) and propidium iodide for dead cells producing red fluorescence (ex/em 535/617 nm). Briefly, hMSC cells were seeded in 6-well plates with approximate 5×10^4 cells per well in 2 mL of medium, and were allowed to grow for 24 h. The cells were then cultured with BGN and ZnO-BGN at concentration of 0.1 and 0.01 mg mL⁻¹ for 24 and 72 h. The medium was then removed, and the cells were washed three times with PBS and fresh medium was added to each well. NucBlue[®] Live reagent and propidium iodide were then added according to the manufacturer's instructions. After another incubation of 15 min at room temperature, the cells were visualized using a laser scanning confocal microscope (Axio Observer Z1, Zeiss, Oberkochen, Germany). The live and dead cells were counted and the cell viability was calculated as below:

$$\begin{aligned} \text{Cell number (\%)} &= \frac{\text{The number of living cells}}{\text{The number of (living cells + dead cells)}} \times 100\% \end{aligned}$$

2.11. ALP activity

The osteogenic differentiation of hMSCs after exposure to BGN and ZnO-BGN was assessed by measuring ALP activity using an ALP assay kit (Abcam, England). Briefly, hMSC were seeded in 6-well plates at 5×10^4 cells per well in 2 mL of medium. After 24 h of stabilization, the medium was replaced with an osteogenic differentiation medium (Lonza, USA) with BGN and ZnO-BGN at concentration of 0.1 and 0.01 mg mL⁻¹. At day 14, cells in different groups were washed with PBS and protein was extracted

using RIPA buffer (Cell Signaling Technology, USA) containing protease and phosphatase inhibitors (Roth, Germany). The protein concentration was detected with the BCA Protein Quantification Kit (Thermo Scientific, USA). ALP activity detection and quantification were performed according to the manufacturer's instructions. ALP activity was expressed per gram of protein. Fluorescence intensity was measured at ex/em 360/440 nm using a fluorescence microtiter plate reader (Promega, USA).

2.12. Statistical analysis

All the quantitative experiments were carried out at least in triplicate, and the data are shown as mean $\pm$ standard deviation (S.D.). For the comparison of quantitative cell biological results, the significance of differences between the control and tested groups was assessed using Student's test and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Morphology of ZnO-BGN

The as-received ZnO QDs were highly dispersed and exhibited a sphere-like shape with a mean size smaller than 10 nm (Fig. S1a and b, ESI[†]). The synthesized BGN were monodisperse and exhibited a spherical shape (Fig. 1a), which is the typical morphology of silica-based nanoparticles prepared *via* the Stöber method.¹⁶ BGN had a mean size of 415 nm with a smooth surface (Fig. 1b). As can be seen in the TEM images, BGN displayed a dense structure without nanopores on their surface (Fig. S1c and d, ESI[†]), since BGN were calcined at 700 °C and this treatment may have eliminated nanoporosity in the particles.³⁴ The isoelectric points (IEP) of ZnO nanoparticles and BGN were ~ 11 ²⁷ and ~ 2 ,³⁵ respectively. This IEP gap suggests that ZnO QDs can be coated on BGN *via* electrostatic interaction in a wide pH range including normal physiological conditions.

Based on the above hypothesis, ZnO QDs were successfully coated on BGN at physiological pH 7.4. The resulting ZnO-BGN (mean size 401 nm) were highly dispersed, spherical, and composed of ZnO QDs clustering uniformly on BGN (Fig. 1c and d). The results suggest that the coating of ZnO QDs had no significant influence on the general morphological characteristics of BGN. TEM results confirmed the spherical and highly dispersed characteristics of ZnO-BGN (Fig. 2a and b). The selected area electron diffraction (SAED) pattern of ZnO-BGN (Fig. 2c) shows that ZnO crystals were present around amorphous BGN. The EDS mapping result (Fig. 2d) indicates a homogenous distribution of ZnO QDs on BGN surface. The EDS result obtained from ZnO-BGN pellets (Fig. 1c) indicates the presence of Zn, Ca and Si elements as well as the molar percentage of ZnO in ZnO-BGN being $\sim 3.12\%$. The zeta-potential values of ZnO-BGN and BGN in PBS (0.01 M, pH 7.4) were ~ -41 mV and ~ -59 mV, respectively, which suggested their stability under physiological conditions. The polydispersity index (PDI) of ZnO-BGN was 0.167 while that of un-coated BGN was 0.112. Although the PDI of particles decreased after coating with ZnO QDs, the low PDI of ZnO-BGN still indicated their high dispersity. All the above morphological,

Fig. 1 Scanning electron microscopy images showing the surface morphology of (a and b) BGN and (c and d) ZnO-BGN; in the insets: particle size distribution diagrams and EDS results showing elements in ZnO-BGN.

Fig. 2 (a and b) Bright field transmission electron microscopy images showing the morphology of ZnO-BGN; (c) the SAED pattern of ZnO-BGN indicating the presence of ZnO crystals; (d) the SEM-EDS map of Si and Zn, showing the presence of Zn on ZnO-BGN (The result shown in figure d does not originate from the particle shown in figure c.).

crystallographic, and elemental results therefore confirmed the successful coating of ZnO QDs on BGN. This covering of BGN by QDs based on electrostatic interaction can thus be an effective way to introduce metallic ions into BGN while retaining the high dispersion and spherical shape of BGN.

3.2. Structural characteristics of ZnO-BGN

Fig. 3a presents the FTIR spectra of BGN and ZnO-BGN showing the characteristic bands of silicate BG at 476 cm^{-1} (Si–O–Si rocking), 809 cm^{-1} (Si–O–Si bending), and in the range $1000\text{--}1200\text{ cm}^{-1}$ (Si–O–Si stretching) cm^{-1} .^{36,37} The band located at 1634 cm^{-1} could be assigned to the bending vibrations of water molecules.³⁷ No significant difference between BGN and ZnO-BGN could be observed in the FTIR spectra, as ZnO QDs were coated on BGN only *via* electrostatic interactions, not covalent bonding. Fig. 3b shows the XRD patterns of BGN and ZnO-BGN. As expected, the as-synthesized BGN exhibited an amorphous nature while ZnO-BGN exhibited obvious diffraction peaks located at 32° and 36° , which could be assigned to the (002) and (101) lattice planes of ZnO, respectively.³⁸ The XRD results are consistent with the electron diffraction patterns of ZnO-BGN (Fig. 2c), confirming the presence of ZnO QDs crystals in ZnO-BGN.³⁸

3.3. Photoluminescence

Fig. 3c shows the photoluminescence (PL) spectra of ZnO QDs, BGN and ZnO-BGN. The emission wavelength of ZnO QDs was at around

380 nm in the violet range, induced by the intrinsic band gap (3.3 eV) of ZnO.³⁹ Notably, BGN showed an emission peak in the range of violet light ($\sim 440\text{ nm}$), which could be attributed to the defect pair consisting of a dioxasilirane and a silylene center in the heated silica-based particles.⁴⁰ As a comparison, ZnO-BGN showed an interesting PL spectrum, in which three PL peaks at ~ 410 , 480 , and 520 nm could be observed. This phenomenon has been found in previous studies on the combination of ZnO and silica-based nanoparticles.^{41,42} The strongest peak located at $\sim 410\text{ nm}$ in the violet range was induced by the presence of ZnO QDs,³⁹ while the emergence of other two peaks in the blue ($\sim 480\text{ nm}$) and green range ($\sim 520\text{ nm}$) could be related to the change in OH groups on the samples^{39,43} and the spherical shape of BGN causing multiple scattering of excitation light,⁴² respectively. After soaking in PBS and SBF for 21 days, ZnO-BGN still exhibited two obvious emission peaks at approximate 380 nm and 440 nm (both were in the violet range), which corresponded to ZnO QDs and BGN, respectively. However, the peak in the blue ($\sim 480\text{ nm}$) region was not observed after soaking while the peak in the green area ($\sim 520\text{ nm}$) was weakly present. Nevertheless, the fluorescence activity of ZnO-BGN could be retained in physiological fluids for up to at least 21 days.

3.4. In vitro mineralization

The as-synthesized BGN have shown apatite-forming ability as reported in our previous study,⁴⁴ and hydroxyapatite (HA) can

Fig. 3 (a) Fourier transform infrared spectroscopy spectra and (b) X-ray diffraction patterns of BGN and ZnO-BGN showing the structural characteristics; (c) PL spectra of BGN, ZnO QDs and ZnO-BGN showing their fluorescence properties; (d) PL spectra of ZnO-BGN after soaking in SBF and PBS for 21 days.

Fig. 4 *In vitro* mineralization of ZnO-BGN in SBF: (a) STEM-BF images of ZnO-BGN after immersion in SBF for 21 days showing the formation of needle-like apatite crystals around the particles, the inset shows a high-magnification image of the needle-like crystals; (b) XRD pattern of ZnO-BGN after soaking in SBF for 21 days demonstrating the characteristic XRD peaks of hydroxyapatite.

form on BGN after immersion in SBF within 7 days. However, the coating of ZnO QDs on BGN inhibited the bioactivity of particles, as no significant formation of HA could be observed in ZnO-BGN upon immersion in SBF for 14 days according to the FTIR spectrum (Fig. S2, ESI[†]). However HA formation could be observed in ZnO-BGN upon immersion in SBF for 21 days, as needle-like crystals clustering the particles were observed in TEM images (Fig. 4a) and this is the typical morphology of formed HA on nanoscale BG.⁴⁵ In addition, two characteristic P–O bands in crystalline Ca–P species located at 560 cm^{-1} and 604 cm^{-1} are seen in the spectra of ZnO-BGN after immersion in SBF for 21 days (Fig. S2, ESI[†]), suggesting the formation of HA. The XRD pattern of ZnO-BGN in SBF for 21 days (Fig. 4b) clearly demonstrates the formation of HA crystals (JCPDS 72-1243). The peaks assigned to ZnO crystals could still be observed in the XRD pattern, suggesting the remaining of ZnO QDs on ZnO-BGN after immersion in SBF for 21 days. Moreover, EDS mapping results (Fig. S3, ESI[†]) show the presence of Ca, P and Zn elements in ZnO-BGN after 21 days immersion in SBF, which also suggests the formation of apatite and the presence of remaining ZnO QDs. It should be noted that needle-like crystals were also found outside the nanoparticles (Fig. 4a). These formed crystals were induced by the released ions from ZnO-BGN leading to a change of local supersaturation towards apatite precipitation.⁴⁶ These formed crystals contained Zn (Fig. S3, ESI[†]) apart from Ca and P, which supported the fact that crystal formation was induced by the released ions. The overall results therefore suggest that the coating of ZnO QDs on BGN caused a delay in the formation of HA upon immersion in SBF, but they also confirm that the apatite-forming ability of BGN was not prevented.

3.5. Ion release from ZnO-BGN

Fig. 5 shows the release of Zn and Ca ions from ZnO-BGN upon soaking under acidic and basic conditions. The release of Zn

ions exhibited an apparent pH-sensitive profile (Fig. 5a): almost all Zn ions ($\sim 33\text{ }\mu\text{g mL}^{-1}$) were released within 8 hours at pH 4, while only a small amount of Zn ions was released ($\sim 3.7\text{ }\mu\text{g mL}^{-1}$) within 21 days at pH 7.4. Additionally, a burst release of Zn ions within 8 h could only be observed at pH 4 while a stable and sustained release of Zn ions was seen at pH 7.4. This phenomenon can be explained by the significant pH sensitivity of ZnO QDs, which are stable at pH 7.4 but rapidly dissolve at $\text{pH} < 5.5$.²⁷ Additionally, the sustained release of Zn ions in PBS suggests the robust bonding between ZnO QDs and BGN after calcination. As a comparison, the release of Ca ions also occurred in a pH-sensitive manner (Fig. 5b), but the difference between two pH conditions was narrow (~ 6.3 and $\sim 9\text{ }\mu\text{g mL}^{-1}$ of Ca ions were released after 21 days at pH 7.4 and pH 4, respectively). A burst release of Ca ions (within 1 day) could be observed under both tested conditions, likely induced by the rapid release of weakly bonded Ca ions on the surface of BGN.⁴⁷ Fig. 5c and d show SEM images of ZnO QDs after soaking in citric acid buffer solution and PBS for 21 days, respectively. No obvious ZnO QDs could be observed on the surface of ZnO-BGN after 21 days of soaking in citric acid buffer solution while ZnO QDs could still be seen on the particles after soaking in PBS. This observation is consistent with the ion release results (Fig. 5a), which indicated that Zn ions were completely released within 3 days under acidic conditions. Notably, smaller particles could be seen after soaking in both solutions, which was the result of the partial degradation of ZnO-BGN in an aqueous solution.⁴⁸ These results suggest that ZnO-BGN were degradable and they could release Ca and Zn ions for up to at least 21 days under physiological conditions.

3.6. Antibacterial activity

Fig. 6 demonstrates the antibacterial effects of ZnO-BGN and BGN towards Gram-positive *S. aureus* and Gram-negative *E. coli*.

Fig. 5 (a and b) Ion release profiles of ZnO-BGN in PBS (pH 7.4) and citric acid buffer solution (pH 4) showing the pH-dependent release of Zn ions from ZnO-BGN; SEM images of ZnO-BGN after soaking in citric acid buffer (c) and PBS (d) for 21 days showing the morphological characteristics.

Fig. 6 Antibacterial effects of BGN and ZnO-BGN towards (a) Gram-positive *S. aureus* and (b) Gram-negative *E. coli* at concentrations of 1, 0.1 and 0.01 mg mL⁻¹.

As can be seen, ZnO-BGN inhibited the growth of bacterial at all tested concentrations. Their strongest antibacterial effect on bacteria was found at the highest concentration of 1 mg mL⁻¹, while no significant difference could be observed between the concentration of 0.1 and 0.01 mg mL⁻¹. The relative viabilities of Gram-positive *S. aureus* and Gram-negative *E. coli* after treated with ZnO-BGN at concentration of 1 mg mL⁻¹ were 34% and 65%, respectively. This result indicated that ZnO-BGN had more pronounced antibacterial effects on Gram-positive than on Gram-negative bacteria, which was consistent with

previous studies showing that Gram-positive bacteria were more susceptible to ZnO than Gram-negative one.^{49,50} BGN also showed antibacterial effects on *E. coli* at all tested concentrations, but the effects were not significant.

3.7. Cell proliferation and cytotoxicity

Two types of cells (hMSC and HepG2) were used in the MTT assay to evaluate the proliferation of cells exposed to BGN and ZnO-BGN (Fig. 7). Compared with the control, BGN had no significant effect on the proliferation of either hMSC or HepG2

Fig. 7 Cytotoxicity assays of BGN and ZnO-BGN towards hMSC and HepG2. The MTT assay showing the effects of tested materials on the proliferation of (a) hMSC and (b) HepG2 cells at concentration of 0.1 and 0.01 mg mL⁻¹. (c) The LDH leakage assay and (d) the live/dead assay showing that BGN and ZnO-BGN had no obvious cytotoxicity towards hMSC.

at the tested concentration (0.1 and 0.01 mg mL⁻¹), while ZnO-BGN showed slight adverse effects on the proliferation of hMSC (Fig. 6a) but no significant effects on HepG2 at both tested concentration (Fig. 6b). In addition, a higher concentration and longer culture time had stronger adverse effects on hMSC in the case of ZnO-BGN (Fig. 7a).

As ZnO-BGN had no significant effect on the proliferation of HepG2, the LDH leakage of hMSC exposed to ZnO-BGN and BGN in cell culture media was measured to better evaluate the cytotoxic effects of ZnO-BGN. As can be seen in Fig. 7c, at concentration of 0.1 and 0.01 mg mL⁻¹, no obvious relative LDH leakage in both BGN-treated cells and ZnO-BGN-treated cells was observed after culture for 24 h. However, after culture for 72 h a slightly higher relative LDH leakage was observed in the ZnO-BGN group while a reduced LDH leakage was observed in the BGN group at concentration of 0.1 mg mL⁻¹. At concentration of 0.01 mg mL⁻¹, no significant differences in LDH leakage were detected among BGN-treated, ZnO-BGN-treated and the control cells. These results indicated that ZnO-BGN had no significant cytotoxic effects on hMSC as evaluated by the LDH leakage assay.

Fig. 7d shows the living and dead cell numbers obtained from the live-dead assay. At concentration of 0.01 mg mL⁻¹, no obvious dead cells could be observed in all groups after both 24 h and 72 h of culture. However, dead cells were found in all tested groups at concentration of 0.1 mg mL⁻¹, but the number was less than 3% of the total cell number. The highest dead cell number (2.5%) was

found in cells treated with ZnO-BGN after culture for 72 h at concentration of 0.1 mg mL⁻¹. Fig. 8 shows representative live/dead images: dead cells (red) are rarely observed under all conditions at concentration of 0.01 mg mL⁻¹ while obvious dead cells can only be seen after ZnO-BGN treatment at concentration of 0.1 mg mL⁻¹. These results were consistent with the results of LDH assay showing that ZnO-BGN had no significant cytotoxicity towards hMSC at concentration of 0.1 and 0.01 mg mL⁻¹.

3.8. Osteogenic differentiation

The ALP activity was measured at day 14 to assess the osteogenic differentiation potential of hMSC after culture with ZnO-BGN and BGN. The activity of ZnO-BGN was significantly higher than that in BGN-treated and control cells at concentrations of 0.1 and 0.01 mg mL⁻¹, while no obvious difference in ALP activity between BGN and the control could be observed at both tested concentrations (Fig. 9). Notably, the ALP activity of ZnO-BGN-treated cells at concentration of 0.1 mg mL⁻¹ was significantly higher than that at concentration of 0.01 mg mL⁻¹.

4. Discussion

In this study ZnO QDs coated BGN (ZnO-BGN) were synthesized for potential application in bone regeneration. The coating of ZnO QDs on BGN can incorporate Zn ions into BGN while

Fig. 8 Representative live/dead images of BGN and ZnO-BGN cultured with hMSC for 24 h and 72 h at concentrations of (a) 0.01 and (b) 0.1 mg mL⁻¹ showing live cells (blue) and dead cells (red).

Fig. 9 The ALP activity of hMSC cultured with BGN and ZnO-BGN at concentrations of 0.1 and 0.01 mg mL⁻¹ for 5 days. Values represent the mean $\pm$ SD. The samples in each experiment were assessed in triplicate. (*) ($P < 0.05$) and (**) ($P < 0.001$) indicate a statistically significance.

maintaining the high dispersion of BGN. The obtained ZnO-BGN showed no cytotoxicity on hMSC and HepG2, as well as could enhance the osteogenic differentiation of hMSC. The morphological characteristics of ZnO-BGN and their lack of aggregation are also beneficial for the further fabrication of nanocomposites or injectable biomaterials.

One of the motivations to develop Zn-containing BGN by coating monodisperse BGN with ZnO QDs was the possibility of avoiding aggregation, as the addition of metallic precursors during conventional synthesis may cause the formation of aggregates making the synthesis of such doped BGN challenging. ZnO-containing BGN have been produced using one-step alkali-mediated sol-gel method with the assistance of ultrasonic dispersion and mechanical agitation.^{17,18} However, the dispersity and uniformity of the synthesized nanoparticles still require improvement. The challenge to produce highly dispersed and uniform BGN in the conventional one-step sol-gel method is

that the addition of salt precursors may destabilize the nanoparticles by changing their surface charge.^{30,51} This destabilization could then lead to the aggregation and non-uniformity of BGN. For the achievement of highly dispersed and uniform BGN, the processing parameters, *e.g.* the molar ratio between silica precursor and metallic precursor,¹⁶ and the addition timing of metallic precursor,³⁰ should be carefully controlled. With proper processing parameters, highly dispersed BGN with binary SiO₂–CaO composition could be relatively easily produced using one-step sol-gel method,^{16,30} but the production of multifold metallic ions containing BGN with high dispersity, uniform shape and size is still challenging considering the complex interaction among the various charged species. Alternatively, post modification of silica nanoparticles or binary BGN to introduce metallic ions has been shown to be able to avoid the aggregation of nanoparticles.^{44,51} In this study, ZnO QDs were used as modification agents to introduce Zn ions. The modification was based on electrostatic interaction that could retain the structure and properties of the involved components. Additionally, the synthesized ZnO–BGN retained high dispersity and spherical shape (Fig. 1), while the size of both ZnO QDs and BGN was not significantly affected (Fig. 1 and 2) during the process. Considering the convenience of controlling the size of BGN with binary SiO₂–CaO composition,³⁰ this proposed method can therefore produce ZnO–BGN with different size by adjusting the size of BGN.

Furthermore, the surface modification of BGN using ZnO QDs can introduce additional properties to BGN. The coating of BGN with ZnO QDs was based on electrostatic interaction that is non-destructive and can thus preserve both the morphology and properties of functionalization agents and matrix materials.⁵² Perhaps the most notable characteristic of ZnO QDs is their unique fluorescence capability. ZnO QDs have shown potential in bio-imaging or drug delivery due to their fluorescence capability,⁵³ however, their emitted violet light is similar to the self-fluorescence of cells that could emit blue color upon UV irradiation.⁵⁴ As a comparison, ZnO–BGN showed three emission peaks (Fig. 3c), located in the wavelengths of violet, blue and green lights. The additional green-light emission could thus distinguish ZnO–BGN from the self-fluorescence of cells. However, the direct use of ZnO–BGN in cell-imaging may not be appropriate, considering the relevant large particle size of ZnO–BGN (~400 nm) causing difficulty for their uptake by cells. Nevertheless, the fluorescence capability of ZnO–BGN can be used to monitor the interaction between cells and materials. In addition, the fluorescence property of ZnO–BGN was stable in physiological fluids for up to at least 21 days. This long-term fluorescence is advantageous. For example, the mineralization process of composites containing ZnO–BGN could be monitored with the assistance of stable fluorescence in physiological fluids.⁵⁵

The combination with ZnO QDs also brought antibacterial activity to BGN. Although BGN have been reported to be able to inhibit bacteria growth, this effect is highly dependent on composition and usually induced by the release of antibacterial ions (*e.g.* silver, cerium ions) or alkaline ions leading to local pH rise.^{56–58} ZnO QDs are effective antibacterial agents, and their antibacterial activity is considered to be from the production of

H₂O₂ from ZnO QDs, the release of Zn ions, or the generation of reactive oxygen species (ROS).⁵⁹ The reported minimum inhibition concentration (MIC) of Zn ions against *S. aureus* was lower than 9 µg mL⁻¹.⁶⁰ In our study, the concentration of Zn ions was ~0.95 µg mL⁻¹ after soaking in PBS for 24 h. In bacterial culture medium, the amount of released Zn ions should be similar to that in PBS, given the similar pH conditions in both media. Although this concentration was lower than the reported MIC of Zn ions against *S. aureus*, our results showed that ZnO–BGN exhibited significant antibacterial effects (Fig. 6). This phenomenon could be due to the synergistic effects with other ions. It has been reported that Zn ions at concentration of 0.05 µg mL⁻¹ exhibited significant antibacterial effects on *S. aureus* in the presence of Ca and Sr ions.⁶¹ ZnO–BGN could release Si and Ca ions in addition to Zn ions, while Zn-free BGN also had antibacterial effects (Fig. 6). The co-existence of Si, Ca and Zn ions could thus account for the antibacterial effects of nanocomposite particles at relatively low concentration of released Zn ions. In addition to the synergistic effects of ions, the presence of produced H₂O₂ and ROS could also improve the antibacterial activity of ZnO–BGN. Notably, the preferred toxicity of ZnO–BGN towards antibiotic-resistant bacteria *S. aureus* could be advantageous in repairing bone defects caused by infections, as *S. aureus* is the major pathogen in bone infections leading to severe and chronic forms of osteomyelitis.⁶²

The coating of ZnO QDs on BGN induced fluorescence and antibacterial properties, but it reduced the intrinsic bioactivity of coated BGN. The doping of BG by ZnO has significant influence on the apatite-forming ability of BG. The presence of ZnO in BG may delay the nucleation of HA crystals during the initial periods of immersion in SBF, but it has no significant effects on HA formation after a long period of immersion.¹¹ The zinc ions released from BG can be adsorbed on the active sites of BG, which therefore inhibits the deposition of apatite. The coating of ZnO QDs on the surface of BGN, playing a similar role in HA formation to the case of Zn-doped BG, delayed but not prevented the HA formation. The homogenous coating of ZnO QDs could inhibit the effective adsorption of Ca²⁺ and PO₄³⁻ from the medium and therefore a delayed formation of apatite took place.⁶³ Here needle-like apatite crystals were observed on ZnO–BGN upon immersion in SBF for 21 days, indicating that the apatite-forming ability of ZnO–BGN was only inhibited but not prevented.

ZnO QDs are considered to be relative low-toxicity materials compared to other types of QDs (*e.g.* CdS), but they still exhibit cytotoxicity at a high concentration.⁵³ The toxicity of ZnO QDs is likely to be induced by the release of Zn ions or the generation of ROS.⁵⁹ To reduce the cytotoxicity of ZnO QDs, silica, considering its high biocompatibility, has been coated on ZnO QDs.⁵⁴ The presence of a silica shell could also increase the stability and hydrophilicity of ZnO QDs. In this study ZnO–BGN did not show obvious cytotoxicity towards hMSC in comparison to BGN and the control, which might be due to the relatively low released concentration of Zn ions. Song *et al.*⁶⁴ reported that the concentration of Zn ions reaching 10 µg mL⁻¹ could induce approximate 50% death of mouse macrophage (Ana-1) cells, which was

similar to the cytotoxicity of ZnCl_2 ($\text{IC}_{50} = 13.33 \text{ Zn } \mu\text{g mL}^{-1}$). Yamamoto *et al.*⁶⁵ found that normal osteoblast function could be inhibited when the concentration of Zn ions reached $5.89 \text{ } \mu\text{g mL}^{-1}$. In this study the concentration of Zn ions in PBS was $\sim 0.95 \text{ } \mu\text{g mL}^{-1}$ when ZnO-BGN were soaked in PBS at concentration of 1 mg mL^{-1} for 24 h (Fig. 5a). Although the soaking medium does affect the degradation behavior of the material, it was estimated that ZnO-BGN in cell culture medium at concentration of 0.1 and 0.01 mg mL^{-1} should not release Zn ions in concentrations higher than $0.95 \text{ } \mu\text{g mL}^{-1}$ and were therefore far below the cytotoxic limit mentioned above. However ZnO-BGN indeed inhibited the relative proliferation of hMSC compared to the control and BGN according to the MTT assay. On the other hand, no inhibition effects were found on HepG2 (Fig. 7). The relatively lower proliferation of hMSC in comparison to HepG2 caused by ZnO-BGN, could be explained by the differentiation tendency of stem cells while cancerous HepG2 tend to proliferate.⁶⁶ It should be noted that the MTT results contradicted the LDH and live/dead results, since ZnO-BGN exhibited cytotoxicity towards hMSC at concentration of 0.1 mg mL^{-1} as indicated by the MTT assay while no obvious cytotoxicity was detected by the LDH and live/dead assays. This difference in cytotoxicity is consistent with previous results on the comparison of MTT and LDH assays,⁶⁷ suggesting that the MTT assay is more sensitive in detecting cytotoxicity compared to the LDH leakage assay.

Bioactive glasses can stimulate the osteogenic differentiation of pre-osteoblasts and stem cells due to the released ions upon dissolution.⁷ It has been reported that glasses containing ZnO could increase the ALP activity of osteoblast cells.⁶⁸ However, it also has been reported that Zn-doped BG scaffolds had no significant effects on the osteogenic differentiation of human adipose stem cells.⁶⁹ This contradiction is likely due to the different concentration of released ions, as Zn ions probably only stimulate the osteogenic differentiation of cells in a narrow range of ion concentration. The synthesized ZnO-BGN could only release a small amount of Zn ions ($\sim 0.95 \text{ } \mu\text{g mL}^{-1}$) in PBS (at concentration of 1 mg mL^{-1} after 24 h) and were thus expected to release a similar amount of Zn ions in cell culture medium. This low concentration of released Zn ions may account for the significant enhancement of ALP activity of hMSC. It has been reported that the concentration of Zn ions between 0.15 to $0.26 \text{ } \mu\text{g mL}^{-1}$ could significantly improve ALP activity, collagen secretion and ECM mineralization of rat mesenchymal stem cells after 14 days culture.⁶⁰ Even at concentrations below $0.01 \text{ } \mu\text{g mL}^{-1}$, Zn ions still could improve the osteogenic activity of mesenchymal stem cells.⁷⁰ Considering the refreshment of cell culture medium during the process, the concentration of Zn ions should be kept at a low level, but it was effective enough to induce osteogenic differentiation of hMSC as indicated by the enhanced ALP activity. Zn ions are able to trigger ERK1/2 signaling that is considered to play a significant role in the osteogenic differentiation of hMSC,⁷⁰ which could explain the enhanced ALP activity of hMSC culture with ZnO-BGN. However, to our knowledge, no conclusive quantitative data have been shown to confirm the effective concentration range of Zn ions leading to

osteogenic effects. In addition, Si and Ca ions may also enhance ALP activity,⁷ which means that the improved ALP activity could be a synergetic effect of released Si, Ca and Zn ions. Nevertheless, ZnO-BGN could induce the osteogenic differentiation of hMSC in comparison to BGN and the control, suggesting their potential in bone-related biomedical applications. In addition, the rapid release of Zn ions from ZnO-BGN under low pH conditions may be advantageous to the inhibition of bacteria in inflammatory tissues that are considered to have lower local pH value, as the relatively high concentration of Zn ions could exhibit stronger antibacterial effects.^{14,61} However, to elucidate the exact concentration range leading to both osteogenic and antibacterial effects, a comprehensive investigation is required. Moreover, the concentration of ZnO in ZnO-BGN can be tailored by tuning the initial coating conditions (e.g. concentration of BGN and ZnO QDs or pH values of coating condition). This possibility of the controlling the Zn concentration is significant to the further application of ZnO-BGN, considering that cytotoxicity and osteogenic activity of ZnO-BGN are dose-dependent. Detailed investigations on the effects of precursor concentration and pH environment on the properties of ZnO-BGN will be performed in future studies.

5. Conclusions

In this work, we developed highly dispersed Zn-containing bioactive glass nanoparticles with uniform spherical shape through a simple and convenient surface modification method. The proposed method was based on electrostatic interaction between building blocks, *i.e.* BGN ($\text{SiO}_2\text{-CaO}$ system) and ZnO QDs, with opposite surface charge. The developed ZnO-BGN particles retained the high dispersion and spherical shape of un-modified BGN. The above morphological characteristics indicated that coating metallic quantum dots on BGN nanoparticles could be a promising method to produce highly dispersed BGN containing therapeutic metallic ions. This combination also allowed the nanocomposite particles to obtain the properties from both components, *i.e.* ZnO-BGN exhibited a pH-sensitive release of Zn ions, fluorescence, antibacterial and osteogenic properties. The synthesized ZnO-BGN are therefore promising building blocks for nanocomposites and can be used for various biomedical applications, particularly in bone regeneration.

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