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Intracellular co-delivery of Sr ion and phenamil drug through mesoporous bioglass nanocarriers synergizes BMP signaling and tissue mineralization

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ABSTRACT

Inducing differentiation and maturation of resident multipotent stem cells (MSCs) is an important strategy to regenerate hard tissues in mal-calcification conditions. Here we explore a co-delivery approach of therapeutic molecules comprised of ion and drug through a mesoporous bioglass nanoparticle (MBN) for this purpose. Recently, MBN has offered unique potential as a nanocarrier for hard tissues, in terms of high mesoporosity, bone bioactivity (and possibly degradability), tunable delivery of biomolecules, and ionic modification. Herein Sr ion is structurally doped to MBN while drug Phenamil is externally loaded as a small molecule activator of BMP signaling, for the stimulation of osteo/odontogenesis and mineralization of human MSCs derived from dental pulp. The Sr-doped MBN (85Si:10Ca:5Sr) sol-gel processed presents a high mesoporosity with a pore size of ~6 nm. In particular, Sr ion is released slowly at a daily rate of ~3 ppm per mg nanoparticles for up to 7 days, a level therapeutically effective for cellular stimulation. The Sr-MBN is internalized to most MSCs via an ATP dependent macropinocytosis within hours, increasing the intracellular levels of Sr, Ca and Si ions. Phenamil is loaded maximally ~30% into Sr-MBN and then released slowly for up to 7 days. The co-delivered molecules (Sr ion and Phenamil drug) have profound effects on the differentiation and maturation of cells, i.e., significantly enhancing expression of osteo/odontogenic genes, alkaline phosphatase activity, and mineralization of cells. Of note, the stimulation is a result of a synergism of Sr and Phenamil, through a Trb3-dependent BMP signaling pathway. This biological synergism is further evidenced *in vivo* in a mal-calcification condition involving an extracted tooth implantation in dorsal subcutaneous tissues of rats. Six weeks post operation evidences the osseous-dentinal hard tissue formation, which is significantly stimulated by the Sr/Phenamil delivery, based on histomorphometric and micro-computed tomographic analyses. The bioactive nanoparticles releasing both Sr ion and Phenamil drug are considered to be a promising therapeutic nanocarrier platform for hard tissue regeneration. Furthermore, this novel ion/drug co-delivery concept through nanoparticles can be extensively used for other tissues that require different therapeutic treatment.

Statement of Significance

This study reports a novel design concept in inorganic nanoparticle delivery system for hard tissues – the co-delivery of therapeutic molecules comprised of ion (Sr) and drug (Phenamil) through a unique nanoparticle of mesoporous bioactive glass (MBN). The physico-chemical and biological properties of MBN enabled an effective loading of both therapeutic molecules and a subsequently sustained/controlled release. The co-delivered Sr and Phenamil demonstrated significant stimulation of adult stem cell differentiation *in vitro* and osseous/dentinal regeneration *in vivo*, through BMP signaling pathways. We consider the current combination of Sr ion with Phenamil is suited for the osteo/odontogenesis of stem cells for hard tissue regeneration, and further, this ion/drug co-delivery concept can extend the applications to other areas that require specific cellular and tissue functions.

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1. Introduction

Bioactive glasses (BGs), clinically proven over the last 40 years, are one of the most impactful biomaterials [1–5]. The applications are primarily hard tissues such as bone, dentin and cementum, but also extend many surrounding soft tissues including ligament, tendon, muscle and skin [6–10]. BGs are basically silica-based containing CaO, P_2O_5, and other oxide(s), although silica-free borate-containing glasses with bioactivity have also been reported [11]. One of the most recent intriguing research inputs on this is the development of nano-scaled BGs, either in the form of nanofibers, nanotubes or nanoparticulates [12–15]. Among them, BG nanoparticles with mesoporosity combine several key assets in nano-bio domain, such as i) loading and delivery of biomolecules, ii) bioactive composition, and iii) nanoscale morphology, providing nanotopological matrix cues for cellular anchorage or delivering therapeutics within cellular compartments.

Even with their infancy, recent studies on BG nanoparticles have shown some promising outcomes. For example, BG nanoparticles treated to dental tissue (carious dentin) were effective in repairing dentin-pulp complex tissue [14,16]. Furthermore, when added to polymer scaffolds or inorganic cements, BG nanoparticles played an essential role in cellular stimulation to an osteogenesis [17,18]. The loading and delivery capacity of BG nanoparticles has also been highlighted; through control over the mesopore size and surface chemistry, different types of biomolecules (from small drug to large plasmid DNA) could be effectively loaded and sustainably released to exhibit therapeutic functions [19,20].

In fact, those features of BG nanoparticles are benefited mostly from the conventional mesoporous silica nanoparticles (MSNs), such as mesoporosity and surface chemistry [21–25]. However, BG nanoparticles incorporate additional ions, primarily Ca (and possibly phosphate) within silica glass network, and this defines unique physico-chemical and biological properties of BG nanoparticles, significantly different from MSNs. For example, the added Ca ions loosen the silica network, making the nanomaterials water-degradable, a prime asset of safe nanocarrier; and the released Ca ions accelerate the deposition of calcium phosphate bone mineral-like phase; and even further, such an ionic release can stimulate cellular functions. A promising aspect is that various ions (Sr^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+} and Ag^{+}) can be doped to the BG nanoparticle structure at proper doses, to target therapeutic functions, such as stimulating cell proliferation, osteogenesis, and angiogenesis, or relieving inflammation and bacterial effect [15,26–28].

Among the ions, Sr^{2+} has been implicated to have profound effects on hard tissue repair processes, such as the stimulation of osteo/odontoblastic functions, the induction of osteo/odontogenesis of progenitor/stem cells, and the inhibition of osteoclastic activities [29,30]. For this reason, Sr^{2+} has been used clinically in the form of strontium ranelate to treat osteoporosis [31–33]. When Sr^{2+} is internalized into cells, it can interact with intracellular signaling molecules to improve and synergize the target biological functions [34]. The stimulating role of Sr^{2+} in hard tissue repair process is known to be through the bone morphogenetic protein 2 (BMP2)/Smad signaling pathway; therefore, the co-administrative approach of Sr^{2+} with BMP2-activating molecules is envisaged to synergize therapeutic effects. Phenamil, known as an irreversible inhibitor of sodium channel, has recently been highlighted as a potent small molecule BMP signaling activator [35]. When phenamil was treated to osteoblasts or odontoblasts, the cellular activity, secretion of ECM molecules and calcification were significantly enhanced through BMP2/Smad signaling pathway [36]. The small molecule activator is relatively stable in physiological conditions, cost-effective, less tumorigenic, and has reduced side effects related with overdoses when compared to biological therapeutic molecules such as growth factors [36–40].

Here we aim to co-deliver those BMP signaling molecules, phenamil and Sr^{2+}, through the BG nanoparticle carrier. While Sr^{2+} constitutes 'intrinsically' the BG nanoparticle chemistry, phenamil is 'extrinsically' loaded onto the mesopores of the nanoparticles. These two molecules are internalized to cells to synergize the osteo/odontogenic stimulating BMP pathway. Human MSCs derived from dental pulp are used as a model cell to examine the co-delivery effects on the odonto/osteogenic differentiation of stem cells and the possible hard tissue matrix production through BMP signaling. The effects are also proven in the *in vivo* mal-calcification condition involving a subcutaneous implantation of extracted tooth defects. This study utilizing 'ion' with 'drug' as the therapeutic molecules in nanocarriers for tissue regeneration is considered to provide informative idea for the design and development of future nano-therapeutic systems.

2. Materials and methods

2.1. Materials

Tetraethyl orthosilicate (TEOS,$C_8H_{20}O_4Si$), strontium nitrate, ($Sr(NO_3)_2$), calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), poly (ethylene glycol) (PEG, $(C_2H_4)_nH_2O$), ammonium hydroxide (NH_4OH), anhydrous methanol (CH_3OH), tris-hydroxymethylaminomethane (Tris–buffer, $NH_2C(CH_2OH)_3$), 1 N hydrochloric acid (IN HCl), nitric acid (HNO_3), phosphate buffered saline (PBS) tablets and highly pure chemicals of simulated body fluid (SBF) were all purchased from Sigma-Aldrich. Phenamil (in methanesulfonate salt form, $C_{12}H_{12}ClN_7O \cdot CH_3SO_3H$) as a small molecular BMP activator was also purchased from Sigma-Aldrich. Ultrapure deionized water (DW, 18.2 MΩ·cm, Millipore Direct-Q system) was used in all experiments.

2.2. Synthesis of Sr-doped mesoporous BG nanoparticles

Mesoporous BG nanoparticles doped with Sr ions (coded as 'Sr-MBN') are based on $85SiO_2$–(15-x)CaO–xSrO glass composition (x = 5 wt%). Mesoporous BG nanoparticles without Sr ions (x = 0 wt%, coded as 'MBN') are used as a comparison group. The Sr-MBN and MBN were synthesized by the ultrasound-assisted sol-gel method (alkali-mediated) using PEG as a structural template [12]. Briefly, 5 g of PEG and 0.189 g of $Ca(NO_3)_2{\cdot}4H_2O$ (for MBN) or 5 g of PEG, 0.126 g of $Ca(NO_3)_2{\cdot}4H_2O$ and 0.031 g of $Sr(NO_3)_2$ (for Sr-MBN) were dissolved in 150 ml of alkaline methanol (around pH 12.5). In parallel, another solution of TEOS was prepared by diluting 0.884 g of TEOS in 30 ml of anhydrous methanol. The TEOS solution was added drop-wise to the first-prepared solution under vigorous stirring and the simultaneous application of ultrasound as described previously [20,41]. The white precipitate produced after 24 h of stirring was separated and washed with DW/ethanol using 3 cycles of centrifugation/redispersion at 5000 rpm for 5 min and then the collected precipitate was dried at 70 °C overnight. The organic template, PEG, was removed by calcination of the dried powder at 600 °C for 5 h in air. Finally, the calcined powder was stored under vacuum for further uses.

2.3. Characterizations of nanoparticles

All data/images were shown as the representative ones from independent triplicate experiments using nanoparticles from different batches. The phase of the nanoparticles was investigated with X-ray diffractometer (XRD, Rigaku, Ultima IV, Japan) with CuKα radiation (λ = 1.5418 °A). X-ray was generated (40 mA and