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Authors
Lee, Dong-Keun
Kim, Sue Vin
Limansubroto, Adelheid Nerisa
et al.

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Nanodiamond—Gutta Percha Composite Biomaterials for Root Canal Therapy

Dong-Keun Lee,*† Sue Vin Kim,† Adelheid Nerisa Limansubroto,† Albert Yen,‡ Akrivoula Soundia,§ Cun-Yu Wang,*,‡ Wenyuan Shi,‡,‡ Christine Hong,† Sotirios Tetradis,§ Yong Kim,†,‡,‡,‡ No-Hee Park,†,‡,‡,‡ Mo K. Kang,‡,‡,‡ and Dean Ho‡,§,‡,‡,‡,‡,‡,‡,‡

ABSTRACT Root canal therapy (RCT) represents a standard of treatment that addresses infected pulp tissue in teeth and protects against future infection. RCT involves removing dental pulp comprising blood vessels and nerve tissue, decontaminating residually infected tissue through biomechanical instrumentation, and root canal obturation using a filler material to replace that space which was previously composed of dental pulp. Gutta percha (GP) is typically used as the filler material, as it is malleable, inert, and biocompatible. While filling the root canal space with GP is the standard of care for endodontic therapies, it has exhibited limitations including leakage, root canal reinfection, and poor mechanical properties. To address these challenges, clinicians have explored the use of alternative root filling materials other than GP. Among the classes of materials that are being explored as novel endodontic therapy platforms, nanodiamonds (NDs) may offer unique advantages due to their favorable properties, particularly for dental applications. This study developed a ND-embedded GP (NDGP) that was functionalized with amoxicillin, a broad-spectrum antibiotic commonly used for endodontic infection. Comprehensive materials characterization confirmed improved mechanical properties of NDGP over unmodified GP. In addition, digital radiography and microcomputer tomography imaging demonstrated that obturation of root canals with NDGP could be achieved using clinically relevant techniques. Furthermore, bacterial growth inhibition assays confirmed drug functionality of NDGP functionalized with amoxicillin. This study demonstrates a promising path toward NDGP implementation in future endodontic therapy for improved treatment outcomes.

KEYWORDS: nanomedicine · dentistry · biomaterials · drug delivery · root canal therapy · gutta percha · endodontics

Conventional root canal therapy (RCT) has been efficacious in salvaging teeth with a history of gross carious lesions, trauma, and fractures that lead to pulp and periradicular infection.1–3 During RCT, the infected or inflamed pulp tissue is removed, and the root canal space is cleaned and shaped before it is filled with gutta percha (GP), a rubber-based filling material with root canal cements. The final obturation step is a crucial step in endodontic therapy because the therapeutic outcome depends on sealing the root canal space along the length of the root canal. While there are many benefits associated with saving natural dentition through endodontic therapies, RCTs may fail due to inadequate sealing of infected root canals, allowing for bacterial regrowth within the root canal system, causing apical periodontitis and abscess.

Although RCT employs powerful intracanal irrigants, e.g., 6% NaOCl, to eradicate microbial infection, it is very difficult to achieve complete disinfection of the complex...
root canal anatomy.\textsuperscript{4,5} Furthermore, secondary intra-
canal structures, e.g., lateral canals and isthmuses, may
harbor established bacterial biofilm and serve as a
source of root canal reinfection, causing treatment
failures. These lateral canals exist throughout most root
canal structures and are poorly detectable in dental
radiographs. Even if detected, lateral canals are often
difficult to instrument, leaving the pre-existing bacterial
biofilms untouched.

While GP offers numerous advantages, including
biocompatibility, cost efficiency, ease of removal, and
a long history of use, its inability to provide adequate
seal to prevent bacterial percolation is a challenge in
endodontic therapy. For instance, inadequate root
canal seal around voids within the obturated root canal
space may allow leakage due to absence of bonding
between GP and the dentinal surface.\textsuperscript{6,7} Such voids
with leakage may allow regrowth of bacterial within
the root canals, including those in dentinal tubules, and
establish reinfection of the root canal space, causing
treatment failures. To address these possibilities of root

canal reinfection, we harnessed the field of nanomedicine
with a broad spectrum of powerful nanoparticle plat-
forms for drug delivery, imaging, and other applica-
tions.\textsuperscript{8–13} We developed a nanodiamond—gutta percha
composite (NDGP) embedded with nanodiamond—
amoxicillin (ND-AMC) conjugates (Figure 1A), which
can reduce the likelihood of root canal reinfection
and enhance the treatment outcomes. NDs are carbon
nanoparticles that are approximately 4–6 nm in di-
ameter; they are waste byproducts that are readily
processed for biomedical applications.\textsuperscript{14–24} Numerous
studies have shown that NDs are biocompatible plat-
forms for drug delivery and imaging, as they possess
surface chemistries suited for electrostatic adsorption
and/or covalent conjugation of various compounds.\textsuperscript{25–46}
Most importantly, NDs themselves have demonstrated
antimicrobial activity.\textsuperscript{47,48} Therefore, the use of biocom-
patible NDs to simultaneously sequester and localize
the activity of amoxicillin as a model therapeutic, as well
as confer enhanced mechanical properties to GP for
improved ease of handling during obturation, provides

Figure 1. Schematic of NDGP embedded with ND-AMC conjugates. (A) Synthesis of NDGP. A polysoprene solution was
prepared by solvating trans-1,4-polyisoprene in chloroform at a 1:71 (w/w) ratio of polyisoprene to chloroform. Zinc oxide
(ZnO2), barium sulfate (BaSO4), and wax were added to this solution at a 3.3:0.55:0.15 ratio. ND-AMC was then mixed into the
polyisoprene solution, and the final mixture was lyophilized to obtain solid NDGP. (B) NDGP can prevent bacterial
contamination after root canal therapy due to the antimicrobial properties of both ND and amoxicillin.
a translationally relevant route toward improving endodontic treatment outcomes.

Our NDGP platform integrates several important attributes into a single filler material. These attributes include improved mechanical properties, relative to those of unmodified GP, and antimicrobial activity against oral bacterial flora (Figure 1B). By taking advantage of the ND surface chemistry, a broad-spectrum antibiotic, such as amoxicillin, can be adsorbed to the ND surface (Figure 1A). Embedding amoxicillin-linked NDs into GP may facilitate the eradication of residual bacteria within the root canal system after completion of obturation. NDGP may also kill bacteria entering through the lateral canals following contact with ND—antibiotic agents. The homogeneous dispersion of NDs throughout the GP matrix also leads to an increase in toughness, as evidenced by mechanical tests comparing the tensile strength of unmodified GP and NDGP. Importantly, root canals were effectively obturated using traditional obturation techniques for both NDGP and unmodified GP. This was validated by performing a conventional lateral obturation technique on human teeth after root canal cleaning and shaping. A comparable quality and diagnostic capability of obturation between unmodified GP and NDGP was confirmed via digital radiography and micro-CT imaging.

RESULTS AND DISCUSSION

ND—Antibiotic Synthesis and Characterization. Two samples of ND-AMC were synthesized by mixing amoxicillin and ND in 2.5 mM NaOH in two distinct ratios: (i) 5:2 w/w ND to amoxicillin (5:2 ND-AMC) and (ii) 5:3 w/w ND to amoxicillin (5:3 ND-AMC). After mixing, the ND-AMC samples were incubated at room temperature for 5–7 days to allow sufficient time for amoxicillin incorporation with ND.

Formation of the ND-AMC complex was assessed using dynamic light scattering (DLS) and ζ-potential analysis. Amoxicillin sequestration by ND was accompanied by a marked increase in hydrodynamic size and decrease in ζ-potential. The hydrodynamic size and ζ-potential of unmodified ND were measured to be \(46.64 \pm 0.17 \text{ nm} \) and \(55.80 \pm 0.37 \text{ mV} \), respectively (Figure 2A). In contrast, 5:2 ND-AMC had a hydrodynamic size of \(143.03 \pm 4.21 \text{ nm} \) and ζ-potential of \(46.08 \pm 1.10 \text{ mV} \), while 5:3 ND-AMC had a hydrodynamic size of \(151.53 \pm 1.22 \text{ nm} \) and ζ-potential of \(40.57 \pm 2.94 \text{ mV} \) (Figure 2A).

The quantity of amoxicillin loaded onto the ND particles was calculated by subtracting the amount of unbound amoxicillin from the initial loading amount after completing ND-AMC synthesis. As shown in Figure 2B, \(1.30 \pm 0.02 \text{ mg/mL} \) of amoxicillin was loaded onto 5 mg/mL of ND from an amoxicillin loading concentration of 2 mg/mL (5:2 ND-AMC), while 2.10 ± 0.05 mg/mL of amoxicillin was loaded onto 5 mg/mL of ND from an amoxicillin loading concentration of 3 mg/mL (5:3 ND-AMC). These concentration values correspond to loading efficiencies of \(\sim 65\% \) for 5:2 ND-AMC and \(\sim 70\% \) for 5:3 ND-AMC, suggesting that ND-AMC synthesis generates amoxicillin-linked NDs.

It is important to note that mere sequestration of amoxicillin by ND is insufficient. The drug must be adsorbed strongly enough such that it does not immediately release after incorporation with ND. An immediate burst release of amoxicillin is obstructive to the combination of ND-AMC and GP, as the drug is lost before the NDGP composite can be synthesized. Cumulative amoxicillin release profiles showed that the amoxicillin was released from ND-AMC in a sustained manner over a week-long period, regardless of the initial amoxicillin concentration (Figure 2C). Due to its greater drug payload, the 5:3 ND-AMC sample released \(274.58 \pm 6.29 \mu \text{g/mL} \) of amoxicillin over this one-week period, a concentration greater than the 218.96 ± 37.86 μg/mL released by the 5:2 ND-AMC sample over the same duration. However, in terms of percentage release, the 5:3 ND-AMC sample eluted \(\sim 13.1\% \) of its total drug payload, whereas the 5:2 ND-AMC sample eluted \(\sim 16.8\% \) of its drug payload. Despite differences in total amounts of released drug, the percentage release values of 5:2 ND-AMC and 5:3 ND-AMC were comparably low, suggesting sustained adsorption of amoxicillin onto the ND surface. This interaction between amoxicillin and ND can prevent loss of amoxicillin during NDGP synthesis.

Fourier transform infrared (FTIR) spectroscopy analysis was performed as a final validation step for amoxicillin loading. Vibrational peaks corresponding to functional groups on ND, AMC, and ND-AMC were compared (Figure 2D). The ND and ND-AMC spectra shared similar peaks, including an \(\text{O}—\text{H} \) bending vibration peak at 1632 cm\(^{-1}\) and \(\text{C}—\text{O} \) stretching vibration peaks from 1700 to 1800 cm\(^{-1}\) corresponding to ketones, esters, lactones, and carboxylic acid groups found on ND surfaces. The spectra of amoxicillin and ND-AMC also overlapped at peaks associated with amoxicillin functional groups. Vibrational bands on the ND-AMC spectra from 800 to 900 cm\(^{-1}\) represented bending vibrations of secondary and primary amine groups on amoxicillin. A series of small amoxicillin peaks from 1000 to 1700 cm\(^{-1}\) are also found exclusively on the ND-AMC spectra and not the ND spectrum. These small vibrational peaks include \(\text{C}—\text{H} \) bending vibrations from 1220 to 1250 cm\(^{-1}\), \(\text{NC}—\text{H} \) and \(\text{COOH} \) bending vibrations from 1300 to 1380 cm\(^{-1}\), \(\text{CH}_3 \) bending vibrations from 1390 to 1400 cm\(^{-1}\), phenolic \(\text{O}—\text{H} \) bending vibrations from 1430 to 1450 cm\(^{-1}\), benzylic \(\text{C}—\text{C} \) stretching vibrations from 1500 to 1590 cm\(^{-1}\), and ketone \(\text{C}==\text{O} \) stretching vibrations from 1680 to 1710 cm\(^{-1}\). These FTIR data confirm successful loading of amoxicillin onto NDs.
Preparation and Evaluation of an NDGP Composite. In order to enhance the antimicrobial and mechanical properties of GP, amoxicillin-loaded NDs were embedded into the GP matrix. Briefly, the GP matrix was prepared by the composition of polyisoprene, ZnO₂, BaSO₄, and wax with a conventional ratio of 3.3:0.55:0.15, respectively. After thoroughly mixing the components, amoxicillin-loaded NDs were dispersed by sonication, followed by overnight lyophilization until all solvents were removed. After the preparation of NDGP, crucial physical properties for endodontic materials, including radiopacity and elastic modulus, were assessed.

In clinical procedures, radiopacity is a critically important property of root canal filling materials. In order to evaluate the quality of obturation, root canal fillers should be radiopaque for the clinicians to visualize the level of root canal obturation and the obturation quality. In order to determine the radiopacity of Figure 2. Characterization of ND-AMC. (A) Dynamic light scattering analyses and ζ-potential measurements for unmodified ND (ND-Pure) and ND-AMC samples synthesized from a 5:2 w/w ratio of ND to amoxicillin (5:2 ND-AMC) and a 5:3 w/w ratio of ND to amoxicillin (5:3 ND-AMC). Top graph: Hydrodynamic sizes of ND-Pure, 5:2 ND-AMC, and 5:3 ND-AMC were measured to be 46.64 ± 0.17, 143.03 ± 4.21, and 151.53 ± 1.22 nm, respectively. Bottom graph: ζ-potentials of ND-Pure, 5:2 ND-AMC, and 5:3 ND-AMC were measured to be 55.80 ± 0.37, 46.08 ± 1.10, and 40.57 ± 2.94 mV, respectively. (B) Standard curve for amoxicillin concentrations (top) and the final amount of amoxicillin successfully loaded onto NDs (bottom). Using the standard curve, the loading efficiency for ND-AMC was determined. For the 5:2 ND-AMC sample, 1.30 ± 0.02 mg/mL of amoxicillin was loaded from an initial amoxicillin concentration of 2 mg/mL (~65% efficiency). For the 5:3 ND-AMC sample, 2.10 ± 0.05 mg/mL of amoxicillin was loaded from an initial amoxicillin concentration of 3 mg/mL (~70% efficiency). (C) Cumulative amoxicillin release profiles for ND-AMC samples synthesized from 5:2 or 5:3 (w/w) ratios of ND to amoxicillin. For both samples, sustained amoxicillin release was observed for 1 week, with some burst release observed during hours 1-5. Although the 5:3 ND-AMC sample exhibits greater total drug release (274.58 ± 6.29 μg/mL) than the 5:2 ND-AMC sample (218.96 ± 37.86 μg/mL), the two samples released similar percentages of their respective amoxicillin payloads (~16.8% for 5:2 ND-AMC and ~13.1% for 5:3 ND-AMC). (D) FTIR spectra of (a) unmodified ND, (b) amoxicillin, (c) 5:2 ND-AMC, and (d) 5:3 ND-AMC. The FTIR spectra of both ND-AMC samples displayed C=C-H out-of-plane bending vibrations at 820 - 840 cm⁻¹ and C=C stretching vibrations at 1560 and 1603 cm⁻¹, which are well matched with similar peaks on the amoxicillin spectrum. A C=O stretching vibration found at 1628 cm⁻¹ on the spectra of both ND-AMC samples also matches well with a similar peak found on the unmodified ND spectrum.
NDGP, we compared the digital X-ray images of NDGP with those of unmodified GP (Figure 3A). Both NDGP (top in both images) and unmodified GP (bottom in both images) showed clear radiopaque X-ray images, which indicates that both materials have comparable radiopacities.

A major goal of the NDGP platform was to improve the mechanical properties of GP, which may lead to improved handling properties during clinical implementation. In general composite materials, additives (e.g., NDs) are associated with organic polymers, reinforcing the mechanical properties by enhancing the interface strength between the additives and polymer. We prepared NDGP with 5 and 10 wt % of NDs to elucidate the effect that NDs would have on the elastic modulus, tensile strength, 0.2% offset yield strength, and percent elongation of GP (Figure 3B–F).

From the initial straight portion of stress–strain curve, the elastic modulus of each sample was derived. As shown in Figure 3C, the addition of 5 and 10 wt % of ND into the GP matrix improves the elastic moduli by 49% and 247%, respectively. In addition, the tensile strength and 0.2% offset yield strength increased 74% and 34% for 5 wt % ND addition and 171% and 85% for 10 wt % ND addition, respectively (Figure 3D and E). However, percent elongation of GP was decreased by 11% upon 5 wt % ND addition and 24% upon 10 wt % ND addition (Figure 3F). Combined, these results showed that the incorporation of NDs into the GP matrix improved the elastic modulus and mechanical strength of the NDGP biomaterial with a minor loss of ductility.

Antimicrobial Efficacy Studies Using NDGP. The primary objective of this study was to confirm amoxicillin functionality following its loading onto ND and incorporation into NDGP. Various bacterial strains can be encountered during reinfection scenarios following root canal therapy. Therefore, a Staphylococcus aureus (S. aureus) strain (ATCC 6538) was selected as the model bacterium for this NDGP antimicrobial efficacy study, as it has been previously observed in endodontic reinfection cases. Recent studies have shown that various S. aureus strains can express beta-lactamase, an enzyme that hydrolyzes beta-lactams. Because of this beta-lactamase expression, reported minimum inhibitory concentrations (MICs) of amoxicillin against S. aureus 6538 have ranged from 31.25 to over 100 μg/mL.

For this efficacy study, an NDGP sample was generated with an initial ND:amoxicillin ratio of 5:6 (w/w), where 8 mg of drug was ultimately incorporated into the NDGP sample. This quantity of loaded amoxicillin far exceeds reported amoxicillin MICs for S. aureus 6538, and NDGP should exhibit an inhibitory effect against this particular strain of bacteria as long as drug functionality is preserved upon incorporation into NDGP.

Two avenues of antimicrobial activity are possible for this NDGP platform: (i) bacterial killing through amoxicillin release from NDGP and/or (ii) contact-mediated inhibition upon bacterial deposition onto the NDGP surface. To determine whether or not amoxicillin is freely eluted from the NDGP composite, an agar diffusion test was conducted with unmodified GP and NDGP. Neither unmodified GP nor NDGP produced a zone of inhibition after incubating on a lawn of S. aureus for 24 h, indicating a negligible amount of amoxicillin elution from NDGP (Figure 4A). This was an important observation because avoidance of elution from NDGP was a prerequisite for contact-mediated inhibition, the desired mechanism of antimicrobial activity for our NDGP platform.

To determine the degree by which S. aureus was killed upon surface contact with NDGP, bacteria were deposited onto both unmodified GP and NDGP. Bacteria were visualized with a live/dead stain. After 70 h of contact, fluorescent images of both the unmodified GP surface and the NDGP surface were generated at 10× magnification. Unmodified GP exhibited no apparent inhibition of S. aureus (Figure 4B, row 1). In contrast, there was a significant amount of bacterial death on the NDGP surface (Figure 4B, row 2). Bacteria on the NDGP surface also formed large aggregates, whereas the bacteria on the GP surface remained dispersed. This effect may have been due to the bacteria’s attempt to minimize exposure to the NDGP surface, thus reducing cell death. When S. aureus was treated for 24 h with amoxicillin (150 μg/mL) or ND (10 mg/mL) alone, bacterial death was also observed, showing that S. aureus responds to both amoxicillin and ND treatment (Figure 4B, rows 3 and 4). Taken together, these results suggest that drug functionality was preserved during NDGP synthesis and that NDGP prevents bacterial contamination through a contact-mediated inhibition mechanism.

It should be mentioned that a key reason for the use of ND particles to sequester and localize amoxicillin activity and mediate contact-based inhibition is that the reduction of antibiotic elution mediated by the NDs may play an important role in reducing antibiotic resistance. In addition, the absence of drug elution coupled with contact-mediated inhibition reduces the dosage of amoxicillin that is loaded into the NDGP. At the same time, the NDs can play a structural role in enhancing the mechanical properties of the NDGP composite. To potentially accelerate NDGP translation toward clinical use, the use of carbon in the oral cavity and digestive tract has been demonstrated using activated charcoal tablets. In addition, coupling antibiotics that have been approved for human use with NDs may serve as a promising route for first-in-human studies of NDGP. As a novel material, the NDGP was designed to remain confined within the root canal to mediate contact-based inhibition. Therefore, the NDGP minimizes contact with dental tissue beyond...
Figure 3. Radiopacity and mechanical properties of NDGP. (A) Left: Photograph of NDGP (top) and unmodified GP (bottom). Right: Digital X-ray image of NDGP (top) and unmodified GP (bottom). An X-ray image showed that the radiopacity of NDGP was comparable to that of unmodified GP. (B) Stress–strain curves of GP and NDGP (5 wt % ND, 10 wt % ND) obtained by tensile test. The tests were performed with a 0.3 cm/min strain rate with sample gauge lengths set at 0.89 cm. They clearly showed the areas under the curves of NDGPs (5% and 10%) are larger than unmodified GP, which means NDGPs are more mechanically robust than unmodified GP. (C) Elastic moduli of GP (240 ± 26.3 MPa), NDGPs with 5 wt % ND (357 ± 52.0 MPa), and NDGP with 10 wt % ND (833 ± 96.5 MPa) calculated by initial linear portions of stress–strain curves. Data are represented as mean ± SD; *, p < 0.05; **, p < 0.01; ***, p < 0.005 (n = 3). (D) Tensile strengths of GP (7 ± 0.3 MPa), NDGP with 5 wt % ND (12 ± 1.2 MPa), and NDGP with 10 wt % ND (19 ± 0.3 MPa) obtained from the highest stress point. Data are represented as mean ± SD; *, p < 0.05; **, p < 0.005; ***, p < 0.00001 (n = 3). (E) 0.2% offset yield strengths of GP (7 ± 0.3 MPa), NDGP with 5 wt % ND (9 ± 0.5 MPa), and NDGP with 10 wt % ND (12 ± 2.0 MPa) obtained from stresses that correspond to the points of intersections of a stress–strain curve and lines that are parallel to the initial port of straight lines. It can be used to approximately determine elastic limits. Data are represented as mean ± SD; *, p < 0.005 (n = 3). (F) Percent elongation of GP (10 ± 1.2 MPa), NDGP with 5 wt % ND (9 ± 0.4 MPa), and NDGP with 10 wt % ND (8 ± 0.7 MPa) measured by changes in lengths from the original length. Data are represented as mean ± SD; *, p < 0.05 (n = 3).
the apex of the tooth that is sometimes observed with conventional sealers. Furthermore, adding a drug directly into the sealer would likely result in drug elution and depletion following burst release, which is not an intended use of the NDGP platform. Subsequent iterations of NDGP may potentially explore the use of emerging antibacterial agents.58–62

**X-ray Imaging of Root Canal Obturation.** Extracted human teeth obturated with unmodified GP and NDGP were imaged using digital radiography (XDR) to compare the quality of obturation between the two different materials. Digital X-ray imaging provides clinicians with a preliminary two-dimensional view of obturated root canals, allowing for detection of voids and quality of the root canal fill.63–65 Root canal obturation of human teeth with NDGP revealed no apparent void formation after conventional technique, e.g., lateral condensation,66 particularly in the critical apical third region (Figure 5). This suggests that NDGP can be used for obturation of root canals using conventional obturation techniques commonly used in clinical settings. In addition to the comparable quality of obturation with NDGP and unmodified GP, the radiopacities of both materials were indistinguishable (Figure 5). These images suggest that NDGP could be clinically implemented using conventional endodontic therapy procedures.

**Microcomputed Tomography Imaging of Root Canal Obturation.** Although digital radiography is used as a clinical standard for determining the quality of obturation and prognosis of endodontic therapies, periapical radiography depicts only a two-dimensional view of the complex root canal structures. We also employed imaging with microcomputed tomography (μCT) to assess the effectiveness of NDGP and unmodified GP in root canal obturation and assessment of void formation. While μCT imaging cannot be utilized during RCT to assess the quality of obturation, enhanced resolution offered by μCT provided important insight as part of this study to compare the effectiveness of NDGP and unmodified GP in root canal obturation. A minimal number of small voids were observed in the canals following obturation with NDGP, comparable to the number of voids also observed in those obturated with unmodified GP (Figure 5). The minimal voids present in the NDGP-filled tooth were in the middle third of the canal, which is a clinically acceptable criteria for a successful root canal treatment.

**CONCLUSION**

This study demonstrated that functionalizing conventional GP cones with amoxicillin-loaded NDs may increase the success rate of endodontic therapies by eliminating pre-existing microbes and preventing reinfection of the root canal system. The mechanical robustness of GP was also increased upon ND incorporation, improving the handling properties during clinical implementation. Future studies will be useful in enhancing the GP interface with the surrounding tooth structure to ultimately improve fracture resistance in weakened endodontically treated teeth. Because of its antimicrobial properties and increased durability,
NDGP may enhance the success rate of conventional endodontic therapies and reduce the need for additional treatments, including retreats and apical surgeries. Because NDGP is compatible with traditional obturation techniques, subsequent studies will focus on the clinical validation of NDGP.

**MATERIALS AND METHODS**

**Materials.** NDs were purchased from the NanoCarbon Research Institute Co., Ltd. (Nagano, Japan) and used to prepare a 50 mg/mL aqueous ND stock solution. Phosphate buffer saline (PBS) was purchased from Thermo Fisher Scientific (Canoga Park, CA, USA), and fetal bovine serum (FBS) was purchased from Gemini Bio Products (West Sacramento, CA, USA). Amoxicillin, trans-1,4-polyisoprene, zinc oxide (ZnO2), barium sulfate (BaSO4), sodium hydroxide (NaOH), and chloroform were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium bromide (KBr) powder, and the mixed powder samples were synthesized by mixing NDs with amoxicillin at 5:2 and 5:3 (w/w) ratios of ND to AMC in 2.5 mM NaOH. The mixture was vortexed and incubated for 5–7 days at room temperature. After incubation, the mixture was centrifuged down and washed with deionized water. The final product was collected via centrifugation at 20000g for 20 min and resuspended in water at a ratio of 5:1 (w/v) ND to water. To quantify amoxicillin loading onto ND, the concentration of unbound amoxicillin recovered after the washing process was used to calculate amoxicillin loading efficiency. The absorbance of the recovered amoxicillin supernatant was measured by UV–vis spectroscopy at 270 nm. The concentration of unbound amoxicillin in the supernatant was calculated from an amoxicillin standard curve with an amoxicillin concentration range of 0 to 200 μg. The amount of unbound amoxicillin was subtracted from the amount loaded to determine the concentration of amoxicillin incorporated onto ND.

To verify amoxicillin binding to ND, Fourier transform infrared spectroscopy was performed with a PerkinElmer FTIR Spectrum 2000 (PerkinElmer, Waltham, MA, USA) over a wavenumber range of 400–4000 cm⁻¹. A 2 mg amount of lyophilized ND-AMC samples was mixed with 100 mg of potassium bromide (KBr) powder, and the mixed powder samples were pressed to a thin disc for FTIR analysis. The FTIR spectra were recorded with a resolution of 1 cm⁻¹ and an accumulation of

![Figure 5. X-ray and microCT (μCT) images of patient-derived teeth samples obturated with unmodified GP and NDGP. (A) Central incisor prepared and obturated with unmodified GP. Left: Three-dimensional rendering of the GP-filled central incisor generated from a μCT image. Middle: A bucco-lingual X-ray view of the incisor revealed no visible voids within the obturated canal. Right: μCT imaging of GP-filled central incisor revealed no visible voids within the apical region of the obturated canal, although minor voids are present in the middle third region of the obturated canal. Coronal, middle, and apical cross sections do not show any voids. (B) Central incisor prepared and obturated with NDGP. Left: Three-dimensional rendering of the NDGP-filled central incisor generated from a μCT image. Middle: A bucco-lingual X-ray view of the incisor also revealed no visible voids within the apical region of the obturated canal and minor voids in the middle third region. There were no discernible differences between the X-ray images of the GP-filled central incisor and the NDGP-filled central incisor. Right: μCT imaging of the NDGP-filled central incisor revealed no visible voids within the obturated canal. Coronal, middle, and apical cross sections also contained no voids. There were no discernible differences between the μCT images of the GP-filled central incisor and the NDGP-filled central incisor. (C) Premolar prepared and obturated with NDGP. Left: Three-dimensional rendering of the NDGP-filled premolar generated from a μCT image. Middle: A bucco-lingual X-ray view of the incisor revealed no visible voids within the obturated canal. Right: μCT imaging of the NDGP-filled premolar revealed no visible voids even within the obturated oval canal. Coronal, middle, and apical cross sections also contained no voids.](image-url)
64 scans. The hydrodynamic size and ζ-potential of ND and ND-AMC suspensions (0.2–0.3 mg mL⁻¹) were also measured with a Zetasizer Nano ZS (Malvern Instruments, United Kingdom). Hydrodynamic size measurements were performed at 25 °C with a 173 μm backscattering angle. Three size measurements were performed. The mean hydrodynamic size and its standard deviation were calculated by averaging the reported z-average size values and determining their standard deviation. The ζ-potential was also measured at 25 °C in automatic mode. Quantifying Drug Release from ND-AMC Conjugates. Amoxicillin release experiments were performed by pipetting 0.5% v/v FBS. A 1 mL amount of ND-AMC solution was centrifuged for 20 min at 14000g to obtain an ND-AMC pellet. This ND-AMC pellet was resuspended in 1 mL of 0.5% v/v FBS/PBS solution by gentle pipetting, followed by incubation for 1 h at 37 °C. At each predetermined time point, the ND-AMC solution was centrifuged for 20 min at 14000g, the supernatant was recovered, and the pellet was resuspended in 1 mL of PBS solution and incubated at 37 °C until the next time point. At the next time point, this procedure was repeated.

To determine the amount of amoxicillin released at each time point, the recovered supernatant was filtered with 0.2 μm centrifugal filters to remove FBS. The absorbance value of the amoxicillin-containing supernatant was subsequently measured with a UV–vis spectrophotometer. The concentration of amoxicillin in the supernatant was calculated according to the manufacturer’s protocol. A 10 μL amount of bacterial suspension was added to the bacterial suspension according to the manufacturer’s protocol. Flattened bacterial suspensions were dropped onto soybean-casein digest agar plates and spread evenly over the entire plate. The plates were incubated at 37 °C until a uniform lawn of bacteria was formed. To assess drug release from NDGP, S. aureus was cultured in soybean-casein digest medium under aerobic conditions at 37 °C until late log phase. Then 200 μL of bacterial suspension was dropped onto soybean-casein digest agar plates and spread evenly over the entire plate. The plates were incubated at 37 °C until a uniform lawn of bacteria was formed.

To assess the viability of bacteria in direct contact with the NDGP surface, S. aureus was cultured in soybean-casein digest medium under aerobic conditions at 37 °C until late log phase. The bacteria were resuspended in 0.85% w/v NaCl, and live/dead BacLight viability assay reagent was added to bacterial suspension according to the manufacturer’s protocol. Flat disks of unmodified GP and NDGP (5:6 w/v ND to AMC, synthesized as previously described) were attached to glass microscope slides. A 20 μL amount of bacterial suspension was pipetted onto the NDGP and GP surfaces. Glass coverslips were placed on the dishes to evenly distribute the bacterial suspensions. The slides were sealed with nail polish and incubated for 70 h. After incubation, the bacteria-coated surfaces of NDGP and unmodified GP were visualized with an Olympus IX81 fluorescent microscope at 10× magnification. Fluorescent images were processed with ImageJ software.

X-ray Imaging. Digital radiographic images were taken with size 2 XDR Digital Intraoral sensors (XDR Radiology, Los Angeles, CA, USA). The images were then transmitted and analyzed by the XDR program. The patient-derived teeth samples were radiographed at 0.016 Gy. This served as a clinically relevant level of radiation that is typically observed for a digital periapical radiograph during a conventional root canal procedure. The X-ray source—object and object–sensor distances were kept constant for all exposures per conventional sample testing protocols.

Computed Tomography Imaging. Teeth samples were imaged using a SkyScan 1172 μCT scanner (Bruker, Billerica, MA, USA) at 20 μm resolution, utilizing 55 kvp, 181 μA, and 0.5 Al filtration. Volumetric image data were converted to DICOM format and imported into the Dolphin Imaging software (Dolphin Imaging & Management Solutions, Chatsworth, CA, USA) to generate 3D and multiplanar reconstructed images.
condensation force. Then, about 1 mm of excess gutta percha was seared off. While the gutta percha was voids, the Glick instrument was heated at its end and then and C. After radiographic confirmation that there were no apical voids, the Master cones all had tugback and reached working length formulation in the apexes.

The lateral condensation obturation technique was used. After thoroughly drying the canal with paper points, master cones were selected. Master cones all had tugback and reached working length after radiographic assessment. The master cone sizes of teeth A, B, and C were 40, 45, and 45. After eugenol Roth sealer (Roth Endodontic Journal 2015, 48, 736–746.

C. After radiographic confirmation that there were no apical voids, the Glick instrument was heated at its end and then the canal orifices were sealed off. While the gutta percha was still warm, an unheated plugger was used to apply a vertical condensation force. Then, about 1–2 mm of excess gutta percha was removed below the cementoenamel junction, and the remaining sealer in the pulp chamber was cleaned with alcohol on a cotton pellet. The teeth were then taken out of the ModuPRO segment for a final radiograph to assess the quality of obturation.

Statistical Analysis. Experiments were conducted in triplicate. Quantifiable results were averaged, and standard deviations were calculated. Analysis of mechanical differences between GP and NDGP was conducted with an unpaired, two-tail Student’s t test. A p-value of <0.05 was considered statistically significant.

Conflict of Interest: The authors declare the following competing financial interest(s): D.K.L., S.V.K., A.N.L., A.Y., and D.H. are co-inventors of a provisional patent pertaining to nanodiamond-linked with Carbon Nanotubes. Biomater. Sci. 2015, 3, 46–58.

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