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Copper-doped mesoporous silica nanospheres, a promising immunomodulatory agent for inducing osteogenesis



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ABSTRACT

The application of mesoporous silica nanospheres (MSNs) loaded with drugs/growth factors to induce osteogenic differentiation of stem cells has been trialed by a number of researchers recently. However, limitations such as high cost, complex fabrication and unintended side effects from supraphysiological concentrations of the drugs/growth factors represent major obstacles to any potential clinical application in the near term. In this study we reported an *in situ* one-pot synthesis strategy of MSNs doped with hypoxia-inducing copper ions and systematically evaluated the nanospheres by *in vitro* biological assessments. The Cu-containing mesoporous silica nanospheres (Cu-MSNs) had uniform spherical morphology (~100 nm), ordered mesoporous channels (~2 nm) and homogeneous Cu distribution. Cu-MSNs demonstrated sustained release of both silicon (Si) and Cu ions and controlled degradability. The Cu-MSNs were phagocytized by immune cells and appeared to modulate a favorable immune environment by initiating proper pro-inflammatory cytokines, inducing osteogenic/angiogenic factors and suppressing osteoclastogenic factors by the immune cells. The immune microenvironment induced by the Cu-MSNs led to robust osteogenic differentiation of bone mesenchymal stem cells (BMSCs) via the activation of Oncostatin M (OSM) pathway. These results suggest that the novel Cu-MSNs could be used as an immunomodulatory agent with osteostimulatory capacity for bone regeneration/therapy application.

Statement of significance

In order to stimulate both osteogenesis and angiogenesis of stem cells for further bone regeneration, a new kind of hypoxia-inducing copper doped mesoporous silica nanospheres (Cu-MSNs) were prepared via one-pot synthesis. Biological assessments under immune environment which better reflect the *in vivo* response revealed that the nanospheres possessed osteostimulatory capacity and had potential as immunomodulatory agent for bone regeneration/therapy application. The strategy of introducing controllable amount of therapeutic ions instead of loading expensive drugs/growth factors in mesoporous silica nanosphere provides new options for bioactive nanomaterial functionalization.

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1. Introduction

The class of mesoporous silica nanomaterials, first developed as MCM-41 in the early 1990s [1,2], were characterized by having extremely high specific surface (up to 700 m² g⁻¹), as well as controllable particle size, large pore volume and low toxicity. Recently, intensive research efforts have been focused on mesoporous silica

nanospheres (MSNs) in order to tailor these materials for applications in biomedicine and diagnostics [3–6]. Application for bone regeneration in particular has garnered considerable interest. Detailed studies of particles as drug/growth factor carriers for osteogenic stimulation of stem cells have been performed [7–9]. Growth factors, such as BMP2, were loaded into MSNs and showed to promote human mesenchymal stem cells (hMSCs) osteogenic differentiation [10]. Alternatively, bioactive silica-based nanoparticles have been found to stimulate osteoblastic differentiation and mineralization [11].

Given the importance of vascularization on the bone regeneration, experiments have been conducted in which angiogenic growth factors (e.g. VEGF) were loaded into MSNs to enhance vascularization [12,13]. The limitations of these studies are that the MSNs as carriers were only applied in a single dose, with an invariably low drug/growth factor loading percentage. Consequently, only a very small fraction of the growth factors tend to reach their targets – something in the order of 1 in 10,000 to 1 in 100,000 molecules according to previous reports [14,15]. Subsequent experiments have sought to prevent the drugs/growth factors from leaching out via encapsulating them in the mesoporous channels by caps covalently bound to the functional groups of the drugs/growth factors [16]. However, the introduction of organic groups to the MSNs introduces additional complication to their physicochemical and biological properties that can result in side-effects on cells and tissues owing to the excessive inflammatory response or oxidative stress [17]. In addition, the high cost and complex fabrication of these cytokines make this strategy less attractive and defeats the whole purpose of developing cost-effective and efficacious synthetic biomaterials with simple chemistries for tissue engineering [13].

Hypoxia describes a condition of insufficient oxygen tension at the cellular level and is known to stabilize hypoxia-induced factors (HIFs), leading to upregulation of the downstream targeted genes, including VEGF, thereby enhancing angiogenesis [18]. Bioactive ions, such as cobalt (Co) and copper (Cu), are reported to have hypoxia-mimicking capacities [19–23], and, unlike drugs/growth factors, generally exert a physiological effect at very low concentrations, and are less specific or sensitive to micro-environmental conditions, including pH value and temperature [17]. The introduced ions and silica would bind covalently and therefore form much stronger chemical bonds compared to hydrogen bonds and *van der waal's* interactions. This could prevent the burst release in the early stages of host tissue interaction that is typical of biomaterials in which the drug has been adsorbed [24–26], which means that the release of the functional components can be sustained over a longer period. In addition, the physiological safety of the particles is improved since no extra organics are needed during the delivery process. The introduction of hypoxia-mimicking bioactive ions has the potential to endow MSNs with both osteogenic and angiogenic capacities, while eliminating the need for the complicated preparation of osteogenic cytokines (such as BMP2) and angiogenic cytokines (such as VEGF), something that would significantly lower cost. These are advantages that will greatly improve translation into clinical applications. Cu, one of the most important trace elements for human beings, is unique among the hypoxia-mimicking ions in that it can enhance bone density by both inhibiting active bone resorption [27]. The presence of Cu ions ($>10^{-6}$ M) was found to inhibit active bone resorption of calvaria from mice. The biosynthesis of E series prostaglandins, which has been proved to enhance bone resorption *in vitro*, could be altered by Cu ions. Further investigation on this issue has revealed that Cu ions could not only impede the activity of exogenous prostaglandins, but also hinder resorption mediated by lysosomal enzymes [28,29]. Cu deficient animals have brittle bones which were thought to be due to reduction of

cross-linking of collagen, the most abundant protein in bone [30]. In humans with dietary or genetic copper deficiency, osteogenesis is affected with patients presenting with thinning of the long bones [31]. Cu could increase the ability of MSCs to differentiate into the osteogenic lineage. It is therefore a promising metal element that could be applied to endow MSNs with functional properties for bone repair materials by enhancing angiogenic and osteogenic stimulation, as well as maintaining the morphology and biocompatibility of the nanospheres.

However, little attention has been paid on foreign ion-incorporation into mesoporous nanospheres. Although there are now a number of reports describing loading metal ions into the mesopores of MSNs, such as aluminum and gadolinium [32,33], or precious metals, such as gold and silver [34,35], which were mostly used as catalyst or contrast agent, there are no reports on the *in situ* introduction of single ions within the network of MSN structure. Here, we describe a modified process in which precursors co-operatively self-assembled to form mesoporous silica structures that were doped with Cu ions to yield Cu-MSNs.

When assessing the cellular response to nanoparticles in the context of bone repair biomaterials, the conventional *in vitro* approach has been focused on the osteoblast cell lineages. This approach often led to results that could not be replicated *in vivo* in pre-clinical trials. The lack of successful translational outcomes is most likely the failure to consider the actual immune response in materials-mediated osteogenesis [36]. There is now a growing appreciation of the effect that a biomaterial has on the immune environment and how this determines the *in vivo* fate of the materials. Among all the immune cells, the macrophage is one of the most important effector cells in both regulating bone dynamic and the materials mediated immune response [36]. It plays an essential role in determining the *in vivo* fate of biomaterials [37]. Therefore, the response of macrophages to biomaterials for bone regeneration must be considered in evaluation of the osteoimmunomodulatory properties. In related studies we have identified osteoimmunomodulation as a key property of bone related biomaterials [37–39]. We have introduced macrophages in the osteogenic assessment of biomaterials to mimic the actual *in vivo* scenario, which were applied in the evaluation of Cu-MSNs.

In this study, we applied a simple and efficient one-pot approach to dope therapeutic Cu ions within the Si–O–Si networks of the MSNs. These Cu-MSNs were then applied to macrophages to determine their effect on immunoreactions. Our results indicate that Cu-MSNs had the effect of stimulating the immune environment in a manner that improved the angiogenic and osteogenic capacities of the macrophages, which in turn enhanced the osteogenic differentiation of bone mesenchymal stem cells (BMSCs). The stimulatory effect of Cu-MSNs on osteogenesis highlights the potential of this composite material for future cell-based therapies or as a source material for biological scaffolds for bone regeneration.

2. Materials and methods

2.1. Synthesis of mesoporous silica nanospheres (MSNs) and copper-doped mesoporous silica nanospheres (Cu-MSNs)

To synthesize pure MSNs, 1.8 g of cetyltrimethylammonium bromide (CTAB) and 3 g of ammonium fluoride (NH_4F) were dissolved in 500 mL of distilled water and stirred for 1 h at 80 °C, after which 9 mL of tetraethoxysilane (TEOS) was added drop wise. The supernatant was collected and then centrifuged at 8000 rpm for 25 min after aging overnight at room temperature. Cu-MSNs were prepared using similar method with some modification. Copper nitrate trihydrate ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$) was first dissolved in a small

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