

Nanoscale Probing of the Enamel Nanorod Surface Using Polyamidoamine Dendrimers

Haifeng Chen,[†] Yunqing Chen,[‡] Bradford G. Orr,^{‡,||} Mark M. Banaszak Holl,^{§,||,⊥} Istvan Majoros,^{||} and Brian H. Clarkson^{*,†}

School of Dentistry, Department of Physics, Department of Chemistry, Program in Macromolecular Science and Engineering, and Center for Biologic Nanotechnology, University of Michigan, Ann Arbor, Michigan 48109-1078

Received July 22, 2003. In Final Form: January 21, 2004

Although it is known that noncollagenous proteins of dental origin bind to the hydroxyapatite crystal surfaces, no measure of their binding strength has been calculated. This experiment used –COOH-capped generation 7 PAMAM dendrimers as nanoprobess of the biological hydroxyapatite nanorod surfaces. Dendrimer distribution was characterized using AFM. The results showed dendrimers to be spaced at intervals along the *c*-axis of the crystals. From these observations and assuming a fully ionized –COOH dendrimer, a mathematical model of the binding capacity of the crystal surface with the dendrimer was developed. The Monte Carlo method was used to simulate the binding process between the dendrimer and crystal surface, and the binding strength of the –COOH groups to the surface was calculated to be 90 ± 20 kJ/mol. These results support the CFM studies which have described alternating bands of charge domains on the crystal surface and that the binding strength will be dependent on both the intensity of the charge on the protein and the crystal surface.

Introduction

Bone and teeth are the main mineralized tissue in the human body, and they have a very similar chemical structure. Both of them consist of nanoscale calcium hydroxyapatites (HAP) and proteins.¹ The deposition and growth of these biological nanocrystals within their respective tissues is thought to be controlled by proteins of the extracellular matrix; however, the precise mechanism of such interactions remains obscure due to the complexity of the tissue.^{1–5}

Dental enamel is the most extreme case of mammalian biomineralization and is the main biomineral source from which naturally derived crystals can be obtained for studies with minimum alteration to their surface characteristics.^{2,5} Dental enamel crystals are nanorodlike with a cross section of 30–80 nm and a length of 200–3000 nm along the *c*-axis.^{2,6} Recently, chemical force microscopy (CFM) studies have revealed that the surfaces of individual developing enamel crystals were comprised of a series of discrete and alternating charge arrays aligned perpendicular to the crystal *c*-axis.⁷ Further atomic force microscopy (AFM) studies have shown that many proteins,

serum albumin, amelogenin, dentin phosphoprotein (DPP), and dentinal sialoprotein (DSP), are able to bind to the crystal surfaces under physiological conditions. This protein binding gave a “banded” appearance to the crystal surfaces, which seemed to be of a similar periodicity to the charge arrays found using CFM.^{5,8,9} All these proteins are expected to carry overall a negative charge at physiological pH. These charge arrays on the crystals are thought to play a key role in the binding of the proteins via electrostatic interactions.⁵ However, the structure of these proteins has not yet been fully determined. This complicates the interpretation of experiments because the size and geometry of the active functional groups are not known. Using a macromolecule with a well-defined and controllable structure allows its functional activity to be measured for a variety of known configurations. Examples of these molecules are the polyamidoamine (PAMAM) dendritic polymers or dendrimers which can be synthesized as spheres of known diameter (1–20 nm). These polymers have been called artificial proteins, and their core structures dictate the characteristics of the molecule (Figure 1). They can be used as protein biomimics because the terminal groups can be functionalized and the dendrimers covalently linked together. This functionalization allows the dendrimers to bind strongly to charged surfaces as well-defined nanospheres which vary in diameter with dendrimer generation.^{10–12}

* Corresponding author. Phone: 734-763-1375. Fax: 734-936-1597. E-mail: bricla@umich.edu.

[†] School of Dentistry.

[‡] Department of Physics.

[§] Department of Chemistry.

^{||} Center for Biologic Nanotechnology.

[⊥] Program in Macromolecular Science and Engineering.

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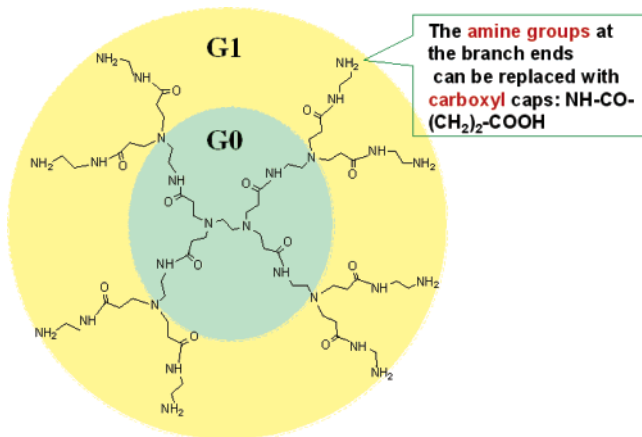


Figure 1. Schematic structure of a polyamidoamine (PAMAM) dendrimer (generation 1). The amine groups at the branch ends can be replaced with carboxylic acid.

In this study, generation 7 PAMAM dendrimers with carboxylic acid terminal groups were employed to probe the specific surface charge arrays on the surface of enamel nanorods. The binding of the dendrimers to the crystals surface was characterized with AFM. The Monte Carlo method was used to simulate the binding process between the dendrimer and crystal surface, and an approximate binding strength between the $-\text{COOH}$ groups of the dendrimer and the crystal surface was calculated.

Experimental Section

Enamel Crystals. Individual crystals from specific stages of enamel development were obtained from the mandibular incisors of 4-week old male Sprague Dawley rats. Particles of enamel were microdissected using the beginning of the white opaque enamel as a marker for the onset of the maturation stage as described previously by Robinson and Hiller.^{13,14} All developmental stages were collected, resulting in three samples: secretory stage enamel, transition stage enamel, and maturation stage enamel. The maturation stage enamel was used in this experiment. Matrix proteins were removed from the enamel crystals using a sequential extraction procedure described by Robinson et al.^{2,6-9,15} For this experiment, the crystals were also treated with 3% hypochlorite to oxidize traces of organic material and washed with distilled water again. The crystals were finally dispersed in HPLC-grade methanol by sonication.

Polyamidoamine (PAMAM) Dendrimers. Generation 7 PAMAM dendrimers capped with carboxylic acid ($-\text{COOH}$) groups were synthesized in the laboratory of the Center for Biologic Nanotechnology at the University of Michigan.^{10,11}

Dendrimer-Crystal Binding. This procedure has been previously described (Chen et al. 2003).¹⁶ Briefly, approximately $5 \mu\text{L}$ of methanol containing the crystals was pipetted onto the mica, the methanol was evaporated rapidly, and the dendrimer solution (285 nM in water, pH 7.4) was then flowed over the crystals. We used a 120 s exposure period for the interaction of the dendrimer solution with the crystals as described by Wallwork et al.⁹ The excess fluid was wicked off, and the specimen was placed in a desiccator for 12 h and subsequently imaged by AFM.

Force Microscopy. All samples were imaged in tapping mode in air, using a Nanoscope IIIa Multimode AFM and controller (Digital Instruments, Santa Barbara, CA) equipped with a $120 \times 120 \mu\text{m}$ J-type scanner. Commercially available tapping-mode

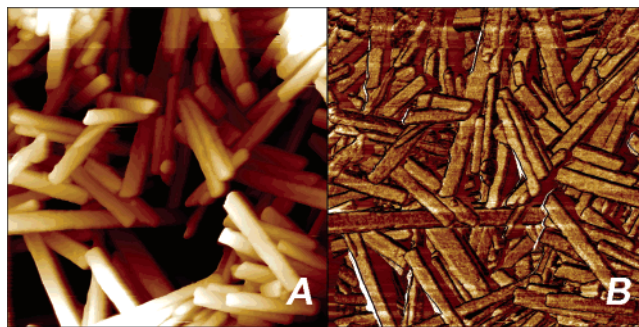


Figure 2. Tapping-mode AFM image of enamel crystals on a mica surface. [All image sizes $1 \times 1 \mu\text{m}$; (A) height image, z-range, 200 nm; (B) phase image, z-range, 30° .]

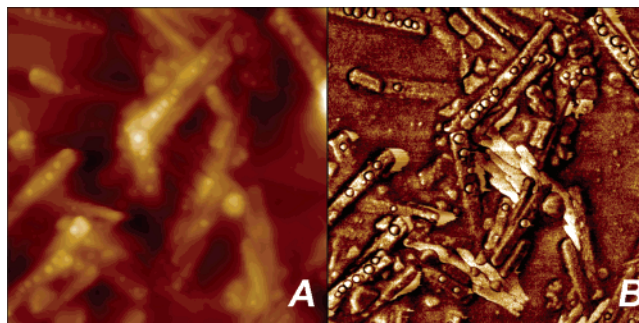


Figure 3. Tapping-mode AFM image of enamel nanorods after binding generation 7 PAMAM dendrimers with carboxylic acid end groups. [All image sizes $1 \times 1 \mu\text{m}$; (A) height image, z-range, 200 nm; (B) phase image, z-range, 30° .]

cantilevers, TESP, were used (Digital Instruments, Santa Barbara, CA) (thickness = $2.6\text{--}4.5 \mu\text{m}$, width = $28\text{--}30 \mu\text{m}$, height = $10\text{--}15 \mu\text{m}$, length = $125 \mu\text{m}$, resonant frequency = $297\text{--}378 \text{ kHz}$, spring constant = $29\text{--}61 \text{ N/m}$, nominal tip radius of curvature = $5\text{--}10 \text{ nm}$). Multiple images for each sample were obtained with scan sizes ranging from $500 \times 500 \text{ nm}$ to $10 \times 10 \mu\text{m}$ at 70–80% of free cantilever amplitude. Widths, heights, and spaces of dendrimers were determined with Digital Instruments off-line section analysis. The measurements of the spaces of every two dendrimers aligned on a single enamel nanorod were based on seven different AFM images. The measurement of each image gave one mean space and one relative standard deviation. We only measured those spaces less than 100 nm as the alternating charge array period of the enamel nanorods was reported to be approximately 50 nm, suggested by Kirkham et al.^{5,7}

Results and Discussion

Figure 2 shows a tapping-mode AFM image of the enamel crystals after being rinsed with distilled water pH 7.4 (control). The crystal surfaces are clean and free of deposits. Flowing $1.5 \mu\text{L}$ of the carboxylic acid ($-\text{COOH}$) capped 285 nM concentration dendrimer solution over the crystals resulted in a sparse covering of dendrimers (Figure 3). The dendrimers on the mica surface are $2.3 \pm 1.0 \text{ nm}$ in height and $15 \pm 3.3 \text{ nm}$ in width; however, those on the hydroxyapatite crystal surface are of $6.9 \pm 1.4 \text{ nm}$ in height and $19 \pm 2.4 \text{ nm}$ in width (Figure 4). Both values are consistent with a previous study of single dendrimer size and shape on different substrates.¹² The G7 dendrimer height ($6.9 \pm 1.4 \text{ nm}$) on the hydroxyapatite crystal surface approaches the theoretical diameter of the single G7 dendrimer (6.7 nm), although the measurement of the width of the dendrimer ($19 \pm 2.4 \text{ nm}$) is much larger. It has been previously shown that dendrimers approaching their theoretical diameter have a sufficiently large aspect ratio that tip convolution affects the measurement of their

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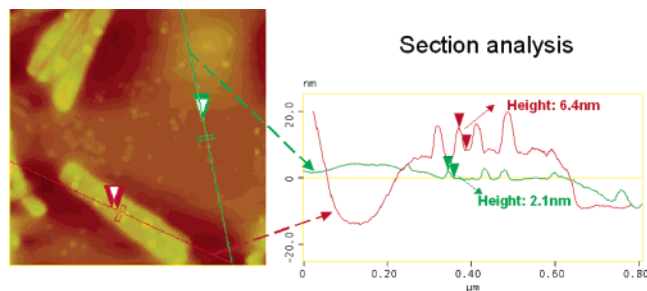


Figure 4. Dendrimers on the mica surface are 2.3 ± 1.0 nm in height and 15 ± 3.3 nm in width; however, those on the hydroxyapatite crystal surface are 6.9 ± 1.4 nm in height and 19 ± 2.4 nm in width.

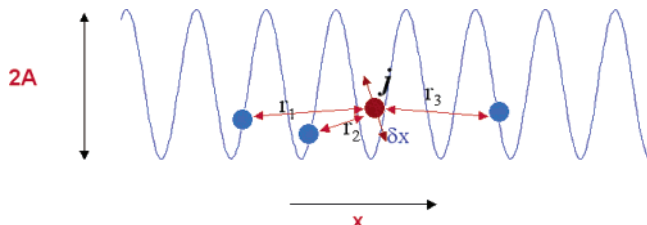


Figure 5. Nanorod surface is modeled as a 1-dimension potential field with a sinusoidal modulation in dendrimer binding ability. The binding energy between the j th dendrimer and the crystal surface $E_{d-c} = -A(\sin 2\pi x + 1)$. The repulsion energy between the j th dendrimer and the other dendrimers on the crystal surface $E_{d-d} = \Sigma(K/r_i)$. The position of the j th dendrimer on the crystal surface is determined by $E(x + \delta x) = E_{d-c} + E_{d-d} = \Sigma(K/r_i) - A(\sin 2\pi(x + \delta x) + 1) = \text{minimum}$.

lateral dimension.¹² However, the height information remains reliable. The dendrimers are spaced regularly, approximately 45 nm apart, along the surface of a crystal; although, there is a large distribution with a relative standard deviation ranging from 0.26 to 0.33 (roughly 12–15 nm) for each crystal. These results are complementary to the chemical force microscopy of Kirkham et al.,⁷ who showed alternating domains of surface charge comprised of broad bands, some 30–50 nm in width. These results are also very similar to the “banded” appearance of the electronegative proteins (e.g., DPP) on the crystal surface that have an average spacing of approximately 50 nm.⁹

The carboxylic-acid-capped dendrimers appear to be fully ionized at the experimental pH 7.4 because the average pK_a of the carboxylic acids are reported to be less than 5.5.^{17–19} This produces a negatively charged globular dendrimer to interact with the nanorod surface. The dendrimer distribution on the crystal surface is determined by the binding energy of the dendrimers to the nanorod surface and the repulsion energy between the dendrimers. The binding energy of the dendrimers is anticipated to be affected by the alternating charge array on the nanorod surface. The repulsion energy between the dendrimers is modeled as the screened Coulomb repulsion of the negative charge on each particle. The nanorod surface is modeled as a 1-dimension potential field with a sinusoidal modulation in the binding potential as shown in Figure 5. Specifically, the binding energy of the dendrimer to the nanorod surface is represented as $E_{d-c} = -A(\sin 2\pi x + 1)$, where $2A$ represents the maximum

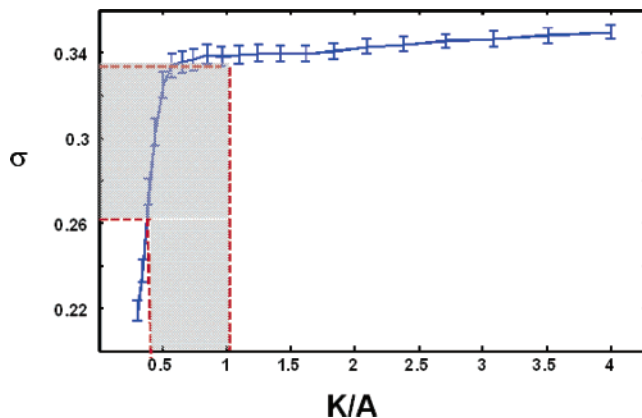


Figure 6. Monte Carlo methods are applied to simulate the binding process of the dendrimers to the crystal surface. We are able to derive a relationship of the relative standard deviation (σ) of the dendrimers distribution on the nanorod surface versus the ratio of the repulsive energy to binding energy, K/A .

binding energy between the dendrimer and the nanorod surface and x represents the binding site along the nanorod surface.

Assuming the dendrimers are spherical globular particles, the potential energy due to the dendrimer–dendrimer interaction can be represented as $E_{d-d} = \Sigma(K/r_i)$, where K is the repulsion energy of two dendrimers spaced at one period of the alternating array and r_i is the distance between the new dendrimer and the dendrimers on the nanorod surface.

Assuming initially that there are no dendrimers on the nanorod surface, dendrimers are introduced into the system sequentially following the principle that the position of the new dendrimer is chosen randomly at first and then its position is allowed to change within a single charge band such that the total energy of the system is minimized.

On the basis of this model, we use Monte Carlo methods to simulate the binding process of the dendrimers to the crystal surface. We are able to derive a relationship for the relative standard deviation (σ) of the dendrimers distribution on the nanorod surface as a function of the dimensionless ratio K/A as shown in Figure 6. Accordingly, as the relative standard deviation (σ) of the dendrimers on the crystal surface ranges from 0.26 to 0.33 in our experimental measurements, the corresponding K/A approximately ranges from 0.4 to 1.0. Using the fact that the dendrimers have a globular structure with fully ionized terminal groups, we can calculate that $K = 110$ eV using Coulomb’s law.

Thus, we find that the binding energy between the dendrimer and crystal surface, $2A$, equals 460 ± 100 eV.

Each generation 7 carboxylic-acid-capped PAMAM dendrimer contains 512 carboxylic acid end groups. Therefore, the binding energy of each $-\text{COOH}$ group exerted upon the crystal surface is 0.9 ± 0.2 eV or 90 ± 20 kJ/mol.

Our results suggest the charge on both the nanorod surface and of the biological protein play a significant role in the binding of the proteins to the nanorod surface. However, relying solely on charge for the binding of proteins to nanorod surfaces may be too simplistic an explanation. This is especially so when the structure of the proteins is not fully understood. It should be pointed out that we do not obtain the same dendrimer “banded” appearances on the nanorod surface when applying amine-capped generation 7 PAMAM dendrimer to interact with

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the crystals.¹⁶ The amine-capped dendrimers seem to bind to the crystal surface more strongly than the carboxylic-acid-capped ones. The pK_a of the amine-group-terminated G7 dendrimer is 8.7 according to our titration data, which means that at the pH 7.4, the amine-group-terminated G7 dendrimers were mostly (95%) protonated and were positively charged. It was reported that the isoelectric point of natural apatite was pH 5.6;²⁰ therefore, we may assume that the enamel crystals are negatively charged at pH 7.4, although the crystal surface may be patterned with charge arrays as reported by Kirkham et al.^{5,7} This will result in an apparent stronger binding capacity of the positively charged amine-terminated dendrimers compared with the negatively charged one ($-COOH$ capped).

Recently Spanos et al.'s investigation suggested that the positively charged amino groups of the phospho-L-serine were located within the inner Helmholtz plane (IHP) of the electrical double layer at the HAP/electrolyte interface while the negatively charged carboxyl groups and phosphate groups of the adsorbed phosphor-L-serine were oriented away from the negatively charged IHP surface of HAP.²¹ Applying these ideas to the amine- and carboxylic-acid-terminated dendrimers, we propose the following hypothesis. The positively charged amine-terminated dendrimers are located within the inner Helmholtz plane maximizing the electrostatic interaction with the negatively charged hydroxyapatite surface. The interaction may also include the hydrogen bonding between the protonated amino group and the oxygen atoms of the anions present on the HAP surface, i.e., hydroxyl (OH^-) and phosphate (PO_4^{3-}) ions. In contrast, in the case of negatively charged carboxylic-acid-terminated dendrimers, both the dendrimer and the surface retain their solvation sphere and the attractive force is dominated by the electrostatic force exerted by those positively charged ions or arrays inside the IHP and/or hydrogen bonding. The AFM data suggests these interactions are relatively weaker. The carboxyl-group-terminated dendrimers mainly stay in the outer Helmholtz plane (OHP). This hypothesis

is consistent with observed stronger binding of the amine-terminated dendrimers to the crystal surface.

It is known that the shape of the adsorbed dendrimer depends strongly on the substrate surface properties.¹² The height of those G7 dendrimers on the hydroxyapatite crystal surface (6.9 ± 1.4 nm) is much larger than those on the mica surface (2.3 ± 1.0 nm). Indeed, the dendrimer height measured is consistent with that measured for quite hydrophobic surfaces.¹² This result is unexpected. This could be because the hydroxyapatite is more hydrophobic than mica, but it could also be that charge differences cause stronger attractive electrostatic forces between the dendrimers and mica than between the dendrimers and hydroxyapatite, as the hydroxyapatite and carboxyl-group-terminated G7 dendrimers might be both negatively charged at pH 7.4. Theoretically, the hydroxyapatite crystal surface is hydrophilic. However, the surface may become hydrophobic if the surface is covered by lipids or other organic material. The extraction treatment employed may not be able to remove all organic material bound to the crystal surface. It is even possible that the charge array patterns result from this layer of organic material on the hydroxyapatite nanorods. Further investigations are ongoing.

In summary, the present work shows the potential of these artificial proteins, dendrimers, to be used as nanoprobe to mimic the pattern and determine the strength of binding of proteins to biological nanorod surfaces. The dendrimers can also be used to probe the surface to establish if there is periodic binding to the nanorod surfaces. This model allows an estimation of the binding energy of each of the protein's chemical groups to the nanorod surface to be determined even though both the nanorod surface and the protein structure have not been fully determined.

Acknowledgment. This investigation was supported in part by USPHS Research Grant DE121899 from the National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD 20892.

LA0303005

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