Official Master's in Applied Microbiology

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Research Proposal

Dental plaque treatment via C16G2 Chitosan nanoparticle

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ABBREVIATIONS

AMP: Antimicrobial peptide

Ch_Np: Chitosan Nanoparticle

CLSM: Confocal laser scanning microscopy

CNM: Collagen binding protein

CSP: Competence stimulating peptide

DD: Deacetylation degree

DNA: Deoxyribonucleic Acid

DSL: Dynamic light scattering

EE: Encapsulation efficiency

EK: Enterokinase

EPS: Extracellular polymeric substances

GBP: Guanylate Binding protein

GMO: Genetic modified organisms

GTS: Glycosyltransferases

HPLC: High-performance liquid chromatography

IMAC: Immobilized metal affinity chromatography

IPTG: Sopropyl β- d-1-thiogalactopyranoside

LC: Loading capacity

LMW: Low molecular weight

MBS: Minimal bactericidal concentration

MCS: Multicloning side

MIC: Minimal inhibitory concentration

MTT: [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay

MW: Molecular weight

NI-NTA: Nickel-nitrilotriacetic acid affinity chromatography

NIH: National Institute of Health

PCR: Polymerase chain reaction

PDI: Polydispersity index

PI: Propidium iodide

PMSF: Phenylmethylsulfonyl fluoride

PTS system: Bacterial phosphotransferase system

SARS-COV-2: Severe acute respiratory syndrome coronavirus 2

SDS-PAGE: Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

STAMP: Specifically targeted antimicrobial peptide

TB: Terrific broth

TEM: Transmissión electron microscope

TPP: Tripolyphosphate

US-FDA: U.S. Food and Drug Administration

UV-Vis: Ultraviolet-visible spectrophotometry

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ABSTRACT

Oral biofilm often represents a source of opportunistic pathogens that can cause, not only oral infections, but also systemic ones and different diseases. The conventional treatment of dental plaque and the excessive use of antibiotics have rendered these techniques obsolete.

In this context, *S. mutans* is identified as one of the principal initiators and stabilizers of oral biofilm. It has been suggested as one of the main targets for dental plaque elimination. Recent findings, indicate and describe a synthetic molecule called C16G2, which has a specific antimicrobial effect against *S. mutans.*

This research proposal describes the construction of C16G2 chitosan nanoparticles which have antimicrobial activity and act as a drug delivery system. Additionally, it presents the recombinant expression of C16G2 antimicrobial molecule in *E. coli* to reduce costs and facilitate the industrial scale-up of the biotherapeutic. To achieve the highest effectiveness and yields, nanoparticle characterization, alongside with different antimicrobial assays, is also described in this work.

Keywords: Antimicrobial activity, dental plaque, chitosan nanoparticle, C16G2 peptide

RESUM

El biofilm oral sovint representa una font de patògens oportunistes que causen no només infeccions orals, sinó també malalties i infeccions sistèmiques. El tractament convencional de la placa dental així com l'ús excessiu d'antibiòtics han fet que aquests procediments comencin a quedar obsoletes.

En aquest context, *S. mutans* s'ha identificat com un dels principals iniciadors i estabilitzadors del biofilm oral. És per això, que s'ha suggerit com una de les principals dianes per a l'eliminació de la placa bacteriana. Recentment s'ha descrit una molècula sintètica anomenada C16G2, la qual té un efecte antimicrobià específic contra *S. mutans.*

Aquesta proposta de recerca descriu la construcció d'una nanopartícula de quitosan-C16G2, la qual funciona com un sistema d'alliberació de fàrmacs. A més, presenta l'expressió recombinant de la molècula antimicrobiana C16G2 en *E. coli* per tal de reduir costos i facilitar l'escalat industrial del nostre bioterapèutic. També es descriu la caracterització de nanopartícules i alguns assaigs antimicrobians amb l'objectiu d'aconseguir la màxima eficàcia i rendiment.

Paraules clau: Activitat antimicrobiana, placa dental, nanoparticules de quitosan, pèptid C16G2

I. BACKGROUND, CURRENT SITUATION, AND JUSTIFICATION OF THE PROPOSAL

1. *STREPTOCOCCUS MUTANS* ROLE IN DENTAL BIOFILM FORMATION

Dysbiotic dental plaque and oral biofilm have emerged as a significant challenge in dentistry. Especially in children, dental caries is a common problem that, if not treated, can result in tooth decay, multiple infections, pain, eating and speaking difficulties and even lead to a reduction in social interactions (1). Caries treatment can be resolve by non-invasive procedures such as sealing, remineralization or by restoration methods (2).
Oral communities represent about 700 species, including opportunistic and commensal microorganisms. Balance disruption within these communities can occur due to sugar ingestion and low oral hygiene, among others (3, 4).

The process of oral biofilm formation begins with the acquired pellicle formation on a clean tooth surface. Following, *Streptococcus sanguinis and Actinomyces viscosus* planktonic cells will bind to the pellicle (5), to promote cell-to-cell interactions with *Streptococcus mutans* (3). In this context, *S. mutans* is a gram-positive acid tolerant bacterium, that once it is adhered to the matrix, will 1) releases insoluble glucans for EPS development (Gts), 2) promotes bacterial adhesion (Gbps, Cnm)(6) and 3) metabolizes sugar through acid lactic cycle by PTS system. This acidification, along with the formation of EPS, contributes to the creation of an acidic environment in which only those microorganisms adapted to biofilm conditions will survive (7).
Mature and complex biofilm will be achieved through multiple microbial interactions between different species.

Moreover, biofilm-surviving bacteria exhibit tremendous amount of antibiotic resistance due to horizontal gene transfer (8). This global health emergency suggests new approaches to reduce caries incidence and oral biofilm propagation. The purpose of this research is to define a molecule that selectively targets *S. mutans* while preserving the commensal population and restores the normal flora with long-term protection.

2. ROLE OF C16G2 AS AN ANTIMICROBIAL PEPTIDE

C16G2 is a selectively target antimicrobial peptide (STAMP), also described as a pathogen selective molecule for *S. mutans*, which is based on the combination of two independent functional domains (CSP16 and G2), which are joined together by a GGG linker sequence. Both domains are responsible for increasing selectivity, potency, and kinetics (9–12).

CSP16 domain is derived from a natural bacterial pheromone called “Competence stimulating peptide” (CSP), which is a C-terminal 16 amino acid sequence. CSP16 maintains both pheromone

and target peptide activity in *S. mutans.* The G2 antimicrobial killing domain is derived from the 16 amino acid sequence of the novispirin G10 antimicrobial peptide (10, 13). Novispirin G10 and their derivates are described as a peptide with low toxicity to mammalian cells and wide antimicrobial activity (9). Therefore, CSP16 will provide specificity towards *S. mutans*, while G2 AMP will contribute to antimicrobial activity.

The C16G2 structural conformation is based on an amphipathic cationic α-helical peptide (12). Scientists have not elucidated the specific process of C16G2- *S. mutans* interaction yet. However, their structural similarities with other AMP suggest their potential mechanisms.
In saliva solution, CSP16 peptide domain tends to adopt a linear conformation, whereas in organic solutions or during membrane interactions, it assumes amphipathic and α-helix conformations. Correct peptide folding is the key for targeting specific *S. mutans*. This conformational interaction between C16G2 and cellular membrane will cause the disruption of the cell membrane. It will allow the secretion of all intracellular molecules, besides the loss of membrane potential, membrane integrity, cell viability, and cell death (12).

Several studies (10, 11) have described the effective antimicrobial activity of both planktonic cell solution and mature *S. mutans* biofilm. Although C16G2 is a selective molecule for *S. mutans*, some studies have shown that C16G2 treatment can reduce other opportunistic oral pathogens, such as *Veillonella, Fusobacterium, Campylobacter, Gemella and Neisseria.* As a result of their commensal relationship, a reduction of these species is directly correlated with *S. mutans* elimination (14). To sum up, the different pathogen communities are known to be influenced by the effect of C16G2 (14, 15).

Consequently, CC16G2 has been accepted by US-FDA as a New investigational Drug (11). Currently, C16G2 is being tested in phase II clinical trial (9).

3. CHITOSAN NANOPARTICLES PROPERTIES

Chitosan is a natural cationic polymer derived from chitin polysaccharide (16), found in crustacean shells aquatic microorganisms, fungal cell walls and insect exoskeleton (17). Chitosan is obtained by the deacetylation of chitin (18). Chitosan nanoparticles consist of linear polymers with an irregular distribution of D-glucosamine and N-acetyl-G-glucosamine, corresponding to the deacetylated and acetylated forms, respectively, and are linked with beta- (1-4) unions (16, 17, 19).

Molecular weight (MW) and deacetylation degree (DD) are the two main parameters for characterising chitosan nanoparticles, typically in the range of 50-1000KDa MW and 30-95 % DD

(17). An equilibrium between these parameters should be established, as a higher DD will reduce biodegradation but at the same time will reduce its solubility (17).
Natural Ch_Np exhibited a size range of 10–100 nm. Therefore, included in the nanoscale category as nanoparticles (20).

ChNp is a non-toxic and biodegradable product with a wide range of properties and applications (21). One important property is that it perfectly works as a drug delivery system for peptides, antibodies, and antibiotics (22). ChNp production methods include ionic cross-linking methods (ion-gelation), emulsion and drying techniques, covalent cross-linking and self-organisation. In addition, ChNp can be administered as ChNp gels, nanofilms, microspheres and fibers (17). For oral biofilm treatment, the principal benefit of ChNp is that their small size particles allow the entrance (23) and the diffusion deep inside the oral biofilm (24), promoting the antimicrobial effect all around them (18). Moreover, C16G2 peptide stability is also improved by Ch_Np (25).

Some of the main properties of ChNp that enhance its effectiveness include anti-inflammatory effects, mucoadhesion, biodegradability, remineralisation, wound healing, bone repair, and pH-responsiveness (16, 19, 20). Some of these properties will be valuable for the experimental plan.

4. PROJECT JUSTIFICATION

The impact of dysbiotic dental plaque not only influences buccal hygiene, but also has been related to different human pathologies, such as different types of cancer, severity of SARS-COV2, Alzheimer's disease, diabetes and different allergies (26–30).

S. mutans is a crucial initial pathogen in dysbotic dental plaque that establishes exponential interactions with other biofilm-forming microorganisms. Moreover, the lack of effective treatments for dental plaque and the continuous problem of antibiotic resistance must suggest new approaches for this global pandemic. Recent advances in drug delivery systems and the possibility to take advantage of the novel biotherapeutic technology have been the baseline for this research proposal.

The experimental plan aims to leverage the small size and high biofilm penetration of nanoparticles, along with the antimicrobial activity and biodegradable property of chitosan. It also seeks to achieve bacterial reduction by introducing a specific antimicrobial peptide against *S. mutans*. The combination of both factors led us to hypothesise that they may have a real impact on reducing oral biofilm.
The original C16G2 antimicrobial peptide had been chemically synthesized and their antimicrobial activity had been analyzed (10, 12). In our research proposal, we aim to express recombinant

C16G2, which will significantly reduce production costs and avoid the use of chemical compounds that could increase cytotoxicity in the final product.

Therefore, the proposed product consists of chitosan nanospheres that include C16G2 recombinant peptide. The construction will be stable under optimal salivary conditions. Once the participle will be inside oral biofilm, chitosan nanoparticle will release C16G2 owing its characteristic acidic pH condition. Chitosan nanoparticles will be pH responsive in oral biofilm and will act as a drug delivery system for C16G2.

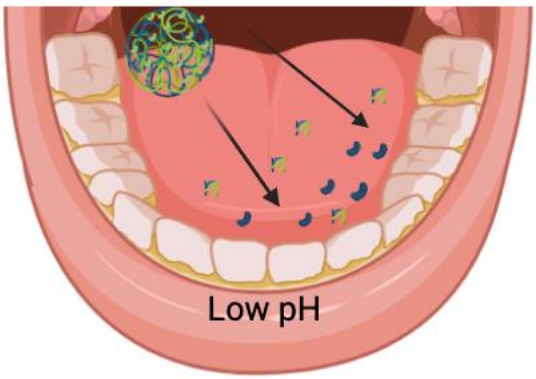

Figure 1: ***Action mechanisms of C16G2 ChNp inside oral biofilms.*** The pH-responsiveness of the chitosan nanoparticles allowed the release of C16G2 inside the oral biofilm

II. HYPOTHESIS AND GENERAL OBJECTIVES

1. HYPOTHESIS

The hypothesis of this project proposal is whether the creation of ChNp_C16G2 can have a synergic and effective antimicrobial activity towards *S. mutans* oral biofilm. ChNp_C16G2 could be used as a potential treatment for dysbiotic dental plaque and dental caries.

2. OBJECTIVES

To achieve the final product ChNp_C16G2, we will have to determine, plan and describe different sub-objectives.

- Define the molecular construction as well as the cloning and expression procedure of C16G2. Also, describe the method for subsequent verification.
- Characterize recombinant C16G2 and its properties.
- Develop Chitosan-nanoparticle as a drug delivery system for recombinant C16G2.
- Define all the methodologies to characterize ChNp_C16G2 properties (including stability, solubility, toxicology and encapsulation studies)

- Describe and quantify antimicrobial activity of active C16G2 and ChNp_C16G2, both in *S. mutans* planktonic cells and biofilm.

3. WORK PLAN

To study the previously explained hypothesis, the research proposal has been divided into four distinct subcategories. Each category will have its own objectives and expected results. By combining the findings from all categories, we would be able to either accept or reject our hypothesis.

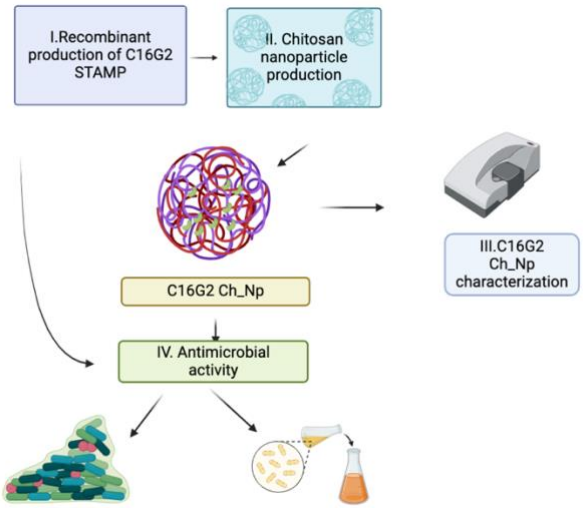

***Figure 2*: Workflow representation of the experimental design.** 1) Recombinant production of C16G2 STAMP. 2) Chitosan nanoparticle production. 3) ChNp characterization. 4) C16G2 Antimicrobial biofilm and planktonic cell activity for *S. mutans*

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III. METHODOLOGY

1. C16G2 RECOMBINANT PROTEIN

1.1. Cloning construction and transformation

Active AMPs are small size particles that can result in rapid degradation due to low solubility and instability in *E. coli* recombinant expression (1). Nevertheless, it can be solved by using a fusion protein. For this research proposal, it is used a pET32a+ plasmid (V010987, Novopro), which contains a thioredoxin fusion protein for C16G2, 6xHist tags for later purification, multiple EK to release the active protein, lac promotor, ORI and ampicillin resistance gene.

The amino acid sequence of C16G2 is TFFRLFNRSFTQALGKGKNLRIIRKGIHIIKKY (2) and will be synthesized and codon optimized for protein expression in *E. coli* as 5'ACA TTT TTC AGA CTA TTT AATAGGTCATTTACCCAGGCGCTGGGTAAGGGCGGTGGCAAGAACTTGCGCATTATCCGTAAAGGTATT CACATCATCAAAAAGTAC 3' (Gene Script; 2024). C16G2 insert and cloned plasmid sequence are described in Supplementary Figure 1-4.

The C16G2 synthesized sequence will include restriction enzyme sites for subsequent cloning, using BamHI 5' restriction site and SalI 3' restriction site, as forward and reverse primers respectively. pET-32a(+), which works both for vector cloning and expression (3), will be digested with the same set of restriction enzymes. Subsequently, T4 DNA ligase will be employed, and cloning will be achieved through a traditional cloning protocol (4).

For optimal cloning, C16G2 sequence will be inserted in the MCS of pET-32a(+) plasmid, flanked by the carrier protein thioredoxin and purification tags. pET-32a(+) C16G2 will be transformed in E. coli BL21 (DE3) competent cells through electroporation technique (3,5). After the recovery phase (1 hour at 37ºC with SOC medium) (6), transformants will be incubated with LB (Sigma-Aldrich) together with ampicillin at 37 ºC overnight (3). The positive transformants need to be further screened to select the correct clone. Sequence verification will be identified through PCR and resulting DNA sequencing. The primers used for PCR verification will include sequences derived from the plasmid and C16G2 (Primer forward F2, Primer reverse R2) (2,3). Positive and negative transformation controls are also necessary to ensure its efficacy (Supplementary Table 5).

1.2. Protein expression

From the culture of positive transformants mentioned before, protein induction will be achieved by reaching bacterial mid log-phase (OD600=0.6). IPTG will be added to induce promoter activity and protein expression, as described elsewhere (2). Centrifugation and lysozyme treatment will be performed to disrupt cellular membranes and harvest cellular proteins. Then, a soluble and

insoluble fraction will be recovered. Thioredoxin_C16G2 is supposed to be in soluble fraction due to Trx-TagT (1,2,3).

To identify the correct protein expression, an SDS-PAGE will be performed (2,3). A 24.026 KDa band, will indicate the presence of the target polypeptide. Subsequently, Western-blot analysis through Anti-Hist 6 tag antibodies, can also be done in case of achieving low soluble peptide concentration (3,7).

1.3. Protein quantification and purification

The collected soluble fraction will be purified via immobilized metal affinity chromatography (IMAC), with niquel-chelating chromatography (Ni-NTA) being the most common. The C-terminal affinity Hist6-tag will be anchored to the membrane for further elution. High imidazole concentrations will be used to improve targeted bindings and reduce co-purification (8,9)

Additional incubation with EK alongside with Urea (1-4 M) will be required for the purification of active C16G2 peptide and enhance cleavage specificity (5). EK treatment will release active C16G2, fusion protein thioredoxin and different tags. After this treatment, SDS-PAGE will be performed to confirm that the active peptide has been released (1), and consequently, a 4.078 kDa band must be found.

Protein quantification can be done with Bradford protein assay to estimate the yield of our recombinant expression system. Total protein concentration will be quantified using Coomassie Brilliant Blue G-250 (2).

1.4. C16G2 protein characterization

Prior nanoparticle's construction, conformational peptide analyses must be done to ensure the correct structure and expression. Earlier SDS-PAGE analysis will indicate whether our protein is in a soluble form or contained within inclusion bodies.

Assuming the peptide is in a soluble form, the analysis will be conducted using HPLC-MS (5, 10). For the HPLC, we will use a SHIMADZU Inertsil ODS-SP analytical column with a flow rate of 1 ml/min. The mobile phase will consist of 0.1% trifluoroacetic acid (TFA) in water and acetonitrile (ACN). At 214 nm, we should observe peaks at 16.843, 17.325, and 17.635 retention times, respectively.

Subsequently, and electrospray ionization mass spectrometry (ESI-MS) will be conducted, using a capillary voltage of -2 KV and a flow rate of 0.2 ml/min. The obtained HPLC and MS spectrum will be compared with those achieved through C16G2 chemical synthesis (11).

2. CH-NP PRODUCTION

Chitosan nanoparticles will be produced via ion gelation through the protocol described elsewhere (11). Chitosan nanoparticles will maintain their stability due to crosslinking interactions with sodium tripolyphosphate (TPP), a non-toxic agent. Cationic chitosan groups will interact with anionic TPP groups (12).

In this case, LMW chitosan polypeptide MW=10,8 KDa, DD=92 % (Sigma Aldrich) will be combined to create a 1 % acetic acid solution, always adjusting pH 3. TPP will be dissolved with deionized water (Mili-Q, ddH20) with a 1 g/mol concentration (13).

C16G2 recombinant peptide will be mixed with chitosan solution, adding 0.4, 0.8, 1.2, 1.6, 2 mg/ml, under constant stirring. TPP solution will be added to the previous one, to start the spontaneous nanoparticle formation. Finally, purified chitosan nanoparticles will be recovered by centrifugation. Subsequently, all concentrations will be analyzed to achieve the highest yields. After characterization we will determine which C16G2 proportion works better for our formulation. ChNp_C16G2 aggregates and nanoparticle size >150 KDa will be excluded, as they present much lower antimicrobial activity (14). If the expected properties are not achieved, alternative protocols may be implemented, as described elsewhere (15, 16).

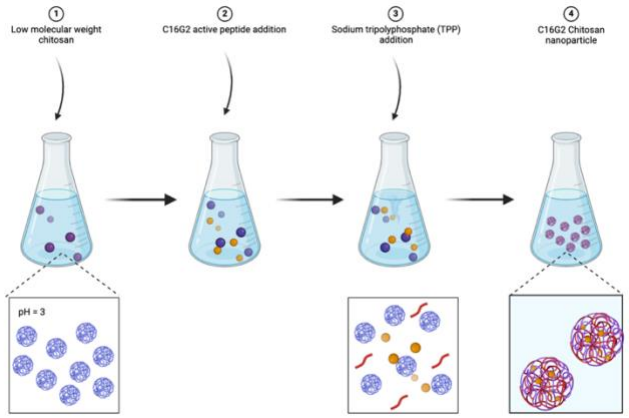

***Figure 3: Steps for C16G2 Chitosan nanoparticles production**. Adapted from (17)*

***Table 1:** C16G2 concentrations for Chitosan nanoparticle production*

	Chitosan (mg/ml)	TPP (mg/ml)	C16G2 (mg/ml)
Ch_Np1	**1**	**1**	**0,4**
Ch_Np2	**1**	**1**	**0,8**
Ch_Np3	**1**	**1**	**1,2**
Ch_Np4	**1**	**1**	**1,6**
Ch_Np5	**1**	**1**	**2**

3. C16G2 Ch-Np CHARACTERIZATION

Particle size and surface charge: Polydispersity index (PDI) together with particle size and zeta potential will be characterized by Dynamic Light Scattering (DSL) technology, using Zetasizer Advanced Range (Malvern Panalytical, UK). These measurements will be performed in triplicate in all C16G2 concentrations to ensure the results (13,18).

Morphology: Nanoparticle morphology will be analyzed through Transmission electron microscope (TEM; FEI Tecnai G2 Spirit BioTwin, USA). Before that, the nanoparticle solution will be incubated and dried overnight at room temperature with a coated cooper grid. TEM analysis will corroborate nanoparticle size as well as determine nanoparticle aggregation and nanoparticle distribution (19, 20).

Colloidal stability of C16G2 ChNp: Nanoparticle colloidal stability along the time can be very difficult to maintain (15) due to changes in pH, temperature, medium, and incubation time. These modifications result in particle aggregation and higher nanoparticle size which can alter the main properties of Np (20, 21).

ChNp stability will be determined over both short and long-time nanoparticle stability. Diluted human saliva solution will be used for colloidal stability studies, while deionized water (Mili-Q, ddH20) solution will act as a negative control.

For short time studies, Np stability will be analyzed at different temperatures, every 5 minutes for 2 hours (22). For long time studies, Np stability will be considered for 20 days, analyzing it every 4 days (12). Np stability will be determined by DSL studies as it has been explained before (12, 20,22).

Table 2: *Conditions for long- and short-time studies*

	Temperature (ºC)	Long time studies	Short time studies
Diluted human saliva	4ºC, 25ºC, 37ºC	4 days / 20 days	5 min / 2 hours
Deionized water (Mili-Q, ddH20)	4ºC, 25ºC, 37ºC	4 days / 20 days	5 min / 2 hours

Protein degradation study: Degradation studies will be done both for the active protein C16G2 and for C16G2 Ch_ Np. As well as for stability studies, we will use the same dispersion media, temperatures and time incubation periods. Both for active protein and C16G2 ChNp the degradation ratios will be quantified by SDS-PAGE as described elsewhere (23).

Cytotoxicity: Human healthy oral fibroblast HOrF (2640, Science Cell Research Laboratories) will be cultured with specific FM (2301, Science Cell Research Laboratories) at 37ºC with 5 % CO2, changing the medium every 24 hours (24). MTS assay (G3582, Promega) will be carried out to determine the possible cytotoxic effects of ChNp. MTS assay will be done with $5 \cdot 10^5$ HOrF cells/ml according to the protocol described elsewhere (21, 25). MTS will allow the quantification of viable cells after the CHNp treatment, by MTS absorbance at 490 nm.

Drug encapsulation efficiency and release kinetics: The encapsulation efficiency (EE) and loading capacity (LC) of C16G2 in chitosan nanoparticles can be quantified by different strategies. Different scientists (18, 19, 26) have suggested the quantification of encapsulated C16G2 through Ultraviolet visible spectroscopy (UV-vis). After the nanoparticle production and purification, the supernatant will be centrifuged and extracted. The C16G2 molecules that remain in the supernatant will be quantified by absorption at 214 nm (11). C16G2 protein will be quantified from a previous quantification curve. The empty C16G2 supernatant will be used as a negative control for our quantification. Another option for the quantification of our encapsulated C16G2 is the use of MicroBCA TM protein assay (Thermo Fisher, IL, USA), as it is described in different articles (13, 15).

In accordance with other authors (18), EE is the amount of C16G2 recovered in C16G2 ChNp, compared to the total amount of peptide used in the nanoparticle production. And LC is the amount of C16G2 per C16G2-Ch_Np dry weight.

4. C16G2 AND C16G2 CH-NP ANTIMICROBIAL ACTIVITY

4.1. Antimicrobial activity on *S. mutans* planktonic cells

Antimicrobial activity in *S. mutans* planktonic cells will be quantified by Microdilution broth method to calculate Minimal Inhibitory Concentration (MIC). Antimicrobial assays will be done for recombinant C16G2 free peptide, ChNp and C16G2_ChNp. *S. mutans* solution will be diluted with BHI broth and concentration will be adjusted at $5 \cdot 10^5$ cfu /ml. Bacterial growth will be measured at 630 nm after 24 hours at 37ºC under anaerobic conditions as described elsewhere (27, 28).

Both positive and negative controls will be present. Negative control will consist of BHI broth while positive control will be chlorohexidine, an antimicrobial molecule for *S. mutans* (29).

Triplicate antimicrobial assays will be necessary to conclude the optimal concentration and a sample data (N) big enough to achieve significant results in our statistics.

4.2. Antimicrobial activity on *S. mutans* biofilm

S. mutans biofilm formations will be achieved by incubation with BHIS (BHI with 1 % sucrose) overnight at 37 ºC at a $5 \cdot 10^5$ cfu/ml. The antimicrobial assays will be determined using 96 well plate microdilution method after incubation with specific concentrations of C16G2 peptide, ChNp and C16G2 ChNp. After 24 hours, the plates will be stained with 0.1 % crystal violet (CV), as described elsewhere (30). A microplate reader tool will measure the stained biofilm's optical density at 590 nm.

Both negative and positive controls will be the same as those used in planktonic cell assays. Also, triplicate assays will be necessary to achieve significant statistical results.

Despite using CV to determine antimicrobial efficacy, it cannot always distinguish between viable and dead cells (31). Reason why further antimicrobial studies will be defined to achieve better results.

Live/Dead bacterial viability assay (SYTO/PI) will take place to conclude which bacterial cells have died due to peptide or nanoparticle activity. The staining protocol is described previously in (30). On one hand, SYTO dye will bind to the total biomass and green color will be shown between the 488-520 nm excitation range. On the other hand, PI dye will bind only to the cells that present membrane damage and dead cell biomass, and absorbance will be emitted at 560-580 nm with red color (30). Absorbance and results will be quantified by CLSM (29) and analyzed by COMSTAT program (32).

IV. CONTINGENCY PLAN AND CRITICAL POINTS

In all research proposals, there is a certain risk that must be assumed at the beginning of the research. The specific methodologies, project design, as well as different techniques, can be crucial for research success. Therefore, this section highlights key points that must be considered for the project's success. Every research proposal requires a contingency plan to implement few variations which might improve efficiency and yields.

The initial challenges that may appear are related to the cloning and transformation process. In the cloning process is very important to confirm ligation efficiency and transformation efficiency. If the ligation efficiency is low, and the plasmid ligates without the insert, the addition of alkaline phosphate should be considered to avoid plasmid religation. In the protein expression process, if the yields and C16G2 concentrations are very low, we should IPTG concentration or induction moment must be changed. Also, if the peptide stability is poor, we may change the fusion peptide, such as SUMO, Intein, Gst among others.

Even with these changes, issues might still arise resulting in low stability and low yields. In this situation, we should eliminate the possibility of C16G2 recombinant expression. Instead, chemically synthetized C16G2 peptide for the nanoparticle construction should be used. Another

consideration involves modifying the principal nanoparticle material, being chitosan. Alternatives such as gold, silver or zinc have been used as a substitute. Nevertheless, these materials lack the biodegradable and non-toxic properties inherent in chitosan, characterized by maintaining low toxicity levels and minimizing bioaccumulation.

V. WORKFLOW

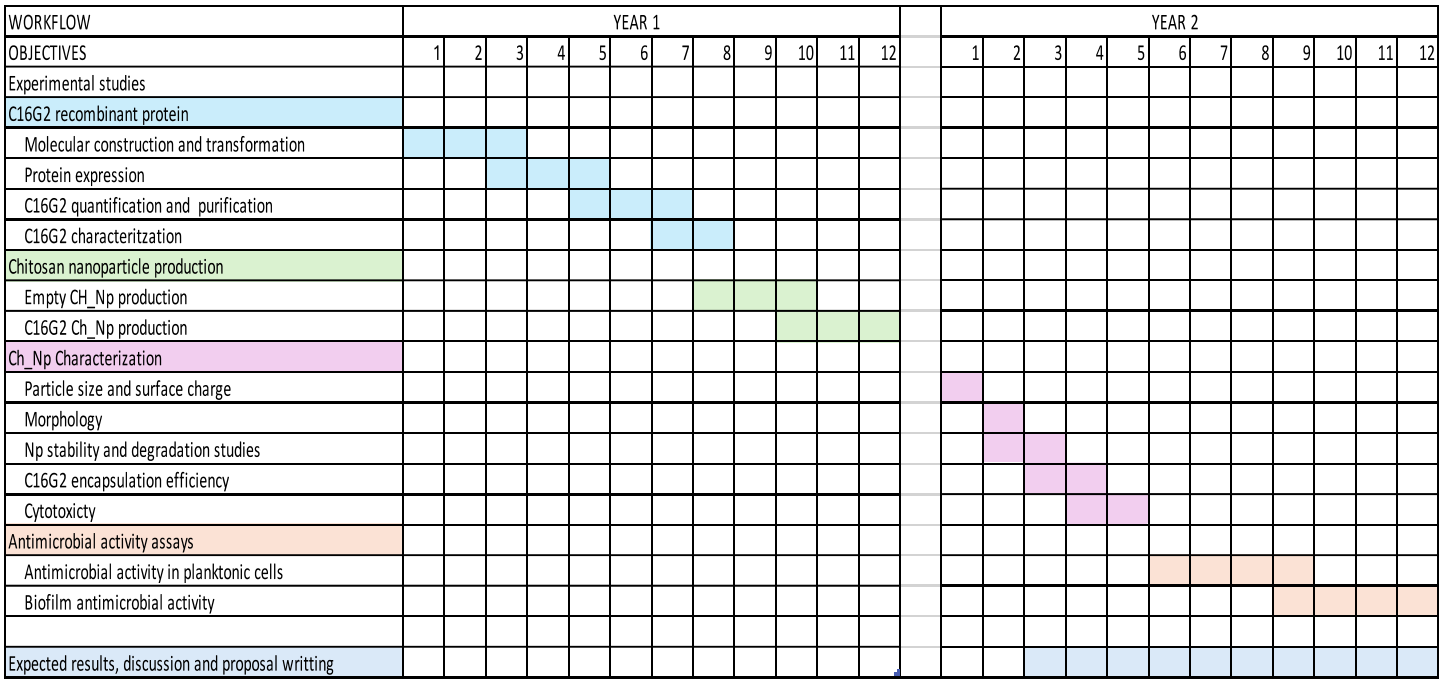

Figure 4: *Research proposal workflow for two years period*

VI. RELEVANCE AND EXPECTED RESULTS

The objective of this research proposal is to define a specific biotherapeutic that aims to act as a product that can deal with dental plaque, and that could have a total inhibition effect in oral biofilm.

C16G2 ChNp is created to be used as a single treatment. However, it could be also used in combination with conventional treatments. The idea is that, regardless of whether single or combined treatment is used, both options will allow the reduction of conventional methods and antibiotic treatment.

As it has been explained previously, the main application of this project is the creation of a therapeutic base on a chitosan nanoparticle which contains the antimicrobial peptide C16G2. A preliminary expected result is that the expression and transformation of our recombinant peptide will be correctly expressed and with high yields. This initial result whether it's combined with the nanoparticle or not, will allow us to reduce manufacturing costs and, consequently, the selling price.

It is expected to achieve optimal antimicrobial concentrations for C16G2 between 5-20 mg/ml (32). Optimal antimicrobial concentration for ChNp is considered between 0.039 to 5 mg /ml, being MIC point 0,625 mg/ ml (26). C16G2 ChNp should have a synergic effect between these antimicrobial concentrations.

It is important to highlight that this research proposal is a proof of concept, and early and preliminary experiments are suggested. To create the biotherapeutic, and before the market's release, further assays will be done to develop the optimal product with statistical results in dental environment. For instance, all the studies that have been explained will be redone, taking into consideration that saliva is the correspondent solution, and mouth is the optimal environment.
Subsequently, also characterization studies as well as antimicrobial assays will be done in multispecies oral biofilm

VII. BIORISK AND ETHICS STATEMENT

Before the initiation of the experiment and regarding risk management, the project must be approved by the Biosecurity Committee of the University or Research Center, which will determine the risk assessment concerning recombinant cells and GMOs. In addition, a containment protocol must be defined in the event of an accident.

The production of the recombinant organisms as well as the generation of the biotherapeutic must be carried out in accordance with National Institute of Health (NIH) guidelines for research involving recombinant and synthetic nucleic acid molecules (34). As it is considered in Regulation (CE) 1272/2008 [CLP], E. *coli* strains used in this research proposal are classified as non-pathogenic and non-dangerous. Therefore, a level 1 biological containment laboratory, containing biological agents of risk group 1 (BSL-1), will be necessary (35).

If successful results are obtained, our University Patent Office shall be consulted in order to ensure our product's patentability. Afterwards, a patentability study might be required.

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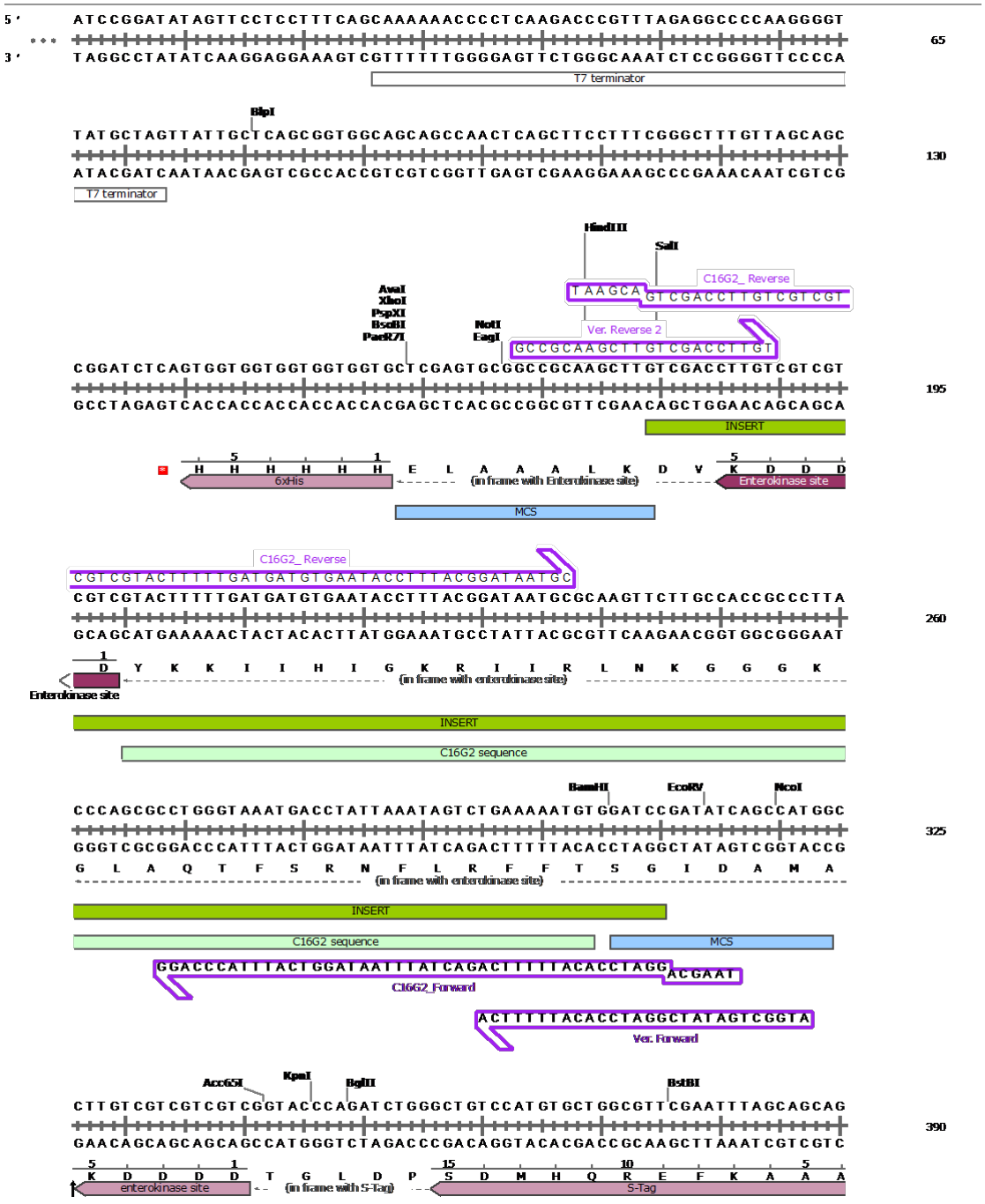
VIII. BUDGET

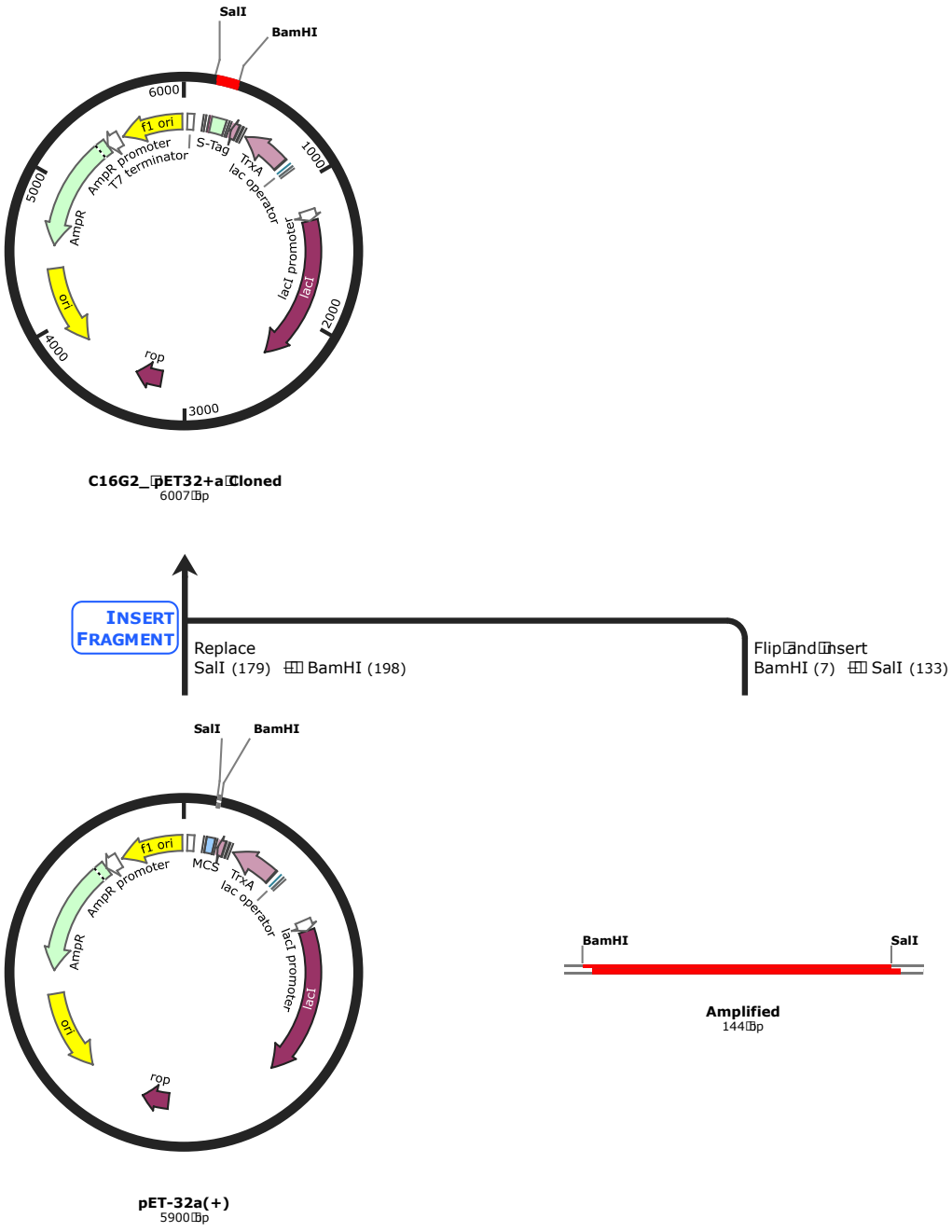
Table 3: Specific budget for this research proposal

BUDGET		
	Description	**Cost (€)**
Human resorceful	2 full-time scientist for two years contract (25000/year)	100000
Expendable material	Molecular biology consumables, kits, antibiotics, primers, enzymes, culture medium, bacterial strains, petri dish, pipette tips…	25000
Equipment	Assuming all equipment is already purchased in the laboratory	0
Patentability studies	The results of the study can be focused on the development of a patent	5000
External services	Gene sequencing and microscopy services	4000
Publication costs	All publication expenses will be charged to the university	0
Science communication services	Travel expenses, conference registration and attendance	5000
		139000
Indirect expenses	25 % of the amount for indirect expenses (water, light, taxes…)	34750
TOTAL		173750

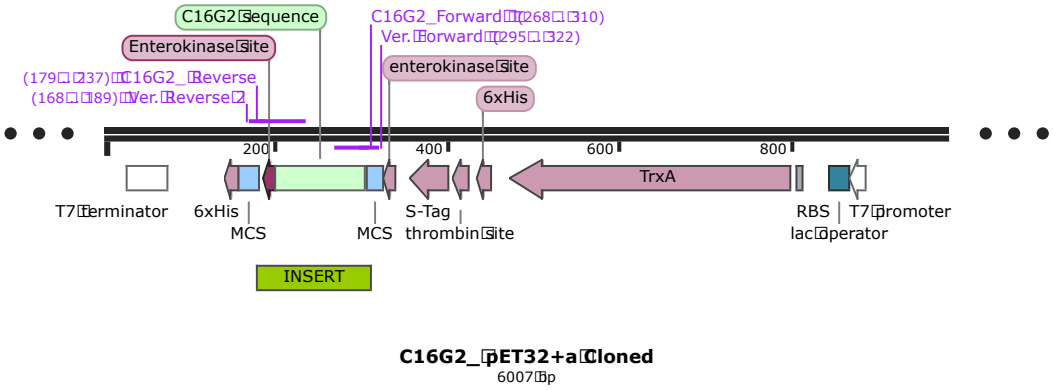
IX. ANNEX



Supplementary figure 1: *Final cloning sequence for pET32a+_C16G2. Performed with SnapGene*



Supplementary figure 2: *Cloning procedure for C16G2 expression. Performed with SnapGene*



Supplementary figure 3: *pET32a with the insert. Performed with SnapGene*

✓ **Ver. Reverse 2** 22-mer 168 .. 189 65°C

/sequence = GCCGCAAGCTTGTCGACCTTGT
59% GC / 6702.4 Da
/note = Verification plasmid

✓ **C16G2_ Reverse** 65-mer 179 .. 237 69°C

/sequence = TAAGCAGTCGACCTTGTCGTCGTCGTCGTACTTTTTGATGATGTGAATACCTTTACGGATAATGC
43% GC / 20017.1 Da
/note = Primer to add EcoRI site

✓ **C16G2_Forward** 49-mer 268 .. 310 63°C

/sequence = TAAGCAGGATCCACATTTTTCAGACTATTTAATAGGTCATTTACCCAGG
37% GC / 15028.9 Da
/note = Primer add to BamHI site

✓ **Ver. Forward** 28-mer 295 .. 322 60°C

/sequence = ATGGCTGATATCGGATCCACATTTTTCA
39% GC / 8553.6 Da
/note = Primer for verification

Supplementary figure 4: *Cloning and verification primers used. Created with SnapGene*

CONTROLS USED IN *E. COLI* TRANSFORMATION	
Positive control	*Electroporation in E. coli* cells with pET32+a, without the insert: bacterial growth should be observed
Negative control	*Electroporation in E. coli* cells without DNA: growth should not be observed
Experimental transformation	*Electroporation E. coli* cells with recombinant DNA with the insert: bacterial growth should be observed

Supplementary figure 5: *Transformation controls in electroporation process*

	C16G2	**ChNp**	**C16G2_ChNp**	**BHI broth**	**Chlorhexidine**
Planktonic *S. mutans* cells	Antimicrobial experimental assay			Negative control	Positive control
***S. mutans* biofilm**	Antimicrobial experimental assay			Negative control	Positive control

Supplementary figure 6: *Controls used in antimicrobial assays*