Potential Penetration of CTAB- and MUDA-coated Gold Nanorods into Tooth Enamel

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ABSTRACT

Aim: Gold nanorods (GNRs) have gained interest as a promising carrier for antibiotics. Gold nanorods may reduce the development of antimicrobial resistance in certain microbial species. Although applications of GNRs to mitigate oral biofilms are under development, their use in the oral cavity may have adverse effects. The aim of this study was to evaluate the potential penetration of GNRs into the tooth enamel structure using confocal laser scanning microscopy (CLSM) and scanning transmission electron microscopy (STEM).

Materials and methods: Our approach was to synthesize GNRs with cationic [cetyltrimethylammoniumbromide (CTAB)] and anionic [11-mercaptopundecanoic acid (MUDA)] surface coatings. We hypothesized that penetration would be surface coating-dependent.

Results: Regardless of the chemical modification of the GNRs of size ~20 nm × 8 nm, exposure of these materials did not result in superficial penetration into the enamel.

Conclusion: Within the limitations of this study, it is concluded that the use of CLSM and STEM is a feasible approach to investigate the penetration of nanomaterials into the tooth structure.

Clinical significance: Exposure of the enamel with chemically modified GNRs of size ~20 nm × 8 nm will not result in superficial penetration into the enamel.

Keywords: Confocal laser scanning microscopy, Enamel, Gold nanorods, Penetration, Scanning transmission electron microscopy.

The Journal of Contemporary Dental Practice (2020); 10.5005/jp-journals-10024-2834

INTRODUCTION

The concept of nanotechnology, which is the ability to control and manipulate atoms and molecules, was introduced by physicist Richard Feynman in 1959.^{1,2} Since then, nanotechnology has revolutionized the scope and depth of medical and dental applications. In dentistry, nanotechnology has enabled a shift from the mechanistic restoration of tooth repair to an emphasis on diagnosis and disease-prevention mechanisms.³

In the era of nanotechnology, bold ideas have been suggested such as formulation of microdental robots that would be programed to survey all teeth surfaces and break down harmful materials, or changing the superficial enamel layer with materials like sapphire or diamond to enhance the toughness and appearance of teeth.⁴ Moreover, there is a time progression for these technologies to transition from laboratory bench to dental applications. This is to ensure safety aspects and timely identification of weaknesses and strengths, which are critical when it comes to introducing a new material into the clinical setting.³

Gold nanomaterials have received widespread interest because of their easy preparation with controlled size, ready conjugation with biomolecules, and highly tunable optical properties. Thus, by varying the shape of the nanogold material, optical and thermal properties can be controlled. This has found applications in photodynamic or theranostic applications where heat can be generated to cure disease by varying the photon energy.⁵ Functional gold nanorods (GNRs) have been used as an alternative delivery vehicle or as a potent antimicrobial agent against multidrug-resistant bacteria.⁶ Therefore, the use of functional GNRs is expected to drive the development of groundbreaking formulations to prevent and treat biofilm-related dental diseases. This is achieved by GNRs affecting the oral biofilm via theranostic and/or alternative delivery vehicle approaches.

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How to cite this article: Kwon SR, Li Y, Walemba EM, *et al.* Potential Penetration of CTAB- and MUDA-coated Gold Nanorods into Tooth Enamel. *J Contemp Dent Pract* 2020;21(5):475–480.

Source of support: Colgate Award for Research Excellence Grant (#2160391).

Conflict of interest: None

The development of biofilm on the tooth surface is a complex process that occurs over a 2- to 3-day period. The process begins with adsorption of a sticky film onto the tooth surface derived from bacterial and host molecules and continues with biofilm maturation and biofilm dispersal.⁷ Once formed, it has been recognized as a virulence factor in many oral infectious diseases, including dental caries, periodontitis, and endodontic infections.⁸ Identification of innovative antimicrobial strategies to control the oral biofilm has been a central issue over the past years. However, the effect of new therapeutic agents and the potential penetration of these agents into the tooth have not been investigated. The human enamel is well known to act as a semipermeable membrane to allow for continuous dynamic ion exchange with the oral biofilm, with calcium

phosphate apatite crystals moving in both directions to maintain a proper mineral balance.⁹ Recent evidence indicates that some organic material in the enamel originates from exogenous sources and becomes part of the organic matrix.¹⁰ The enamel prisms though compactly organized and structured have pore sizes of approximately 2–6 nm that would make this interchange feasible.¹¹

The use of nanomaterials in the oral cavity is promising but may cause certain effects on the tooth enamel that are not documented. The purpose of this study was to evaluate potential penetration of GNRs into the tooth enamel structure. The aim was to synthesize GNRs with cationic [cetyltrimethylammoniumbromide (CTAB)] and anionic [11-mercaptoundecanoic acid (MUDA)] surface coatings. We characterized the formation of cationic and anionic GNRs and evaluated the penetration of differently charged GNRs into extracted human teeth. We hypothesized that accumulation and penetration into the enamel would be dependent on the type of chemical modification of GNRs as determined with confocal laser scanning microscopy (CLSM) and scanning transmission electron microscopy (STEM) imaging.

MATERIALS AND METHODS

The study was performed at Loma Linda University School of Dentistry. The use of extracted human teeth with no identifiers was approved by the Loma Linda University Institutional Review Board (IRB).

Synthesis of CTAB-coated Gold Nanorods (3–5 Aspect Ratio)

The ammonium hydroxide (28–30%), NaOH ($\geq 98\%$), silver nitrate ($\geq 99\%$), gold (III) chloride hydrate ($\text{HAuCl}_4 \cdot x\text{H}_2\text{O}$; 99.999% trace metals basis), ascorbic acid (reagent grade), and sodium borohydride ($< 90\%$) were used as received (Sigma-Aldrich, Milwaukee, WI, USA). Single-crystal GNRs of aspect ratio 3:5 were synthesized as per published methods.¹² In the GNR synthesis, the solutions were maintained at 27–30°C using a water bath. Gold nanoparticle seeds were first synthesized, and then added to the CTAB growth solution. To make the seeds, 25 μL of 50 mM HAuCl_4 solution was added to 4.7 mL of 0.1 M CTAB solution and the mixture thoroughly mixed and left in the water bath for 5 minutes. Then, 300 μL of a freshly prepared 10 mM NaBH_4 (7.6 mg NaBH_4 in 20 mL water under ice) solution was rapidly injected into the CTAB solution, while it was being vortexed. After 10–20 seconds, the seed solution was stirred mildly (400 rpm). For the growth solution, HAuCl_4 (100 μL , 50 mM) was added to CTAB (10 mL, 100 mM); the mixture was gently shaken and kept for 10 minutes in a water bath to ensure complexation between gold salt and CTAB. Afterward, ascorbic acid solution (75 μL , 100 mM) was added to the mixture, which was gently shaken for few seconds (the solution turns colorless). A solution of AgNO_3 (80 μL , 5 mM) was added to the growth solution and shaken for few seconds. Finally, the seed solution (120 μL) was added to the mixture and the solution vigorously shaken for the last time and then left undisturbed at 27°C for 30 minutes in a water bath.

Chemical Modification to MUDA-coated Gold Nanorods

The chemical classes of materials were modeled by chemical modification of the GNRs (~2–5 nm) using alkyl thiols. The traditional approach is via the thiol functionality on the alkyl chain (RSH), which binds chemically to gold to form thiolate

Au-S-R. We adopted this approach with GNRs to chemically modify CTAB coating with 11-mercaptoundecanoic acid. The method of diafiltration was used to purify the CTAB-coated GNRs.¹³ After washing, the samples were left at room temperature overnight to precipitate out the excess CTAB crystals (which are only soluble above room temperature) and the supernatant decanted. Then, 5 mL of the GNR supernatant was added to a glass vial and mixed with 5 mL of MUDA solution, which is comprised of 4 mL of water (pH adjusted to 9.0) and 1 mL of 10 mM of MUDA in ethanol. The mixture was heated with stirring in a water bath to about 80°C for 10 minutes to allow efficient ligand replacement of CTAB with MUDA. After cooling at room temperature, the solution was transferred to microcentrifuge tubes and sonicated for 1 hour. The MUDA GNR solutions were then centrifuged twice at 10,000 rpm for 20 minutes and suspended in 10 mM Tris buffer.

Characterization of Gold Nanorods

Both CTAB-coated and MUDA-coated GNRs were characterized by atomic force (AFM) and electron microscopies as well as UV-vis and dynamic light scattering (DLS). Au NRs solutions were dried on silanated mica discs and examined by atomic force microscopy with a Multimode 8 scanning probe microscope (Bruker, Santa Barbara, CA, USA) in the peak force tapping mode and using ScanAsystTM ($k = 0.4 \text{ N m}^{-1}$, $f = 70 \text{ kHz}$) air probes. Samples were prepared for electron microscopy measurements by centrifuging ($10,000 \times g$) twice to remove excess reagents and suspended in water followed by dropping 5–10 μL of solution onto a carbon-coated Cu grid (Ted Pella 200 mesh) and allowing the samples to air dry. UV-vis spectra were recorded using a Varian Cary 300 spectrophotometer equipped with a temperature controller. All UV-vis measurements were made using a quartz cell (1 cm path length, 25°C) unless otherwise indicated. Particle sizes were determined using a Nicomp Z3000 Zeta Potential/Particle Sizer (PSS Nicomp, USA) equipped with a He–Ne laser wavelength of $\lambda = 638 \text{ nm}$ and a power output of 60 mW. All data were collected at 25°C and at a scattering angle of 90° with a square acrylic cuvette (3 mL volume) containing a suspension of diffusible particles. Values of the refractive index, $n = 1.33$, and viscosity, $\eta = 8.9 \times 10^{-4} \text{ N s m}^{-2}$, for water were assumed to be applicable to the solutions. Prior to measurements, all samples were centrifuged ($8,000 \times g$, 30 minutes) to remove the excess surfactant and suspended in deionized water. Zeta potential measurements (average of three replicates taken at 1-minute intervals) were done using 20 μL sample and diluting in 2 mL 10^{-2} M NaCl solution.

Treatment of Human Enamel Slabs

Extracted sound human third molars ($N = 18$) were collected and stored in 0.2% sodium azide solution at 4°C. Teeth were cleaned of gross debris, sectioned, and the crown portion cut to a rectangular shape of $4 \times 6 \times 4 \text{ mm}^3$. The sectioned specimens were then embedded in acrylic resin to expose the enamel surface only. The enamel specimens were immersed in 500 μL of 0.1 M Tris-buffer (Control, $N = 6$), 500 μL of 0.1 M CTAB-coated GNRs (CTAB, $N = 6$), and 500 μL of 0.1 M MUDA-coated GNRs (MUDA, $N = 6$) for 3 weeks at 37°C. The solutions were replenished every week. The concentration of the GNRs was determined by the method of Orendorff and Murphy.¹⁴ Half of the specimens from each group were used for confocal laser scanning microscopy and the other half of each group was used for scanning transmission electron microscopy imaging.

Confocal Laser Scanning Microscopy

On completion of 3 weeks' immersion in respective solutions, specimens were rinsed with Milli-Q water and immersed in 100 μ M Rhodamine B solution for 24 hours to counterstain the dental hard tissue. The images of gold nanorod penetration into enamel were acquired in a laser scanning confocal microscope (Zeiss LSM 710 NLO, Jena, Germany). A 750 nm fs Ti:Sapphire laser (Coherent Chameleon Vision II Ti) was used as the excitation source for the gold nanorod imaging while the dental hard tissue was imaged with a laser at 561 nm. An oil immersion objective (Plan-Apochromat 63 $\times$ /1.40 Oil DIC M27) and a matched pinhole were used in all experiments. With the z-scan function, 3D images were obtained in ZEN computational software to evaluate the penetration depths of the GNRs into the enamel tissue.

STEM Sample Preparation

On completion of 3 weeks' immersion in respective solutions, STEM lamellae were prepared from a polished cross-section of the tooth specimen following established procedures with a dual-beam scanning electron microscope and FIB instrument (Quanta 200i 3D, ThermoFisher Scientific). First, a strap of 5- μ m-thick carbon layer was deposited over a region of interest on the polished cross-section using the ion beam (30 kV, 0.5 nA). Two trenches were then milled out (30 kV, 10 nA) on either side of a 15- μ m-thick slice of material. The slice of material was reduced to 1 μ m thickness (30 kV, 5 nA). The slice was then cut free from the substrate on three sides, leaving only a small connecting bridge. An *in situ* tungsten nanomanipulator probe (Autoprobe200, Oxford Instruments Inc.) was attached to the free side of the substrate by depositing Pt (2 kV, 8 nA). The remaining connection to the substrate was milled away (30 kV, 0.5 nA) and the probe was retracted with the sample. The sample was then welded to a copper TEM half-grid (Omniprobe) using Pt deposition and the connection to the probe was milled away (30 kV, 0.5 nA).

The lamella was thinned to 500 nm at 30 kV, 1 nA on both sides at $\pm 4^\circ$ incidence angle grazing milling condition. To reduce surface amorphization and gallium implantation, final milling at 5 kV and 0.5 nA was used to thin the sample to approximately 80 nm.

STEM Imaging and Analysis

STEM imaging was performed at 300 kV accelerating voltage in a ThermoFisher Scientific Titan Themis 300 instrument, fitted with X-FEG electron source, 3 lens condenser system, and S-Twin objective lens. STEM images were recorded with Fischione Instruments Inc. Model 3000 High angle annular dark field (HAADF) Detector with probe current of 150 pA, frame size of 2,048 $\times$ 2,048, dwell time of 15 μ sec/pixel, and camera length of 245 mm.

Energy-dispersive X-ray spectroscopy (EDS) analyzes and elemental mapping were obtained in the STEM at 300 kV, utilizing the ThermoFisher Scientific SuperX system equipped with 4 $\times$ 30 mm² window-less SDD detectors symmetrically surrounding the specimen with a total collection angle of 0.68 sr, by scanning the thin-foil specimens. Elemental mapping was performed with an electron beam probe current of 350 pA at 1,024 $\times$ 1,024 frame resolution. EDS spectra were extracted from the mapped areas and processed using the Bruker Instruments Esprit 1.9 software. Quantification is based on the Cliff-Lorimer approach using calculated k-factors and correcting for absorption by estimating foil thickness.

RESULTS

Synthesis and Characterization of Gold Nanorods

Figure 1 illustrates the methodology used in this study, from GNRs synthesis to application on the tooth enamel surface. Figure 2 shows the normalized UV-vis spectra of the CTAB- and MUDA-coated GNRs suspended in 10⁻² Tris buffer. Two plasmon peaks are observed at $\sim$ 524 (transverse) and $\sim$ 744 nm (longitudinal) in the

Fig. 1: Graphical illustration of study design

UV-vis. The longitudinal plasmon peak position is dependent on the aspect ratio of the NR; for nanorods with higher-aspect ratios, the longitudinal plasmon peaks will red-shift to longer wavelength. Chemical modification by the MUDA red-shifted the longitudinal plasmon peak from ~ 744 to 756 nm. This is because the refractive index around the GNR interface is changed due to the MUDA coating. The aspect ratio and morphology were determined by STEM. The size of GNRs was consistent and determined to be ~ 20 nm $\times$ 8 nm (Fig. 3). Average zeta potential measurements of -1.50 and -28.74 mV were recorded for initial CTAB- and MUDA-modified GNRs, respectively. The significantly negative zeta potential for MUDA-coated GNRs is from the deprotonated carboxylic acid groups, where the pH is above pK_a of MUDA (5.5).

Confocal Laser Scanning Microscopy

Figure 4 show the 3-D image of the human enamel stained with Rhodamine B to contrast the images of MUDA- and CTAB-coated

GNRs. The enamel is primarily composed of hydroxyapatites in the rod and interrod enamel. In the enamel, Rhodamine B penetrated along the interprismatic spaces so that the longitudinal orientation of the rods was clearly visible. Surface striations formed by the polishing process were visible on all enamel samples. Accumulation of scattered MUDA- and CTAB-coated GNRs was limited to the outer surface while the control group showed a clean enamel surface.

STEM Imaging and Analysis

STEM samples of ~ 80 nm thickness were prepared from a polished cross-section of the tooth specimen with a dual-beam scanning electron microscope and focus ion beam instrument. Additionally, the EDS analysis was obtained in STEM (Fig. 5). The micrographs in Figure 5 represent enamel specimens treated with Tris buffer the negative control where no GNRs were visible (Fig. 5) and detectable with EDS (Fig. 5). Immersion of enamel specimens in CTAB GNRs solution showed localized accumulation of GNRs onto the surface,

Fig. 2: UV-vis spectrum of cetyltrimethylammoniumbromide and 11-mercaptoundecanoic acid coated gold nanorods

Fig. 3: Scanning transmission electron microscopy image of gold nanorods

Figs 4A to C: Three dimensional confocal images of teeth exposed to Tris buffer or gold nanorods; (A) Tris buffer solution; (B) Cetyltrimethylammoniumbromide-coated gold nanorods solution; (C) 11-Mercaptoundecanoic acid-coated gold nanorods solution

Figs 5A to F: Scanning transmission electron microscopy (STEM) and EDS images of enamel slabs: (A) Tris buffer solution STEM image; (B) Tris buffer solution energy-dispersive X-ray spectroscopy (EDS) image; (C) Cetyltrimethylammoniumbromide-coated gold nanorods solution STEM image; (D) Cetyltrimethylammoniumbromide-coated gold nanorods solution EDS image; (E) 11-Mercaptoundecanoic acid-coated gold nanorods solution STEM image; (F) 11-Mercaptoundecanoic acid-coated gold nanorods solution EDS image

which was consistent with the micrographs obtained with CLSM. High-magnification micrographs of samples immersed in MUDA GNRs solution showed the accumulation localized to the outer enamel surface. GNRs were visible on subsurface pore areas (Fig. 5), which may be attributed to preexisting defects within the enamel substrate or cracks formed during the experimental procedure. The results of STEM imaging indicated that exposure of the enamel with chemically modified GNRs of size $\sim 20 \text{ nm} \times 8 \text{ nm}$ would not result in superficial penetration into the enamel.

DISCUSSION

Novel diagnostic and therapeutic approaches using nanomaterials is a promising field in dentistry and medicine. This is because of the change in existing physicochemical properties as the size of the materials reduces to the nanometer scale. Gold nanoparticles (Au NPs) distinguish themselves from other nanoplateforms such as semiconductor quantum dots and magnetic and polymeric nanoparticles by their unique surface plasmon resonances.

These plasmonic resonance induced by photons enhances all the radiative and nonradiative properties of the nanoparticles. This offers multiple application modalities for biological and medical settings.^{6,15,16}

The purpose of this study was to evaluate the potential penetration of GNRs into the tooth enamel using confocal laser scanning microscopy and scanning transmission electron microscopy. The rationale was to determine whether functional GNRs can penetrate the dental enamel creating unexpected changes in the tooth surface. Our study showed that our team was able to successfully synthesize and characterize GNRs exhibiting different surface charges. Additionally, the approach of utilizing CLSM and STEM for the evaluation of potential penetration of the nanomaterial into the tooth structure proved to be novel and feasible.

The dental enamel is the most highly mineralized and hardest tissue of the body. It is approximately 96% mineral, 3% water, and 1% organic matter by weight. The enamel hydroxyapatite is primarily composed of phosphate ions (PO_4^{3-}) and calcium ions (Ca^{2+}). Under normal conditions, there is a stable equilibrium between the calcium and phosphate ions in saliva and the crystalline hydroxyapatite. We hypothesized that the chemical modification made to the GNRs would make a difference in the accumulation and penetration, with the anionic MUDA-coated GNRs having better affinity and penetration into the enamel. However, both qualitative CLSM and STEM images have demonstrated no difference between the two chemically modified GNRs in penetration into the enamel.

It is important to note that NPs most commonly used in food or personal care products have particle sizes that are less than 100 nm.¹⁷ Despite the fact that studies are ongoing on potential toxicity of nanomaterials, there have been no studies on the ability of nanomaterials to adhere or permeate to the enamel.¹⁸ The flux into the enamel is dependent on many factors such as solubility, concentration, capillary forces, and osmotic gradients.^{19–21} The most critical factor seems to be the particle size. The main limitation of this study was the size of the GNRs. The synthesis of GNRs, that are as small as enamel pore sizes of approximately 2–6 nm, could have provided better insight on size dependency. Additionally, the absence of dentinal fluid flow produced by intrapulpal pressure in vital teeth may have affected our results. With the rapid introduction of nanomaterials as a means to treat oral biofilm-related diseases, it is crucial that further studies are carried out on the potential effects of these materials on the tooth structure.

CONCLUSION

Within the limitations of this study, it is concluded that the use of CLSM and STEM is a feasible approach to investigate the penetration of nanomaterials into the tooth structure. Regardless of the chemical modification of the GNRs of size $\sim 20 \text{ nm} \times 8 \text{ nm}$, exposure of these materials did not result in superficial penetration into the enamel.

ACKNOWLEDGMENTS

This research project has been made possible through a grant from the Colgate-Palmolive Company (#2160391). The authors thank Dong An Yang at the University of Riverside Clean Facility for helping with the SEM/STEM and Monica Romero from LLUSM AIM facility for helping with the CLSM imaging.

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