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pH-sensitive nano-crystals of carbonate apatite for smart and cell-specific transgene delivery

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The treatment of a human disease at a genetic level by either providing a cell with a functional gene or a nucleic acid sequence to precisely silence a harmful gene, is a powerful approach that could revolutionise clinical medicine. Despite the existence of both genetically engineered viral vectors and synthetically designed lipid- or polymer-based nanocarriers, an ideal delivery system in terms of safety and efficacy is still lacking. This editorial reports on the development of biocompatible, inorganic nanoparticles of carbonate apatite, which has the unique features essentially required for smart delivery, as well as for the expression of a genetic material in a mammalian cell.

Keywords: carbonate apatite, DNA release, endosomal acidification, gene delivery, nano-crystal, particle dissolution, targeted delivery, transgene expression

Expert Opin. Drug Deliv. (2007) 4(3):193-196

1. Introduction

Introducing nucleic acids (genes or gene-silencing elements) to mammalian cells is a powerful way to modulate the cellular functions and signal transduction mediated by proteins. Following delivery to the cytoplasm, a foreign gene enters the nucleus and is transcribed to the corresponding mRNA, which is subsequently transported to the cytoplasm for translation into a specific protein. However, a gene-silencing element, such as an antisense onligonucleotide or a small interfering RNA blocks the transcription of a target mRNA [1]. Nucleic acid delivery has been an essential tool to turn on and off the expression of a particular gene in basic research laboratories, and is highly promising for the development of new therapeutic concepts, such as gene therapy and DNA vaccination, which are likely to have an impact on clinical medicine and biotechnology in this century [1-3].

Traditionally, gene delivery systems have been divided into viral and non-viral vectors. Although viral systems are, at present, the most effective means of DNA delivery, achieving high efficiencies for both transgene delivery and expression, some major immunogenicity and carcinogenicity limitations have resulted in intensive research into gene delivery by non-viral vectors, the majority of which are synthetic, such as cationic polymers, lipids and peptides [1-3]. Negatively charged DNA molecules are usually condensed with cationic reagents to allow formation of complexes carrying net positive charges. The resulting complexes can interact electrostatically with anionic heparan sulfate proteoglycans (syndecans) on the cell surface and reach the cytoplasm in the form of endosomes, through endocytosis [4]. The extremely low pH, as well as the enzymes within the late endosomes, usually brings about the degradation of entrapped DNA and the associated complexes. Finally, DNA that survives both endocytic processing and cytoplasmic nucleases must dissociate from the condensed complexes either before or after nuclear translocation through a nuclear pore or during cell division [3]. The precise mechanism of DNA release from macromolecular complexes is yet to be fully

elucidated. However, the competitive binding of anionic lipids, heparin, RNA and proteins in the endosomal membrane or in the cytosol with the cationic vectors can help DNA unpackaging [5,6].

Many therapeutic applications require a vehicle with the capability of delivering a transgene(s) to only a selected cell type, in order to increase the expression efficacy and prevent any side effects. A common strategy in non-viral cases involves the attachment of a targeting moiety to a polycation (lipid or polymer) backbone, which finally condenses the DNA through ionic interactions. Polylysine, the first backbone used for gene delivery, has been conjugated to a diverse set of cell-specific ligands, such as asialoorosomucoid [7], transferrin [8], EGF [9], mannose [10], fibroblast growth factor (FGF) [11] and antibodies [12] in order to target, respectively, hepatocytes via asialoglycoprotein receptors, transferrin receptor-positive cells, EGF receptor-carrying cells, macrophages through membrane lectins, FGF receptor-bearing cells and lymphocytes via surface-bound antigens. In a similar way, polymers such as polyethylenimine (PEI) and liposomes have been coupled to other cell-surface receptor-specific ligands in addition to those described above, such as integrin-binding peptide conjugated to PEI to target integrins on cell surfaces [13] and vitamin folate conjugated to liposomes through a polyethylene spacer to target folate receptor-bearing cells [14].

2. Molecular aspects of DNA delivery with nano-crystals of carbonate apatite

Being inspired by the need to develop a superior non-viral approach in terms of both efficiency and biocompatibility, the development of an inorganic DNA nanocarrier based on *in vitro*-synthesised carbonate apatite, a major component of hard tissues in the body, has recently been reported [15]. Particles are generated in the presence of DNA in a bicarbonate-buffered solution or medium containing appropriate concentrations of phosphate and calcium salts through incubation at a high temperature (37 – 55 ° C). The chemical reaction required for particle formation takes place with Ca^{2+}, PO_4^{3-} and HCO_3^-, and DNA that is negatively charged can be electrostatically associated with the cationic (Ca^{2+} rich) domains of the particles [16]. The number of particles, as well as their size, two crucial factors for determining transfection potency, are dramatically influenced by the concentrations of calcium, phosphate and bicarbonate and, additionally, by the pH of the medium (or solution) and the incubation temperature and time. Increasing the concentration of any of the reactants (calcium, phosphate and bicarbonate), in general, accelerates particle formation by providing a stronger driving force for the chemical reaction while the other parameters (pH, incubation temperature and time) are constant. On the other hand, an increase in pH and temperature (or incubation time) shifts the ionisation equilibrium of phosphate and bicarbonate towards the forward direction and consequently favours particle generation by increasing the rate of the reaction. The induction of particle formation is generally accompanied by growth and aggregation of the particles, leading to larger-sized crystals [16]. However, bicarbonate, which is a minor component of the final apatite structure, $Ca_{10}(PO_4)_{6-X}(CO_3)_X(OH)_2$, prevents particle aggregation and generates small-sized crystals in a dose-dependent manner. Due to the small size of the crystal, which has an average diameter of 50 – 300 nM, and strong binding with DNA (almost 100% efficiency), carbonate apatite can be rapidly internalised with the associated DNA into the cell by endocytosis, following close contact with the cell surface through electrostatic forces. The efficiency of cellular uptake of DNA has been estimated to be at least 10-times more rapid than that achieved by the classical calcium phosphate method [15,16].

3. Transfection efficiency and the role of crystal dissolution

Transgene expression results only after multiple barriers are overcome, such as endocytosis, DNA release from the particles and the endosomes, and translocation to the nucleus. Once inside the endosomes, exposure to an increasingly acidic environment results in the consumption of the excess H^+ by phosphate and carbonate ions in the particle, leading to particle dissolution and consequential DNA release. Therefore, inorganic crystals with high acid solubility or low crystallinity would enable quicker DNA release in endosomes, compared with crystals with low solubility or high crystallinity. In addition, the high dissolution rate may contribute to the destabilisation of endosomes, to achieve DNA release into the cytoplasm, as vacuolar proton-pumping ATPase-driven massive proton accumulation that causes crystal dissolution could also lead to passive chloride influx to endosomes and subsequent endosome swelling and rupture. Finally, released DNA can enter the nucleus either through the nuclear pore or during cell division and be heavily expressed both in primary and cancer cell lines with an efficiency 5- to 100-times higher than with existing techniques.

4. Bio-recognition devices associated with the inorganic crystals for cell-targeted delivery

As mentioned before, a gene delivery system should have a precise cell-targeting capability for many therapeutic applications. To achieve this with the inorganic nanocarrier now under discussion, which delivers transgenes in a non-specific manner depending on ionic interactions between the particles and the cell surface, an organic–inorganic hybrid nanocarrier was synthesised by coating the DNA-embedded particle surface. The surface was coated sequentially with a cell-recognisable protein, such as asislofetuin for asislo-glycoprotein receptors on hepatocytes or transferrin for transferrin receptors on several cancer cell lines and a highly hydrophilic protein, such as serum albumin for preventing

non-specific interactions of the particle surface with cell-surface proteoglycans, other receptors or serum proteins [17]. The composite particles, with dual surface properties, have been shown to accelerate transgene delivery and expression only in the corresponding receptor-bearing cells. Moreover, mimicking the natural mineralisation process, extracellular matrix proteins, such as collagen or fibronectin, have been successfully attached to the nano-crystals for integrin-specific efficient delivery of DNA [18]. A remarkable success has been achieved for transfecting embryonic stem cells, where particles initially did not show any significant transfection activity owing to the lack of particle interactions with the cell surface. However, when the particles were complexed with a naturally occurring fibronection and genetically engineered E-cadherin-Fc together with DNA, a synergistic effect resulted in a dramatic enhancement both in transgene delivery and expression in mouse embryonic stem cells that possessed both transmembrane fibronectin-specific integrin and E-cadherin [19].

5. Conclusion

The present author's group has developed an advanced technology for gene delivery to mammalian cells based on the properties of carbonate apatite, which prevent crystal growth, for the generation of nano-scale particles. These properties are beneficial for efficient endocytosis and the high-affinity binding of DNA and protein molecules for delivering DNA to the cell in either a specific or non-specific manner, and allow a fast dissolution rate in endosomal acidic compartments to facilitate DNA release from the particles and endosomes. These novel approaches could pave the way to the wide potential applications of carbonate apatite, from laboratories to clinical medicine, and provide insights to create a new era for inorganic crystal-based gene and drug delivery.

6. Expert opinion

Despite intensive efforts for the last three decades, there has been a lack of proper understanding of the molecular and cellular barriers to gene delivery, which could assist in developing a superior non-viral technique. Calcium phosphate precipitation, which is based on hydroxyapatite and has been widely used for > 30 years, is a good example of where attention was rarely focused on the regulation of crystal growth at the molecular level so as to generate nanosized particles for effective DNA delivery or on the elucidation of a method to achieve the escape of bound DNA from apatite particles for final protein expression. The newly developed carbonate apatite, as with hydroxyapatite, adsorbed DNA, but, unlike the latter, it can prevent the growth of its crystals to a significant extent, enabling the synthesis of nanosized crystals to effectively carry the associated DNA across the cell membrane. It also possesses a high dissolution rate in endosomal acidic pH, leading to the rapid release of the bound DNA for a subsequent high level of protein expression.

The effectiveness of viral particles is a result of its highly evolved and specialised structure, which is basically composed of a protein coat surrounding a nucleic acid core. A non-viral approach to the delivery of therapeutics that has beneficial virus-like properties, but lacks disadvantageous ones, would emerge as the most attractive strategy. As described in this paper, this ideal approach was kept in mind in the design of an universal and novel strategy of electrostatically coating the surfaces of DNA-bound nano-crystals with a cell-recognisable protein, and subsequently with a hydrophilic protein, with the aim to prevent non-specific interactions.

Carbonate apatite is a natural component of the body, and is usually found in the hard tissue, such as bone and teeth. Moreover, because of their nanosize dimensions and sensitivity to low pH, particles of carbonate apatite are quickly degraded when taken up by cells in their acidic vesicles, without any indication of toxicity. Therefore, these materials appear to be clinically safe – one of the major criteria for practical applications in human body. However, rigorous *in vivo* assessment is required to establish the therapeutic efficacy and potential toxicity of these inorganic nanoparticles. The nanoparticles can theoretically be targeted to many tissues or cells through passive or active targeting. Passive targeting to the tumour can be made possible by means of precisely controlling the size of the nanomedicine by regulating the initial concentrations of carbonate prior to particle preparation. Active targeting can be made by coupling one or more cell-recognisable protein(s) and by making a hydrophilic protein layer onto the nano-particle surface.

Thus, a high affinity for nucleic acids and proteins, and excellent colloidal stability at physiological conditions, but extreme instability at the low pH of cellular endosomal compartments, make these apatite-based inorganic particles superior candidates for therapeutic delivery in the 21st century, compared with polymer- or lipid-based systems that are generally non-biodegradable and inefficient.

Disclosure

This study was financially supported by special coordination funds for promoting science and technology from the Ministry of Education, Culture, Sports, Science and Technology (MEXT).

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