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**Original Article**

# Capsule-Conjugate Vaccination Against Porphyromonas gingivalis Confers Protection in a Preclinical Model of Periodontal Bone Loss

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## ABSTRACT

Periodontal disease is a chronic inflammatory condition of the supporting structures of the teeth, characterized by gradual degradation of both soft and hard tissues, and stands as the leading cause of tooth loss in adults. In the United States, more than 100 million people are estimated to be affected. The disease is driven by subgingival bacterial communities that trigger and sustain inflammation. Among the diverse microbial species implicated in periodontitis, Porphyromonas gingivalis has been identified as a central contributor to disease progression. Although current clinical management emphasizes rigorous maintenance to slow the advancement of disease, these approaches frequently fail to completely prevent tissue destruction, underscoring the need for an effective vaccine. To address this, we developed a conjugate vaccine consisting of purified P. gingivalis capsular polysaccharide linked to eCRM®, an optimized variant of the CRM197 carrier protein with engineered conjugation sites (Pg-CV). In mice, immunization with the alum-adjuvanted Pg-CV elicited robust IgG responses against the whole bacterium, surpassing the immune response induced by the unconjugated polysaccharide alone. In a murine oral bone loss model, animals vaccinated with the capsule-conjugate formulation showed significant protection against bone resorption induced by P. gingivalis. These results indicate that targeting capsular polysaccharide through a conjugate vaccine can effectively mitigate oral bone loss, the primary clinical hallmark of periodontal disease. The findings provide a strong rationale for advancing the development of a P. gingivalis capsule-based vaccine as a preventive intervention for periodontal disease.

**Keywords:** Porphyromonas gingivalis, Periodontal disease, Conjugate vaccine, Oral bone loss, Capsular polysaccharide

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## Introduction

Periodontal disease is a persistent inflammatory condition of the oral tissues, triggered by shifts in the subgingival microbiota, which leads to the gradual breakdown of the soft and hard structures supporting teeth. In advanced cases, it is the leading cause of tooth loss among adults [1]. In the United States, roughly 42% of adults are affected, making periodontal disease one of the most widespread chronic infectious disorders globally [2]. The associated economic impact, including loss of productivity, was estimated at $54 billion in 2013 [3]. While most prevalent in adults, the disease can also manifest in children and adolescents and is common among elderly populations. Periodontitis has additionally been linked to systemic conditions, such as diabetes, cardiovascular disease, rheumatoid arthritis, cancers of the orodigestive tract, and Alzheimer's disease [4-7]. Despite intensive clinical management aimed at halting disease progression and preserving remaining bone, these strategies often fail to prevent further tissue destruction, highlighting the need for novel preventive measures, including vaccination.

Although periodontitis involves multiple microbial species, Porphyromonas gingivalis frequently becomes dominant during disease onset and is recognized as a principal pathogen driving disease progression [8]. This Gram-negative anaerobic bacterium expresses several virulence factors, including fimbriae, dual forms of lipopolysaccharides, gingipains, and a polysaccharide capsule, which collectively enhance its pathogenicity [9-15]. Vaccine development efforts targeting these components have met with variable success [16-21]. While P. gingivalis exhibits significant strain variability, encapsulated strains are particularly associated with invasive infections [9, 22]. Six distinct capsular serogroups have been identified [23], and the genetic locus responsible for capsule biosynthesis has been mapped [24, 25]. Structural and compositional analyses of the capsular polysaccharide (CPS) have also been reported [26, 27]. CPS appears to protect the bacterium from host immune recognition, as capsulated strains induce weaker immune responses than isogenic non-encapsulated mutants [9]. Interestingly, isolated CPS can provoke inflammatory responses in immune cells [28]. Previous studies, including our own, have demonstrated that immunization with purified CPS can confer protection against oral bone loss following P. gingivalis infection in mice [20, 29].

Polysaccharide antigens typically elicit T cell-independent responses, generating mainly B-cell-mediated immunity without durable immunological memory [30]. Even when antibodies are produced, booster doses often do not enhance immunity and can sometimes reduce antibody levels compared to a single immunization [31]. Some polysaccharide vaccines, such as Bacteroides fragilis CPS, can induce T-cell responses [32], but generally, polysaccharide-only vaccines are limited in their long-term efficacy. Conjugating polysaccharides to carrier proteins—such as tetanus toxoid, Haemophilus influenzae protein D, or diphtheria toxoid (CRM197)—enhances immunogenicity and induces robust, long-lasting responses across age groups [33-35].

Vaxcyte, Inc., has developed an enhanced carrier protein (eCRM®), an optimized variant of CRM197, which retains the glycine-to-glutamic acid mutation at position 52 to eliminate toxicity [36]. eCRM® includes engineered conjugation sites, enabling precise attachment of polysaccharides via site-specific copper-free click chemistry. Traditional conjugation methods react randomly with lysine residues on CRM197, which may interfere with human T-cell epitopes and reduce vaccine efficacy [37-39]. eCRM® and its site-directed conjugation strategy thus represent a significant advance in polysaccharide vaccine design.

In this study, we evaluated a novel conjugate vaccine composed of P. gingivalis CPS linked to eCRM® (Pg-CV) for its ability to prevent oral bone loss in a murine infection model. Immunized mice mounted strong IgG responses targeting the whole bacterium and, importantly, were protected against oral bone resorption induced by P. gingivalis. These findings provide strong support for further development of a CPS-based conjugate vaccine as a potential preventive and therapeutic strategy for periodontal disease.

## Materials and Methods

*Cultivation of P. gingivalis*

For all experiments, we employed P. gingivalis strain A7436, which was cultured under anaerobic conditions following previously established protocols [21]. In short, bacteria from frozen stocks were streaked onto brain-heart infusion (BHI) blood agar containing yeast extract. Colonies obtained from these plates were then expanded in BHI-YE broth until reaching the late logarithmic growth phase. To prepare capsular material, 2 liters of broth-cultured bacteria were collected, frozen, mechanically disrupted, and washed three times with saline. The bacterial pellets were centrifuged, collected, and stored at −80°C prior to capsular polysaccharide extraction using hot phenol, as described below.



For oral bone loss studies, overnight BHI-YE broth cultures were harvested, washed three times with pyrogen-free saline, and concentrated tenfold to an OD660 of 1.0. In parallel experiments, bacteria grown in broth were fixed with formaldehyde, washed, and used to coat 96-well plates for subsequent ELISA assays.

*Hot phenol-water/ether extraction, purification of capsular polysaccharide, and cell harvest*

Capsular polysaccharide (CPS) from P. gingivalis was prepared using a hot-phenol/ether-water extraction procedure, largely following previously established methods with modifications [20, 26, 40, 41]. Frozen bacterial pellets were first treated with hot phenol water, and the aqueous fraction was carefully recovered. To remove any remaining phenol, the aqueous extract underwent two successive extractions with ether. The resulting crude CPS was extensively dialyzed and stored at −80°C.

Prior to use, the frozen CPS extract was thawed and subjected to enzymatic treatments to remove contaminants. Specifically, samples were incubated twice with a nuclease mixture containing DNase I (0.005 mg/ml) and RNase A/T1 (0.01 mg/ml) to degrade nucleic acids, followed by two rounds of

proteinase K treatment (0.05 mg/ml) to eliminate residual proteins. The solution was then concentrated using a centrifugal filtration device with a 30,000 molecular weight cutoff. Two successive ethanol precipitations were performed to further purify the CPS. The precipitated material was air-dried at room temperature and subsequently stored at −80°C until further purification steps were carried out.

*CPS fractionation using size exclusion chromatography*

To purify the capsular polysaccharide (CPS) from P. gingivalis and remove lipopolysaccharide (LPS) contamination, gel filtration chromatography was employed using a modified method based on Schifferle *et al*. [26]. The crude CPS was dissolved at 5 mg/ml in a glycine-based buffer (0.05 M glycine, 0.5% deoxycholate, 0.001 M EDTA, pH 9.8) and applied to a pre-equilibrated GE HiPrep Sephacryl S-400 HR column (GE Healthcare, Chicago, IL). Elution was carried out with the same buffer on an Akta Avant system (Cytiva Life Sciences, Marlborough, MA), while UV absorbance was monitored at 214, 254, and 280 nm using Unicorn 7.1.0 software.

Fractions were collected and further analyzed by analytical size-exclusion HPLC coupled with multi-angle light scattering (MALS), refractive index detection, and UV detection at 280 nm as previously described [42-44]. The HPLC system (Agilent 1100, Agilent, Santa Clara, CA) included a degasser, a temperature-controlled autosampler (4°C), and a column compartment maintained at 25°C. Three columns were connected in series: a TSKgel Guard PWXL (6.0 mm × 4.0 cm, 12 µm), a TSKgel 6000 PWXL (7.8 mm × 30 cm, 13 µm), and a TSKgel 3000 PWXL (7.8 mm × 30 cm, 7 µm; Tosoh Bioscience, Tokyo, Japan). Detection employed a UV-VIS diode array detector (Agilent 1100), a Wyatt DAWN-HELEOS 18-angle MALS, and an Optilab T-rEX differential refractive index detector (Wyatt Technology, Santa Barbara, CA). Samples of 40–50 µg were run at 0.5 ml/min in 0.2 µm-filtered PBS containing 5% acetonitrile (pH 7.6) over 60 minutes. Data acquisition and analysis were managed with Agilent OpenLab and Wyatt Astra 7 software for molecular weight and protein-conjugate assessment.

For molecular weight calculations, the UV extinction coefficient and dn/dc values were set at 0.9 ml/(mg·cm) and 0.185 ml/g for eCRM®, respectively. For native CPS and the Pg-CV conjugate, dn/dc values of 0.133 and 0.155 ml/g were applied. Alternate fractions were examined by SEC-MALS to identify high-mass material with minimal UV signal, corresponding to early-eluting CPS, which was pooled as the final product. This separation approach exploits the ability of deoxycholate to disrupt LPS aggregates, delaying their elution relative to CPS and allowing effective isolation of the large polysaccharide component [20, 40]. The pooled CPS was scanned at $A_{260}$ and $A_{280}$ to evaluate residual nucleic acid and protein contamination.

*Polysaccharide size reduction with high-pressure homogenizer*

The high molecular weight of the purified CPS (>10 MDa) prevented direct conjugation to the eCRM® carrier. To overcome this, the polysaccharide was mechanically sheared using a PandaPlus 2000 homogenizer (GEA, Dusseldorf, Germany), with multiple passes at pressures exceeding 10,000 psi. This process reduced the average molecular weight to approximately 270 kDa, as monitored in real time by SEC-MALS. The processed CPS was then concentrated and diafiltered through a 30,000 Da cutoff membrane. Subsequent characterization included SDS-PAGE for purity assessment (data not shown), size and structural verification by SEC-MALS, and detailed carbohydrate profiling via HPAEC-PAD [45].

*Profiling monosaccharides using pulsed amperometric detection on anion-exchange chromatography*

The purified CPS was analyzed to determine its monosaccharide composition using a Thermo Scientific™ Dionex ICS-6000 ion chromatography system, which includes a gradient pump and an electrochemical detector with a gold electrode and an Ag/AgCl reference. Separation was achieved on a Thermo Scientific™ Dionex CarboPac PA20 analytical column (3 × 150 mm) with an attached guard column. Three different eluents were used: high-purity deionized water (18.5 MΩ·cm) filtered through a 0.45-µm membrane, 100 mM sodium hydroxide, and 100 mM sodium hydroxide containing 1 M sodium acetate, applied via a custom gradient at 0.5 mL/min over 30 minutes.

To release monosaccharides, the CPS was hydrolyzed in 10 N trifluoroacetic acid (TFA) at 121°C for 2 hours. After cooling on ice, the hydrolyzed material was lyophilized and then dissolved in the same volume of filtered deionized water. This solution was injected into the HPAEC-PAD system for analysis. Monosaccharide identities were confirmed by comparing their retention times with those of a reference mixture, which included glycerol phosphate (GroP), glycerol (Gro), rhamnose (Rha), glucosamine (GlcN), galactose (Gal), and glucose (Glc).

*CDAP activation of size-reduced P. gingivalis CPS and attachment of DBCO-PEG4 linker*

The reduced-size CPS was chemically activated using 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) [46, 47] to enable its covalent attachment to the azide-modified (pAMF) eCRM® carrier protein via a PEG linker through copper-free click chemistry. In this procedure, 2 mM of P. gingivalis CPS was dissolved in 100 mM borate buffer and treated with three molar equivalents of CDAP in acetonitrile under vigorous stirring. After 5 minutes, 0.5 molar equivalents of the dibenzocyclooctyne-amine PEG4 (DBCO) linker were introduced, and the reaction was allowed to proceed at room temperature for 1 hour. Unreacted cyanate esters were subsequently neutralized by adding glycine (pH 8.35) to a final concentration of 200 mM, followed by an additional hour of incubation. The resulting DBCO-functionalized polysaccharide was purified using a desalting spin column to remove any residual reactants. To evaluate the modification process, reverse-phase HPLC was employed to monitor DBCO absorbance at 309 nm throughout the reaction and to confirm complete removal of unreacted DBCO following desalting (data not shown). This step ensured that remaining DBCO-PEG4-Amine linker and DMAP were fully eliminated. Polysaccharide content was determined using the anthrone assay [48, 49], and DBCO concentration was measured spectrophotometrically at 309 nm. By combining these measurements, the fraction of polysaccharide molecules successfully modified with DBCO could be calculated.

*Conjugation of PEG4-linked P. gingivalis polysaccharide to eCRM®*

The eCRM® protein employed in this study is a modified form of the detoxified diphtheria CRM197 carrier, engineered to include the non-native amino acid para-azidomethyl-L-phenylalanine (pAMF) at designated positions. The mechanically size-reduced CPS was first functionalized with a PEG4-DBCO linker and then conjugated to eCRM® in a site-specific fashion. For the conjugation, the PEG4-DBCO-CPS was dissolved in potassium phosphate buffer containing sodium chloride and thoroughly mixed. A portion of the eCRM® protein was added to this solution, and the mixture was left to react at room temperature overnight to allow conjugation.

To characterize the conjugate, SEC-MALS analysis was performed to measure its weight-average molar mass. The composition of the conjugate was determined using a differential approach that compares the ultraviolet signal, representing the protein content, with the refractive index signal, representing total mass, allowing quantification of the relative amounts of CPS and eCRM® and the percentage of each in the final product [42]. This conjugate was subsequently formulated to prepare the vaccine doses administered to mice for the bone-loss experiments. Additionally, ELISA assays were conducted on samples of the full-length P. gingivalis CPS, the mechanically reduced polysaccharide, the PEG4/DBCO-modified CPS, and the final CPS/eCRM® conjugate to confirm their identities.

*Endotoxin assessment*

The presence of endotoxins was evaluated throughout the purification and conjugation steps, with particular attention to the finished vaccine formulation, using the Limulus amebocyte lysate (LAL) test kit (Endosafe, Charleston, S.C.).

*Oral bone loss modeling*

To assess the impact of P. gingivalis infection on oral bone loss and evaluate vaccine efficacy, we employed the murine model of oral bone loss described by Baker and colleagues [20, 21, 50]. All procedures involving animals were performed in accordance with IACUC-approved protocols at the University of Florida. Six-week-old Balb-C mice (Jackson Labs) were randomly divided into four groups (n = 10 per group): untreated controls, mice subjected only to oral P. gingivalis exposure, mice vaccinated with Pg-CV prior to oral challenge, and mice receiving a CPS-only control vaccine before oral challenge **(Figure 2a)**.

Vaccination involved intramuscular injections of 50 µL containing 7.5 µg of either Pg-CV or purified CPS, emulsified with Imject (Sigma). Booster injections of the same dose and volume were administered two and four weeks after the initial immunization. During this period, the untreated and oral-challenge-only groups received no interventions. Two weeks after the second booster, sera were collected from all mice, and oral challenge with P. gingivalis was performed on all vaccinated and CPS-only groups three times within a one-week period using a slurry in pyrogen-free saline containing 2% carboxymethylcellulose.

Six weeks following the final oral challenge, mice were humanely euthanized. Serum samples were collected, and heads were harvested and carefully cleaned of soft tissue. Oral bone loss was measured morphometrically, as previously described [18, 21]. Maxillae were stained with 1% methylene blue, oriented using buccal landmarks, and imaged with a stereo microscope (Leica Microsystems, Germany). Distances between the alveolar bone crest (ABC) and the cement-enamel junction (CEJ) were measured at seven sites per

hemimaxilla (14 sites per animal) using Leica Application Suite Software Version 4.12.0. Group means were calculated from individual measurements, and standard errors of the mean were determined using PRISM (v8; GraphPad Software, San Diego, CA).

*ELISA-assessment of serum levels of P. gingivalis whole organism-specific IgG*

Serum IgG responses in mice were quantified using an ELISA according to previously published methods [20]. For the assay, 96-well plates coated with formaldehyde-fixed P. gingivalis A7436 were blocked with 2% BSA. Mouse sera were applied either undiluted or in two-fold serial dilutions and incubated for 2 hours at room temperature. Following incubation, the wells were washed with PBS-Tween and gently tapped dry, then incubated for an additional 2 hours with alkaline phosphatase-conjugated anti-mouse IgG (1:1,000; Sigma-Aldrich, USA). After a second wash, pNPP substrate (Bio-Rad, USA) was added to each well, and the enzymatic reaction was terminated with NaOH. Absorbance was measured at 405 nm using a Synergy H1 plate reader (Biotek, USA). EC50 values for IgG specific to whole P. gingivalis cells were calculated from the absorbance versus dilution data, as previously described [21].

*Statistical analysis*

All data were recorded and managed using PRISM statistical software, where both descriptive and comparative analyses were conducted. Statistical comparisons were performed using either t-tests or ANOVA, with a significance threshold set at $P < 0.05$.

## Results and Discussion

*SEC-MALS analysis for molecular weight*

The molecular weights of the vaccine components were characterized using size exclusion chromatography coupled with light scattering, refractive index, and ultraviolet detection, which allowed tracking of changes across the production process—from the native full-length CPS, through the mechanically reduced polysaccharide, to the final polysaccharide-protein conjugate. As summarized in **Table 1**, the native P. gingivalis CPS had a weight-average molecular mass of 30.3 MDa. Due to its large size, direct conjugation to the monomeric eCRM® protein (~58.4 kDa) would be inefficient, necessitating a reduction in polymer length. High-pressure homogenization was employed to achieve a target molecular weight below 500 kDa, producing a processed CPS with a final weight-average molecular mass of 270 kDa.

This size-reduced CPS was then conjugated to eCRM® to form the final Pg-CV conjugate, which displayed a weight-average molecular mass between 2.2 and 2.7 MDa. The conjugate was formulated at 0.95 mg/mL in PBS (pH 7.4) and stored at −80°C. In the final vaccine product, the CPS content was 0.49 mg/mL **(Figure 1d)**, accounting for approximately 48% of the total conjugate mass.



**Table 1.** SEC-MALS Mwt summary.

| Material | Molecular Weight (Mw) [kDa] | Protein Content (%) | Polysaccharide Content (%) | dn/dc (PS) [mL/g] | dn/dc (Protein) [mL/g] | Extinction Coefficient (280 nm) |
|---|---|---|---|---|---|---|
| **Native PGPS** | 30,315 | n/a | n/a | – | – | – |
| **Sized PGPS** | 270 | n/a | n/a | 0.133 | – | 0.0 mL/(mg·cm) (PS) |
| **Sized PGPS–eCRM Conjugate** | 2,185 | 51.2 | 48.7 | 0.133 | 0.185 | 0.9 mL/(mg·cm) (eCRM protein) |

The molecular weights of P. gingivalis capsular polysaccharide (CPS) were assessed at multiple stages throughout the vaccine production process. The final conjugate vaccine's content of polysaccharide versus protein was estimated by comparing the signals obtained from the differential refractive index (dRI) and UV detectors, as detailed in the text.

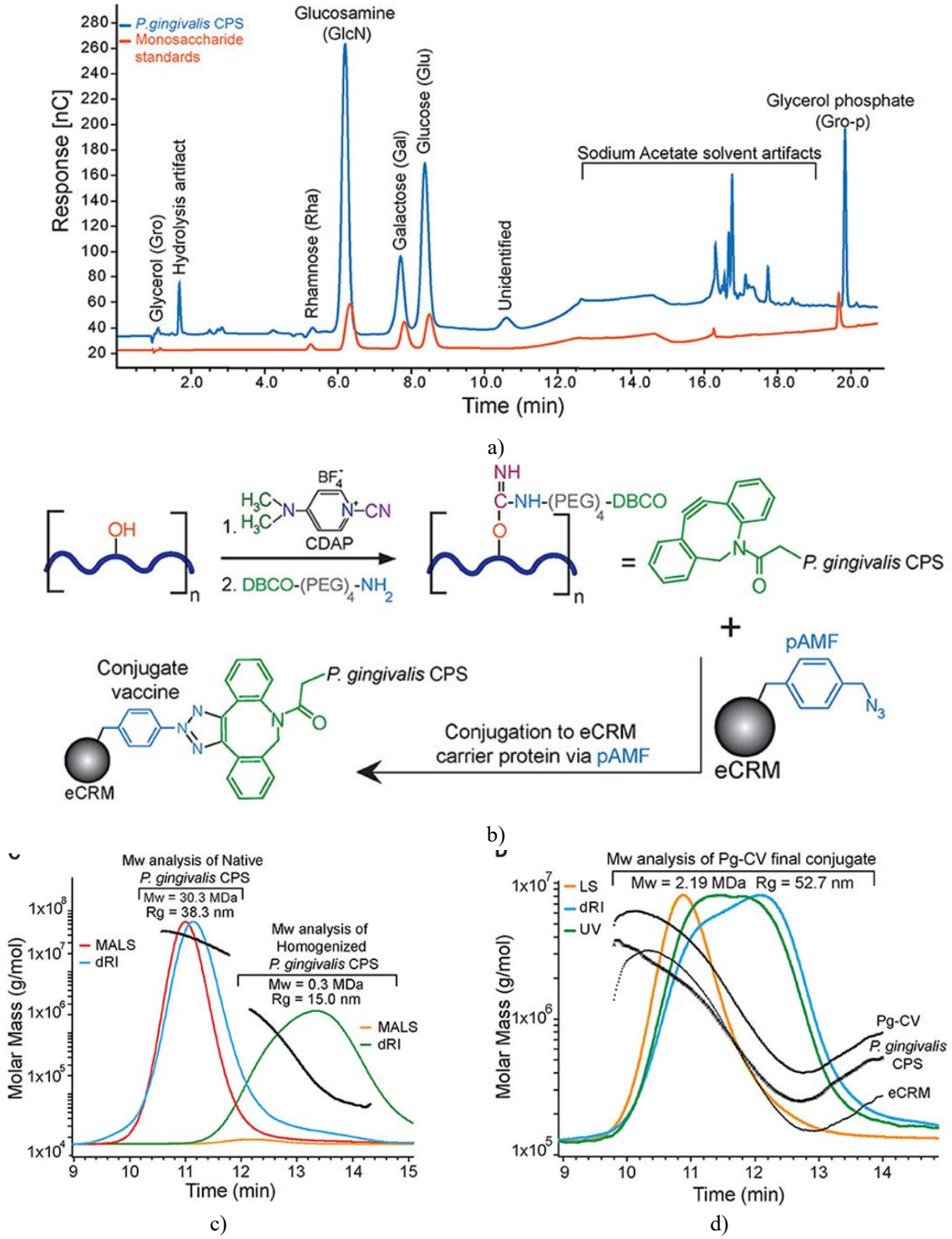



**Figure 1.** Analysis of Porphyromonas gingivalis A7436 CPS, conjugation approach, and characterization of Pg-CV. (a) Monosaccharide composition was examined using HPAEC-PAD. Shown are the chromatograms of Pg-CPS (blue) and a standard monosaccharide mixture (orange), with major peaks identified based on retention times relative to the reference. (b) Illustration of the conjugation strategy. Hydroxyl groups (red) on the Pg-CPS repeat units (thick dark blue line) were activated with CDAP to generate cyanoester intermediates. These intermediates reacted with the terminal amine of a DBCO-PEG4-NH2 linker, forming amide bonds that attached the DBCO moiety (green) to the polysaccharide. The eCRM® protein (gray sphere) containing a representative pAMF site then binds the DBCO, producing the final covalently linked polysaccharide-protein vaccine, Pg-CV. (c) HPLC/SEC-MALS-RI profiles for full-length and partially degraded Pg-CPS. Light scattering is represented in red and refractive index in blue. Later-eluting peaks (orange and green, 12–14 min) correspond to light scattering and refractive index signals, respectively, with

calculated molecular weights for each fraction indicated in black. (d) HPLC/SEC-MALS-RI-UV analysis of the completed Pg-CV. Light scattering is shown in orange, refractive index in blue, and UV absorbance at 280 nm in green. The average molecular weight of the Pg-CPS/eCRM® conjugate is 2.19 MDa, with protein conjugate molecular weight data overlaid in black.

*Endotoxin assessment*

Testing confirmed that both the purified CPS fractions and the final vaccine contained less than 100 EU/ml of endotoxin/LPS, indicating they were essentially free of contamination (data not shown).

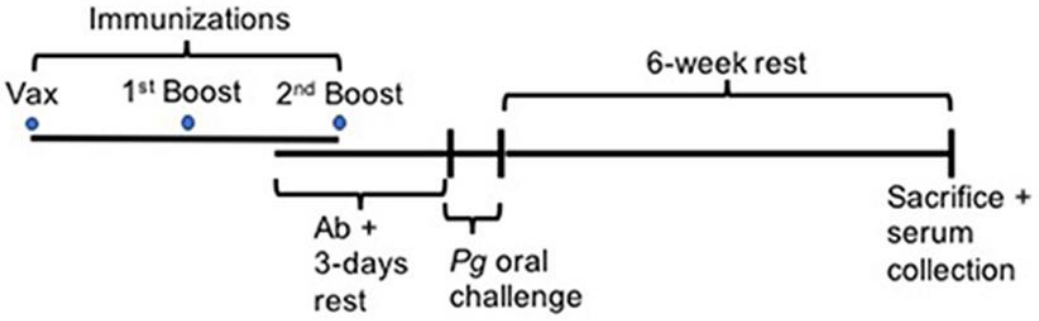
*HPAEC-PAD analysis for capsule monosaccharide composition*

Monosaccharide analysis by HPAEC-PAD showed that the purified P. gingivalis CPS shared many features with previously reported CPS compositions [26], though strain-specific variations were observed. **Figure 1a** displays a representative chromatogram of the purified CPS alongside a standard monosaccharide mixture. Four major monosaccharides were identified in the isolated CPS: glucosamine, galactose, glucose, and glycerol phosphate, in an estimated molar ratio of 5:1:3:1 (per PSRU). Additional minor peaks included a potential rhamnose signal at 4.25 min and an unidentified peak at 10.5 min, while signals between 12–19 min were likely due to either hydrolysis byproducts or buffer background. The absence of mannose and N-acetylgalactosamine indicates that the CPS was free from the main bacterial LPS contaminants—O-LPS, which contains O-antigen tetrasaccharide repeats, and A-LPS, which consists of anionic polysaccharide repeats [51, 52]. Interestingly, the overall sugar composition of P. gingivalis CPS resembles that of some pneumococcal polysaccharides, such as S. pneumoniae serotype 15B, whose PSRU consists of 1GlcNAc:3Gal:1Glc:1Gro [53]. This resemblance suggested that the activation and conjugation methods developed for pneumococcal 15B could potentially be applied to P. gingivalis CPS and Vaxcyte eCRM®, even in the absence of prior optimization **(Figures 1b and 1c)** [36].

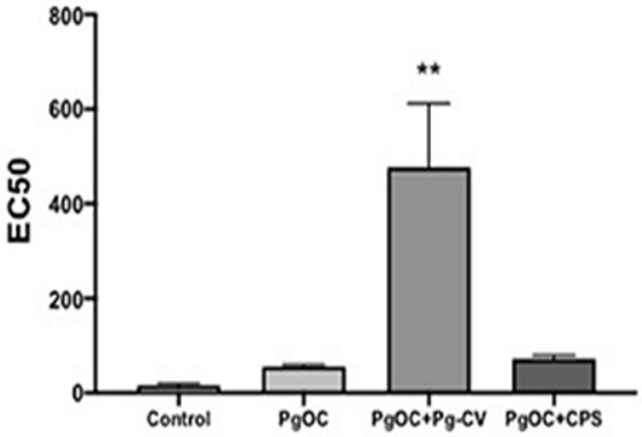
*Pg-CV conjugate vaccine elicits a robust IgG response from mice*

Conjugating capsule polysaccharides to a carrier has been shown to improve the immunogenicity of these molecules compared with the polysaccharides alone. In prior work, we observed that purified P. gingivalis CPS provided some protection in mice following oral challenge with the bacterium but elicited only a weak IgG response relative to a heat-killed whole organism [20]. To evaluate the effectiveness of the Pg-CV vaccine in preventing periodontal disease, we employed a murine oral challenge model. Serum levels of IgG specific to whole P. gingivalis were measured by enzyme-linked immunosorbent assay (ELISA). At the start of the study, all mice exhibited very low circulating levels of P. gingivalis-specific IgG (data not shown). Following three rounds of vaccination but prior to oral challenge, sera from non-vaccinated control mice and mice designated for P. gingivalis challenge remained largely negative for IgG against the bacterium. In contrast, mice immunized with Pg-CV developed a strong IgG response that effectively recognized whole P. gingivalis, whereas mice receiving CPS alone showed only a minor increase in IgG levels. At the time of sacrifice, control animals continued to exhibit negligible IgG, and mice challenged orally with P. gingivalis showed only a modest rise compared with the unvaccinated/uninfected group. In contrast, mice vaccinated with Pg-CV maintained significantly elevated IgG titers against the organism relative to all other groups ($P < 0.05$; **Figure 2b**), whereas the CPS-only group demonstrated only a limited IgG response to the whole bacterium.






a)



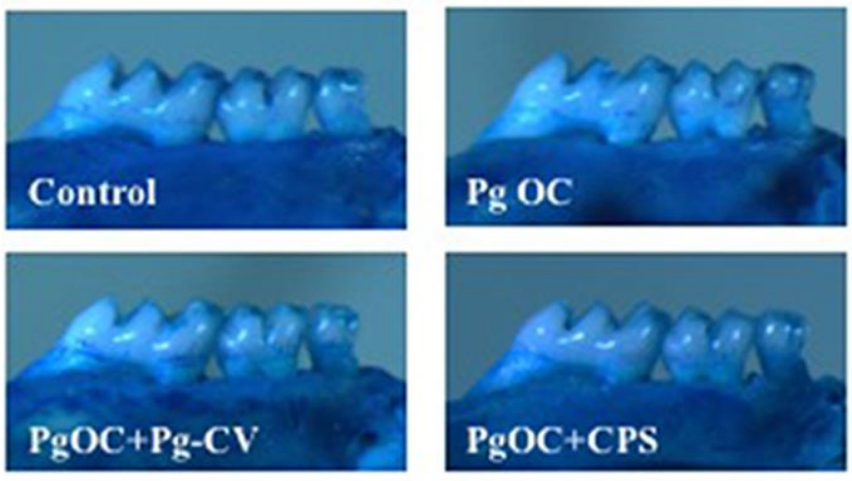

b)

**Figure 2.** Pg-CV induces a strong IgG response specific to whole P. gingivalis. (a) Experimental timeline for the animal studies. Mice (n = 10 per group) were either left untreated or received intramuscular immunizations with Pg-CV or purified P. gingivalis CPS three times over a 4-week interval. After oral antibiotic treatment (Ab), the animals were orally challenged three times within one week with P. gingivalis strain A7436 suspended in 2% carboxymethylcellulose. Six weeks later, mice were euthanized, serum samples were collected, and oral bone loss was measured using digital microscopy. (b) EC50 values for each group at the time of sacrifice were calculated from initial serum dilutions of pooled samples (2–3 mice per pool, 4 pools per group). Statistical analysis was performed by ANOVA with Tukey's post-test; **P < 0.01 versus Control, PgOC, and PgOC + CPS groups (all other comparisons were not significant).

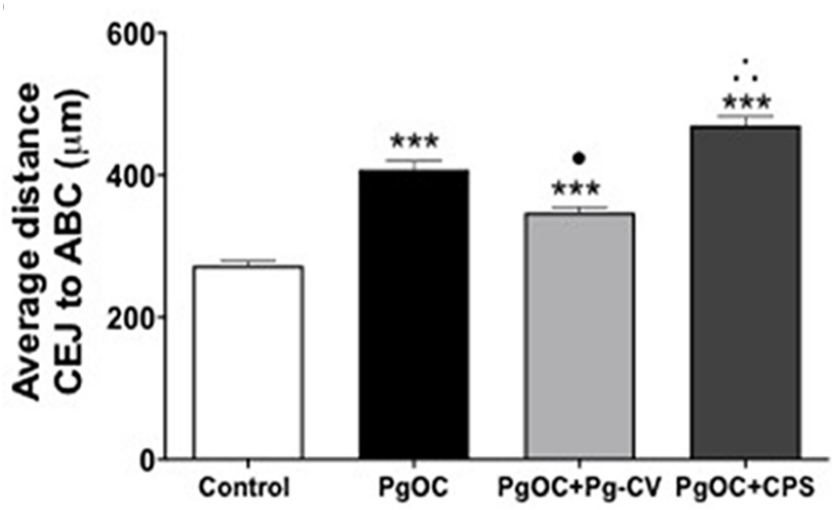
*Vaccination with the conjugate vaccine limits P. gingivalis-elicited oral bone loss*

Our group and others have successfully utilized this mouse model to evaluate the preclinical efficacy of various vaccine candidates in preventing oral bone loss [20, 21]. To investigate the effect of Pg-CV on P. gingivalis-induced bone destruction, maxillae were harvested after sacrifice, carefully cleaned, stained, and analyzed to determine bone loss for each experimental group **(Figure 3a)**. Measurements from the alveolar bone crest (ABC) to the cementoenamel junction (CEJ) in control animals established baseline bone levels. As expected, oral infection with P. gingivalis caused significant alveolar bone loss compared with controls (P < 0.05; **Figure 3b**). Administration of Pg-CV markedly reduced bone loss in challenged mice. Surprisingly, mice immunized with CPS alone exhibited a slight increase in ABC-to-CEJ distance relative to the orally challenged group (P < 0.05; **Figure 3b**).






a)




b)

**Figure 3.** Vaccination with Pg-CV mitigates bone loss caused by oral P. gingivalis infection. (a) Example images of maxillae from four groups: untreated controls, mice challenged orally with P. gingivalis alone (Pg OC), mice challenged and immunized with Pg-CV (PgOC + Pg-CV), and mice challenged and immunized with purified P. gingivalis CPS (PgOC + CPS). (b) Assessment of alveolar bone loss. The distance between the alveolar bone crest (ABC) and the cementoenamel junction (CEJ) was measured at 14 anatomical landmarks per mouse (7 per hemimaxilla; n = 10 mice per group). Measurements were combined to calculate group means ± SEM. Statistical comparisons were performed using ANOVA with Tukey's post hoc test: ***P < 0.001 versus Control, •P < 0.05 versus Pg OC (decrease), ∴P < 0.05 versus Pg OC (increase).

Conjugate vaccines have proven to be an effective strategy for reducing the burden of several major human diseases, including S. pneumoniae and other pneumococci [54]. Periodontal disease is a chronic inflammatory condition that is notoriously difficult to manage clinically, with limited long-term success in preventing progressive tissue destruction and tooth loss due to the lack of effective therapies. Consequently, novel approaches aimed at preventing or slowing disease progression are highly desirable and could substantially reduce the impact of this condition in humans. Various P. gingivalis antigens have been explored for their vaccine potential, with mixed results; however, several components, including the capsular polysaccharide (CPS), have demonstrated protective effects against infection-induced oral bone loss [20, 29].

Early research showed that encapsulated P. gingivalis strains produced more severe infections in mice compared with non-encapsulated strains [22]. Specifically, encapsulated strains caused systemic dissemination, with bacteria recoverable from blood, spleen, and kidneys, while non-encapsulated strains (381 and 33,277) generated localized abscesses, suggesting that the capsule contributes to bacterial spread. Aduse-Opoku *et al*. [25] identified the capsule biosynthesis locus, and Davey and Duncan [55] demonstrated that capsule production inhibited surface attachment and biofilm formation. Further, cloning the CPS locus (PG0106–PG0120 and upstream promoter region) into the plasmid PT-COW and introducing it into a non-encapsulated 381Δ0106 strain restored capsule expression and suppressed biofilm development, highlighting the capsule's role in modulating fimbrial adhesion. Collectively, these studies underscore the capsule's central role in defining P. gingivalis surface properties.



Although P. gingivalis strains can be classified based on serologic differences in their capsules [23, 56, 57], consensus on CPS structure remains unclear due to varying reports of repeat unit composition [26, 27]. Importantly, Choi *et al*. [58] demonstrated that in SCID mice reconstituted with human peripheral blood leukocytes, a conjugate vaccine comprising P. gingivalis CPS and fimbrial proteins elicited a strong human IgG response and protected the animals from subsequent subcutaneous challenge. Similarly, Gonzalez *et al*. [20] showed that purified CPS alone could protect mice from oral bone loss, although the induced organism-specific IgG was lower than that generated by immunization with killed whole bacteria. In our studies, the Pg-CV conjugate elicited a significantly stronger P. gingivalis-specific IgG response than CPS alone.

The purified CPS initially had a high molecular weight (~30.3 MDa), which hindered efficient conjugation, necessitating size reduction. Achieving this required prolonged homogenization under very high shear

pressure (20,000 PSI). Early attempts using acetic acid hydrolysis were largely ineffective (data not shown). These observations are consistent with the presumed biological function of the capsule, including environmental protection and pH shielding in vivo. Additional studies using LS-MS and NMR are ongoing to fully define the CPS linkage and structure.

Most bacterial capsules are T cell-independent antigens, typically eliciting only low-level IgG responses following immunization. Conjugation strategies, however, can substantially enhance specific antibody production [59, 60]. Consistent with this, our findings indicate that the Pg-CV conjugate is a more potent inducer of whole organism-specific IgG than CPS alone. Our initial studies focused on IgG production and protection against P. gingivalis-induced oral bone loss. While one animal died during the study, it was in the group receiving only oral challenge, indicating no observable toxicity of Pg-CV. Interestingly, CPS alone did not confer protection and appeared to slightly worsen oral bone loss. Differences from prior studies [20], which reported CPS alone as protective, may relate to several factors: fewer injections in our study (half as many), a lower CPS dose per injection (>10-fold lower, 7.5 μg vs. 100 μg), and a different administration route (intramuscular vs. subcutaneous). Vaccine-associated enhanced disease [61, 62] could also play a role, although the mechanisms remain unclear. Further work is needed to optimize antigen dose and immunization schedule. Importantly, the present study shows that conjugation of CPS to eCRM® effectively protects against oral bone loss following P. gingivalis challenge.

We selected P. gingivalis strain A7436, a K1 capsule serotype, as the vaccine target. Capsule-expressing strains generally cause more severe infections than K- strains [22], and K1 strains are more prevalent in periodontitis patients [63], though other K serotypes are also implicated [23]. Targeting the K1 capsule thus represents a viable strategy for vaccine development. In our hands, immunization with K1-based Pg-CV conferred protection against homologous oral challenge, providing proof-of-concept for its potential in periodontal disease. Future vaccine development will likely require inclusion of multiple K serotypes to ensure broad coverage.

## Conclusion

In summary, our results demonstrate that Pg-CV, administered in a preclinical model using intramuscular injection with alum adjuvant, elicits a robust IgG response recognizing P. gingivalis and protects against oral bone loss. These findings support Pg-CV as a promising candidate for preventing the severe periodontal tissue destruction characteristic of human disease.

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**Conflict of Interest:** AB, AS, NK, LP, and JF are current employees of Vaxcyte Inc.; JF is a founding member of Vaxcyte. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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