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Diagnostic evaluation of a nanobody with picomolar affinity towards the protease RgpB from *Porphyromonas gingivalis*

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Abstract

Porphyromonas gingivalis is one of the major periodontitis-causing pathogens. *P. gingivalis* secretes a group of proteases, termed gingipains, and in this study we have utilized the RgpB gingipain as a biomarker for *P. gingivalis*. We constructed a naïve camel nanobody library and used phage display to select one nanobody towards RgpB with picomolar affinity. The nanobody was used in an inhibition assay for detection of RgpB in buffer as well as in saliva. The nanobody was highly specific for RgpB as it did not bind to the homologous gingipain HRgpA. This indicated a presence of a binding epitope within the immunoglobulin-like domain of RgpB. A subtractive inhibition assay was used to demonstrate that the nanobody could bind native RgpB in the context of intact cells. The nanobody bound exclusively to the *P. gingivalis* membrane-bound RgpB isoform (mt-RgpB) and to secreted, soluble RgpB. Further cross-reactivity studies with *P. gingivalis* gingipain deletion mutants showed that the nanobody could discriminate between native RgpB and native Kgp and RgpA in complex bacterial samples. This study demonstrates that RgpB can be used as a specific biomarker for *P. gingivalis* detection and that the presented nanobody-based assay could supplement existing methods for *P. gingivalis* detection.

Keywords

Porphyromonas gingivalis; nanobody; periodontitis; cysteine protease; gingipain

1. Introduction

Periodontitis is a chronic inflammatory disease caused by bacterial infection. Several hundred microbial taxa have been identified in the human oral cavity, but only few bacterial species are associated with development of periodontitis. Among those bacteria,

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Porphyromonas gingivalis, *Treponema denticola* and *Tannerella forsythia* are considered the major periodonto-pathogens [1]. The common feature of these pathogens is production of high levels of extracellular proteolytic activity [2; 3; 4; 5; 6; 7; 8], which directly and/or indirectly contributes to disease symptoms manifested by loss of attachment between the tooth and the gingiva due to degradation of bone and soft tissues surrounding a root of a tooth. The end-result is formation of deep periodontal pockets and teeth loss [9]. In addition, there is accumulating evidence, which suggests that periodontitis is an important factor in the development of rheumatoid arthritis [10]

The gram negative anaerobic rod bacteria *P. gingivalis* secretes cysteine proteases called gingipains, which contribute to the pathology of periodontitis by deterring host immune system and degrading tissue and plasma proteins thereby providing nutrients needed for *P. gingivalis* growth [11]. Two types of gingipains exist, one specific for Arg-Xaa bonds (Arg-gingipain, Rgp) and the other specific for Lys-Xaa bonds (Lys-gingipain, Kgp) [12]. The Rgps (RgpA and RgpB), encoded by separate genes, have a conserved structure with a pro-fragment, a caspase-like catalytic domain and an immunoglobulin-like domain [12]. At the amino acid sequence level RgpB and RgpA share a high similarity consisting of, 72% for the pro-fragment, 99 % for the catalytic domain and 52 % for the immunoglobulin-like domain [12]. The Rgps have been shown to destroy endogenous inhibitors of proteases [13] and activate the kallikrein/kinin pathway and coagulation cascade [14] leading to bradykinin release and prothrombin activation, respectively. Bradykinin and thrombin are strong proinflammatory mediators contributing to inflammation progression and alveolar bone loss in periodontitis disease development [9]. The excreted RgpB has a molecular weight of 48 kDa, but posttranslational modifications of RgpB also results in a membrane-bound form of RgpB, which is modified with lipopolysaccharide (LPS) for membrane attachment. This membrane-bound form is called mt-RgpB [12].

Detection of *P. gingivalis* is important for identifying patients at risk for developing periodontitis as well as for monitoring disease progression during therapy [15]. Anaerobic culturing is most commonly used to detect and quantify *P. gingivalis* in patient samples, but these methods have several drawbacks, because they are insensitive and time-consuming [15]. In recent years the general trend in pathogen detection is moving towards faster molecular detection techniques [16]. Indeed, several real-time PCR methods have been developed for *P. gingivalis* detection in biological matrices [15; 17; 18]. Although PCR-based methods are highly sensitive they often require extensive sample preparation that can introduce variability between measurements [19]. Antibody-based pathogen detection methods are typically faster due to no, or limited, sample preparation and are, therefore, attractive in the context of bacterial detection [16]. Antibodies (Abs) for detection of pathogens should preferably be directed towards protein targets that are either exposed on the pathogen-surface or excreted from the organism. Furthermore, the protein target should be constitutively expressed [16]. Monoclonal Ab fragments have overtaken hybridoma-derived intact IgG's for many diagnostic applications in recent times [20]. Nanobody (nAb) technology, in particular, has shown promising potential. nAbs are derived from camelid heavy-chain Abs and the single domain VHH (also termed nanobody) sequences can easily be cloned into phagemid vectors for phage display selection of nAb binders. In addition to simplified Ab library construction, nAbs also have the advantage of their smaller size (approx. 15 kDa, 1/10th of intact IgG's) and ease of production in *E. coli*. Furthermore, the very special convex paratope found in nanobodies means that cryptic epitopes can be targeted, which are not easily accessible for Fab fragments, scFv's and intact Abs. Hard accessible cryptic epitopes are present on the surfaces of intact pathogens and nAbs can therefore be particularly useful for pathogen detection [20; 21; 22]. At present no antibody-based *P. gingivalis* detection methods have been described in the literature, most likely due to a lack of specific probes. The *P. gingivalis* proteinases can be biomarkers for disease

detection and progression, because gingipains have important activities in the pathogenesis of periodontitis. In this study we have consequently developed a high affinity camel nanobody towards RgpB and demonstrate for the first time that RgpB can be used as a biomarker for *P. gingivalis* detection.

2. Materials & methods

2.1 Rgps purification

HRgpA, a non-covalent complex of a catalytic domain and hemagglutinin-adhesin domains was purified from culture medium of *P. gingivalis* HG66 by acetone precipitation, gel filtration on Sephadex G-150, and affinity chromatography on arginine-Sepharose [23]. RgpB with the C-terminal His-tag was produced by the *P. gingivalis* W83 strain bearing the modified *rgpB* gene. The sequence coding for six His was inserted in frame at the junction between the Ig-like domain and C-terminal domain of proRgpB. Such modification results with secretion into the media of the soluble RgpB with the C-terminal His-tag. RgpB-6xHis was purified to homogeneity from the growth medium by subsequent affinity and ion-exchange chromatography on Ni-Sepharose and Mono Q, respectively (manuscript in preparation).

2.2 *P. gingivalis* strains

Creation of single gingipain mutants have been reported previously [24; 25]. The RgpA-RgpB inactivation mutant was created by truncation of RgpB at position 410aa to remove the Ig-CTD domain using deletional mutagenesis as previously described [25]. To create the gingipain-null mutant W83/K/RgpAB, the *ermF*/AM cassette of the plasmid construct pREC-KgpΔIg/Cterm/HA [24] was swapped for a tetQ cassette [26] and transformed into the RgpA-RgpB mutant above. The pertinent regions of all mutants were sequenced to confirm the correct construction and gingipain assays were. Characteristics of *P. gingivalis* strains are found in table 1.

P. gingivalis strains were grown on the anaerobe blood agar plate (3% soy tryptone, 1.5% agar, 0.5% yeast extract, 0.05% L-cysteine, 0.01% DTT, 0.00005% vitamin K, 0.0005% hemin, 5% defibrinated sheep blood). All strains were incubated for 7 days at 37°C in an anaerobic chamber MACS500 (Don Whitley Scientific Limited, Frederick, MD, USA) in an atmosphere of 80% N₂, 10% CO₂, 10% H₂. Afterwards, bacteria were inoculated into 40 ml of appropriate broths and grown overnight under the same conditions. For growth selection of mutants on solid medium, 1 µg/ml tetracycline or 5 µg/ml erythromycin was used.

2.3 Camel mRNA isolation and DNA amplification

The nanobody library was constructed as described [21; 27] with some modifications. Blood from a healthy *Camelus bactrianus* (10 ml) was supplied to us by the Copenhagen Zoological Garden. Messenger RNA (mRNA) was isolated (QIAamp RNA blood mini kit, QIAgen) from the peripheral blood lymphocytes and converted to cDNA (iScript™ cDNA synthesis kit, Bio-rad). The 5' part of the immunoglobulin heavy chains were amplified with the primers: VHBACA6 (5'-GAT GTG CAG CTG CAG GCG TCT GG(A\G) GGA GG-3') and CH2FORA4 (5'-CGC CAT CAA GGTACC AGT TGA-3'). The amplified product, of approximately 600 bp, was purified by agarose gel electrophoresis (gel purification kit, QIAgen) and used as a template for the second round of PCR. To accommodate insertion of the VHH sequences into our pFab74 phagemid [28] we used a modified version of the original VHFOR36 primer. The primers used for VHH amplification was VHFOR36.pFab74 (5'-GGA CTA GTG CGG CCG CTG AGG AGA CGG TGA CCT G-3') and VHBACA4: 5'-CAT GCC ATG ACT CGC GGC CCA GCC GGC CAT GGC CGA (G\T)GT (G\C)CA GCT-3'). The PCR program used for both reactions was, 94°C for

5 min. followed by 30 cycles of 94°C for 1 min., 60 °C for 1 min., 72 °C for 1 min and ending with 72 °C for 10 min. The VHH product was approximately 400 bp and was gel-purified followed by *Sfi* I/*Not* I digestion and again gel-purified. All primers were from Eurofins MWG-Operon.

2.4 Nanobody library construction

Sfi I/*Not* I digested Pfab74 was isolated as a single DNA band after agarose gel electrophoresis (Qiagquick gel extraction kit). The ligation was performed with 2 µg *Sfi* I/*Not* I digested pFab74 and 500 ng *Sfi* I/*Not* I digested VHH using T4 DNA ligase (Invitrogen). The ligation mix was ethanol precipitated and redissolved in MQ water. The ligation mix was transformed into ultra-competent TG1 cells prepared on the same day. Twenty-five separate electroporations were done and pooled thereby creating a nanobody library with a size of approximately 5×10^7 . The library was expanded in 500 ml LB-media (Sigma-Aldrich) containing 50 µg/ml ampicillin (Calbiochem) over night at 37 °C. From the overnight culture glycerol stocks were made and stored at -80 °C. Nanobody-phages were produced by dilution the overnight culture to a start OD₆₀₀ of 0.05, followed by incubation at 37°C to a density of OD₆₀₀ 0.6. Then a 100-fold excess of the R408 helper phages (Promega) were added and the culture was incubated for 20 min at 37°C. Induction with 1 mM isopropyl β-D-thiogalactoside (IPTG, VWR) was performed and the nanobody-phages were expressed overnight at 22°C. The following day nanobody-phage were precipitated from the cell supernatant with phage precipitation buffer (20% (w/v) PEG6000), 2.5 M NaCl) and redissolved in PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7.4). The nanobody-phages were stored at -20 °C and used for panning experiments.

2.5 Panning

Purified RgpB was immobilized on maxisorp wells (Nunc) overnight in PBS at 4 °C for panning experiments. During each panning round a decreasing amount of RgpB was immobilized; round 1 (50 µg/ml), round 2 (25 µg/ml) and round 3 (12.5 µg/ml). Wells were washed 3 times in PBS-T and blocked in 4% (w/v) skimmed milk powder/PBS for 1 hour. Wells were again washed and incubated for 2 hours with approximately 10^{11} nanobody-phage in 2 % (w/v) skimmed milk powder/PBS. Wells were washed 15 times in PBS-T and phages were eluted with 100 µl 10 mM glycine-HCl pH 2.0 for 10 minutes followed by addition of 1 µl 2 M TRIS-base for neutralization. The eluate was used to infect exponentially growing TG1 cells for 20 minutes. Next ampicillin was added to 50 µg/ml and the culture was incubated overnight at 37 °C. From the overnight culture nanobody-phage were produced, as described above, and used for the next panning round.

2.6 Clone screening by phage ELISA

Single clones were isolated and superinfected for nanobody-phage production overnight as described above. Overnight cultures were centrifuged at $10,000 \times g$ to remove cells and the supernatants were diluted 1:1 in 4 % (w/v) skimmed milk powder/PBS and applied directly to RgpB-coated and blocked maxisorp wells (coating concentration was 10 µg/ml). The nanobody-phages were allowed to bind for 2 hours at room temperature followed by washing 5 times in PBS-T. Nanobody-phage binding was detected by a monoclonal anti-M13 antibody-HRP conjugate (diluted 1/1000, GE-healthcare) and OPD as the substrate (DAKO).

2.7 Nanobody expression and purification

pFab74 phagemid harboring the RgpB.VHH7 sequence fused to protein III was isolated by maxiprep (QIAGEN). The phagemid was digested with the restriction enzyme *Eag*I to remove the protein III sequence [28] and re-ligated with T4 DNA ligase. The ligation mixture was

transformed into chemo-competent TG1 cells, individual clones were isolated and sequenced to verify removal of protein III DNA. A delta protein III clone was isolated and used for RgpB.VHH7 expression. The RgpB.VHH7 clone was expanded in LB-media supplemented with 10 mM MgCl₂ and when an OD₆₀₀ 0.6 was reached it was induced with 1 mM IPTG for 3 hours at 30 °C. The periplasmic fraction was isolated [29] and extensively dialysed into 20 mM sodium phosphate, 500 mM NaCl, pH 7.4 (buffer A). The dialyzed fraction was applied to a 1 ml HiTrap chelating HP column charged with NiSO₄ (GE Healthcare). The hexa-His tagged RgpB.VHH7 was eluted using a linear gradient of buffer B (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). RgpB.VHH7 purity was confirmed by SDS-PAGE (Pierce Precise 4–20 % pre-cast gradient gel).

2.8 Quantification of recombinant RgpB in saliva by inhibition assay

Nunc maxisorp plates were coated with 10 µg/ml RgpB and blocked with 4 % (w/v) skimmed milk powder/PBS as described in section 2.6. A titration curve revealed the RgpB.VHH7 concentration that gave half maximum response and this concentration (50 ng/ml) was used for the inhibition assay. The inhibition assay was performed essentially as previously described [30]. Briefly, the RgpB.VHH7 was incubated with decreasing concentrations of RgpB for one hour with extensive mixing. The mix was added to coated/blocked wells (10 µg/ml RgpB) and the unbound RgpB.VHH7 was allowed to bind for one hour. Wells were washed with PBS-T five times and incubated with rabbit anti-camel whole serum pAb (Bethyl laboratories, diluted 1/1000) for one hour, followed by PBS-T washing five times. The final step was incubation with a goat anti-rabbit IgG-HRP conjugate (diluted 1/1000, Sigma-Aldrich) for one hour and development with OPD substrate (Kem-En-Tec). The absorbance values were measured at 490 nm after 30-min incubation at 22°C. Absorbance values at each RgpB concentration (A) were divided by the absorbance measured in the presence of zero RgpB (A₀), which yielded normalized values (A/A₀). The normalized values were plotted against the RgpB concentration to construct the inhibition curve. The assay was performed in triplicate measurements to generate standard deviations. The RgpB concentration required for 50 % inhibition (IC₅₀) was identified and used as an estimated antibody affinity as described previously [30]. For cross-reactivity studies towards HRgpA we performed the inhibition assay at a single concentration of 10 nM but otherwise the protocol used was as described here.

2.9 Subtractive inhibition assay for *P. gingivalis* native RgpB detection

As above wells were coated with 10 µg/ml RgpB and blocked in skimmed milk. Fifty ng/ml RgpB.VHH7 was incubated with decreasing amounts of *P. gingivalis* W83 wild type, W83/ΔrgpA, or W83/ΔRgpA/RgpBΔ495 cells (volume 200 µl) for 30 min. on an orbital shaker. Samples were centrifuged at 5,000 × g for 5 min. and 100 µl was transferred to RgpB-coated wells (coated and blocked as above) and incubated for 30 min. Surface-bound RgpB.VHH7 was detected with rabbit anti-camel whole serum pAb and goat anti-rabbit IgG-HRP conjugate as described above. Absorbance values at each *P. gingivalis* concentration (A) were divided by the absorbance measured in the presence of zero *P. gingivalis* (A₀), which yielded normalised values (A/A₀). The normalized values were plotted against the *P. gingivalis* concentration to construct the subtractive inhibition curve. For cross-reactivity studies towards mutant strains of *P. gingivalis* we performed the assay, as above, at a cell concentrations of 10⁸ cells/ml.

2.10 Determination of kinetic rate constants by surface plasmon resonance

Biacore binding experiments were performed using a Biacore 3000 instrument with CM5 sensor chips and HBS-EP running buffer (Biacore GE Healthcare, Uppsala, Sweden). Prior to analysis, the instrument was cleaned using a super desorb procedure [31] and a fresh CM5 chip that was preconditioned with two consecutive injections each of 100 mM HCl, 50 mM

NaOH and 0.5% (w/v) SDS for 10 seconds per injection. Purified RgpB.VHH7 was immobilized on the surface of a CM5 sensor chip using the Biacore 3000 immobilization wizard to a target level of 250 RU. The carboxymethyl dextran surface of the CM5 chip was activated with a mixture of 400 mM EDC and 100 mM NHS for 10 minutes at a flowrate of 10 μ l/min. RgpB.VHH7 was diluted in sodium acetate, pH 4.4, to a final concentration of 20 μ g/mL and subsequently passed over the activated chip surface at a flowrate of 10 μ l/min. One molar ethanolamine was then passed over the chip surface for 7 minutes resulting in the capping of any un-reacted amine groups. A total of 192 RU of RgpB.VHH7 was immobilized onto the sensor chip surface. Regeneration studies were performed and 5 mM NaOH selected for regeneration as it provided efficient and reproducible binding profiles without affecting the surface integrity. For kinetic analysis, antigen diluted in running buffer was injected at concentrations of 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.195 nM. The 25, 6.25 and 1.56 nM concentration were run in duplicate and a zero concentration included, enabling double referencing. Association and dissociation phases were monitored for 3 and 20 minutes respectively. Sensorgrams were fitted globally to a 1:1 interaction model using BiaEvaluation software.

2.11 Tests for inhibitory function of RgpB.VHH7

The amidolytic activity of RgpB was determined with N-Benzoyl-L-Arginine-p-Nitroanilide (L-BAPNA) (Bachem). RgpB (20 nM) was incubated with RgpB.VHH7 (900 nM) in assay buffer (200 mM Tris, 5 mM CaCl₂, 150 mM NaCl, 0.02% NaN₃, pH 7.6) supplemented with 1 mM L-Cysteine (Sigma). After 1 hour at 22°C, 0.5 mM of the substrate L-BAPNA was added and the formation of p-nitroanilide was monitored spectrophotometrically at 405 nm (Spectrophotometer plate reader Spectramax M5, Molecular device). Alternatively, to eliminate a possibility of reducing an essential disulphide bridge in nAbs, glutathione (GSH; 1 mM) was used to activate gingipain or pre-activated RgpB (10 min in activity buffer supplemented with L-cysteine 10 mM) was mixed with nAbs. In the latter conditions the final concentration of L-cysteine during the incubation was 0.01 mM).

The second assay used for evaluating the potential inhibitory effect of RgpB was based on the natural ability of RgpB to cleave fibrinogen. RgpB was preincubated with a range of nAb titers. The molar ratios ranged from an excess of RgpB over nAb (5:1), through a number of intermediates up to an excess of nAb over RgpB (highest molar ratio equal 20:1, respectively). After one hour incubation an equal corresponding volume of assay buffer (200 mM Tris, 5 mM CaCl₂, 150 mM NaCl, 0.02% NaN₃, pH 7.6) supplemented with 1 mM L-Cysteine (Sigma-Aldrich) was added to the mixture for one hour.

A solution of 7.5 mg/ml fibrinogen (Sigma) was added to each reaction tube and incubated for an additional hour at 22°C. All samples were subjected to SDS-PAGE (Pierce 4–20 % pre-cast gradient gel) and developed by Coomassie blue staining. The RgpB-induced fibrinogen degradation pattern was used to monitor any inhibitory activity of RgpB.VHH7.

Results and discussion

3.1 Selection and expression of RgpB nanobodies

A naïve nanobody library was constructed from mRNA isolated from non-immunized healthy *Camelus bactrianus* blood lymphocytes. The library was constructed using the modified primer pairs described and the pFAB74 phagemid [28]. The nAb library consisted of $\sim 5 \times 10^7$ clones and the size lies within the $1.6 \times 10^5 - 5 \times 10^9$ clones that have been reported for naïve camel/llama nanobody libraries [32; 33; 34; 35; 36]. The 50 kDa RgpB with a C-terminal hexa-Histidine tag was homogenous as determined by SDS-PAGE (data not shown). The naïve camelid nAb library was screened for binders towards immobilized

RgpB. After three rounds of panning on decreasing amounts of RgpB and high washing stringency, the difference in the number of eluted phage between sample (RgpB) and control (panning on skimmed milk) was > 1000 (data not shown). A similar difference was found by phage ELISA (data not shown). Forty single clones were analyzed by phage ELISA for RgpB binding and as seen in figure 1A, eight clones were defined as positive (OD above 0.5). The eight clones (termed RgpB.VHH5,7,11,12,16,17,27,36) were all sequenced and found to be identical (figure 1B). We applied a very stringent selection scheme (washing 15 times and decreasing antigen coating concentration) during the three panning rounds and this procedure could have led to the limited diversity of only one binder. To test if high stringency eliminated nAb diversity, the library was re-panned on RgpB under less stringent conditions (50 µg/ml coating concentration and only 7 washes), but this procedure did not lead to isolation of any binders. It is likely that only one binder was present in the library or alternatively the nanobody-phages in the library are sticky and stringent washing is needed to achieve a large difference in eluted phages between the control well (skimmed milk) and RgpB. Indeed, other panning studies of naïve libraries have also used high washing frequencies (15–20 times) [34; 35; 36].

The RgpB.VHH sequence has the long CDR3 region (16 amino acid residues), which is typically seen in camelid nanobodies [37]. Furthermore, cysteine residues were present in CDR1 and CDR3 and it was shown that often a cysteine bridge is formed to stabilize the rather long CDR3 loop (figure 1B) [37]. Camelid VHH domains normally contain the amino acid substitution L11S, V37F/Y, G44E, L45R and W47G compared to mouse/human VH domains, which increases the solubility of isolated VHH's [37]. In the case of the RgpB.VHH sequence isolated, only the L11S, V37Y, and G44E substitutions are seen. A tryptophan is found in position 45 and in position 47 a serine. The RgpB.VHH7 clone was chosen for large scale nanobody expression and from 2 L of culture approximately 2 mg nanobody of high purity, as demonstrated by SDS-PAGE (figure 1C). The nanobody is very soluble, easy to work with and stable over time at 4 °C.

3.2 RgpB.VHH7 characterization

Purified RgpB.VHH7 was used to establish an inhibition ELISA for detection of soluble RgpB. This served to demonstrate that the epitope targeted by RgpB.VHH7 was native and accessible on RgpB in solution (figure 2). From figure 2 a clear dose-response relationship is seen and the IC₅₀ value was used to estimate the dissociation constant to the low nanomolar range by the methods described previously [30]. We expanded the kinetic characterization by detailed surface plasmon resonance analysis. A common and useful approach for kinetic analysis is to use a capture assay format whereby a capture antibody (such as an anti-tag antibody) is immobilized onto the sensor surface and used to capture the ligand of interest (the nanobody in this case). This helps to orientate the ligand and retain ligand activity while also permitting the re-use of the surface for screening multiple ligands and, thus, facilitating higher throughput [38]. However, as RgpB and the RgpB.VHH7 both contained His-tags (and no other unique tag) and only one antibody needed to be characterised, this format was not evaluated. Instead direct immobilization of the RgpB.VHH7 on the sensor surface was used. To minimize mass transfer limitations and rebinding, a low density nanobody surface was prepared (192 RU of RgpB.VHH7 was immobilised onto the CM5 sensor chip; data not shown). Purified RgpB was analysed by size exclusion-HPLC prior to kinetic analysis to confirm that it was monomeric with no signs of aggregation and, therefore, could be fitted to a 1:1 binding model. A large peak with a retention time of 10.3 minutes (similar to that of ovalbumin, molecular weight 44 kDa) was observed confirming the monomeric identity of RgpB (data not shown). For kinetic analysis, RgpB was diluted in assay running buffer to minimize refractive index differences and passed over the nanobody surface at varying concentrations (figure 3A). RgpB was injected at a high flowrate for 3 minutes (30 µl/

minute) and allowed to dissociate in buffer for 20 minutes. Association and dissociation curves were obtained (figure 3A) and a 1:1 binding model was applied which fitted well to the data ($\chi^2 = 0.457$) as shown in figures 3C and 3D. Surface regeneration while maintaining ligand integrity is of paramount importance for accurate kinetic analysis. In this experiment the nanobody surface was efficiently regenerated with 5 mM NaOH (figure 3B) with less than 2 RU change in baseline level over the course of the experiment. The rate constants and affinity were determined from the fitted data (figure 3C) indicating that the nanobody had a very high affinity of 362 pM (figure 3D). Due to the relative ease of obtaining naïve camel blood compared with immunized blood, we selected this option. In terms of affinity RgpB.VHH7 can easily rival nanobodies selected from immunized libraries [22]. In a recent study Monegal *et al.* selected llama nanobodies from a naïve library on six different antigens in parallel and found binders with affinities ranging from 0.15 to 1970 nM [34]. This suggests that the type of antigen used for nanobody selection is important when panning with naïve libraries. We have panned our library on other targets of rat and human origin and have only isolated low affinity (μ M) binders.

3.3 Detection of recombinant RgpB in saliva and cross reactivity

Matrix effects often interfere with analysis of authentic biological samples [39]. Indeed, it was found that saliva interferes with detection of *P. gingivalis* in PCR-based assays [40]. To investigate the effects of saliva in the RgpB.VHH7-based inhibition ELISA the assay was performed in this matrix. As seen in figure 2, detection was clearly possible in saliva since a clear dose-response relationship is seen. However, the estimated IC_{50} value is increased about two-fold suggesting that matrix effects are present. Furthermore, the coefficients of variation (CV) did generally increase in the saliva assay (0.1–10.3%) compared to the assay performed in PBS (0.9–6.6%) (figure 2). However, the CV's for the assay in saliva are still very acceptable for diagnostic assays and overall the assay could be performed efficiently in a salivary matrix. To further characterize the nanobody we used the inhibition assay in a cross-reactivity study towards the homologous HRgpA. When tested in a single concentration around the ELISA IC_{50} values for RgpB (10 nM) HRgpA led to insignificant inhibition suggesting a high specificity for RgpB (figure 4A). From alignment of the RgpB and HRgpA sequences it is evident that the differences between the two are located in the immunoglobulin-like domains, as the catalytic domains are composed of practically the same amino acids (figure 4B). This suggests that the RgpB epitope resides within the immunoglobulin-like domain.

3.4 Subtractive inhibition assay for detection of native RgpB/mtRgpB in *P. gingivalis* samples

We utilized a subtractive inhibition approach for RgpB.VHH7-based detection of *P. gingivalis* cells. Subtractive inhibition assays have previously been used for detection of fungal spores and bacterial cells in ELISA and Biacore formats [41; 42; 43; 44]. This method allows binding of antibody-probes to intact cells in solution, which is an advantage compared to assays based upon immobilization of cells in microtiter wells because cell surface antigens are in their native conformation. The assay was performed in saliva and, as seen in figure 5, we did find that normalized values decreased as a result of increasing wild type *P. gingivalis* (W83) concentration, thereby illustrating that the assay can be used for *P. gingivalis* detection. The detection limit of W83 cells in the subtractive inhibition assay was calculated as the lowest point exhibiting >10% inhibition (IC_{10} value), as suggested previously [45]. This gave an approximate detection limit of 7.81×10^6 cells/ml (the A/A0 value is 0.9 for this concentration). Furthermore, excellent CV's were found ranging from 0.4% – 6.6 %, illustrating that the assay is robust when performed in saliva.

We further tested a RgpA deletion mutant strain of *P. gingivalis* (W83/ Δ RA) and found that these cells performed in a manner similar to W83, which confirmed the lack of cross-reactivity with HRgpA, (Figure 5). No inhibition was also observed by the double RgpA and RgpB knock-out strain (W83/ Δ AB) which again illustrates the nAb specificity towards RgpB (Figure 5). As seen in figure 6, RgpB.VHH7 did not bind the W83/ Δ KRAB mutant strain, deficient of all gingipains. This confirms that the RgpB.VHH7 does bind native gingipains, but does not exclude that the nanobody could bind the lysine gingipain Kgp. To exclude any Kgp binding we further tested a Kgp deletion mutant (W83/ Δ K) and found RgpB.VHH7 binding on the same level as for the parental strain. The same results were observed with a RgpA/Kgp double deletion mutant (W83/ Δ KRA). Taken together these findings prove the specificity of the nanobody towards RgpB and firmly confirm that RgpB.VHH7 can bind native RgpB. However, from these experiments we were not able to say whether, RgpB.VHH7 binds soluble RgpB excreted from *P. gingivalis* cells or it binds the membrane-associated (mt-RgpB). To answer this question *P. gingivalis* W83 cells were collected from the growth medium by centrifugation, washed, re-suspended to the original volume in buffer and then tested in the subtractive inhibition assay. As clearly seen in figure 7, RgpB.VHH7 bound the re-suspended washed bacterial cells to the same extent as control W83 cells directly in the growth media. This result suggests that the effect of cells we observe in the subtractive inhibition assay is primarily caused by nanobody binding to the membrane associated RgpB (mt-RgpB) in keeping with finding that in *P. gingivalis* W83 the majority of RgpB is found on the bacterial surface [[46]].

The detection limit for W83 *P. gingivalis* is slightly higher than those found when using subtractive inhibition assays for detection of fungal spores (1.5×10^5 to 2.2×10^6 spores/ml) [41; 42; 43] and bacteria (1×10^5 cells/ml) [44]. Antibodies for detection of pathogens should preferably be directed against a single target molecule that is exposed on the pathogen-surface and constitutively expressed so that environmental factors do not influence target expression [16]. In the case of mt-RgpB there is currently no knowledge about the density level of the gingipain molecules on the bacterial surface. It is only apparent from the recent studies that gingipains are evenly distributed on the surface of *P. gingivalis* in an electron dense surface layer composed of gingipains and polysaccharide [47]. In the case of mt-RgpB there is currently no knowledge about the surface distribution and molecule density level. Therefore, the relatively low detection level (7.81×10^6 cells/ml) in the RgpB.VHH7-based assay suggests that the mt-RgpB surface-density is low. Alternatively it is possible that only a part of mt-RgpB molecules on the *P. gingivalis* surface has the Immunoglobulin-like domain accessible to interaction with nanobody, while in the other part of the gingipain molecules, the Immunoglobulin-like domain is buried in the electron-dense surface layer and cannot interact with antibodies. Published real-time PCR assays for *P. gingivalis* detection have reported detection limits as low as 200 cells/ml [15], so as is the case for most immunoassays the nanobody-based assay cannot compete with real-time PCR's in terms of sensitivity. However, the nanobody-based assay can be a useful supplement to existing tests.

3.10 Evaluation of nanobody inhibitory activity towards RgpB

Due to the important activities of RgpB in the pathogenesis of periodontitis, RgpB can be a target for periodontal disease therapy [9]. Consequently, several strategies have been explored for RgpB inhibition, including, chlorhexidine, benzamidine and peptides [48; 49; 50]. nAbs have a globular/convex binding paratope and the CDR3 loop is considerable longer than the mouse/human equivalent. These structural features mean that nAbs favour binding into crevices on molecules [51] and enzyme nAb inhibitors have been isolated that acts by active site targeting [52; 53; 54; 55]. Similarly, nAbs have been selected for cell surface receptor antagonism [56] and as inhibitors of receptor function [57]. From the data

above RgpB.VHH7 does likely not bind in the area surrounding the catalytic dyad of RgpB as these amino acids are conserved between RgpA and RgpB. But allosteric effects on RgpB activity upon nanobody binding to the Immunoglobulin-like domain could occur. To investigate this, the ability of RgpB.VHH7 to inhibit the amidolytic activity of RgpB towards the chromogenic substrate L-BAPNA, was tested. As all cysteine proteases, RgpB must be in reducing conditions to be active (the cysteine of the active site must be reduced to its thiolate form $-S^-$). Consequently, the activity assay of RgpB is commonly performed in the presence of 10 mM L-cysteine. However, due to the likely presence of a disulfide bridge in RgpB.VHH7, which can be sensitive to reducing agents, we decreased the cysteine concentration to 1 mM. Using this condition, the incubation of RgpB with a large excess of VHH7 (molar ratio enzyme/nAb:1/45) did not affect hydrolysis of the substrate as indicated by the comparable increases of the absorbance at 405 nm (figure 8A). To be sure that the reducing condition during the incubation is not responsible of the lack of inhibition, the assay was repeated with a less powerful reducing agent, the reduced glutathione (1 mM), or at a very low cysteine concentration (0.01 mM). In the latter case RgpB was pre-activated in 10 mM L-cysteine then diluted 1000-fold in reaction mixture with RgpB.VHH7. In accordance with results of the first experiment no effect of RgpB.VHH7 on RgpB activity was observed in these conditions (figure 8B). We also further used direct ELISA to confirm that 1 mM cysteine in the buffer did not affect the RgpB binding of RgpB.VHH7 (data not shown). Taken together, these results indicate that VHH7 is not able to inhibit the amidolytic activity of RgpB against a small synthetic chromogenic substrate. However, due to the size of these substrates, from the data we could not exclude that RgpB.VHH7 could inhibit proteolysis of large protein substrate due to a direct steric hindrance effect independent of the active site. However, we performed extensive experiments to test whether RgpB.VHH7 could inhibit the proteolytic activity of RgpB towards fibrinogen, a large protein substrate, but no inhibition was observed, (data not shown). Due to the likely binding of RgpB.VHH7 to the immunoglobulin-like domain, RgpB.VHH7 could however be a useful research tool to study the autoproteolytic maturation process of proRgpB.

Conclusions

Taken together this study demonstrates for the first time that RgpB can be used as a specific biomarker for *P. gingivalis* infection. The very high affinity nanobody can prove useful in the context of the *P. gingivalis* detection and the nanobody-based assays presented could supplement existing methods for *P. gingivalis* detection. Furthermore, the nanobody can likely be used in structure-function studies of RgpB.

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Figure 1.

A) Phage ELISA test of forty selected clones after 3 panning rounds. Optical density responses detected after the binding of nanobody-phages to immobilized RgpB are shown. Clones with signals above 0.5 were deemed positive and further sequenced. **B)** Clones RgpB.VHH5,7,11,12,16,17,27,36 all have the same sequence. Highlighted are the three CDR regions, the conserved disulfide bridge and the cysteine residues in CDR1 and CDR3 which may form a disulfide bridge. **C)** SDS-PAGE evaluation of RgpB.VHH7 purity after IMAC-purification. Lane 1 shows RgpB.VHH7 as a single band with an apparent molecular weight of 10–15 kDa. Lane 2 PageRuler prestained marker.

Figure 2. Inhibition assay for detection of RgpB in PBS and saliva

Normalized responses (A/A_0) for increasing concentration of RgpB are shown. Data represents three assays performed on the same day with error bars to depict standard deviations. A clear dose-response relationship is seen for RgpB detection in PBS (filled circles). The IC_{50} value for detection in PBS is estimated to be in the low nM range. Matrix effects for RgpB detection in saliva (open circles) are apparent and the IC_{50} value increases to approximately 20 nM. Each data set is fitted to a logistic 4-parameter equation (sigma-plot). Coefficients of variation (CVs) for each RgpB concentration were calculated and are seen in the inserted table. CV's are defined as standard deviations divided by the mean from each assay ($n = 3$) $\times 100$ %.

Figure 3. Association and dissociation curves at different concentrations of RgpB

A) Varying concentrations of RgpB were diluted in HBS-EP buffer (same as the running buffer to minimise refractive index changes) and passed randomly over the surface. RgpB at 25, 6.25 and 1.56 nM were passed over the surface in duplicate to determine assay reproducibility. **B)** The surface was regenerated with 5 mM NaOH which generated a very reproducible baseline and reproducible binding signals (shown in the replicate analysis). **C)** A simple 1:1 binding model was fitted to the association and dissociation data and the rate constants determined using BiaEvaluation software. **D)** Summary of kinetic constants and binding affinities calculated for the anti-RgpB nanobody fragment that bound recombinant RgpB. [^] The number in brackets represents the standard error in the last significant digit of k_a . ⁺ The number in brackets represents the standard error in the last significant digit of k_d .

Figure 4. Cross-reactivity of RgpB.VHH7 towards HRgpA

A) The inhibition assay is used to show the cross-reactivity profile of RgpB.VHH7. At 10 nM no inhibition is seen for HRgpA illustrating that RgpB.VHH7 is specific for RgpB. **B)** Differences in the amino acid sequence (low case font) between RgpB and the RgpA catalytic domain (both from strain W83). Black and blue font, caspase-like and Immunoglobulin-like domains, respectively. The catalytic dyad of Cys and His is in red font. Low and high degree of similarity between of amino acid residues is marked by "." and ":", respectively. It is evident that the catalytic domains share an almost identical sequence, while there are many amino acid variations between the Immunoglobulin-like domains of RgpA and RgpB.

Figure 5. Subtractive inhibition assay for *P. gingivalis* cell detection

This illustrates normalized values for increasing *P. gingivalis* concentrations. The wildtype W83 cells (solid circles) display a dose-response inhibition profile illustrating that RgpB.VHH7-based detection of *P. gingivalis* is possible. Inhibition is also possible with the RgpA deletion mutant (W83/ Δ RA, open circles), suggesting that RgpB.VHH7 does not bind RgpA. No inhibition is seen when using the double *rgpA* and *rgpB* genes deletion mutant (W83/ Δ AB, black triangles). Coefficients of variation (CVs) for each *P. gingivalis* concentration (wildtype W83) were calculated and are seen in the inserted table. CV's are defined as standard deviations divided by the mean from each assay ($n = 3$) $\times 100$ %.

Figure 6. Cross-reactivity studies with other *P. gingivalis* proteases

The subtractive inhibition assay is used to study RgpB.VHH7 binding to various *P. gingivalis* deletion mutants. Inhibition is not seen in the strain where all gingipain genes have been deleted (W83/ΔKRAB) and the same is seen in the W83/ΔAB strain. Inhibition is however seen in the strains with either the single *kgp* gene deletion (W83/ΔK) or with both *rgpA* and *kgp* genes deleted (W83/ΔKRA). Taken together this data set confirms that i) RgpB.VHH7 binds native RgpB and ii) RgpB.VHH7 is specific for RgpB.

Figure 7. RgpB.VHH7 binds the membrane bound mt-RgpB isoform

Wild type *P. gingivalis* cells (W83) was collected by centrifugation, washed and re-suspended to 2×10^8 cells/ml in PBS and then analyzed in the subtractive inhibition assay. The re-suspended, washed bacteria cells perform in manner similar to unwashed wild-type cells suggesting that RgpB.VHH7 primarily bind the membrane-associated mt-RgpB isotype.

Figure 8. RgpB.VHH7 do not possess inhibitory properties versus RgpB

A) VHH7 was incubated with recombinant RgpB (nAB/Enzyme: 45/1) in activity buffer supplemented with 1 mM. L-cysteine. After 1 hour at room temperature, the substrate L-BAPNA was added and the absorbance at 405 nm was recorded (representative data of triplicate determinations). **B)** Alternatively, to eliminate a possibility of reducing an essential disulphide bridge in nAbs, glutathione (GSH; 1 mM) was used to activate gingipain or pre-activated RgpB (10 min in activity buffer supplemented with L-cysteine 10 mM) was mixed with nAbs (0.01 mM final cysteine concentration). The residual RgpB activity on L-BAPNA was then recorded at 405 nm. (n = 3, mean $\pm$ S.D.).

Table 1

Description of *P. gingivalis* strains used in this study

<i>P. gingivalis</i> strain	Characteristics	RgpA	RgpB	Kgp	Ref.
W83	Wild type	+	+	+	[58]
W83/ΔRA	<i>rgpA</i> Cm ^r	–	+	+	[25]
W83/ΔK	<i>kgp</i> ^{Δ602} Em ^r	+	+	–	[24]
W83/ΔKRA	<i>kgp</i> ^{Δ598} Δ <i>rgpA</i> Tc ^r , Cm ^r	–	+	–	[59]
W83/ΔAB	Δ <i>rgpA</i> Δ <i>rgpB</i> ^{Δ495} Cm ^r , Em ^r	–	–	+	[25]
W83/ΔKRAB	<i>kgp</i> ^{Δ598} <i>rgpA</i> <i>rgpB</i> ^{Δ410} Tc ^r , Cm ^r , Em ^r	–	–	–	[59]