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Clindamycin-modified Triple Antibiotic Nanofibers — A Stain-free Antimicrobial Intracanal Drug Delivery System

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Abstract

Introduction—A biocompatible strategy to promote bacterial eradication within the root canal system following pulpal necrosis of immature permanent teeth is critical to the success of regenerative endodontic procedures. This study sought to synthesize clindamycin-modified triple antibiotic (metronidazole/MET; ciprofloxacin/CIP; clindamycin/CLIN) polymer (Polydioxanone, PDS) nanofibers and determine *in vitro* their antimicrobial properties, cell compatibility, and dentin discoloration.

Methods—CLIN only and triple antibiotic CLIN-modified (CLIN-m, minocycline-free) nanofibers were processed via electrospinning. Scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), and tensile testing were carried out to investigate fiber morphology, antibiotic incorporation, and mechanical strength, respectively. Antimicrobial properties of CLIN only and CLIN-m nanofibers were assessed against several bacterial species by direct nanofiber/bacteria contact and over time based on aliquot collection up to 21 days. Cytocompatibility was measured against human dental pulp stem cells (hDPSC). Dentin discoloration upon nanofiber exposure was qualitatively recorded over time. The data were statistically analyzed ($p<0.05$).

Results—The mean fiber diameter of CLIN-containing nanofibers ranged between 352±128 nm and 349±128 nm and was significantly smaller than PDS fibers. FTIR analysis confirmed the presence of antibiotics in the nanofibers. Hydrated CLIN-m nanofibers demonstrated similar

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tensile strength to antibiotic-free (PDS) nanofibers. All CLIN-containing nanofibers and aliquots demonstrated pronounced antimicrobial activity against all bacteria. Antibiotic-containing aliquots led to a slight reduction in DPSC viability but were not considered toxic. No visible dentin discoloration upon CLIN-containing nanofiber exposure was observed.

Conclusion—Collectively, based on the remarkable antimicrobial effects, cell-friendly, and stain-free properties, our data suggest that CLIN-m triple antibiotic nanofibers might be a viable alternative to minocycline-based antibiotic pastes.

Keywords

Electrospinning; antibiotic; nanofibers; clindamycin; disinfection; regeneration; stem cells

Introduction

Tooth loss in young children as a result of deep caries or trauma-induced pulpal necrosis can lead to complications in craniomaxillofacial growth and development, thus impacting their psychosocial well-being (1, 2). From a clinical standpoint, the management of pulpal necrosis in immature permanent teeth is challenging due to the abrupt interruption of root development resulting in thin dentinal walls, wide-open apices, and increased risk of cervical fracture (3–6). Calcium hydroxide and mineral trioxide aggregate apexification have been widely used to treat immature permanent teeth with necrotic pulps in an effort to obtain an aseptic environment and a calcified apical barrier (7). However, neither apexification therapy has induced complete root development (length and thickness) (5, 6), which compromises the long-term mechanical integrity of the tooth (3, 5–7).

A fairly novel alternative approach to apexification is regenerative endodontics, which aims to promote periapical healing, restitution of pulpal function, and root maturation through a combinatorial disinfection and intracanal stem cell recruitment approach with the use of antibiotic pastes (e.g., triple antibiotic paste [TAP]) and evoked bleeding from the periapical tissues, respectively (7, 8). A seminal study by Sato et al. (9) demonstrated significant bacterial elimination in deep root canal dentin when using a mixture of metronidazole (MET), ciprofloxacin (CIP), and minocycline (MINO) in a paste-like consistency. Specifically, MET is a bactericidal imidazole that is highly effective against obligate anaerobic bacteria (10), CIP is a bactericidal broad-spectrum synthetic quinolone (11), and MINO is a bacteriostatic broad-spectrum tetracycline (9, 12). Despite the documented clinical efficacy associated with the use of TAP (1 g/mL), recent evidence demonstrates not only toxic effects on various cell types (13–15), but also significant dentin discoloration (9, 16, 17) and potential anti-angiogenic activity due to the presence of MINO (18–20). Meanwhile, clindamycin (CLIN), a bacteriostatic lincosamide (21, 22) known for its efficacy against a broad spectrum of endodontic bacteria (i.e., gram-positive aerobes and most anaerobic bacteria), seems a clinically viable alternative to MINO. Thus, this study sought to synthesize clindamycin-modified triple antibiotic polymer nanofibers as a biocompatible, stain-free and potentially pro-angiogenic intracanal drug delivery system for regenerative endodontics.

Materials and Methods

Synthesis and Characterization of CLIN-containing Antibiotic Nanofibers

CLIN only and CLIN-modified (CLIN-m, minocycline-free) triple antibiotic (CLIN, CIP, and MET) nanofibers were processed via electrospinning. Polydioxanone suture filaments (PDS II®, Ethicon, Somerville, NJ, USA) were cut into pieces and soaked in dichloromethane (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 48 h to remove the sutures' purple color (23–25). Next, undyed PDS suture filaments were dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, Sigma-Aldrich) at 10 wt.% under stirring conditions. CLIN- and CLIN-m-containing polymer (PDS) solutions were separately synthesized by dissolving 210 mg (i.e., 35 wt.% relative to the total PDS weight, 600 mg) of each antibiotic, followed by 48 h of vigorous stirring. Electrospinning under optimized parameters (1.5–2.0 mL/h, 18 cm distance, and 18 kV) was performed using a laboratory designed apparatus (26). Antibiotic-free PDS fibers (control) were synthesized, as previously reported (23–26). After electrospinning, the fibers were vacuum dried (48 h), followed by storage at 4°C until used (27).

Fiber morphology was evaluated using a field-emission scanning electron microscope (FE-SEM, Model JSM-6701F, JEOL, Tokyo, Japan). The samples were mounted on Al stubs and sputter-coated using Au-Pd prior to imaging. The mean fiber diameter was calculated from 25 single-fibers per image (4 images/group) using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (24). Fourier transform infrared spectroscopy (ATR/FTIR-4100, JASCO, Easton, MD, USA) was performed for each antibiotic powder and the processed fibers to confirm incorporation of the chosen antibiotics (24). The mechanical strength of the CLIN-containing fibers (15×3 mm^2, n = 10/group) was gauged under dry and wet conditions (24 h incubation in phosphate-buffered saline, PBS) was determined by tensile testing (26).

Antimicrobial Properties

The antimicrobial efficacy of electrospun nanofibers and antibiotics-containing aliquots generated through nanofiber samples' incubation (over time assessment) were evaluated against *Actinomyces naeslundii* (*An,* ATCC 43143), *Enterococcus faecalis* (*Ef,* ATCC 29212), *Aggregatibacter actinomycetemcomitans* (*Aa,* ATCC 33384), and *Fusobacterium nucleatum* (*Fn,* ATCC 25586) through agar diffusion-based assays (28).

Disc-shaped (ϕ = 5 mm) samples were weighed and disinfected by UV light (30 min each side). *Fn* and *Aa* were anaerobically cultured for 24 h in 5 mL of Brain heart infusion supplemented with 5 g/L of yeast (BHI+YE) and 5% vol Vitamin K + hemin. Meanwhile, *Ef* and *An* were aerobically cultured for 24 h in 5 mL of Tryptic soy broth (TSB). 100 μL of each broth was swabbed onto blood agar plates to form a bacterial lawn that was then divided into 3 zones: 10 μL of 0.12% chlorhexidine (CHX; positive control), 10 μL of distilled water (negative control), and the fiber disc-shaped samples (23, 29). After 2 days of incubation, the zones of growth inhibition were measured (in mm).

For the aliquots, square-shaped (15×15 mm) samples (n=3/group/bacteria, 4.0±0.2 mg) from each nanofibrous mat were cut, disinfected, and rinsed (2×) with sterile PBS. Each

sample was placed in an individual glass vial with sterile PBS (5 mL at 37°C). 500 μL aliquots were drawn on days 1, 7, 14, and 21 and replaced with an equivalent amount of fresh PBS. The aliquot samples were stored at −20 °C until used. Bacterial plates were prepared and cultured as aforementioned and after 2 days of either aerobic or anaerobic incubation, the diameters (in mm) of the clear zones of growth inhibition were measured (23, 28).

Colony-Forming Units (CFU/mL)

An and *Ef* were specifically selected, based on their association with immature trauma-induced pulpal necrosis (30). Square-shaped (15 × 15 mm) electrospun samples (n=6/group/specie) were cut, disinfected, fixed to a plastic sample mount (CellCrown; Scaffdex Ltd., Tampere, Finland), and placed individually into 24-well plates. Both *An and Ef* were aerobically cultured overnight in 50 mL of TSB and 2 mL of inoculated broth was placed into each well to be aerobically incubated for 3 days (28). The samples were removed, rinsed with saline (2×), and placed in 3 mL vials with PBS (n=4/group/specie), which were sonicated and vortexed to remove biofilm bacteria for enumeration. A 1:100 saline dilution was prepared. 100 μL of dislodged biofilm solution was spiral plated onto blood agar plates, which were aerobically incubated (37°C for 24 h) and counted. Two samples per group were fixed in buffered 2.5% glutaraldehyde solution (Sigma-Aldrich) and dehydrated in ascending ethanol solutions prior to SEM imaging.

Cytocompatibility

UV light-disinfected rectangular-shaped (4.0±0.2 mg; n=4/group) samples were individually placed into the wells of 24-well plates containing 5 mL of sterile alpha-Modified Eagle's Medium (α-MEM, Gibco Invitrogen Corporation, Grand Island, NY, USA), supplemented with 10% FBS (Atlanta Biologicals Inc., Flowery Branch, GA, USA), and incubated at 37°C. Aliquots (500 μL) were collected at 1, 7, 14, 21, and 28 days to evaluate cell toxicity over time (23). Human dental pulp stem cells (hDPSCs, Lonza, Walkersville, MD, USA) obtained from permanent third molars were cultured in low glucose DMEM containing 10% FBS and 1% penicillin–streptomycin (Sigma-Aldrich) in a humidified incubator at 37°C with 5% CO_2. The cells were seeded at a density of 3×10^3/well (100 μL cell suspension) on 96-well tissue culture plates. After 4 h of incubation, the media was removed and replaced with the collected aliquots (100 μL) that were adjusted to 10% FBS and 1% penicillin–streptomycin. After incubation, 40 μL of CellTiter 96 AQueous One Solution Reagent (Promega Corporation, Madison, WI, USA) was allowed to react with the media for 2 h prior to reading the absorbance at 490 nm in a microplate reader (BioTek Instruments Inc., Winooski, VT, USA) against blank wells. The DPSCs cultured with the media was used as the positive control (23).

Dentin Discoloration

Antibiotic-free (PDS), CLIN only, and CLIN-m electrospun fibers were processed, as detailed previously. The electrospun samples (n=3/group) were disinfected by UV light (30 min each side) and individually mounted in plastic inserts (CellCrown) (27). Fifteen human, caries-free, non-restored canines were used in conformity with the rules and guidelines of the Indiana University Institutional Review Board (IRB #1407656657). The teeth were

longitudinally sectioned using a diamond disk mounted on a straight low-speed handpiece (27). All specimens were rinsed in saline solution for 10 min. The tooth slices were placed in 24-well plates and exposed to the electrospun fibers. A rubber ring was used to standardize the distance between the fibers mounted in CellCrowns and the tooth slice surface (27). Next, 2 mL of PBS was pipetted into the wells containing the CellCrown/nanofiber (i.e., CLIN only, CLIN-m, and antibiotic-free PDS fibers). Triple antibiotic paste (TAP, Champs Medical, San Antonio, TX, USA) was also prepared at 1 g/mL (MET, CIP, and MINO in equal proportions) and pipetted (2 mL) into the wells for the TAP group. Tooth slices were also incubated with PBS (control). PBS and TAP were replaced at each interval (17). Macrophotographs of each group were taken at days 1, 7, 14, and 21 to qualitatively demonstrate the non-staining properties of the clindamycin-containing nanofibers when compared to the TAP and control group (antibiotic-free nanofibers).

Statistical Analysis

Two-way analyses of variance (ANOVAs) followed by Holm-Sidak's multiple comparisons were used to evaluate differences tensile properties, antimicrobial properties (colony forming unit assays), and cell viability (cytotoxicity). The significance level was set at $p<0.05$.

Results

Characterization of CLIN-containing Nanofibers

SEM micrographs demonstrated a similar fiber diameter for the CLIN (352±128 nm) and CLIN-m (349±128 nm) fibers. Antibiotic-free PDS fibers showed a significantly ($p<0.05$) larger diameter (847±172 nm). The inset graphs show the fiber diameter distribution for all groups (Figure 1A). FTIR confirmed the antibiotic's incorporation into CLIN and CLIN-m nanofibers (Figure 1B). The dry tensile strength of PDS and CLIN were similar (6.3±2.0 and 7.2±1.0 MPa, respectively) and significantly higher than the CLIN-m fibers (2.60±1.6 MPa) (Figure 1C). However, upon 24 h of hydration, the tensile strength of the CLIN nanofibers decreased significantly (1.3 ± 0.26 MPa) compared to the CLIN-m and antibiotic-free PDS fibers.

Antimicrobial Properties

The antimicrobial effects of the CLIN and CLIN-m nanofibers were dependent on the bacterial species (Figure 1D). Both nanofiber groups inhibited the bacterial growth of all species tested. The CLIN-m nanofibers demonstrated greater inhibition zones when tested with *Aa* and *Ef*; whereas, the CLIN nanofibers exhibited larger inhibition zones when tested with *An*.

The antimicrobial properties were also evaluated over time. Aliquots containing CLIN (CLIN only) or a mixture of CLIN, CIP, and MET (CLIN-m) obtained through nanofibers' incubation over 21 days were tested. A fairly homogeneous and consistent antimicrobial activity with a slight decrease in inhibition over 21 days was seen (Figure 2A–D). Overall, CLIN-m demonstrated greater bacterial growth inhibition than CLIN and CHX against the endodontic bacteria tested.

Colony-Forming Units (CFU/mL)

CLIN-m nanofibers significantly ($p<0.05$) inhibited biofilm growth of both *Aa* and *Ef* (Figure 2E). SEM analysis provided further qualitative evidence that CLIN-m antibiotic nanofibers inhibited growth of the bacterial species tested (Figure 2F). No significant biofilm growth inhibition was observed for the antibiotic-free PDS and CLIN groups, regardless of the bacteria tested.

Cytotoxicity

A significant decrease in hDPSC viability was observed after exposure to the aliquots obtained from CLIN and CLIN-m nanofibers when compared to PDS and the control groups (Figure 3A). However, both CLIN and CLIN-m nanofibers showed cell viability above 70% and 50% respectively, at all time points.

Dentin Discoloration

Representative macrophotographs attesting no discernible dentin discoloration for the CLIN-containing groups (i.e., CLIN and CLIN-m) are shown in Figure 3B. Meanwhile, significant color changes can be seen on dentin specimens treated with triple antibiotic paste (TAP), particularly when comparing the initial macrophotograph (Day 1) taken after sample preparation with the image recorded after 3 weeks (dark-brown color).

Discussion

Regenerative endodontics has drastically changed the management and overall clinical prognosis of immature teeth with necrotic pulps (7, 8). The current regenerative-based therapy, also known as the “evoked bleeding” method, is comprised of a thorough disinfection of the root canal system (RCS), followed by intentional laceration of the periapical tissues, allowing stem cell-rich blood invasion into the RCS to stimulate regeneration of the pulp-dentin complex (7, 8).

Historically, the successful resolution of endodontic-related infections has required a combination of multiple antibiotics (4–7, 9, 10). MET and CIP together have an excellent antibacterial spectrum with established effects against obligate anaerobes and gram-negative bacteria, respectively (9). Nonetheless, despite the proven clinical efficacy associated with antibiotic mixtures containing MINO (i.e., TAP), serious adverse effects that not only offset the regenerative potential due to toxicity to dental stem cells (13–15) and potential inhibition of angiogenesis have been identified (18–20). A recent *in vitro* study demonstrated MINO as having a similar inhibition of angiogenesis to that of cortisone and heparin (31). Furthermore, MINO is thought to negatively affect angiogenesis by decreasing vascular endothelial growth factor (VEGF) secretion, which suppresses the neovasculogenesis of endothelial cells (20). Worth mentioning, in regenerative endodontics, oxygen and nutrient transport to DPSCs via angiogenesis is critical to recreation of the dentin-pulp complex (32).

In the late 1990’s, the first effort of using clindamycin-impregnated fibers as an intracanal drug delivery system was reported (33). The total amount of clindamycin present in a 1-cm-long ethylene vinyl acetate (EVA) fiber was 1.3 mg. The authors reported a continuous

release of antibiotic and significant antimicrobial properties over 1 week. The clindamycin/EVA fibers were shown to be effective in reducing growth of several endodontic pathogens using an *in vitro* extracted human teeth model. No discoloration was observed upon treatment (33). To the best of our knowledge, the present study is the first reporting on the synthesis of CLIN-modified triple (CLIN, MET, and CIP) antibiotic nanofibrous-based drug delivery system. Thus, the use of CLIN may be a promising substitute to MINO, not only based on its broad antibacterial spectrum and stain-free properties, but also due to reported *in vitro* pro-angiogenic activity (22).

The SEM micrographs presented the clindamycin-containing nanofibers as being smaller in fiber diameter than the antibiotic-free PDS control. Smaller fibers have been claimed to be superior, since they provide more surface area, which could allow more drug release over time (24). FTIR spectra confirmed the CLIN, CIP, and MET incorporation into the CLIN and CLIN-m nanofibers. The mechanical properties of the clindamycin-modified triple antibiotic nanofibers were evaluated through the employment of tensile tests under both wet and dry conditions. The nanofibers were determined to be able to mechanically withstand handling, which suggests its potential to endure placement in clinical conditions.

The antimicrobial activity of CLIN-containing nanofibers was measured against *Aa, An, Ef, and Fn*, which were selected based on their role in endodontic bacterial infections. Specifically, *Ef* is often associated with asymptomatic, persistent endodontic infections due to difficulty in bacterial eradication during traditional endodontic treatment (34). Agar diffusion-based assays confirmed the incorporation and release of antibiotics from the polymer nanofibers. Overall, both CLIN and CLIN-m nanofibers provided bacterial inhibition significantly greater than that of chlorhexidine for all bacteria tested, with varying degrees of success based on the bacterial specie. Specifically, the antimicrobial effects of both nanofibers on *Fn* were significantly greater than any other bacteria tested potentially due to *Fn* being an anaerobic, gram-negative bacterium, which CLIN has a well-established antimicrobial effect against. CLIN-m triple antibiotic nanofibers and aliquots demonstrated a significantly ($p < 0.05$) stronger antimicrobial efficacy against *Aa* and *Ef* when compared with the CLIN group due to the multiple antibiotics mixture. Moreover, the incorporation of MET and CIP, in addition to CLIN, demonstrated being essential to inhibiting the biofilm growth of both *Aa* and *Ef*.

Analysis of our cell viability data for CLIN-containing nanofibers (i.e., CLIN and CLIN-m) revealed slight toxicity of CLIN-m nanofibers to DPSCs (ranging from ~52% at Day 1 to ~63% at Day 28), with the CLIN only nanofibers producing a significantly ($p<0.05$) more cell-friendly effect compared to CLIN-m over 28 days (Figure 3A). This observation is likely due to the absence of MET and CIP being released from the CLIN nanofibers. Although the present study did not investigate the kinetics of drug release, the demonstrated long-term antimicrobial activity, in addition to an increase in cell viability over time, suggests a similar antibiotic release pattern, i.e., burst release, followed by sustained maintenance of the antimicrobial properties, as previously reported by similar studies involving the use of PDS polymer nanofibers as a drug delivery system (24). We previously demonstrated that human dentin treated with antibiotic-containing nanofibers support cell adhesion/proliferation (15). Cell spreading was comparable in antibiotic-free and triple

antibiotic eluting nanofibers, whereas, cells on TAP-treated dentin did not spread. Cell proliferation on Day 7 was significantly higher on dentin treated with antibiotic nanofibers compared to TAP. Taken together, the present data suggest that since CLIN and CLIN-m nanofibers could carry pro-angiogenic effect to cells it could make a superior candidate to TAP and triple antibiotic (minocycline-containing) nanofibers in regenerative endodontics.

In spite of the clinically proven antimicrobial properties associated with antibiotic mixtures containing MINO, the intracanal use of TAP has led to severe crown discoloration. In this study, dentin discoloration was qualitatively evaluated (i.e., macrophotographs) based on long-term (3 weeks) exposure to CLIN, CLIN-m, and TAP. As expected, neither CLIN nor CLIN-m nanofibers demonstrated any discernible dentin discoloration after 3 weeks, which further confirms the association between MINO and tooth discoloration, as well as enlightening the stain-free properties of the proposed CLIN-modified triple antibiotic nanofibers. The findings of the present study corroborate with a previous investigation over the discoloration potential of tetracycline-based (minocycline or doxycycline) antibiotic pastes or nanofibers (17). In brief, two distinct triple antibiotic pastes containing either MINO or DOX, in addition to four nanofibers-based 3D tubular-shaped drug delivery constructs formulated with MINO or DOX at distinct concentrations, were quantitatively evaluated (CIEL*a*b* parameters) over 28 days. The incorporation of MINO or DOX into 3D nanofibrous constructs produced similar dentin discoloration when compared with their respective triple antibiotic systems, although less pronounced discoloration was seen for DOX-containing paste or nanofibers (17).

Taken together, this study demonstrated that CLIN-m triple antibiotic nanofibers can be successfully processed via electrospinning. Furthermore, as highlighted in our previous and comprehensive review on the clinical perspective of the proposed strategy (35), the rationale for using biodegradable antibiotic-containing nanofibers as a three-dimensional (3D) intracanal drug delivery construct is the localized release of the antimicrobial agents at much lower concentration and in a predictable fashion onto the dentinal walls. Lastly, it should be acknowledged that although the angiogenic potential of the proposed CLIN-m nanofibers needs to be further investigated, the data gathered herein demonstrates its clinical potential in regenerative endodontics based on significant antimicrobial effects, low cytotoxicity, discoloration-free properties, and potential angiogenic effects. Thus, the clinical relevance of the proposed intracanal drug delivery system should be further assessed using preclinical animal models of periapical disease, which in turn could provide the means to test its efficacy in humans.

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Highlights

- Clindamycin-containing nanofibers were successfully synthesized
- Clindamycin-modified triple-antibiotic fibers (CLIN-m) showed great antimicrobial effects
- CLIN-m nanofibers did not promote significant cell death
- CLIN-containing nanofibers did not cause any visible dentin discoloration

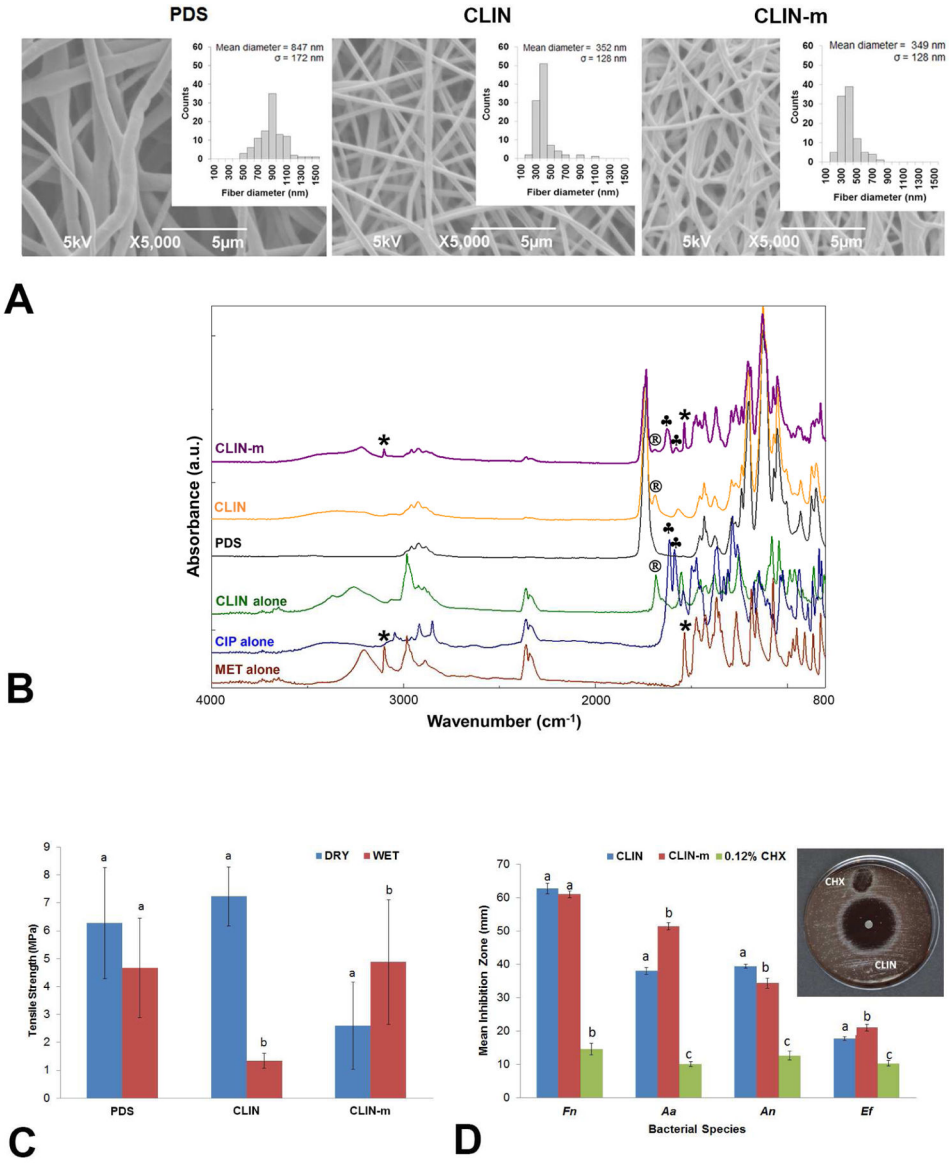

Figure 1.

(A) Representative SEM micrographs of antibiotic-free (PDS), CLIN, and CLIN-m electrospun fibers. The fiber diameter distribution and mean fiber diameter (±SD) are given in the inset; (B) FTIR spectra confirming CLIN, CIP, and MET incorporation into the CLIN and CLIN-m nanofibers; (C) Tensile strength of the obtained fibers under dry and wet conditions; (D) Mean inhibition zones of CLIN-containing fibers against *Ef, An, Aa*, and *Fn*. (D-Inset) Representative blood agar plate showing *Fn* growth inhibition.

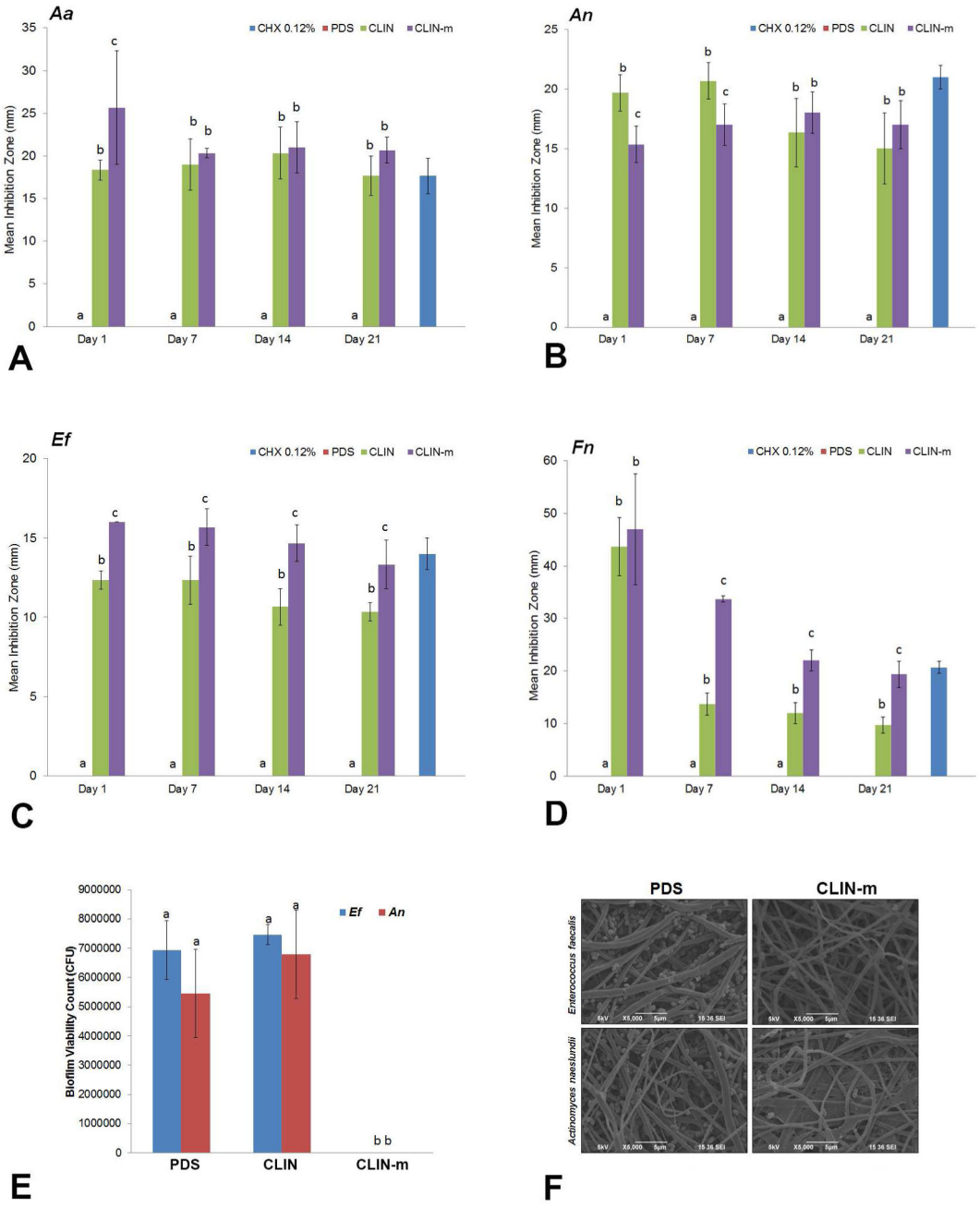

Figure 2.

Effects of CLIN-containing electrospun nanofibers on the growth of bacteria. Results from agar diffusion assays are represented as mean inhibition zone (in mm) against the different bacteria tested: (A) *A. actinomycetemcomitans* (B) *A. naeslundii* (C) *E. faecalis*, and (D) *F. nucleatum*. Same letters indicate non-significant difference compared with the results of the same day of aliquots. (E) Spiral plating was used to calculate CFU/mL of samples of dislodged *A. naeslundii* and *E. faecalis*. Significant difference is denoted with a different letter (*$p < .05$) when compared with the control. Representative SEM micrographs showing growth inhibition of *An and Ef* on CLIN-m electrospun nanofibers when compared to PDS.

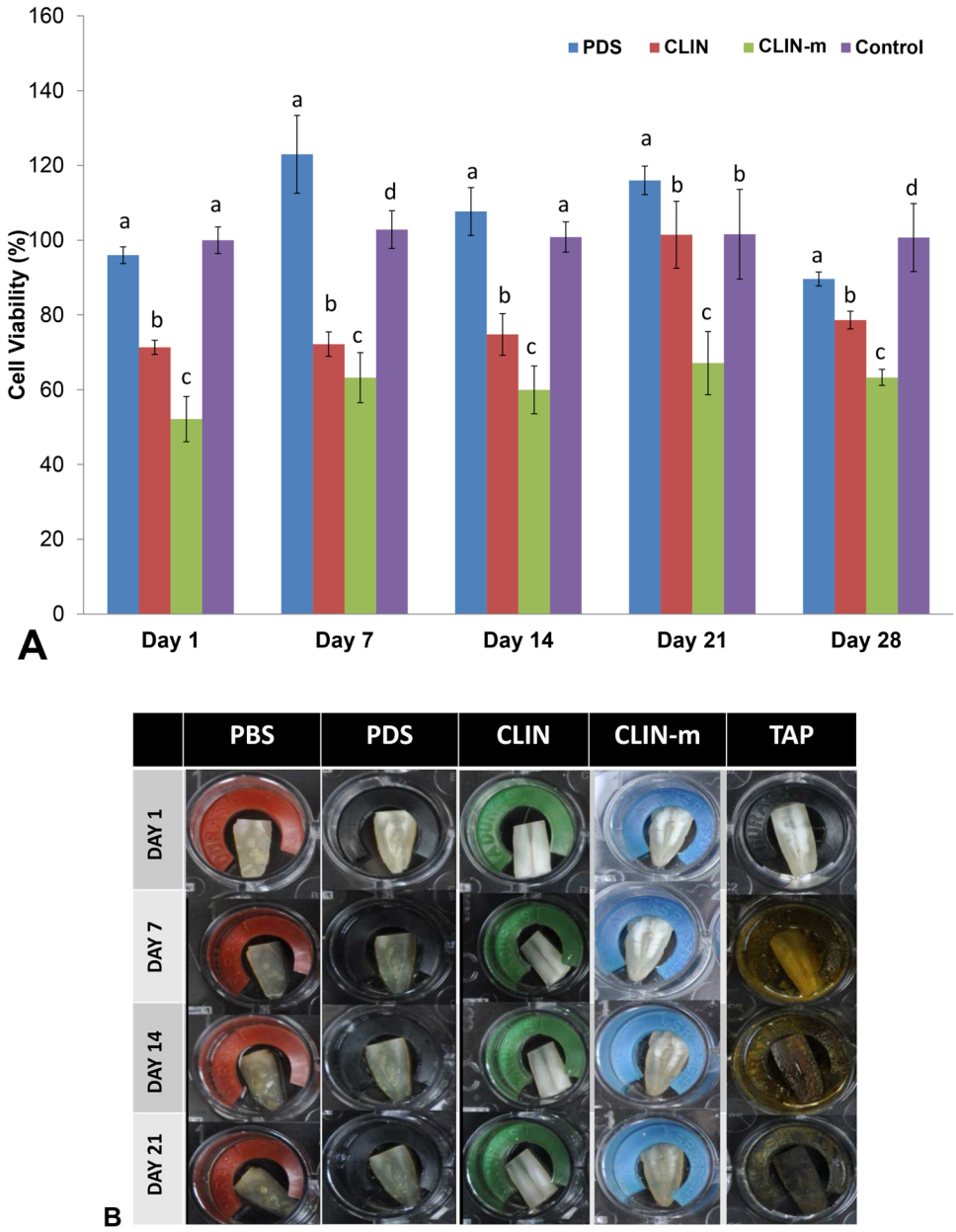

Figure 3.

(A) hDPSCs viability in response to aliquots on days 1, 3, 7, 14, 21, and 28 from electrospun nanofibers. Statistical analyses were compared with the same-day results. (B) Representative macrophotographs showing human dentin color stability/change after 1, 7, 14, and 21 days of exposure to control (PBS), antibiotic-free (PDS), CLIN, and CLIN-m nanofibers and triple antibiotic paste (TAP).