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Proteoliposomes as matrix vesicles' biomimetics to study the initiation of skeletal mineralization

A.M.S. Simão^{1,2}, M.C. Yadav¹, P. Ciancaglini² and J.L. Millán¹

¹Sanford Children's Health Research Center,
Sanford-Burnham Medical Research Institute, La Jolla, CA, USA

²Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto,
Universidade de São Paulo, Ribeirão Preto, SP, Brasil

Abstract

During the process of endochondral bone formation, chondrocytes and osteoblasts mineralize their extracellular matrix by promoting the formation of hydroxyapatite (HA) seed crystals in the sheltered interior of membrane-limited matrix vesicles (MVs). Ion transporters control the availability of phosphate and calcium needed for HA deposition. The lipidic microenvironment in which MV-associated enzymes and transporters function plays a crucial physiological role and must be taken into account when attempting to elucidate their interplay during the initiation of biomineralization. In this short mini-review, we discuss the potential use of proteoliposome systems as chondrocyte- and osteoblast-derived MVs biomimetics, as a means of reconstituting a phospholipid microenvironment in a manner that recapitulates the native functional MV microenvironment. Such a system can be used to elucidate the interplay of MV enzymes during catalysis of biomineralization substrates and in modulating *in vitro* calcification. As such, the enzymatic defects associated with disease-causing mutations in MV enzymes could be studied in an artificial vesicular environment that better mimics their *in vivo* biological milieu. These artificial systems could also be used for the screening of small molecule compounds able to modulate the activity of MV enzymes for potential therapeutic uses. Such a nanovesicular system could also prove useful for the repair/treatment of craniofacial and other skeletal defects and to facilitate the mineralization of titanium-based tooth implants.

Key words: Alkaline phosphatase; Biomineralization; Calcification; Lipids; Pyrophosphate; ATP

Matrix vesicles

Mineralization of cartilage and bone occurs by physicochemical and biochemical processes that facilitate the deposition of hydroxyapatite (HA) in specific areas of the extracellular matrix (ECM). Experimental evidence has pointed to the presence of HA crystals along collagen fibrils in the ECM (reviewed in Ref. 1) and also within the lumen of chondroblast- and osteoblast-derived matrix vesicles (MVs) (reviewed in Ref. 2). Investigators in the bone mineralization field are usually divided in supporting the collagen- versus the MV-mediated mechanism of mineralization, but we see no incompatibility between these mechanisms. Our working model is that bone mineralization is first initiated within the lumen of MVs and, in a second step, HA crystals grow beyond the confines of the MVs and become exposed to the extracellular milieu where they continue to propagate along collagen fibrils. This process is coordinated by the

balanced action of promoters and inhibitors of calcification (reviewed in Ref. 3).

It is currently believed that MVs have at least two principal roles in initiating calcification: 1) MV enzymes regulate the ratio of phosphate (P_i) to inorganic pyrophosphate (PP_i) in the intra- and extravascular fluid, and 2) MV proteins and lipids, including acidic phospholipids, serve as nucleation sites for apatite deposition (4). PP_i , derived both from ectoplasmic PC-1/nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1)-catalyzed production from extracellular nucleoside triphosphates (5) and by intracellular export via the ankylosis protein (ANK) transporter, inhibits matrix mineralization (6). This inhibition is released through the action of tissue-nonspecific alkaline phosphatase (TNAP), which hydrolyzes PP_i , thus simultaneously removing the inhibitor and providing additional P_i for mineral formation (7,8). Mineralization is said to proceed in two phases: an initial formation of apatite within MVs, and a subsequent

Correspondence: J.L. Millán, Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA. Fax: +1-858-646-3195. E-mail: millan@burnham.org

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propagation phase in the matrix (2,9). According to this mechanism, Ca^{2+} enters MVs via an annexin channel and phosphate enters via a type III Na^+ -dependent phosphate transporter (P_iT), and possibly other P_i -transporters, to form apatite within MVs (10,11). Acidic phospholipids and other MV components are thought to nucleate these intravesicular nanocrystals (12-14). Subsequently, the intravesicular mineral grows beyond the confines of MVs onto a collagenous matrix aided by a number of promoters and inhibitors of calcification (3).

MV enzymes, channels and transporters

MVs are complex nanovesicles. Three recent proteomic papers have provided a first indication of just how diverse their proteomic constitution is (15-17). Here we will only refer to some of the enzymes, channels and transporters that have been implicated in the biological function of MVs to date. Figure 1 illustrates the proposed role of the molecules that are described in more detail below.

TNAP is the only tissue-nonspecific isozyme of a

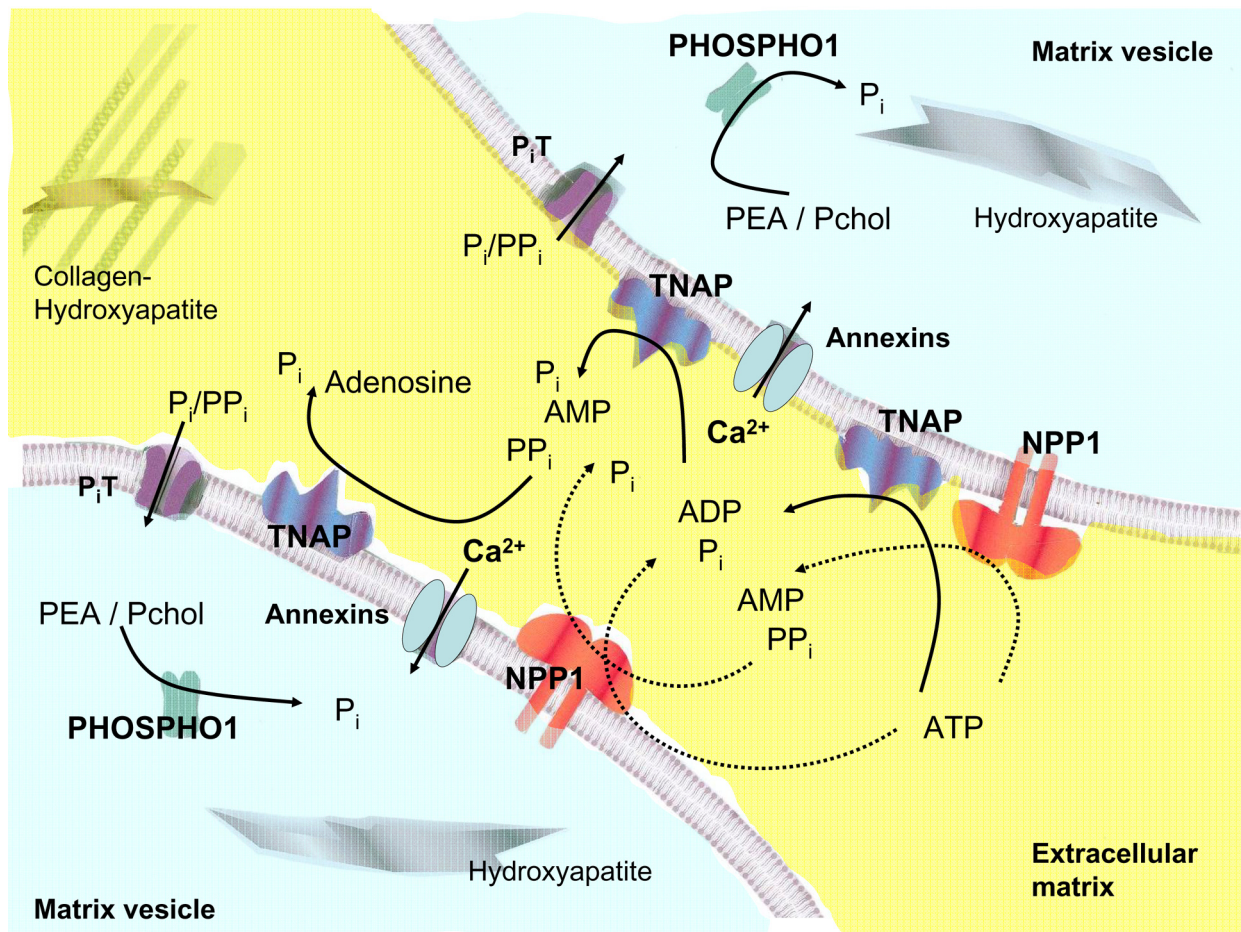


Figure 1. Schematic representation of the molecules involved in matrix vesicle (MV)-mediated calcification including the most favored enzymatic reactions of their putative physiological substrates. This is a modified figure based on the one published in Ciancaglini et al. (54). Hydroxyapatite seed crystals are formed in the sheltered interior of MVs favored by the inorganic phosphate (P_i)-generating ability of PHOSPHO1, which uses phosphocholine (Pcho) and phosphoethanolamine (PEA) as substrates, as well as by the function of P_i -transporters (P_iT) and annexins that mediate Ca^{2+} ion influx. Tissue-nonspecific alkaline phosphatase (TNAP) has a major role both as a PP_i ase and as an ATPase/ADPase and thus participates in the calcification process by restricting the concentration of extracellular PP_i while also contributing to the P_i pool available for calcification. PC-1/nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) can act as a backup PP_i ase and also ATPase, particularly in the absence of TNAP. Thick lines indicate the most favored reactions, while dotted lines indicate alternative backup enzymatic reactions. Outside the MVs, collagen acts as a primary scaffold for the deposition of hydroxyapatite crystals.

family of four homologous human AP genes (EC. 3.1.3.1) (reviewed in Ref. 18). Expressed as an ecto-enzyme anchored via a phosphatidylinositol glycan moiety, TNAP has been demonstrated to play an essential physiological role during bone matrix mineralization. A deficiency in the TNAP isozyme causes the inborn error of metabolism known as hypophosphatasia and the study of this disease has provided the best evidence of the importance of TNAP for bone mineralization (19). The severity of hypophosphatasia is variable and is modulated by the nature of the TNAP mutation (18,19). The clinical severity of hypophosphatasia patients varies widely. The different syndromes, listed from the most severe to the mildest forms, are: perinatal hypophosphatasia, infantile hypophosphatasia, childhood hypophosphatasia, adult hypophosphatasia, odontohypophosphatasia, and pseudohypophosphatasia (19). These phenotypes range from complete absence of bone mineralization and stillbirth to spontaneous fractures and loss of deciduous teeth in adult life. Inactivation of the mouse TNAP gene (*Akp2*) phenocopies the infantile form of human hypophosphatasia (18). Biochemically, hypophosphatasia is diagnosed by a low serum TNAP level as well as accumulation of the phosphocompounds inorganic pyrophosphate (PP_i), pyridoxal-5'-phosphate and phosphoethanolamine (PEA) (18,19).

In bone, TNAP is confined to the cell surface of osteoblasts and chondrocytes, including the membranes of their shed MVs (2). It has been proposed that the role of TNAP in the bone matrix is to generate the inorganic phosphate needed for HA crystallization. However, TNAP has also been shown to hydrolyze the mineralization inhibitor PP_i , thus facilitating mineral precipitation and growth (2). Electron microscopy revealed that TNAP-deficient MVs, in both humans and mice, contain apatite crystals, but that extravesicular crystal propagation is delayed (20,21). This growth retardation could be due to either the lack of TNAP's pyrophosphatase function or the lack of inorganic phosphate generation. Our recent studies have provided compelling proof that the major function of TNAP in bone tissue consists of hydrolyzing PP_i to maintain a proper concentration of this mineralization inhibitor to ensure normal bone mineralization (7,8,22).

A primary inhibitor of ECM mineralization is extracellular PP_i (23), primarily generated by the members of the nucleotide pyrophosphatase/phosphodiesterase (NPP) family of isozymes. Nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) is plasma membrane-bound, whereas autotaxin (NPP2) is secreted and B10 (NPP3) is abundant in intracellular spaces (5). All three isozymes are expressed in a wide variety of tissues, including bone and cartilage and they all share the ability to hydrolyze diesters of phosphoric acid into phosphomonoesters, primarily ATP to AMP and PP_i , and/or ADP to adenosine (5). Similar to skeletal TNAP expression, NPP1 is highly abundant on the surfaces of osteoblasts and chondrocytes as well as on the

membrane of their MVs (5). NPP1 has a role in inhibiting HA precipitation by its PP_i -generating property (5).

Similar to NPP1, ANK has a role in mineralization by contributing to the extracellular supply of PP_i . However, unlike NPP1, ANK appears to function as a transmembrane PP_i -channeling protein, allowing PP_i molecules to pass through the plasma membrane from the cytoplasm to the outside of the cell (6). ANK protein is detectable in many tissues, yet its expression is particularly strong in the cartilage of joints (6). Cell surfaces of osteoblasts and chondrocytes appear to be abundant in ANK protein.

Mice deficient in NPP1 (*Enpp1*^{-/-}) or ANK (*ank/ank*) develop soft tissue calcification, including vascular calcification, resulting from the reduced production or transport of PP_i (7,8,22), while mice deficient in TNAP function (*Akp2*^{-/-}) display rickets and osteomalacia due to an arrest in the propagation of HA crystals outside the MVs caused by an increase in extracellular PP_i concentrations (2,7,8,21). HA crystals are still present in TNAP-deficient MVs and it has been proposed that the soluble MV phosphatase PHOSPHO1 might be involved in increasing the local intravesicular concentration of P_i to change the P_i/PP_i ratio to favor precipitation of HA seed crystals (24).

PHOSPHO1 is a member of the haloacid dehalogenase superfamily of Mg^{2+} -dependent hydrolases, first identified in chick embryos, that is expressed at levels 100-fold higher in mineralizing chondrocytes than in non-mineralizing tissues and shows high phosphohydrolase activity towards PEA and phosphocholine (PCho) (25). The use of small compounds to inhibit its activity in *Akp2*^{-/-} MVs leads to a significant decrease in *in vitro* MV-mediated calcification (24), implicating PHOSPHO1 function in the initiation of skeletal calcification. Since PHOSPHO1 is specific for PEA and PCho (24,25), it is possible that P_i is generated from PEA and PCho through the enzymatic action of PHOSPHO1 during the mineralization process in order to generate the P_i concentration needed to establish a P_i/PP_i ratio permissive for the initial formation of HA crystal inside the MVs. The very low K_m values for both PEA and PCho (3 and 11.4 μM , respectively) (25) suggest that under physiological conditions both molecules would be rapidly hydrolyzed by PHOSPHO1, indicating that these compounds are therefore likely to be natural substrates of the enzyme.

Annexins also play an important role in MV function (26-28). Annexins are Ca^{2+} and phospholipid binding proteins, which under the right conditions can form calcium channels through MV membranes (26). These conditions include membranes with a high content of acidic phospholipids (26). MVs contain annexins II, V and VI, and in such membranes, MV annexins appear to be associated with Ca^{2+} transport. Moreover, annexin V binds to type II and X collagen and these interactions stimulate its Ca^{2+} channel activities leading to increased influx of Ca^{2+} into liposomes and matrix vesicles (20,28).

The inorganic phosphate (P_i) transporter (P_iT) fam-

ily comprises Na⁺-dependent P_i symporters, e.g., the mammalian type III NaP_i symporters, P_iT1 and P_iT2 (29). Moreover, the broad tissue distribution of P_iT1 and P_iT2 together with their transport characteristics suggests that they constitute the housekeeping phosphate uptake system between cells and the extracellular fluid (29). In addition, they have been implicated in chondroblastic and osteoblastic mineralization (30) as well as in transdifferentiation of vascular smooth muscle cells to osteoblast-like cells in the process of vascular calcification under hyperphosphatemic conditions (3). Despite the essential role of P_i for life and the specific roles of P_iT1 and P_iT2 in supplying mammalian cells with their general P_i needs as well as the increasing evidence for the role of type III transporters in normal and pathologic calcification, only a few studies have addressed the physiologic function of members of the P_iT family. Recently, Festing et al. (31) successfully generated conditional and null P_iT1 mouse alleles to determine the *in vivo* role of P_iT1, and demonstrated that P_iT1 is necessary for embryonic development and is non-redundant, since P_iT2 could not compensate for the loss of P_iT1 and allow development into adulthood. Other studies have indicated that P_i transporters other than P_iT1/2 might be involved in endochondral ossification (11).

Lipid composition of MVs

In order to understand the physiological interplay between important MV-associated enzymes in the initiation of biomineralization, it is important to keep in mind the microenvironment in which these enzymes function, which can have a profound effect on their biological properties, since phospholipids play an important role in the initiation of the biomineralization process (4,20). Early studies of MV lipid composition have determined that there are significant differences between the lipid content of MVs and the plasma membranes from which they arose. Wuthier (32) reported

these differences and hypothesized that the increase in acidic phospholipids in MVs was somehow associated with MV calcification. Further studies from his group, along with those of Boyan et al. (33) have proposed that MV lipids could act as a nucleation site for HA formation (27,34,35). This premise has been extensively developed under the idea of a “nucleational core complex”, which describes the interactions of MV phospholipids, Ca²⁺, PO₄³⁻ and some MV proteins to form a molecular architecture that nucleates apatite crystallization (27,36). The acidic phospholipids can bind Ca²⁺ but, more importantly, it has been shown that they facilitate calcium-dependent annexin binding, and are permissive for annexins to form calcium channels through the membrane (12,26). Several lines of evidence suggest that glycosylphosphatidylinositol (GPI)-anchored proteins are commonly found to be enriched within putative cholesterol- and (glyco)sphingolipid-enriched platforms called “rafts” and that such association is required for the expression of their biological function (37,38).

Liposome systems

Recent data (39) suggest that the location of TNAP on the membrane of MVs plays a role in determining substrate selectivity in this micro-compartment. These data suggest that assays of TNAP bound to MVs or to liposome-based systems might be more biologically relevant than assays done with solubilized enzyme preparations, particularly when studying the hydrolysis of organophosphate substrates. The ability of synthetic or natural vesicles (40,41) to mimic the organizational structure and function of biomembranes makes these structures an advantageous and convenient experimental model to help in advancing our understanding of MV-mediated calcification. Table 1 lists some advantages and disadvantages of working with liposome systems when compared to the use of native MVs. While MVs represent the native nanovesicular system

Table 1. Comparison of the advantages and disadvantages of the use of proteoliposomes as biomimetics for matrix vesicles in the study of mechanisms of initiation of skeletal mineralization.

System	Advantages	Disadvantages
Matrix vesicles	Native membrane structure Native protein composition Relatively stable	Variable size Undefined proteomic composition Need live animals for isolation Laborious isolation procedure
Proteoliposomes	Easy to prepare Controlled lipid composition Controlled reconstitution of proteins Uniform size Controlled electrolytes	Artificial system Limited stability

with a full complement of active proteins, their isolation demands the use of animals and a laborious procedure that can lead to the production of membrane artifacts in the process. In addition, the proteomic composition of these MVs is still incompletely defined, although some proteomic studies have recently been published (15-17). On the other hand, proteoliposomes are easy to prepare and can be manufactured using different methods and with controlled lipid and protein composition, electrolytes and sizes, representing a convenient experimental model to mimic the organizational structure and function of natural biomembranes and to reproduce some essential features of the biomineralization process (42,43). But since proteoliposomes are artificial systems, it is important to consider also the stability of the vesicles, which may affect the structure and function of the reconstituted enzymes. An important experimental consideration is the need to establish the appropriate lipid composition, especially the cholesterol content, to be used in the construction of the proteoliposomes since the amount of cholesterol modulates the fluidity of the bilayer and influences membrane features, such as curvature, that can affect the activity of the enzymes in different ways, depending on the substrates used (40). Moreover, the lipid membrane plays an important role as a nucleation agent in the biomineralization process (44,45), as a protective and/or activation agent (46).

Proteoliposomes can be obtained by different preparation techniques, such as mechanical dispersion, sonication, extrusion, solvent dispersion, co-solubilization with detergents and reverse-phase evaporation as well as direct insertion after removal of the detergent (47,48). In fact, enzyme reconstitution varies depending on the methodology used for enzyme extraction, detergent used, solubilization method and also an adequate proportion of the lipids used (49-51). The method of first forming mixed micelles of various lipids and detergent followed by the use of appropriate resins to remove the detergent has proved to be an effective means to consistently synthesize homogeneous, unilamellar vesicles containing transmembrane proteins and with large encapsulated volumes (52). In this case, it is important to have the protein reconstituted with the right orientation in the membrane, since a random orientation would confound the design and interpretation of experiments aimed at elucidating the biological role of these proteins in the initiation of calcification (52). The method of direct insertion is more useful for proteins containing GPI anchors, since it allows the reconstitution of the enzymes into preformed liposomes by direct insertion of the anchor into the system. A combination of both liposome methodologies could be used for the construction of more complex systems containing different types of proteins, with the co-solubilization method being used for the incorporation of transmembrane and cytosolic proteins, while the GPI-anchored proteins could then be integrated into this preformed system by direct insertion of the anchor portion of the molecule.

Two of the main lipids found in the MV membranes are dipalmitoylphosphatidylserine and dipalmitoylphosphatidylcholine (DPPC) and many studies have revealed that they play a crucial role in the biomineralization process, regulating both calcium entry into the MVs and formation of HA crystals (4,26). Using the direct insertion method and DPPC liposomes, Simão et al. (53) described the production and characterization of proteoliposomes harboring TNAP alone, NPP1 alone and TNAP + NPP1 together as MV biomimetics to further understand the interplay between these enzymes in the utilization of physiological substrates as a means of understanding the more complex interplay of these enzymes in intact MVs, crucial during early events of skeletal mineralization. The data show that MV enzymes can be reconstituted into liposomes of predefined composition. Such proteoliposomes proved to be useful in the study of the kinetic interplay of two enzymes present together on biomembrane mimetics when presented with physiological substrates relevant to the biomineralization process. These studies were validated by simultaneous studies on isolated MVs. Thus, Ciancaglini et al. (54) examined substrate utilization by isolated osteoblast-derived MVs, where these and other enzymes are present together in a physiological biological compartment, reporting the relative ability of wild-type MVs, as well as MVs deficient in TNAP, NPP1 or PHOSPHO1 to utilize the substrates ATP, ADP and PP_i under physiological conditions. TNAP- and PHOSPHO1-deficient MVs showed reduced calcification ability, while NPP1-deficient MVs hypercalcified, demonstrating that the cooperativity as well as the competition of TNAP, NPP1 and PHOSPHO1 for the biomineralization substrates provides an additional level of regulation of metabolite flow for the control of the calcification process.

The size of natural osteoblast- and chondrocyte-derived MVs varies from 20 to 300 nm in diameter and it is not known if a single cell produces multiple sub-classes of MVs or only one class at a time (39,55). The size of TNAP proteoliposomes reconstituted from DPPC was 300 nm, as determined by dynamic light scattering (53), and thus is comparable to the median size of natural MVs (39) and can adequately serve as a vesicular mimetic system to examine TNAP function in the context of a lipid membrane environment that mimics the MV environment. Electron microscopy of empty DPPC liposomes (Figure 2A) and TNAP-proteoliposomes (Figure 2B) showed that enzyme reconstitution does not affect the morphology of the liposomes.

Simão et al. (53) showed that the specific phospholipid microenvironment of MVs strongly influences the catabolism of the various physiological substrates of biomineralization by TNAP and NPP1, as well as their relative weight in catabolism. It further suggests that the mechanistic study of these enzymes and their pathological mutants in biomineralization would, therefore, be better conducted in relevant liposomes rather than in solution. Most previous work on MVs has focused on their disassembly to determine

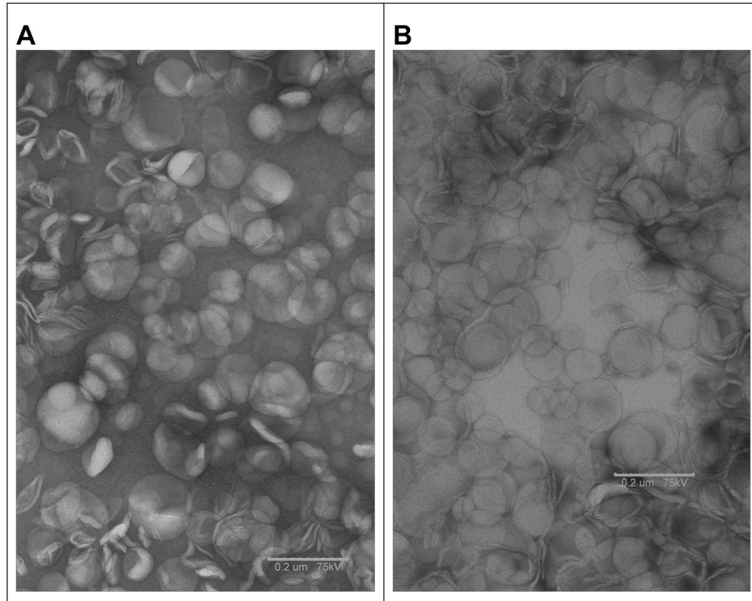


Figure 2. Electron microscopy using negative staining of: A, Liposomes consisting of dipalmitoylphosphatidylcholine (DPPC) and B, tissue-nonspecific alkaline phosphatase reconstituted in DPPC liposomes, both with magnification of 50X. A 5- μ L suspension of liposomes or proteoliposomes was placed on carbon-coated copper grids for 1 min to sediment the sample. The excess buffer was removed and exchanged for 2% (w/v) of an aqueous solution of uranyl-acetate for 15 s; excess uranyl-acetate was removed, grids were air-dried for 2-5 min and examined with a Hitachi H600A transmission electron microscope at 75 kV. Images were collected with an L9C cooled CCD, 11.2 megapixel camera (Special Achievement in innovation - SIA)

chemical composition, and on assays to elucidate how they function in mineralization. The proteoliposome system provides a means of reconstituting lipid vesicles that will function like MVs. There have been previous attempts to simulate MV mineralization using multilamellar liposomal systems (44,45,56,57). More recently, liposomes containing various proportions of anionic and neutral phospholipids and cholesterol were used to study the ability of annexin V to facilitate Ca^{2+} uptake (26). However, an accurate model of MVs must entail a unilamellar system closely approximating the size, lipid, electrolyte, and protein composition of native MVs that is capable of inducing mineralization.

Proteoliposomes as MV biomimetics

Such an MV biomimetic proteoliposome system would be useful for many important translational applications. The enzymatic defects associated with disease-causing mutations in the TNAP molecule, such as those found in

hypophosphatasia (58) could be further elucidated in a membrane vesicle that better mimics their *in vivo* biological environment. Since this artificial vesicular system adequately mimics the kinetic behavior of the enzymes in the natural vesicular MV environment (53), this proteoliposome system can be used for the screening of small molecule compounds able to modulate (inhibit or activate) the activity of MV enzymes for potential therapeutic uses (59,60). Such an approach seems indicated, especially when these compounds bear organic moieties capable of interacting with membrane phospholipids, directly or indirectly via Ca^{2+} ions present in the mineralizing microenvironment. A liposome environment will mimic the phospholipid-modified availability of organic substrates, inhibitors and modulators to membrane-bound enzymes, thus allowing the study of enzyme catalysis in a more physiological manner than with solubilized enzymes.

This nascent *in vitro* experimental system will also allow the construction of progressively more complex proteoliposomes containing PHOSPHO1, $\text{P}_i\text{T}1/2$, ANK, annexins, etc. that closely simulate the lipid, size, electrolyte, and protein composition of native MVs, and that also reproduce the kinetic properties of native MV in the formation of calcium phosphate minerals, with the

ultimate goal of replicating *in vitro* the key events leading to the initiation of HA crystal formation in chondrocyte- and osteoblast-derived MVs. Once the proteoliposomes have been built and characterized, these vesicles can be added to fixed amounts of MVs, wild-type or deficient in specific enzymes, as a way of modulating their *in vitro* calcification properties. Those experiments would validate the use of these nanovesicles in promoting or delaying calcification, and such an artificial nanovesicular system could also potentially prove useful for the repair/treatment of craniofacial and other skeletal defects and to facilitate the mineralization of titanium-based tooth implants.

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