Published in final edited form as:

Biopolymers. 2015 February ; 103(2): 96–108. doi:10.1002/bip.22573.

Interactions of Amelogenin with Phospholipids

Sowmya Bekshe Lokappa¹, Karthik Balakrishna Chandrababu¹, Kaushik Dutta², Iva Perovic², John Spencer Evans², and Janet Moradian-Oldak^{1,*}

¹Center for Craniofacial Molecular Biology, University of Southern California, School of Dentistry, Los Angeles, California 90033

²Laboratory for Chemical Physics, Division of Basic Sciences and Craniofacial Biology, New York University, New York, New York 10010

Abstract

Amelogenin protein has the potential to interact with other enamel matrix proteins, mineral and cell surfaces. We investigated the interactions of recombinant amelogenin rP172 with small unilamellar vesicles as model membranes, towards the goal of understanding the mechanisms of amelogenin-cell interactions during amelogenesis. Dynamic light scattering (DLS), fluorescence spectroscopy, circular dichroism (CD) and nuclear magnetic resonance (NMR) were used. In the presence of phospholipid vesicles, a blue shift in the Trp fluorescence emission maxima of rP172 was observed (~334 nm) and the Trp residues of rP172 were inaccessible to the aqueous quencher acrylamide. Though in DLS studies we cannot exclude the possibility of fusion of liposomes as the result of amelogenin addition, NMR and CD studies revealed a disorder-order transition of rP172 in a model membrane environment. Strong FRET from Trp in rP172 to DNS-bound-phospholipid was observed, and fluorescence polarization studies indicated that rP172 interacted with the hydrophobic core region of model membranes. Our data suggest that amelogenin has ability to interact with phospholipids and that such interactions may play key roles in enamel biomineralization as well as reported amelogenin signaling activities.

Keywords

Amelogenin; intrinsically disordered protein; enamel; lipid vesicles; cell membrane

Introduction

A number of key extracellular matrix ECM proteins have been identified as controlling enamel formation^{1–3}. Amelogenin, an intrinsically disordered protein, is the most abundant of these, accounting for >90% of the total extracellular organic matrix⁴. It is a hydrophobic protein comprised of an N-terminal tyrosine-rich domain (TRAP), a central proline-rich domain, and a C-terminal hydrophilic telopeptide⁵. The N- and C- termini of secreted amelogenin are highly conserved domains in the vertebrates in which they have been studied⁶. Deletion of the conserved domains in the amelogenin sequence has been associated with formation of defective enamel⁷. Mutations in the amelogenin sequence affect self-

*Corresponding Author: Prof. Janet Moradian-Oldak; Tel: 323-442-1759, Fax: 323-442-2981, joldak@usc.edu.

assembly and are associated with *Amelogenesis Imperfecta*, a condition in which abnormal enamel formation is observed^{8–10}. Amelogenin monomers adopt an extended conformation in acidic solution, with a large fraction of polyproline-type II (PPII) helices^{11–14}. Under physiological pH conditions *in vitro*, amelogenin self-assembles to form nanospheres, which further aggregate into chain-like higher-order structures^{15–17}. Although the exact functional mechanisms by which amelogenin monomers, oligomers and nanospheres direct enamel formation are not clear, several *in vitro* mineralization experiments have shown that amelogenin plays a significant role in regulating the morphology and organization of apatite crystals, similar to the organization observed in enamel rods^{18–22}.

During the secretory stage of enamel formation, ameloblasts participate in dynamic interactions with each other, as well as with the ECM, and they migrate as they retract from the dentin-enamel junction²³. Although knowledge of the environment of amelogenin during mineralization *in vivo* is limited, the presence of phosphorylated, glycosylated and sulfated proteins, proteinases and lipids in the ECM has been documented³. Due to this heterogeneity, complex protein-protein, protein-mineral and protein-cell interactions can be envisaged during amelogenesis. Because amelogenin is intrinsically disordered, it can bind to differently shaped targets by structural accommodation. Since amelogenin binds to hydroxyapatite and is present in the organic matrix of developing enamel^{24,25}, it may mediate the adhesion of ameloblasts and other cell types to the extracellular mineralizing matrix of a developing tooth²⁶. Amelogenin is also known to participate in signaling activities in a variety of *in vitro* cell culture models. Lectin-like activity has been proposed to orient amelogenin nanospheres to the secretory ameloblasts²⁷. Biochemical investigations have established the presence of various classes of lipids in dental tissues^{28–30}. However, little is known about the possible roles of phospholipids in amelogenesis. Because amelogenin is synthesized by the ameloblast cells and secreted via matrix secretory vesicles, the study of its structure in the presence of cell membrane or through membrane-mimicking models can give more insight into its function during amelogenesis.

Here we applied fluorescence spectroscopy, CD, NMR and DLS to investigate binding between recombinant amelogenin and lipid vesicles. We used both zwitterionic (POPC) and negatively-charged lipid vesicles (POPG) to investigate the contribution of electrostatic interactions (Table 1). Additional vesicles were prepared using a mixture of different lipids to mimic the apparent lipid composition of the ameloblast membrane^{28–30}. We propose that the potential of amelogenin to interact with phospholipids can provide detailed insight into mechanisms of amelogenin-cell interactions during amelogenesis, as well as into the signaling function of amelogenin^{31–33}.

Results

Intrinsic fluorescence analyses of rP172 assembly

Recombinant porcine amelogenin (rP172) exists in monomeric, oligomeric and assembled forms at different pH values⁹. We studied rP172 monomers at pH 3.5 and rP172 nanospheres at pH 8.00. Amelogenin has three tryptophan (W or Trp) residues, two of which are localized in the N-terminus with the remaining one in the C-terminus. The characteristic fluorescence emission properties of tryptophan are sensitive to the polarity of its local

environment and proximity of other residues, and can therefore be exploited to investigate the assembly of amelogenin. At pH 3.5 the Trp emission maximum of rP172 was 347 nm, indicating that the Trp residues were in a more hydrophilic environment than at pH 8.00, where a blue shift in λ_{max} and enhancement in intensity were observed (Fig. 1).

Amelogenin monomers interact with lipid vesicles at pH 3.5

In order to gain insight into the membrane binding ability of amelogenin, we investigated the interaction of rP172 with various lipid vesicles as models (Table 1). To investigate the contribution of electrostatic interactions, we used both zwitterionic (POPC) and negatively-charged vesicles (POPG).

In order to mimic the primary lipid composition of the ameloblast membrane, a mixture of lipid vesicles was used (Table I). We refer to these as ameloblast cell membrane-mimicking lipid vesicles (ACML)²⁸. We employed dynamic light scattering to determine the particle size distribution of rP172 monomers, lipid vesicles, and mixtures of both at pH 3.5 (Table 2). The hydrodynamic radii (R_H) of rP172 monomers ranged from 1.3–1.8 nm, which is in agreement with the reported hydrodynamic radii^{9,34}. The hydrodynamic radii of lipid vesicles ranged from 10–13 nm, which is within the expected range for sonicated small unilamellar lipid vesicles (Table 2). Heterogeneity was apparent when rP172 was mixed with anionic POPG, zwitterionic POPC or ameloblast-mimicking ACML lipid vesicles. The addition of lipid vesicles resulted in disappearance of monomeric amelogenin (R_H =1.3 nm particles) and formation of particles that could be divided into three different size groups, suggesting the formation of rP172-lipid complexes, although fusion or aggregation of lipid vesicles as the result of their mixing cannot be ruled out (Table 2).

The emission spectra of rP172 in the presence of lipid vesicles with varying lipid compositions were examined at a fixed rP172 concentration. At pH 3.5, the maximal Trp fluorescence emission wavelength (λ_{max}) of rP172 was ~347 nm, which is indicative of exposure of Trp residues to an aqueous environment (Fig. 2a). A blue shift in Trp emission (λ_{max}) and enhancement in fluorescence intensity were observed for rP172 in the presence of anionic POPG (334 nm) and ACML (334 nm) vesicles (Fig. 2a). The data presented are maximal changes observed at a lipid/protein molar ratio of 30:1. Similarly, in the presence of POPC vesicles, a blue shift of ~5 nm accompanied by an increase in fluorescence intensity was observed (Fig. 2a) and the data presented are maximal changes observed at a lipid/protein molar ratio of 100:1. The increase in fluorescence emission intensity and the shift of the emission maximum to shorter wavelengths were both markedly larger when rP172 interacted with anionic (POPG and ACML) as opposed to zwitterionic membranes. Stern-Volmer plots indicate shielding of Trp residues from acrylamide in the presence of POPG and ACML lipid vesicles. A small degree of protection is observed with POPC vesicles, as demonstrated by shallow slope of the curve relative to control rP172 (Fig. 2b).

The conformation of rP172 at pH 3.5 and in the presence of lipid vesicles was examined by CD (Fig. 3). In buffer the spectrum of rP172 shows a minimum at 201 nm and a weak n- π^* region (215–230nm), suggesting a population of both unordered and polyproline type II structures¹². However, rP172 exhibited negative minima at 208 nm, having crossover at 195 nm in the presence of POPG and ACML lipid vesicles, suggesting that rP172 adopted more

helical conformation in a membrane environment (Fig. 3a and 3b). In agreement with the fluorescence data, changes in secondary structure were not observed upon addition of rP172 to zwitterionic POPC vesicles (Fig. 3c).

Using NMR spectroscopy, we confirmed that POPG lipid vesicles associate with rP172 and introduce changes in protein backbone dynamics. Using the published ^1H , ^{15}N rP172 monomeric assignments¹³, we were able to unambiguously assign a subset of residues that are located in different regions of the protein, including the conformationally sensitive N-terminal (P2-W45) and C-terminal (D155-D173) regions^{13,35} (Fig. 4). An overlay comparison of ^1H , ^{15}N HSQC spectra obtained for aqueous (AQ- Black) and 10:1 POPG: rP172 (Grey) samples at pH 3.5 reveals that significant changes occur to the amelogenin protein, as evidenced by significant resonance frequency shifts for all locations in the protein. This indicates that the introduction of POPG vesicles elicits a global backbone conformational response in the rP172 molecule. Further, NMR resonance shifts specific for the N- and C-terminal regions of the protein are noted as well, indicating that the POPG-induced conformational perturbation affects these domains.

We also note the attenuation of some of the ^1H , ^{15}N resonances and the presence of additional crosspeak resonances. Selective loss or broadening of NMR signals was also observed in studies involving rP172 in detergents³⁴ and alcohols³⁵. Thus, the attenuation of cross peaks reflects that these residues are undergoing conformational exchange on an intermediate time scale. Furthermore, the presence of additional cross peaks in the ^1H , ^{15}N HSQC spectra of the rP172: POPG sample most likely arise from either the very slow *cis-trans* isomerization of the proline residues or from slow time scale conformational exchange^{13,35} in the presence of the lipid vesicles, both of which have also been reported for rP172 in the presence of SDS³⁴. We believe that this alteration in protein conformational exchange occurs in response to a number of factors, including membrane-induced protein backbone interactions and dynamics. In summary, our NMR studies have identified that POPG induces conformational change and alternations in backbone dynamics on both global and regional scales (i.e., at the N- and C-termini) within the amelogenin molecule.

Amelogenin interacts with lipid vesicles at pH 8.00

We determined the particle size distribution of the rP172 nanospheres, lipid vesicles and a mixture of both using a dynamic light scattering approach. The hydrodynamic radius of ~10.5–13.16 nm of rP172 without lipid vesicles indicated the formation of nanospheres (Table 3). Upon mixing rP172 with 300 μM of POPC, POPG or ACML lipid vesicles, particles of three different groups of sizes were formed, suggesting that at pH 8.00 amelogenin has an affinity for lipid vesicles (Table 3). In all cases, the majority of the mixture (54–83% mass) appeared as particles ranging from 13.9 to 21.6 nm. The second size group (3–11.4% mass) ranged from ~133 to 136 nm, and large particles of 279–338 nm (2.6–6.6%) appeared as separate peaks. These large particles hinted at the association of rP172 with POPC, POPG, and ACML although fusion or aggregation of lipid vesicles cannot be ruled out. (Table 3).

At pH 8.00, the maximal Trp fluorescence emission wavelength of rP172 was ~336 nm, indicating the presence of Trp residues in a hydrophobic pocket even without the addition of

lipids (Fig. 5a). In order to compare the properties of rP172 in different phospholipid environments, we used a range of lipid-to-rP172 molar ratios to assure that the number of lipid vesicles was sufficient to allow all of the rP172 to associate with the lipids. In Fig. 5a, the fluorescence spectra shown are maximal changes observed at a lipid/protein molar ratio of 100:1. Blue shifts of 1 nm and 2 nm were observed upon titration of rP172 with POPG and ACML vesicles, respectively, accompanied by substantial quenching of Trp fluorescence (Fig. 5a). Upon interaction with POPC lipid vesicles, rP172 shifted the Trp emission λ_{max} by 1 nm at higher lipid concentrations.

The accessibility of Trp residues of rP172 in Tris buffer in the presence of POPG and ACML SUVs was explored by fluorescence quenching using acrylamide (Fig. 5b). At pH 8.00, when rP172 nanospheres are predominantly present, the Trp residues of rP172 are less accessible to acrylamide when associated with POPG and ACML vesicles (decreased slope), as compared to free and POPC-associated rP172 (higher slope). As shown in Fig. 6a and 6b, CD spectra of the rP172 in anionic POPG lipid vesicles and ACML lipid vesicles demonstrate that rP172 was promoted to some extent to adopt an alpha-helical conformation, with characteristic double minima at 208 and 222 nm. In contrast, in the presence of POPC, rP172 did not show much conformational change (Fig. 6c). The CD spectra shown are maximal changes observed at a lipid/protein molar ratio of 100:1.

To further explore the interaction of rP172 nanospheres with lipid bilayers, the extrinsic probe 8-ANS, which reports on the status of hydrophobic regions, was used³⁶. ANS fluorescence decreased after adding 100 μM POPG lipid vesicles to 10 μM rP172 at pH 8.00 (Fig. 7a), suggesting disassembly of amelogenin nanospheres upon interaction with the lipid surface. Further increasing the lipid concentration did not change the ANS fluorescence, indicating avid association of rP172 with POPG vesicles. Though ANS fluorescence decreased upon interaction of rP172 nanospheres with 100 μM ACML lipid vesicles, further increase in the lipid concentration resulted in an increase in ANS fluorescence, (Fig. 7b). Thus, the fluorescence decrease observed in Fig. 7a and 7b can be attributed to structural changes in the rP172 after the addition of lipids. This disassembly of rP172 nanospheres upon association with lipids was also confirmed using FRET (Fig. 7c and 7d). FRET between Trp and ANS has been used previously to monitor several protein processes³⁷. This approach is particularly useful for membrane proteins because the ANS fluorescence emission maximum does not overlap with the Trp emission maximum. Fig. 7c and 7d show the fluorescence emission spectra obtained for rP172 in Tris buffer at pH 8.00 in the presence of ANS. The addition of 100 μM POPG or ACML lipid vesicles induced a decrease in the maximal fluorescence of ANS (peak at 470 nm) concomitant with an increase in Trp fluorescence (peak at 335 nm), indicating disassembly of nanospheres upon interacting with lipid surfaces (Fig. 7c and 7d).

Analysis of amelogenin-lipid interactions by FRET and fluorescence polarization

We further studied the binding of rP172 to lipid vesicles using protein-lipid FRET. Since DLS, fluorescence and CD studies revealed that POPC is weakly associated with amelogenin, we excluded it from the FRET studies. Because the emission spectrum of tryptophan and the absorption spectrum of dansyl overlap, resonance energy transfer of Trp

in rP172 to dansyl in lipid vesicles can be used to study the association of rP172 with lipid vesicles. Varying degrees of energy transfer were obtained between rP172 and POPG or ACML lipid vesicles. The FRET signal shown on the Y-axis in Fig. 8 is calculated as $I-I_0$, where I is the intensity at 520 nm and I_0 is the intensity for a solution of the lipid alone, without protein (Fig. 8a and 8b). The data indicate that there is extensive transfer of energy from Trp residues of rP172 to dansyl of POPG and ACML lipid vesicles at pH values of both 3.5 and 8.00. However, at pH 8.00 rP172 associated more strongly with ACML lipid vesicles than with POPG, as documented by the difference in the slopes of the plotted data (Fig. 8b). The influence of rP172 on membrane fluidity was studied by fluorescence polarization (Fig. 9). To analyze the depth of rP172 penetration into the membrane, two probes that locate at different positions in the bilayer were used. DPH has a rigid and highly hydrophobic structure that is mainly localized in the internal core of bilayers. It has been used to monitor chain-packing order in that region³⁸. TMA-DPH, due to its polar moiety, remains located at the surface of the bilayer in such a way that the nonpolar part is inserted below the phosphate head group region. The degree of DPH or TMA-DPH fluorescence polarization reflects the extent to which the corresponding molecule reorients while in an excited state. Fluorescence polarization studies of rP172-lipid vesicles with a TMA-DPH probe give us information about the interaction of rP172 at the lipid interfacial region, whereas similar studies of rP172-lipid vesicles with a DPH probe give us information about the interaction of rP172 with the hydrophobic acyl chains of lipid vesicles.

Fig. 9 shows changes in DPH and TMA-DPH polarization as a function of rP172 protein concentration when associated with POPG and ACML lipid vesicles. The results show that rP172 increased packing in the acyl chain region in POPG and ACML vesicles, as evidenced by an increase in fluorescence polarization of DPH at pH 3.5 and 8.00 (Fig. 9a and 9b). ACML lipid vesicles are more rigid than POPG lipid vesicles, which was revealed by the increase in polarization values of both DPH and TMA-DPH before the addition of the rP172 (Fig. 9a–d). However, rP172 influenced the polarization of TMA-DPH to a lesser extent in both POPG and ACML lipid vesicles (Fig. 9c and 9d).

Discussion

In this study we have investigated the interactions of a recombinant amelogenin with three different types of phospholipids in order to explore mechanisms of amelogenin-cell interactions. Since it has been previously reported that amelogenin assembly strongly depends upon pH⁹, two different pH values were selected in order to study interactions of monomeric amelogenin (pH =3.5) and amelogenin nanospheres (pH=8) with liposomes. We used dynamic light scattering to analyze the distribution of particle sizes in amelogenin-lipid mixtures, fluorescence spectroscopy to assess exposure or shielding of Trp residues in amelogenin following its interaction with the lipid bilayer, FRET to detect direct amelogenin-lipid association, and NMR and CD spectroscopy to analyze changes in the secondary structures of amelogenin following their interactions with lipids. We demonstrated that amelogenin has the ability to interact with zwitterionic and negatively charged liposomes via electrostatic as well as hydrophobic interactions.

Amelogenin-lipid interactions at pH 3.5

At pH 3.5, amelogenin is monomeric, extended and flexible, and the Trp residues of rP172 are highly exposed to an aqueous environment. With amelogenin in this form, addition of small unilamellar vesicles of POPG (negatively charged) and ACML resulted in greater burial of the Trp residues of rP172, as shown by the changes in emission maxima as well as more substantial decreases in acrylamide quenching and FRET. As documented by CD spectra, this hydrophobic membrane environment induced a structural transition of rP172 from random coil to alpha helix. NMR studies support the notion of conformational changes and alterations in backbone dynamics within the amelogenin molecule, and hint that such changes may be concentrated at the N- and C-termini. Research is in progress in our laboratory in order to identify the specific regions in the amelogenin sequences that are involved in their interactions with lipids (Lokappa et al, in preparation).

Electrostatic interactions between amelogenin and phospholipids may dominate at acidic pH values as histidines of rP172 are protonated and attract negatively-charged lipid vesicles⁹. Several studies have reported that protonation of lipids is not affected across different pH values^{39,40}. The studied phospholipids remain negatively charged at pH 3.5 because their ionization constants are close to or below 3.5.⁴¹

Based on FRET as well as quenching data, we conclude that rP172 monomers bind more avidly with ACML than with POPG lipid vesicles. We interpret these data to suggest that there are differences in the nature of the interaction and orientation of rP172 with respect to these vesicles. Moreover, dynamic light scattering data highlight that the mode of interaction between rP172 monomers and anionic POPG or ACML is different from the mode by which these monomers interact with zwitterionic POPC. In all cases, following addition of rP172 to liposomes at pH 3.5, complexes larger than rP172 alone or the liposomes alone were formed. Note that the mass majority of particles (58%) obtained with the rP172-POPC mixture (~18.5 nm) were different from that of the particles formed with the rP172-POPG mixture (~131.8 nm) (58%) or with the rP172-ACML mixture (~130.5 nm) (58%) (Table 2). The mass percentage of large particles of either rP172-POPG or rP172-ACML formed at low pH is higher than the mass percentage of the large particles formed at pH 8. Since amelogenin will not aggregate at pH 3.5, these data suggest stronger association between amelogenin and POPC and ACML at low pH compared to pH 8. Looking at DLS data we cannot exclude the possibility of fusion of liposomes. However, based on conformational changes to amelogenin observed using CD and the blue shift in fluorescence of Trp residues as the result of liposome addition, as well as the result of our quenching experiments, our data collectively suggest that amelogenin interacts with liposomes. The large particles detected in DLS therefore may be complexes of rP172 and liposomes, the stoichiometry of which cannot be known. Further fluorescence polarization studies using DPH confirmed that rP172 monomers interact with hydrophobic acyl chains of anionic lipid vesicles.

The ACML lipid vesicles, which were made based on lipid composition information available in the literature for enamel²⁸, are composed of zwitterionic phospholipids (POPC and POPE) and anionic phospholipids (POPS). The basic residues of rP172 at the C-terminus might also contribute to electrostatic interactions between rP172 and the ACML lipid vesicles. Our data collectively indicate that electrostatics between the anionic lipid

head groups and the positively-charged rP172 monomers (14 positively-charged His residues) are indispensable for driving rP172 to the membrane surface to achieve primary binding under acidic conditions.

Amelogenin-lipid interactions at pH 8

In the absence of lipids, under more physiologically relevant pH conditions (8.00) where amelogenin is highly soluble and forms nanospheres⁹, the wavelength of the maximal intrinsic fluorescence emission was 15 nm shorter than at pH 3.5. The lower fluorescence emission maximum of rP172 at pH 8 indicates that its Trp residues were in a more hydrophobic environment because of nanosphere assembly.

At pH 8.00, although rP172 interaction with negatively charged POPG and ACML lipid vesicles did not show a blue shift in the fluorescence spectra, Stern-Volmer plots indicated that rP172 interacted with lipid vesicles. A decrease in ANS fluorescence upon interaction of rP172 with anionic lipid vesicles confirmed that nanospheres disassemble upon interaction with lipids. Further evidence for the disassembly of the nanospheres in the presence of lipids is provided in our present study by DLS analysis. As detected by DLS, mixing amelogenin with POPG and ACML lipid vesicles resulted in the formation of complexes that were smaller than those formed at pH 3.5. Note that the mass majority (>50%) of the particles had radii of ~21.6 and ~18.3 nm for POPG and ACML, respectively (Table 3). It is possible that upon interacting with negatively-charged lipid vesicles, amelogenin nanospheres initially disassemble into monomers, but later re-assemble depending on protein concentration and equilibrium. Similar observations were also made when rP172 nanospheres interacted with anionic SDS micelles³⁴. The disassembly of amelogenin nanospheres on inorganic (rigid) and organic (flexible) surfaces with different charges and polarities has been also well documented^{42,43}. Our recent *in situ* AFM studies have revealed that the assembly state of amelogenin molecules absorbed to the surface is highly dependent on the properties of the substrate surface¹⁷. Notably, on negatively-charged bare mica, rP172 formed a film of monomers at pH 8.00.

Adding neutral POPC lipid vesicles to rP172 at pH 8.00 induced no blue shift and had little effect on acrylamide quenching. This suggests that there was only a weak interaction between the rP172 and POPC lipid vesicles, and a limited insertion of rP172 into the hydrophobic region of the lipid vesicles. The mass majority (>70%) of the particles detected in the POPC-rP172 mixture by DLS had radii similar to that of POPC, hinting at a lack of complexation.

The biological significance of amelogenin-lipid interactions

Amelogenin is secreted from the ameloblasts by secretory vesicles into a complex biological microenvironment where a number of different targets such as other matrix proteins^{4, 44}, mineral^{45–47} and charged cell surfaces¹⁷ participate in a dynamic process. The study of amelogenin-phospholipid interaction may well be relevant to secretory vesicles as well as ameloblast surface interactions. Moreover, numerous reports utilizing both *in vitro* and *in vivo* systems document the ability of amelogenin to act as a signaling molecule. The biological relevance of amelogenin-phospholipid interactions may well be expanded to the

strong potential of amelogenin to promote osteogenesis, chondrogenesis and periodontal tissue regeneration^{31–33,48,49}.

Like many other intrinsically disordered proteins (IDPs), amelogenin lacks a unique 3D structure in its unbounded and monomeric form¹³. The structural flexibility of amelogenin was further documented in our most recent studies where we show that rP172 undergoes significant changes in secondary structure at its N-terminal upon interaction with anionic SDS micelles³⁴. This lack of structure provides amelogenin with a larger interaction surface area than globular proteins of similar length and also with conformational flexibility that allows it to interact with the above-mentioned targets during the complex process of enamel biomineralization.

We envisage that under close-to-physiological pH conditions (pH 8), the hydrophobic interaction between the non-polar surface of rP172 helices and lipid fatty acyl chains (cell surfaces) will stabilize amelogenin monomers and may even promote rP172 insertion into the hydrophobic core of the phospholipid. We therefore suggest that the tendency of amelogenin to associate with phospholipids may play a role in enamel mineralization.

During the maturation stage of enamel mineralization, the pH of the extracellular matrix fluctuates between mildly acidic and neutral. In calves, acidic values of pH 5.8–6 were reported⁵⁰, while a pH range of 6.2–6.9 were reported in case of rats⁵¹. Such conditions would create a localized mildly acidic environment, ensuring that any amelogenin in the vicinity would become slightly protonated. Although not physiologically relevant, our current analysis at pH 3.5 reveals the importance of electrostatic interactions between amelogenin and lipids in a monomeric form. It is possible that enamel matrix-phospholipid interactions can be facilitated by amelogenin and influenced or even controlled by the cyclical pH changes in the extracellular matrix during enamel maturation^{9,50,52}. Not only an acidic environment but also a negatively charged surface (like that of a liposome or cell surface) can stabilize amelogenin monomers¹⁷. This combination may result in the breakdown of the amelogenin nanospheres into oligomers or monomers, increasing the available surface area of amelogenin for binding to the cell surface as well as to apatite crystals, and exposing additional domains for binding to other targets.

Conclusions

Utilizing four independent techniques including dynamic light scattering, fluorescence spectroscopy, circular dichroism and nuclear magnetic resonance, we showed that rP172 possesses membrane-binding ability with a disordered-to-ordered conformational change. The evidence comes from (a) the appearance of larger-sized particles upon the interaction of rP172 and lipid vesicles, (b) a blue shift in Trp fluorescence emission maxima in a lipid environment (~334 nm), (c) the inaccessibility of Trp residues of rP172 to aqueous quencher acrylamide in lipid vesicles, (d) circular dichroism studies revealing a disorder-order transition of rP172 in a membrane environment, (e) strong FRET from Trp in the rP172 to DNS-bound phospholipid and f) an increase in fluorescence polarization, indicating rigidity of the hydrophobic core region of the membrane upon interaction with rP172. We suggest

that amelogenin-lipid interactions may not only play key roles in enamel biomineralization but also may be involved in reported amelogenin signaling activities.

Materials and methods

Protein Expression and Purification

Recombinant porcine amelogenin (rP172) was expressed in *E. coli* strain BL21-codon plus ((DE3)-RP, Strategene, LaJolla, CA), and precipitated by 20% ammonium sulfate⁵³. The precipitate was dissolved in 0.1% TFA. Protein purification was accomplished on a reverse phase C4 column (10 × 250 mm, 5 μm) mounted on a Varian Prostar HPLC system (ProStar/Dynamics 6, version 6.41 Varian, Palo Alto, CA) and fractionation was performed using a linear gradient of 60% acetonitrile at a flow rate of 1.5 mL/min. Uniformly triple-labeled recombinant rP172 [U-²H, ¹³C, ¹⁵N] (hereafter referred to as DCN-rP172) was produced via recombinant bacterial overexpression in the presence of ¹⁵NH₄Cl, ¹³C-Glucose, and 100% D₂O (Cambridge Isotope Laboratories, Andover, MA). The extent of triple labeling was verified to be 98.9% using ESI-MS TOF (Mass=21777.0 Da). Both the labeled and unlabeled versions of rP172 lack the N-terminal methionine and the phosphate in the serine at the 16th position when compared to their wild type analogues.

Preparation of Small Unilamellar Lipid Vesicles

Small unilamellar vesicles were prepared using appropriate lipids purchased from Avanti Polar Lipids, Birmingham, Al, USA (Table 1). Appropriate amounts of the lipids were mixed in chloroform/methanol to obtain the desired lipid composition followed by evaporation of the solvent under a stream of nitrogen to obtain a thin lipid film. The film was subjected to overnight desiccation and subsequently hydrated with either 25 mM sodium acetate buffer (pH 3.5) or 25 mM Tris buffer (pH 8.00). Lipid vesicles were prepared by sonication at 42 kHz in a water bath until a clear solution was obtained.

Protein Sample Preparation for CD, DLS and Fluorescence Experiments

rP172 stock solutions were prepared by dissolving 3–4 mg of lyophilized protein in water. The sample was placed on a rocker at 4 °C for 24–36 h. Clear solution was obtained by centrifuging the sample at 10,000 g for 1 h at 4 °C and only 95% of the supernatant was used in order to avoid un-dissolved aggregates. Concentration of the protein was estimated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). Two different buffer solutions, 25 mM Sodium acetate at pH 3.5 and 25 mM Tris pH 8.00, were prepared and used to dilute the protein stock to a final concentration of 10 μM. The concentration of lipid vesicle stock used was 10 mM. Titration experiments were carried out by preparing individual samples with specific protein/lipid ratios. The final reaction volume used was 100 μL in all the fluorescence and DLS experiments and 200 μL for CD measurements.

Binding of rP172 to Lipid Vesicles by Intrinsic Tryptophan Fluorescence Spectroscopy

Fluorescence spectra were recorded on a PTI QuantaMaster QM-4SE instrument (Photon Technology International, Birmingham, NJ). Lipid-protein interactions were studied by monitoring the changes in Trp fluorescence spectra of the protein upon addition of small

unilamellar lipid vesicles of various lipid compositions at pH 3.5 and pH 8.00. Appropriate volumes of POPG and ACML lipid vesicles were added to 25 mM Sodium acetate buffer (pH 3.5) containing 10 μ M of rP172 from 10 mM lipid stock to obtain a lipid concentration range of 10–300 μ M. For each lipid-rP172 concentration, experiments were carried out separately. Corresponding solutions without rP172 were used to correct for background and SUV scattering contributions to the fluorescence signal, and spectra were also corrected for dilution effects. The data presented are maximal changes observed at a lipid/protein molar ratio of 30:1. An appropriate volume of POPC lipid vesicles was added to 25 mM Sodium acetate buffer (pH 3.5) containing 10 μ M of rP172 from 10 mM lipid stock to obtain a lipid concentration range of 10–1000 μ M. The data presented are maximal changes observed at a lipid/protein molar ratio of 100:1. At pH 8.00, amelogenin-lipid binding studies were carried out by adding appropriate volumes of lipid vesicles from lipid stock of 10 mM into sample containing 10 μ M rP172 to obtain a concentration range of 10–1000 μ M. The data presented are maximal changes observed at a lipid/protein molar ratio of 100:1. Emission spectra were recorded with an excitation wavelength of 295 nm and emission range of 310–400 nm with excitation and emission slit-widths of 5 nm. For each measurement, baseline spectra recorded in the absence of protein were subtracted from the protein spectra. The change in intensity and shift in wavelength were monitored at different lipid to protein molar ratios.

Quenching of Amelogenin Tryptophan Fluorescence and Determination of Net Accessibility Factor

Solvent accessibility of the Trp residues of rP172 was assessed by monitoring quenching of fluorescence by acrylamide in the absence or presence of lipid vesicles by adding 1 μ L of acrylamide each time from a stock solution of 5 M acrylamide to obtain concentrations ranging from 0.05 M to 0.25 M acrylamide. The concentration of the protein used was 10 μ M. Stern-Volmer plots were created from the data for analysis using Eqn1:

$$F_0/F = 1 + K_{SV}[Q]$$

where F_0 is the fluorescence intensity in the absence of quencher, F is the fluorescence intensity in the presence of quencher, $[Q]$ is the concentration of the quencher, and K_{SV} is the Stern-Volmer quenching constant. NAF was calculated from the ratio of K_{SV} obtained from quenching of tryptophan fluorescence in the presence and absence of lipid vesicles⁵⁴.

Amelogenin-ANS Binding Experiments

ANS is a reporter of hydrophobic pockets of proteins. rP172 was added to prepare a final concentration of 10 μ M to 25 mM Tris (pH 8.00) containing 3 mM of ANS. ANS fluorescence was registered between 400 and 600 nm following excitation at 380 nm with excitation and emission slit-widths of 7 nm. Amelogenin-lipid binding studies were carried out by titrating appropriate volumes of lipid vesicles from 10 mM lipid stock into a sample containing 10 μ M rP172 and 3 mM ANS to get a concentration range of 100, 500 and 1000 μ M of lipid vesicles. Spectra were corrected for solvent and lipid scattering contributions as well as protein dilutions. Fluorescence resonance energy transfer from the Trp residue to the

ANS chromophore was determined by setting the excitation monochromator at 295 nm and monitoring the fluorescence emissions between 310 and 550 nm.

Binding of rP172 to Lipid Vesicles by Fluorescence Resonance Energy Transfer (FRET)

FRET of Trp in rP172 to dansyl (DNS) in lipid vesicles was used to study the association of rP172 with lipid vesicles. Lipid vesicles of POPG and ACML doped with a 2-mole percentage of dansyl PE in either 25 mM Sodium acetate buffer (pH 3.5) or 25 mM Tris (pH 8.00) were used in the experiment. FRET from the Trp residue to the DNS chromophore was determined by setting the excitation monochromator at 295 nm and monitoring the fluorescence emissions between 300 and 550 nm. For data in Fig. 8, an appropriate volume of rP172 was added to 200 μ M labeled POPG lipid vesicles to obtain a concentration range of 1–10 μ M rP172. Similarly, an appropriate volume of rP172 was added to 200 μ M labeled ACML lipid vesicles to obtain a concentration range of 1–20 μ M rP172 and the final reaction volume used in the experiment was 100 μ L. Spectra were corrected for solvent background, lipid scattering and dilution effects.

Fluorescence Polarization Measurements

Two different probes were employed in the fluorescence polarization experiments, namely 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) and 1-[4-(trimethylammoniumphenyl)-6-phenyl]-1, 3, 5-hexatriene (TMA-DPH). DPH was dissolved in tetrahydrofuran and TMA-DPH was dissolved in methanol. The lipid/probe molar ratio was 100:1. For data in Fig. 9, an appropriate volume of rP172 was added to 200 μ M labeled POPG lipid vesicles to obtain a concentration range of 1–10 μ M rP172. Similarly, an appropriate volume of rP172 was added to 200 μ M labeled ACML lipid vesicles to obtain a concentration range of 1–20 μ M rP172. Spectra were corrected for solvent background, lipid scattering and dilution effects. The final concentrations of rP172, lipid vesicles, DPH and TMA-DPH were 10, 200, 2 and 2 μ M, respectively. The excitation monochromator was set at 365 nm, and the emission monochromator set at 425 nm.

Polarization values were calculated from Eqn 2:

$$P = (I_{VV} - GI_{VH}) / (I_{VV} + GI_{VH})$$

Where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented respectively. G is the grating correction factor, and is equal to I_{HV} / I_{HH} . The spectra were recorded at 22°C.

Dynamic Light Scattering

Freshly prepared lipid vesicles were mixed with rP172. The final concentration of rP172 used in the experiment was 10 μ M and that of the lipid vesicles was 300 μ M. Subsequently the hydrodynamic radius (R_H) of rP172 in the presence and absence of lipid vesicles was measured using a Wyatt DynaProNanostar DLS instrument (Wyatt Technology, Santa Barbara, CA). Data were analyzed using Dynamics 7.0 software by performing regularization fit using the Dynals algorithm on the resultant autocorrelation functions. All

experiments were recorded at 22 °C either in 25 mM Sodium acetate buffer (pH 3.5) or in 25 mM Tris (pH 8.00).

Circular Dichroism

CD measurements were carried out at 22 °C on a JASCO J-715 spectropolarimeter between 195 and 250 nm in a quartz cell with a path length of 1 mm. Appropriate volumes of POPG and ACML lipid vesicles were added from 10mM lipid stock to 25 mM Sodium acetate buffer (pH 3.5) containing 10 μ M rP172 to obtain a concentration range of 10–300 μ M of lipid and the final reaction volume used in each experiment was 200 μ L. The data presented are maximal changes observed at a lipid/protein molar ratio of 30:1. An appropriate volume of POPC lipid vesicles was added to 25 mM Sodium acetate buffer (pH 3.5) containing 10 μ M rP172 from 10 mM lipid stock to obtain a concentration range of 10–1000 μ M and the final reaction volume used in each experiment was 200 μ L. The data presented are maximal changes observed at a lipid/protein molar ratio of 100:1. In 25 mM Tris buffer (pH 8.00), amelogenin-lipid binding studies were carried out by adding appropriate volumes of lipid vesicles from lipid stock of 10 mM into sample containing 10 μ M rP172 to get a concentration range of 10–1000 μ M. The data presented are maximal changes observed at a lipid/protein molar ratio of 100:1. Spectra were corrected for solvent and lipid scattering contributions as well as protein dilutions. Four spectra were accumulated for each sample, and the contribution of the buffer was always subtracted. Data are represented as mean residue ellipticities. Appropriate blank subtraction was done in all the measurements.

NMR Experiments

NMR data on rP172 (80 μ M) in the presence of POPG vesicles (rP172 : POPG = 1:10) were collected at 25 °C using a Bruker AVANCE 800 MHz spectrometer equipped with a 4-channel 5 mm cryoprobe. The operating Larmor frequencies were 800.234 MHz for proton, 201.255 MHz for carbon, and 81.096 MHz for nitrogen. HSQC experiments (512 and 128 complex points with sweep widths of 12 and 24 ppm in the ^1H and ^{15}N dimensions respectively) were performed on each sample and also used to monitor if there was any aggregation of the sample during the course of data collection by following the line width of the peaks. No aggregation was observed during data collection. NMRPipe⁵⁵ software was used to process all data and NMRView⁵⁶ was used for sequential assignments. The spectra were referenced with respect to the temperature-corrected water resonance and ^{15}N chemical shifts were referenced on the basis of the ^1H IUPAC guidelines using the unified chemical shift scale.

Acknowledgements

The study was supported by NIH-NIDCR grants DE-020099 and DE-013414 to JMO. We acknowledge Nanobiophysics Core Facility at the University of Southern California for circular dichroism spectropolarimetry and fluorescence spectroscopy. Portions of this work (NMR spectroscopy) were supported by a grant from the U.S. Department of Energy, Office of Basic Energy Sciences, Division of Materials Sciences and Engineering under Award DE-FG02-03ER46099 to JSE. The data collection at NYSBC was made possible by a grant from NYSTAR, ORIP/NIH facility improvement Grant CO6RR015495, NIH Grant P41GM066354, the Keck Foundation, New York State, and the NYC Economic Development Corporation. This paper represents contribution number 71 from the Laboratory for Chemical Physics, New York University.

Abbreviations

POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPA	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
POPI	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoinositol
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
TRAP	tyrosine-rich amelogenin polypeptides
ACML	ameloblast cell membrane-mimicking lipid vesicles
NAF	net accessibility factor
FRET	fluorescence resonance energy transfer
ECM	extra cellular matrix
DPH	1, 6-diphenyl-1, 3, 5-hexatriene
TMA-DPH	1-[4-(trimethylammoniumphenyl)-6-phenyl]-1, 3, 5-hexatriene
ANS	1-aniline-8 naphthalenesulfonate
DLS	dynamic light scattering
CD	circular dichroism
NMR	nuclear magnetic resonance
SUVs	Small Unilamellar Vesicles

References

1. Fincham AG, Moradian-Oldak J, Simmer JP. J Struct Biol. 1999; 126:270–299. [PubMed: 10441532]
2. Moradian-Oldak J. Front Biosci-Landmark. 2012; 17:1996–2023.
3. Bartlett JD, Ganss B, Goldberg M, Moradian-Oldak J, Paine ML, Snead ML, Wen X, White SN, Zhou YL. Curr Top Develop Biol. 2006; 74:57–115.
4. Lakshminarayanan, R.; Moradian-Oldak, J. Amelogenins: Multifaceted Proteins for Dental and Bone Formation and Repair. Goldberg, M., editor. Oak Park, IL: Paris: Bentham Science Publishers Ltd; 2010. p. 106-132.
5. Margolis HC, Beniash E, Fowler CE. J Dent Res. 2006; 85:775–793. [PubMed: 16931858]
6. Delgado S, Ishiyama M, Sire JY. J Dent Res. 2007; 86:326–330. [PubMed: 17384026]
7. Paine ML, Luo W, Zhu DH, Bringas P, Snead ML. J Bone and Miner Res. 2003; 18:466–472. [PubMed: 12619931]
8. Lakshminarayanan R, Bromley KM, Lei YP, Snead ML, Moradian-Oldak J. J Biol Chem. 2010; 285:40593–40603. [PubMed: 20929860]
9. Bromley KM, Kiss AS, Lokappa SB, Lakshminarayanan R, Fan D, Ndao M, Evans JS, Moradian-Oldak J. J Biol Chem. 2011; 286:34643–34653. [PubMed: 21840988]
10. Hart PS, Aldred MJ, Crawford PJM, Wright NJ, Hart TC, Wright JT. Arch Oral Biol. 2002; 47:261–265. [PubMed: 11922869]
11. Goto Y, Kogure E, Takagi T, Aimoto S, Aoba T. J. of Biochemistry. 1993; 113:55–60.

12. Lakshminarayanan R, Fan D, Du C, Moradian-Oldak J. *Biophys J*. 2007; 93:3664–3674. [PubMed: 17704165]
13. Delak K, Harcup C, Lakshminarayanan R, Sun Z, Fan YW, Moradian-Oldak J, Evans JS. *Biochemistry*. 2009; 48:2272–2281. [PubMed: 19236004]
14. Lakshminarayanan R, Yoon I, Hegde BG, Fan DM, Du C, Moradian-Oldak J. *Proteins Struct Func Bioinform*. 2009; 76:560–569.
15. Moradian-Oldak J, Du C, Falini G. *Eur J Oral Sci*. 2006 May; 114(Suppl 1):289–296. discussion 327–9, 382. [PubMed: 16674701]
16. Wiedemann-Bidlack FB, Beniash E, Yamakoshi Y, Simmer JP, Margolis HC. *J Struct Biol*. 2007; 160:57–69. [PubMed: 17719243]
17. Chen CL, Bromley KM, Moradian-Oldak J, DeYoreo JJ. *J Amer Chem Soc*. 2011; 133:17406–17413. [PubMed: 21916473]
18. Wang LJ, Guan XY, Yin HY, Moradian-Oldak J, Nancollas GH. *J Phys Chem C*. 2008; 112:5892–5899.
19. Beniash E, Simmer JP, Margolis HC. *J Struct Biol*. 2005; 149:182–190. [PubMed: 15681234]
20. Fan YW, Sun Z, Wang RZ, Abbott C, Moradian-Oldak J. *Biomaterials*. 2007; 28:3034–3042. [PubMed: 17382381]
21. Kwak SY, Wiedemann-Bidlack FB, Beniash E, Yamakoshi Y, Simmer JP, Litman A, Margolis HC. *J Biol Chem*. 2009; 284:18972–18979. [PubMed: 19443653]
22. Fang PA, Conway JF, Margolis HC, Simmer JP, Beniash E. *Pro Nat Acad Sci*. 2011; 108:14097–14102.
23. Paine ML, White SN, Luo W, Fong H, Sarikaya M, Snead ML. *Matrix Biology*. 2001; 20:273–292. [PubMed: 11566262]
24. Moradian-Oldak J, Bouropoulos N, Wang LL, Gharakhanian N. *Matrix Biology*. 2002; 21:197–205. [PubMed: 11852235]
25. Moradian-Oldak J, Tan J, Fincham AG. *Biopolymers*. 1998; 46:225–238. [PubMed: 9715666]
26. Hoang AM, Klebe RJ, Steffensen B, Ryu OH, Simmer JP, Cochran DL. *J Den Res*. 2002; 81:497–500.
27. Ravindranath RMH, Moradian-Oldak J, Fincham AG. *J Biol Chem*. 1999; 274:2464–2471. [PubMed: 9891017]
28. Goldberg M, Septier D. *Crit Rev Oral Biol Med*. 2002; 13:276–290. [PubMed: 12090465]
29. Prout RES, Odutuga AA, Tring FC. *Arch Oral Biol*. 1973; 18:373–380. [PubMed: 4578478]
30. Shapiro IM, Wuthier RE, Irving JT. *Arch Oral Biol*. 1966; 11:501–512. [PubMed: 5226757]
31. Veis A. *Cell Mol Life Sci*. 2003; 60:38–55. [PubMed: 12613657]
32. Haruyama N, Hatakeyama J, Moriyama K, Kulkarni AB. *J Oral Biosci*. 2011; 53:257–266. [PubMed: 23914134]
33. Olivares-Navarrete R, Vesper K, Hyzy SL, Almaguer-Flores A, Boyan BD, Schwartz Z. *Eur Cell Mater*. 2014; 28:1–10. [PubMed: 25017640]
34. Chandrababu KB, Dutta K, Lokappa SB, Ndao M, Evans JS, Moradian-Oldak. *Biopolymers*. 2014; 101:525–535. [PubMed: 24114119]
35. Ndao M, Dutta K, Bromley KM, Lakshminarayanan R, Sun Z, Rewari G, Moradian-Oldak J, Evans JS. *Protein Sci*. 2011; 20:724–734. [PubMed: 21351181]
36. Cattoni DI, Kaufman SB, Flecha FLG. *Biochim Biophys Acta-Proteins and Proteomics*. 2009; 1794:1700–1708.
37. Daniel E, Weber G. *Biochemistry*. 1966; 5:1893–1900. [PubMed: 5963431]
38. Lentz BR. *Chem Phys Lipids*. 1993; 64:99–116. [PubMed: 8242843]
39. Johnson JE, Xie M, Singh LMR, Edge R, Cornell RB. *J Biol Chem*. 2003; 278:514–522. [PubMed: 12401806]
40. Tocanne J. Teissie, J. *Biochem. Biophys Acta*. 1990; 1031:111–142.
41. Tsui FC, Ojcius DM, Hubbell WL. *Biophys J*. 1986; 49:459–468.
42. Tarasevich BJ, Lea S, Bernt W, Engelhard M, Shaw WJ. *J Phys Chem B*. 2009; 113:1833–1842. [PubMed: 19199690]

43. Tarasevich BJ, Lea S, Bernt W, Engelhard MH, Shaw WJ. Biopolymers. 2009; 91:103–107. [PubMed: 19025992]
44. Lakshminarayanan R, Fan DM, Du C, Moradian-Oldak J. Biophy J. 2008; 94:715–715.
45. Moradian-Oldak J, Bouropoulos N, Wang L, Gharakhanian N. Matrix Biol. 2002 Mar; 21(2):197–205. [PubMed: 11852235]
46. Beniash E, Simmer JP, Margolis HC. J Dent Res. 2012; 91:967–972. [PubMed: 22933608]
47. Lu JX, Xu YS, Shaw WJ. Biochemistry. 2013; 52:2196–2205. [PubMed: 23477367]
48. Warotayanont R, Frenkel B, Snead ML, Zhou Y. Biochem Biophys Res Comm. 2009; 387:558–563. [PubMed: 19615979]
49. Huang Z, Newcomb CJ, Zhou Y, Lei YP, Bringas P, Stupp SI, Snead ML. Biomaterials. 2013; 34:3303–3314. [PubMed: 23398885]
50. Sasaki S, Takagi T, Suzuki M. Arch Oral Biol. 1991; 36:227–231. [PubMed: 1877895]
51. Smith CE, Issid M, Margolis HC, Moreno EC. Adv Dent Res. 1996; 10:159–169. [PubMed: 9206332]
52. Lacruz RS, Hilvo M, Kurtz I, Paine ML. Biochem Biophys Res Comm. 2010; 393:883–887. [PubMed: 20175995]
53. Ryu OH, Fincham AG, Hu CC, Zhang C, Qian Q, Bartlett JD, Simmer JP. J Den Res. 1999; 78:743–750.
54. De Kroon AI, Soekarjo MW, De Gier J, De Kruijff B. Biochemistry. 1990; 29:8229–8240. [PubMed: 2252886]
55. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax AJ. Biomol Nmr. 1995; 6:277–293.
56. Johnson BA. Methods Mol Biol. 2004; 278:313–352. [PubMed: 15318002]

Figure 1.
Intrinsic fluorescence approach to analyze rP172 assembly (10 μ M) at pH 3.5 and pH 8.00.

Figure 2.

A) Fluorescence emission spectra of Trp residues of rP172 (pH 3.5) in the presence of lipid vesicles. Concentration of rP172 used was 10 μ M. The data presented are maximal changes observed at a lipid : rP172 ratio of 30:1 for POPG and ACML lipid vesicles. A lipid : rP172 ratio of 100:1 was used for POPC lipid vesicles. B) Stern-Volmer plots of rP172 (pH 3.5) in the presence of lipid vesicles.

Figure 3.
CD spectra of rP172 (pH3.5) in the presence of A) POPG, B) ACML and C) POPC lipid vesicles.

Figure 4.

^1H , ^{15}N HSQC spectra of native DCN-rP172 recorded at 800 MHz in the monomeric aqueous state (black) and 10 : 1 POPG : protein (grey). Partial assignments are given for the N-terminal (P2-W45) and C-terminal (D155 – D173) regions. “x” symbol denotes the presence of additional crosspeaks not found in the aqueous state. Asterisks denote resonances with multiple crosspeaks arising from conformational exchange.

Figure 5.

A) Fluorescence emission spectra of Trp residues of rP172 in the presence of lipid vesicles at pH 8. B) Stern-Volmer plots of rP172 (pH 8.00) in the presence of lipid vesicles.

Figure 6. CD spectra of rP172 in the presence of A) POPG, B) ACML and C) POPC lipid vesicles. Concentration of rP172 used was 10 μ M.

Figure 7.

Fluorescence changes in rP172 and lipid vesicle-bound rP172 in the presence of an extrinsic probe, 1-aniline-8-naphtalenesulfonate (ANS), at pH 8.00. ANS and Trp fluorescence were registered after adding 3 mM ANS exciting at: A) and B), 380 nm (ANS); C) and D), 295 nm (Trp-ANS-FRET)

Figure 8. Protein-lipid FRET measured from intrinsic Trp of rP172 to the DNS probe of the DNS-PE-containing POPG and ACML lipid vesicles at pH 3.5 (A) and pH 8 (B).

Figure 9.

Fluorescence polarization of A, B) DPH and C, D) TMA-DPH-doped POPG and ACML lipid vesicles as a function of rP172 protein concentration. Increasing concentrations of rP172 were added to lipid vesicles composed of POPG-DPH, ACML-DPH, POPG-TMA-DPH and ACML-TMA-DPH. The spectra were recorded at 22 °C.

Table 1

Lipids used in the present study

Lipids used in the study	Referred in text as
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoglycerol	POPG
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine	POPC
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoethanolamine	POPE
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphoinositol	POPI
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phospho-L-serine	POPS
1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphate	POPA
Cardiolipin	CA
(Ameloblast Cell Mimicking Lipid Vesicles)	ACML
POPC:POPE:POPI:POPS:POPA:Cardiolipin (1:2:2:2:1:2 mol/mol)	

Table 2

Hydrodynamic radii (R_H) (nm) and mass percentages of particles of amelogenin (rP172) bound to POPG, ACML and POPC lipid vesicles at pH 3.5 as determined by DLS analysis.

rP172 10μM		POPG 300μM		rP172-POPG (1:30)	
R_H (nm)	Mass (%)	R_H (nm)	Mass (%)	R_H (nm)	Mass (%)
1.3±0.2	98.4±0.9	13.2±0.4	96.2±1.0	44.5±2.4	28.2±1.3
				130.5±0.9	57.9±5.8
				241.6±11.4	13.9±4.9
rP172 10μM		ACML 300μM		rP172-ACML (1:30)	
R_H (nm)	Mass (%)	R_H (nm)	Mass (%)	R_H (nm)	Mass (%)
1.6±0.3	95.2±2.5	10.6±0.2	99.2±1.4	18.5±2.3	22.0±0.9
				131.8±0.3	57.6±3.7
				249.8±9.2	20.4±3.2
rP172 10μM		POPC 300μM		rP172-POPC (1:30)	
R_H (nm)	Mass (%)	R_H (nm)	Mass (%)	R_H (nm)	Mass (%)
1.8±0.1	95.1±3.1	10.7±0.2	97.6±2.5	18.5±2.3	55.9±2.3
				134.4±0.3	23.9±2.9
				304.5±39.8	20.2±3.4

Table 3

Hydrodynamic radii (R_H) (nm) and mass percentages of particles of amelogenin (rP172) bound to POPG, ACML and POPC lipid vesicles at pH 8.00 as determined by DLS analysis.

rP172 10µM		POPG 300µM		rP172-POPG (1:30)	
R_H (nm)	Mass (%)	R_H (nm)	Mass (%)	R_H (nm)	Mass (%)
13.16±0.4	99.2±0.3	14.3±0.7	94.3±4.4	21.6±0.4	82.6±3.1
				134.6±0.9	11.4±0.9
				279.1±33.6	6.0±2.9
rP172 10µM		ACML 300µM		rP172-ACML (1:30)	
10.5±0.6	97.1±1.2	12.2±0.2	93.1±7.1	18.3±0.7	53.9±3.7
				136.5±1.2	2.9±0.5
				296.9±45.9	2.6±1.6
rP172 10µM		POPC 300µM		rP172-POPC (1:30)	
11.5±0.2	95.7±2.3	13.5±0.2	93.8±1.2	13.9±0.3	77.8±11.4
				133.1±0.4	7.1±0.2
				338.2±15.5	6.6±0.7