Final summary report STEP “Sensing peri-implant disease”

Grant agreement no: 314911

Contents

Executive Summary

Peri-implant disease is a true threat in today`s advancement of implant reliability and performance. The aim of the STEP project (Sensing peri-implant disease) is to develop a new and easy to use diagnosis tool for early detection of peri-implant diseases. The delayed recognition of peri-implant complications which are typically recognized only once clinical signs appear – a stage at which the disease course may be irreversible and lasting complications may prevail at the site of the implant zone. A treatment at this late stage is difficult and results often in irreversible bone degradation. Early diagnosis of peri-implant disease would avoid irreversible bone loss and permanent complications and help to apply existing therapies. The STEP project is a multidisciplinary approach that combines innovative technologies emerging from biochemistry, surface engineering, and dentistry. The diagnosis is based on biomarker recognition and aims to allow self-monitoring by the patient. This fact renders the system truly disruptive and radically opens novel opportunities for early diagnosis and treatment modalities of peri-implant disease.

Project description

A recent consensus meeting has concluded that peri-implant disease occurs in 12 – 40% of dental implants [4]. This renders peri-implant disease a true threat in today's advancement of implant reliability and performance [5]. The proposal is addressing this challenge by deploying the human sense of taste / gustatory system for surveillance of connective tissue degradation, which marks the borderline between gingivitis/mucositis and periodontitis/peri-implantitis (**Figure 1**). This radically new and easy to use diagnostic tool, will identify and stratify subjects at risk for development of peri-implant disease, opening a new window of opportunity for medical risk assessment and, therefore, possible intervention at an early stage. This early on detection will allow pre-emptive, successful, non-complex and well tolerated treatment. The strategy followed within this consortium is disruptive in terms of shifting current point-of-care (PoC; i.e. the practitioner's office) diagnosis to self-monitoring, allowing consultation of one's dentist in diseases stages which are clinically unapparent and within which relatively moderate therapeutic intervention suffice to prevent further destruction of the implant and surrounding tissues and in contrast to more radical interventions necessary at later stages (**Figure 1**).

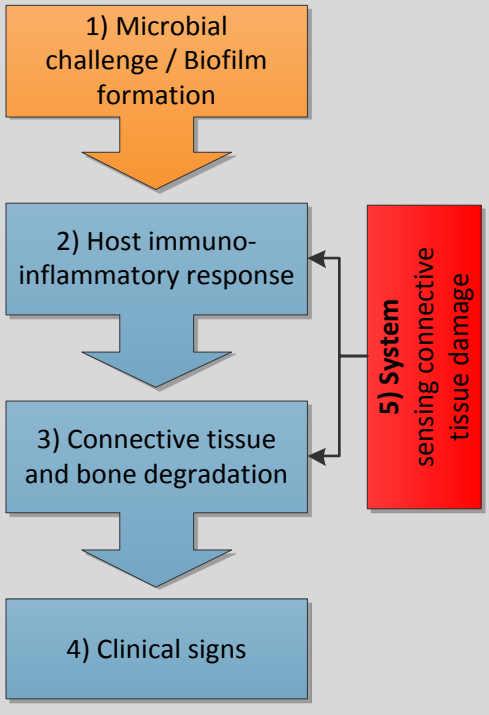

***Figure 1**: The diagnostic approach followed in this grant for early diagnosis of risk factors for the development of peri-implant infections (modified from [1]). The system (box 5) is sensing Matrix metalloproteinase (MMP) regulation indicative for connective tissue degradation as present at the borderline of gingivitis/mucositis and periodontitis/peri-implantitis. Note that today complications are typically recognized once clinical signs appear (box 4) a stage at which the disease course may be irreversible and lasting complications may prevail. Today, single assessments can be done in the dentist's office using point of care systems which measure preferentially MMP-8 from sulcus fluid samples collected around the affected implant (e.g. [3]). Naturally, these single assessments provide limited information. The proposed systems are designed to allow on-demand, self-monitoring. Therefore, the patient is providing continuous monitoring and based on this the dentist can diagnose complications early on. It is this self-monitoring which we believe renders the system truly disruptive and radically changes the time of diagnosis and preventive treatment of peri-implant infection.*

The disease course of peri-implant infection and its most severe form, peri-implantitis, commences from microbial challenge and biofilm formation. The microbial challenge triggers a defensive host response (release of cytokines and other signals) in response to bacterial mediators e.g. lipopolysaccharides (LPS; component of the cell wall of gram$^-$ bacteria), leading to massive infiltration of macrophages (Φ). It is these Φ which are capable of releasing various

proteolytic enzymes, including MMPs. Among many other MMPs tested, MMP-8 - also known as type II collagenase – has demonstrated impressive prognostic power to predict clinical progression through enhanced pocket formation, attachment loss, bone resorption, gingival recessions, increased tooth/dental implant mobility and finally tooth/dental implant loss [6] and [7, 8]. MMP-8 is disrupting the dense tissue collagen network thereby allowing efficient Φ infiltration as a prerequisite for bacteria removal – in other words, MMP-8 activity is directly linked to the first and clinically fully reversible stage of gingival connective tissue destruction (**Figure 1**, second and third boxes from top [1, 9]). MMP-8 upregulation in peri-implant infection is massive as compared to other inflammatory dental disease [10] and therefore, MMP-8 is an ultra-sensitive, prognostic biomarker for sensing peri-implant disease long before more severe disease states are attained, such as peri-implantitis. The challenge is to enable the subject for self-monitoring of MMP-8 activity using the system (conceptually presented in **Figure 1**) and functionality is achieved as outlined in **Figure 2**.

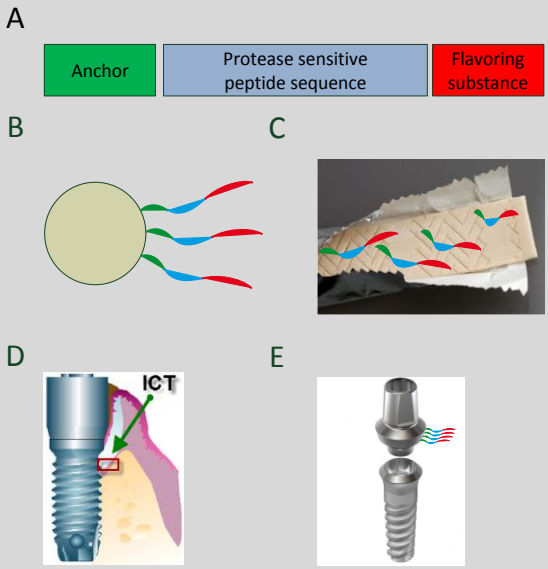

***Figure 2**: (A) The functionality is linked to the MMP-8 protease sensitive peptide sequence (blue) located in between an anchor (green) and a flavoring substance (red). (B) The sequence is linked to particles, referred to as 'system' in this proposal. Upon contact with MMP-8 at specific levels, the peptide sequence is cleaved and the off-coming flavoring substance triggers an intensive taste recognized by the patient. (C) The system is formulated into a chewing gum. During chewing, the self-monitoring is on for connective tissue damage being a prognostic factor for developing peri-implant disease. The chewing gum is providing a full mouth profile. Clearly, when activated, a dentist must perform a thorough diagnosis to identify the root cause of the positive signal as tasted by the patient. (D) Schematic illustration of a peri-implantitis lesion – an inflammatory cell infiltrate (ICT) is typically located as highlighted by the red box (copied from. (E) The peptide sequence sensitive to MMP-8 is covalently linked to the region of the abutment where the ICT is typically located to provide most sensitive self-monitoring.*

The gustatory system principally has four primary taste sub modalities recognizing sweet, sour, salty, and bitter. Maximal sensitivity is provided for bitter taste and bitter taste can be calibrated for control of inter-patient variability using methods outlined in the European Pharmacopoeia. Quinine sulfate (bitter) is sensed down to 0.0004 mM. Within the context of this project it is important, that certain short peptides can be typically sensed down 0.05 to 6 mM and this

insight is deployed by designing peptide sequences for the system which result in bitter taste following cleavage. By this strategy, the coupling of a flavoring substance can be eventually avoided as the cleaved peptide sequence itself will mediate a bitter sensation recognized by the affected patient.

In conclusion, the MMP-8 protease sensitive system provides the necessary power to the dentist for early detection and continuous surveillance of peri-implant diseases. The MMP-8 sensitive system recognizes early connective tissue damage. The system provides radically new, easy to use tools to the dentist and patient for early on monitoring of peri-implant diseases with immediate relevance on patient oral health. The system is radically shifting monitoring of peri-implant infection from assessments involving complex machinery to self-monitoring using the human tongue as a sensitive detector. Instead of restricting the monitoring of the oral health status to visits at the dentist, the approach supports frequent self-monitoring such that in case of positive signal, the subject can visit the dentist's office to get a thorough diagnosis. Performance research indicators were identified and listed, accordingly.

Main objectives

To realize the end products, the chewing gum and the coated abutment, a series of objectives was defined. First of all, *the sensitive system* has to be synthesized. Using solid phase chemistry, sensor molecules composed of (I) anchor coupled to (II) sensitive peptide sequence coupled to (III) flavoring substance are synthesized. These sensor molecules are provided to other partners of the consortium. The synthesized system has to be anchored in the next step. Therefore, strategies to immobilize sensitive peptide sequence flavoring substance conjugates to abutment/implant surfaces have to be established and the formulation of a spherical system into a chewing gum has to be realized. In parallel, MMP-8 concentrations in sulcus fluid from peri-implant disease sites are determined. Sulcus fluid from peri-implant diseased patients in different disease stages and from healthy peri-implant pockets are collected to determine specific threshold MMP-8 concentrations by correlation of measured MMP-8 concentrations with clinical diagnosis for this site. Using the obtained data, the systems MMP-8 performance regarding specificity and sensitivity is established by *in vitro* testing. The Pre-clinical assessment is performed in minipigs. A proof of principle experiment will investigate if the MMP-8 system is linked to disease staged transmucosal dental implants. Histological analysis of peri-implant hard and soft tissue structures are performed to gain detailed information. Ultimately, the system is tested in *Patient acceptance trials* to evaluate system functionality of the chewing gum in patients. In this frame, patient acceptance is assessed, as well as gustatory sensitivity of flavoring substances.

Main results

D 1.1. At least 30 sequences of MMP-8 sensitive systems

A peptide library with 248 different amino acid sequences was developed and synthesized by solid phase peptide synthesis (SSPS) in an effort to provide a broad platform for the selection of at least 30 systems with different protease sensitive peptide sequences. Two negative controls based were included and taken from previous contributions [11, 12] Bitterness of oligopeptides correlates with the hydrophobicity of the peptide sequence. For screening purposes, the Q value of a peptide sequence is used. The Q value characterizes the average hydrophobicity of a peptide, with Q > 1400 cal/mol being a threshold for possible bitter taste [13-15]. Based on this approach, the amino acids valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan were selected and introduced into MMP-8 sensitive sequences [11, 12].

Additional test peptides were synthesized to determine the cleavage conditions. Test conditions were defined based on (i) MMP-8 levels in sulcus fluid from peri-implant diseased patients in different diseases stages and from healthy peri-implant pockets (as determined in WP 3), (ii) different MMP-8 concentrations (25 - 200 nmol) and (iii) incubation times (1, 2 or 20 h). Analysis was based on electrospray ionization (ESI) and liquid chromatography–mass spectrometry (LC-MS). The peptide sequence GPQGIAGQ was used as a model peptide and incubated with different MMP-8 concentrations. Cleavage of the cleavable linker (CL) as a function of increasing MMP-8 concentrations followed a simple exponential pattern for the intact CL and the corresponding fragments followed a 3 parameter sigmoidal fit. 50% cleavage was achieved in less than 60 minutes at concentrations of 900 nM MMP-8.

Suitable examples of flavoring substances were selected having a known bitter or sweet taste. An example for a bitter tasting substance is denatonium benzoate - the bitterest compound known. It is widely used as an additive for alcohol, detergents, disinfectants or pesticides etc. to prevent swallowing by mistake. Neotame is a sweet-tasting substance which exhibits 7000 - 13000 times higher sweetness than sucrose. The sweetener is accepted by the EU as food additive with the number E 961 (**Figure 3**).

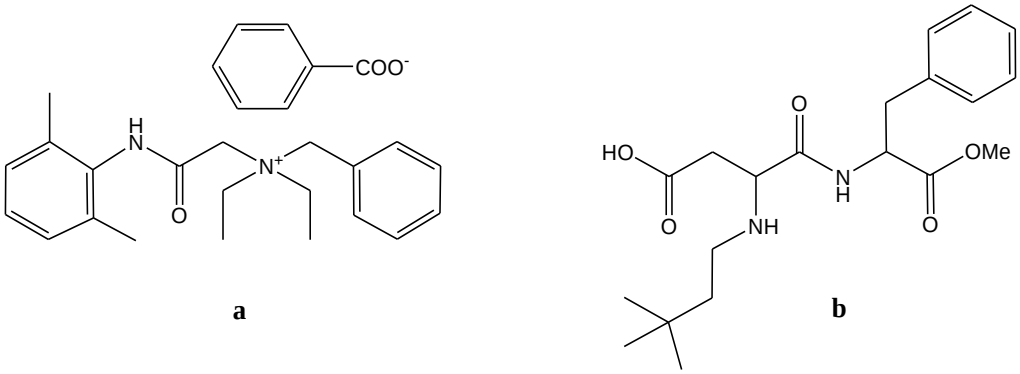

Figure 3: *Selected flavoring substances, a: denatonium benzoate, b: neotame*

The preferred method of coupling the flavoring substance to the sensitive system is the use of conventional coupling agents including but not limited to carbodiimides. Obviously, it is mandatory that upon coupling the functionalized flavoring substance has a bitter or sweet taste after cleavage of the sensitive system by MMP-8. One of the challenges was to preserve the bitter taste upon cleavage and in spite of the fact that by virtue of the MMP cleavage mechanisms, two or more amino acids remain at the flavoring substance and in identifying coupling strategies as well as flavoring molecules and peptide sequences alike to address this issue.

During project research bitter-tasting compounds could be synthesized introducing a carboxyl group enabling sophisticated ways of synthesis (**Figure 4**). Despite substitution with further functional groups the novel substances exhibit bitter taste and are suited for coupling reactions.

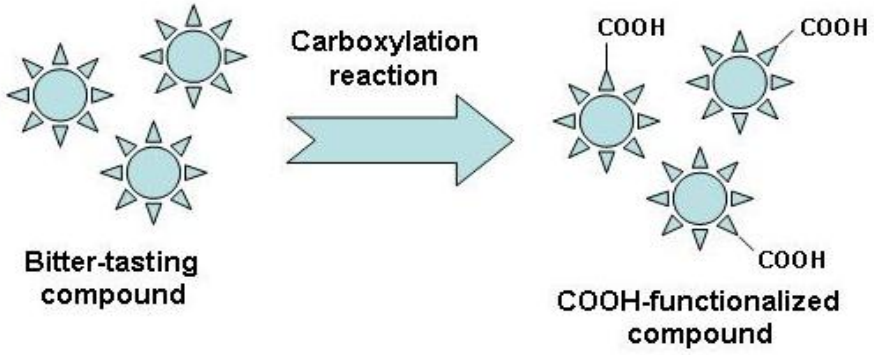

Figure 4: *Principle of synthesis of carboxylic group containing bitter-tasting compounds*

The characterization of the bitter-tasting Compounds was performed by mass spectrometry, IR and NMR spectroscopy as well as elemental analysis. The sweet tasting substance neotame was also linked to the sensitive peptide by using the carbodiimide coupling.

The coupling of flavoring substances to the MMP-8-sensitive peptide was performed by solid phase supported synthesis. Carboxylic acid group containing flavors were coupled to the amino group of the resin-bound peptide using a suitable carbodiimide in a polypropylene-reactor. The cleavage of the protecting fmoc group of the last amino acid was performed with piperidine in DMF. The syntheses as well as coupling reaction of the bitter-tasting compounds / sweetener is not disclosed in detail due to a pending patent application and was optimized in a sense that the reaction proceeded effectively and the resulting product was obtained with sufficient purity.

D 1.2. Optimization of peptide sequence according to feedback: Optimized peptide sequences according to feedback from in vitro studies

In vitro testing of spherical systems as described in D 4.1 was done and confirmed the cleavability of the sequence GPQGIAGQ, as well as the cleavability of modified sequences based on the model peptide GPQGIAGQ.

During the animal study it turned out that auto-fluorescence of certain natural contents in the saliva and sulcus fluid impacted the measurement and analysis of the samples by flow cytometry. With increasing inflammatory reaction, also the amount of auto-fluorescent particles accumulated. Therefore a reasonable and concluding evaluation was not possible.

A solution to this issue is an initial centrifugation and filtration of the saliva and sulcus fluid before incubation of the beads (**Figure 5**). In further experiments this has to be carefully considered.

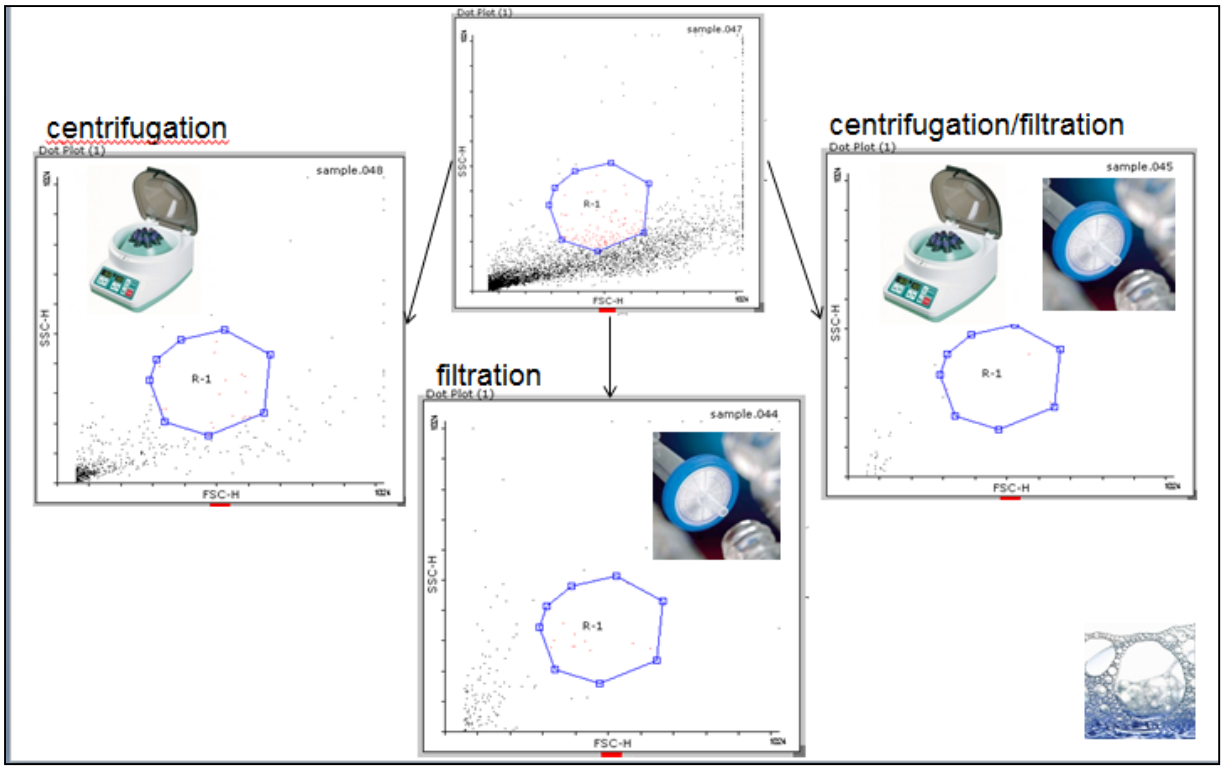

Figure 5: *Flow cytometry experiments of human saliva revealed auto-fluorescent particles disturbing measurements. Centrifugation and filtration removes these particles.*

D 2.1. Dental abutments/implants with surface-immobilized peptide-flavoring substance conjugates

Titanium disks with diameter of about 10 millimeters and dental abutments were supplied by THOMMEN MEDICAL AG. For further functionalization the disc surfaces were activated by treatment with the Pyrosil® technology, a combustion chemical vapor deposition process. During the activation the titanium surface is treated with the oxidizing part of an organosilicon containing gas jet. This organosilicon is pyrolyzed and builds up a hydrophilic layer of amorphous silicate of a thickness in nanometer range containing an increased number of reactive sites (Si-OH) on the surface. The coating of the activated surface was realized by the sol-gel-technology. The sol was prepared using different amino substituted organosilicon compounds (3-aminopropyltriethoxysilane (APTES) or trimethoxysilylpropyldiethylentriamine) in ethanol. For sol formation the ethoxy or methoxy groups were partially hydrolyzed by use of water and acetic acid. Then the sol was aged at room temperature for 20 hours. At this time hydrolysis and condensation reactions occur. The activated titanium disks were dip coated in the sol of the organosilicon for about 10 seconds. Afterwards the disks were dried at room temperature and after that at 160 °C. This leads to evaporation of ethanol and water and a gel-layer is formed on the surface of the titanium disks whose reactive sites of the Pyrosil®-

activation reacts with remaining non-hydrolyzed silicon attached ethoxy or methoxy groups of the sol. The present amino groups in the gel are linkable to the sensitive peptide system. For characterization the silicon was detected by energy-dispersive X-ray spectroscopy (EDX, **Figure 6**).

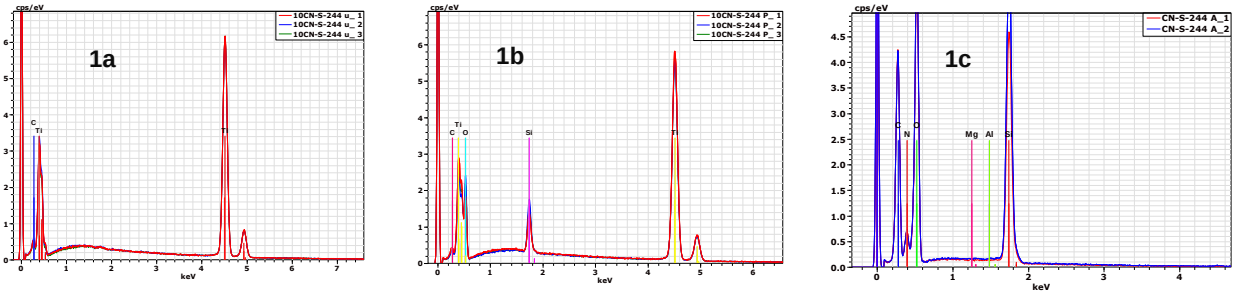

Figure 6: *EDX-spectroscopy of a: uncoated titanium samples, b: titanium disks coated with Pyrosil® and c: titanium samples coated with Pyrosil® and APTES-gel*

At the uncoated titanium sample only titanium and traces of carbon (amount dependent on the quality of vacuum during the measurement) were found. After the Pyrosil® coating silicon, oxygen and carbon can be found whereas titanium also is detectable. At the APTES-gel coated samples the silicon is much higher and nitrogen is additionally detectable whereas titanium cannot be found because of the thickness of the gel layer and penetration depth of the electron beam.

The thickness of the gel layer was determined in the case of sol-gel-coating with an alkyne functionalized organosilicone using 2-propyn-1-yl [3-(triethoxysilyl)propyl]carbamate. The procedure was carried out as mentioned before (**Figure 7**).

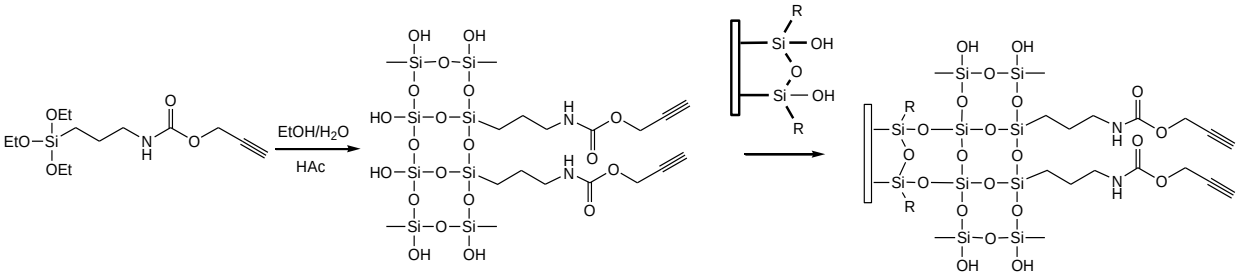

Figure 7: *Reaction scheme of coating of an activated titanium surface using a sol of 2-propyn-1-yl [3-(triethoxysilyl)propyl]carbamate*

In this case the triple bonds are available for click reactions with MMP 8 sensitive peptide systems which contain azide groups. For the determination of the thickness the Dektak 3SD (Veeco) was used. During gel coating the half sample surface was masked with an adhesive strip which was removed before measurement. The thickness ranges between 1 and 10

microns dependent on the amount of alkoxy silicone in the sol. During different storing experiments in 0.9 wt% NaCl-solution it was found, that a pre-activation with Pyrosil® to form a hydrophilic nanolayer of silicate with reactive Si-OH groups on the Ti disks is not necessary to form stable sol-gel-layers. The silicon of the gel layer still can be detected by EDX-spectroscopy even at layers of about 1 micron after a storing time of 168 days in medium.

As a surrogate for the peptide-flavoring substance a fluorocarbonic acid was coupled to an amino functionalized titanium surface by the carbodiimide coupling strategy. The fluorine was characterized by XPS-spectroscopy (**Figure 8**), indicating, that the coupling reaction was successful.

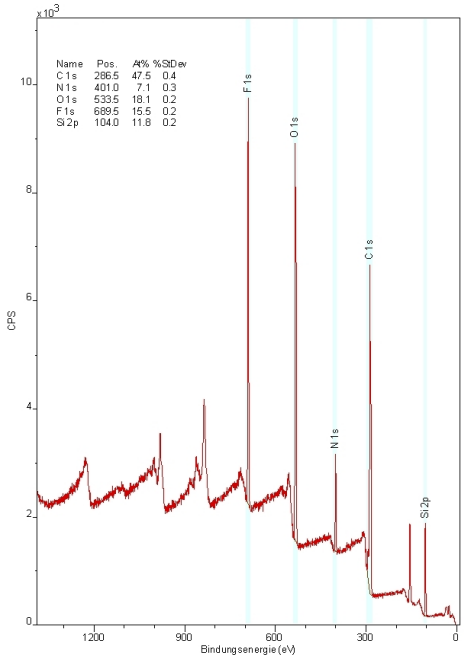

Figure 8: *XPS spectrum of amino-silicone modified Ti with surface immobilized fluorocarbonic acid*

Peptide conjugates containing bitter tasting denatonium moieties on the one hand and neotame as sweetener on the other hand were coupled on modified Ti surfaces. We do not show reaction schemes at present due to pending patent applications. The neotame bearing peptide with an azido group on one amino acid was coupled via copper catalyzed click reaction on a functionalized titanium surface containing triple bonds. The denatonium bearing peptide was coupled by carbodiimide reaction on amine functionalized Ti disks. **Table 1** shows the XPS-results of measured Ti surfaces before and after coupling of the peptide-flavoring substance conjugate.

Table 1: *Atom % of elements on surface of silicone modified Ti before and after immobilization of peptide-flavoring substance conjugate*

	Peptide-denatonium		Peptide-neotame	
	Before coupling	After coupling	Before coupling	After coupling
C	51,5 %	52,7 %	60,5 %	60,9 %
N	12,7 %	12,5 %	7,7 %	8,5 %
O	26,0 %	25,5 %	22,9 %	22,1 %
Si	9,8 %	9,2 %	9,0 %	8,3 %

Due to the used small amount of the peptide-flavoring substance conjugate (about 1 mg from solid phase synthesis) only thin substance layers were generated on the titanium surface. Consequently changes of the elemental composition of the surface are low. However the reduced silicon percentage gives a strong hint at a successful peptide coupling.

D 2.2 Chewing gum loaded with spherical systems

Bioresponsive system design and tableting

A carboxyfluorescein modified amino acid was N-terminally integrated into the peptide chain (cleavable linker (CL)) consisted of glycine, proline, glutamine, isoleucine and alanine, with a modified alanine carrying an azido group. This chain was clicked to PMMA beads, which were previously surface decorated with ethynyl groups. The conjugate consisted of the PMMA bead, the CL and the flavoring molecule / fluorescent surrogate is referred to as ‘system’ here within (**Figure 9 A**). PMMA beads with a diameter of 5 µm were homogenously distributed within the blend for tableting (**Figure 9 B**). Tableting resulted in mechanically stable pharmaceutical forms (**Figure 9 C**). Upon chewing, a pleasant chewing gum was readily formed.

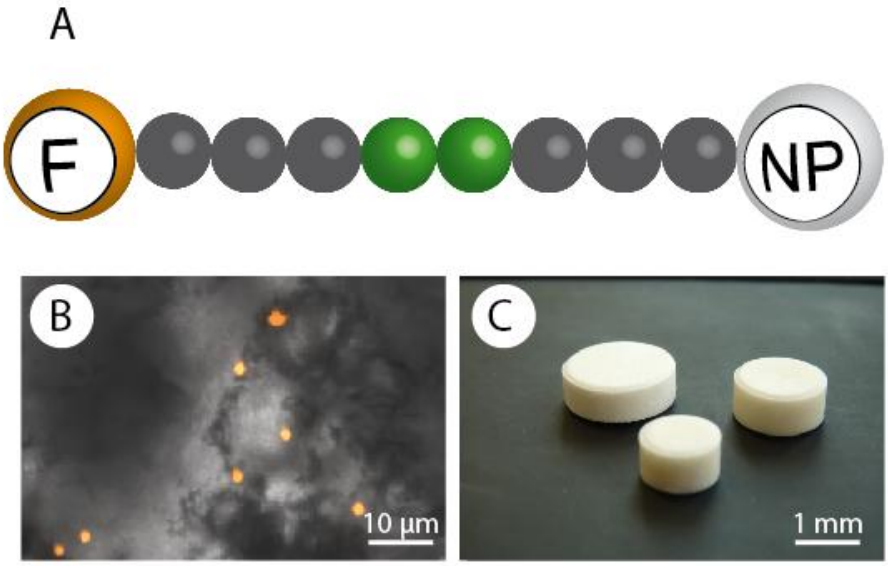

Figure 9: *(A) Schematic view of the system, consisting of a flavor molecule (F), a cleavable linker peptide chain (cleavage site highlighted by the green balls) and the particle (NP). (B) Distribution of the system within the chewing gum matrix and (C) tableted chewing gums of different sizes.*

Chewing gum and pressing

For the production of medicated chewing gum the directly compressible powder (PWD) (Health in Gum (HIG) ® Basics CAFOSA GUM SAU, Barcelona, Spain) was used in combination with PMMA-beads differing in bead-size. HIG composition and manufacturer's suggested recipes are mentioned in **Table 2**.

Table 2: *Composition of several HIG-PWD-bases and recipes*

HIG PWD-01		**HIG PWD-02**		**HIG PWD-03**	
gum base	22-26%	gum base	28-32%	gum base	33-37%
xylitol	8-12%	xylitol	8-12%	sorbitol	4-8%
softener	< 1,5%	softener	< 1,5%		
E-551 (silicon dioxide)	< 2,0%	E-551 (silicon dioxide)	< 2,0%	E-551 (silicon dioxide)	< 2,0%
sorbitol	ad 100%	sorbitol	ad 100%	isomalt	ad 100%
recipe 01		**recipe 02**		**recipe 03**	
HIG PWD-01	97,5 %	HIG PWD-02	97,5 %	HIG PWD-03	97,5 %
aerosil®	1 %	aerosil®	1 %	aerosil®	1 %
magnesium-stearat	1,5 %	magnesium-Stearat	1,5 %	magnesium-Stearat	1,5 %

Powder characterization of the Health in Gum ® Basics and the manufacturer's suggested recipes include the study of angle of repose α, Hausner ratio, compressibility index (Carr index) and the powder flow rate and speed from an orifice. These last assays were done with a flow funnel (Pfrängle), corresponding to a conical orifice in accordance to the requirements of the *European Pharmacopoeia*. A defined amount of HIG powder flows through the funnel to a centrally positioned film with concentric circles (with increasing radius of 1 cm). The radius and the height of the formed powder cone determine the angle of repose and can be obtained from the following relation:

$$\tan\alpha = \frac{h}{r} \quad \text{bzw. } \alpha = tan^{-1}\left(\frac{h}{r}\right)$$

α = angle of repose [°]

h = Height of powder cone [cm]

r = Radius of powder cone [cm]

To determine the time of powder outlet, a time measurement was done after opening the flow judge until the powder has completely flowed out.

Hausner factor and the compressibility index (Carr index) were determined by filling the powder vibration-free into a scaled measuring cylinder. After compressing the powder by jolting volumeter PT -TD 200 (PHARMA TEST Apparatebau AG, Hainburg, Germany) until the volume reduction is 0%, the compacted volume was determined. This residual volume is the compacted volume. Based on the bulk (flow) and tapped (pitch) volume the Hausner ratio (HR) and the Carr index (CI) can be calculated as follows:

$$HF = \frac{V_{flow}}{V_{pitch}}$$

V_{flow} = Flow volume [16]

V_{pitch} = Pitch volume [16]

$$CI = \frac{V_{flow} - V_{pitch}}{V_{flow}} \cdot 100\%$$

Table 3: *Composition of several HIG-PWD-bases and recipes*

Gum base	**HIG PWD-01**	**HIG PWD-02**	**HIG PWd-03**
angle of repose α [°]	30,36	29,06	27,10
Hausner ratio []	1,0917	1,1543	1,1794
Carr-Index [%]	8,37	13,37	15,21
recipe	**recipe 01**	**recipe 02**	**recipe 03**
angle of repose α [°]	28,06	24,35	24,38
Hausner ratio []	1,1858	1,1543	1,1669
Carr-Index [%]	15,67	13,36	14,31

In order to make a decision on the most appropriate basis for the production of chewing gum, all three formulations were compressed with the instrumented eccentric press EK0 (Korsch AG, Berlin, Germany). During the pressing process "PMA3 eccentric" the upper punch force-way and the upper punch force were recorded with the aid of the program. From the recorded data using an upper punch force-way diagram was created for each recipe. For better comparison, the individual curves were plotted on a graph (**Figure 10**). Tableting of HIG PWD-03 resulted in mechanically stable pharmaceutical forms, with break forces exceeding 100 N.

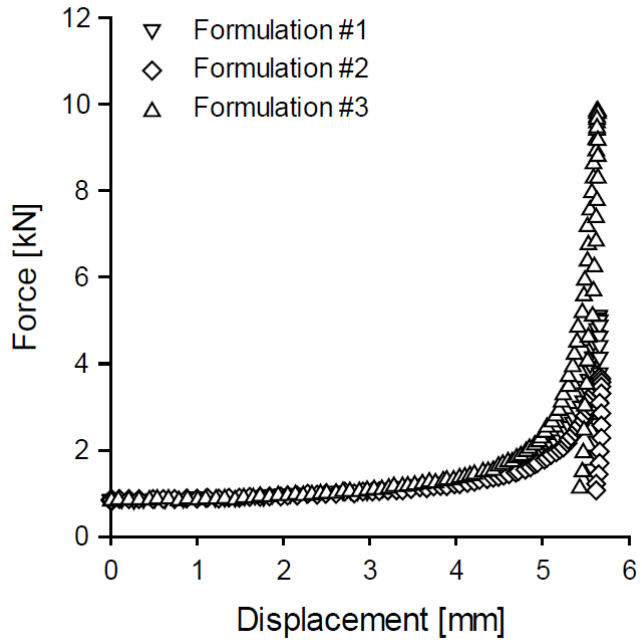

***Figure 10**: Comparison of force-displacement diagrams of the HIG recipes.*

Distribution experiments and spherical system

Experiments were performed to find the PMMA-bead-size, which show homogenous distribution and no destruction when formulated in chewing gum. Distribution experiments were analyzed using fluorescence microscopy (**Figure 11**). Beads with and without surface modifications (decorated with ethynyl groups) were analyzed.

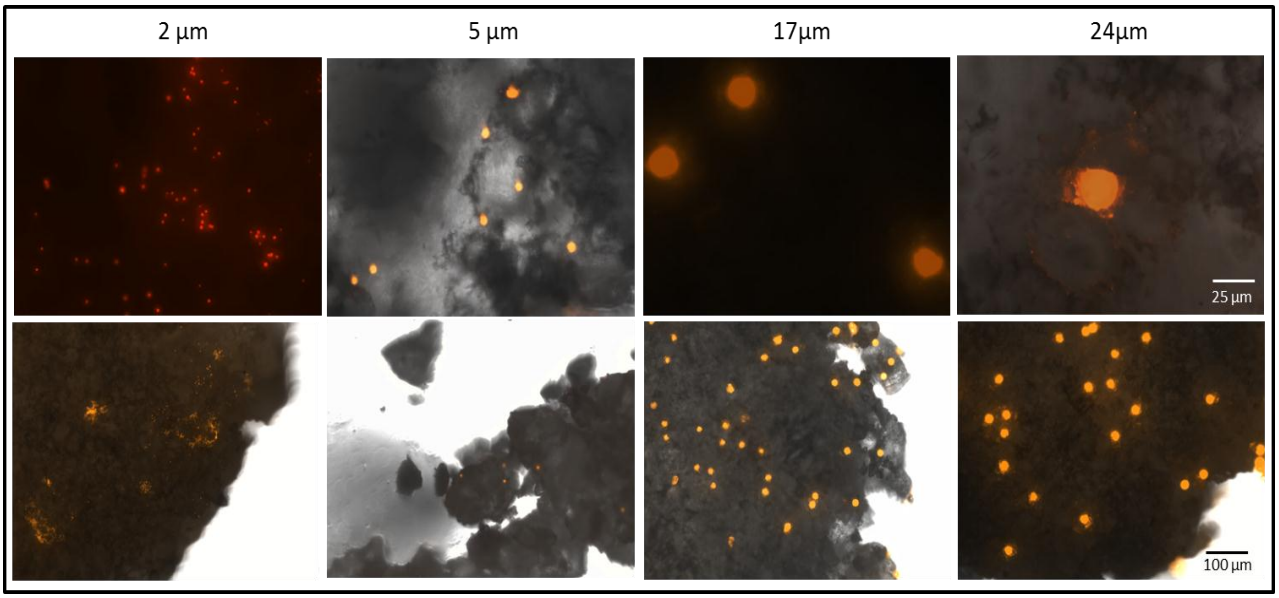

***Figure 11:** Distribution experiments of PMMA-beads in chewing gum. Different bead-sizes and modification were tested and analyzed via fluorescence microscopy*

Table 4 summarizes the results of the PMMA-bead distribution experiments of modified and not modified beads.

***Table 4**: Distribution experiments of PMMA-beads in chewing gum after pressing*

PMMA-bead size	Surface modification	No surface modification
2µm	inhomogeneous distribution, no destruction	inhomogeneous distribution, no destruction
5µm	homogeneous distribution, no destruction	homogeneous distribution, no destruction
17µm	homogeneous distribution, no destruction	homogeneous distribution, partial destruction
24µm	inhomogeneous distribution, partial destruction	Not tested

PMMA-beads with a size of 5 µm show a homogeneous distribution and no destruction during the pressing process and were selected for further experiments.

Following synthesis, peptides were successfully coupled to ethynyl group decorated PMMA beads via click chemistry (**Figure 12**).

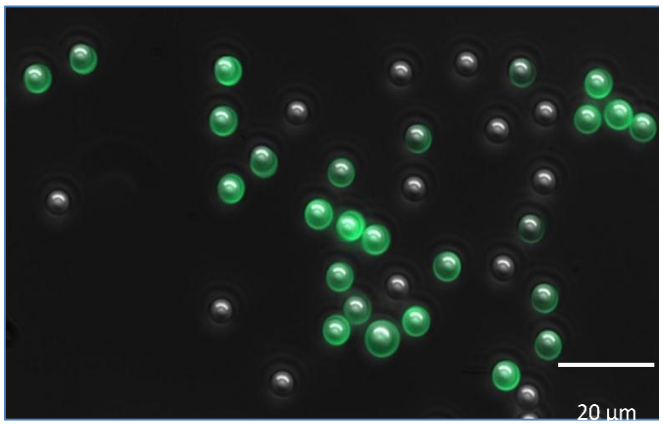

***Figure 12:** Click-reaction, 5 µm PMMA-beads with alkine monomer surface coupled to Carboxyfluorescein (Cf) labeled peptides: Cf-G-P-Q-G-I-A-G-Q-A(N3)-Q (green) with and (transparent as control) without Cu I, λ_{ex} 492 nm; λ_{em} 517 nm*

D 3.1. MMP-8 concentration in sulcus fluid from different peri-implant disease states

For the collection of sulcus fluid from peri-implant diseased patients, a cross-sectional study with 3 groups of patients was initiated. Each 20 patients with either a mucositis or a peri-implantitis were selected. Additionally 20 patients with healthy peri-implant conditions were chosen as control group. The study was approved by local authorities and is conducted according to Declaration of Helsinki. All patients enrolled in the study had to give their written consent. General inclusion criteria were an age above 18 years and patients had to show up at least one titanium dental implant. General exclusion criteria were pregnancy or lactating period, patients with a history of systemic disorders, antibiotics/anti-inflammatory drug administration within the last 30 days, substance abusers, symptoms of acute illness (e.g., fever, sore throat, diarrhea), presence of an oral mucosal inflammatory condition (e.g., aphthous, lichen planus, oral injuries, leukoplakia and oral cancer). Sulcus fluid was collected from the peri-implant zone using standardized MMP-8 collection strips (5-GCF/PISF strips, Dentognostics GmbH, Jena, Germany). The operator took 4 samples from 4 different sites at each implant (samples for Dentognostics laboratory in Germany). Additionally to the sulcus fluid sampling, a further saliva sampling was performed in the patients. Unstimulated saliva was collected using an absorbent device (Salimetric Oral Swab). With the help of supplementary saliva sampling further information about MMP-8 concentration and distribution in the oral cavity should be gained. The examiners of the laboratories were blinded to group assignment. Kruskall-Wallis test was applied to the group as the explicative variable. Post-hoc comparisons were performed using the Steel-Dwass tests. Receivers operating characteristic (ROC) curves were used to discriminate peri-implantitis versus peri-implant mucositis and healthy implants for aMMP-8 sulcus fluid and saliva fluid.

40 patients were clinically evaluated and samples were sent to the labs. Data from the analysis of the stripes by the manufacturer clearly showed a characteristic conformity between the clinical findings in patients and the absolute MMP-8 results. MMP-8 concentrations were well fitting to the clinical diagnosis of mucositis, peri-implantitis or a healthy state. Median sulcus fluid concentrations of aMMP-8 were 4.5 ng/mL for healthy implants, 9.0 ng/mL for peri-implant mucositis and 38.5 ng/mL for peri-implantitis. The differences were statistically significant (P = 0.0020). In particular, the estimated difference between medians of peri-implantitis and healthy implants was 31 ng/mL (CI95%: 7; 54 ng/mL P = 0.0014).

Also data from saliva sampling were evaluated. Data supported the MMP-8 levels of sulcus fluid collection. Median salivary concentrations of aMMP-8 were 34.8 ng/mL for healthy

implants, 68.1 ng/mL for peri-implant mucositis and 65.0 ng/mL for peri-implantitis. The differences were not statistically significant (P = 0.0634).

Overall, the area under the ROC curve (AUC) was 0.74 (CI95%: 0.61; 0.85) for sulcular aMMP-8 and only 0.56 (CI95%: 0.42; 0.68) for salivary aMMP-8. Correlation coefficient between the logarithm of sulcus aMMP-8 and the logarithm of saliva aMMP-8 was 0.37.

The explorative analysis showed positive correlation between the logarithm of sulcus aMMP-8 and Pocket Depth (PD), Full-Mouth Bleeding Score (FMBS), afternoon sampling and a negative correlation with age. In addition the analysis showed positive correlation between the logarithm of aMMP-8 in the saliva and the presence of peri-implantitis.

In conclusion: 1. Concentration of sulcus fluid of aMMP-8 was higher at peri-implantitis sites than at healthy implant sites. 2. No significant differences were detected in salivary concentration of aMMP-8 between patients with peri-implantitis, mucositis or healthy implant. 3. Area under curve, sensitivity and specificity for peri-implantitis were moderately elevated for sulcus fluid of aMMP-8. A good threshold could be 11 ng/mL of aMMP-8. Area under curve, sensitivity and specificity for peri-implantitis were low for salivary aMMP-8 concentrations. 4. The logarithm of sulcus fluid concentration of a-MMP-8 and the logarithm of salivary concentration of aMMP-8 were slightly positively correlated. 5. Higher concentrations of sulcular aMMP-8 could be detected in deeper peri-implant pockets, in younger patients, and in patients with higher FMBS. In addition, the sulcular concentration of aMMP-8 could vary during the day. Higher concentration of salivary aMMP-8 could be detected in periodontitis patients. Hypothetically, the salivary concentration aMMP-8 was related to the total periodontal and peri-implant condition of the patient.

D 4.1. In vitro testing of at least 30 spherical and 10 implant systems for MMP-8 sensitivity

Overview – synthesized peptide sequences for *in vitro* testing

Several peptides (**Table 5**) were synthesized for *in vitro* testing. After introducing an azido group into the peptide sequence, click chemistry was used for covalent immobilization to alkyne decorated beads. PEGylation and glycine-linker were inserted to improve the MMP-efficiency. Carboxyfluorescein was attached to the N-terminus of the peptides in order to observe the functionalized beads/surfaces by fluorescent microscopy. Moreover, online monitoring of the course of the cleavage of the bead-peptide construct by flow cytometry was performed.

Table 5: *For various experiments different peptides were used. The universal one letter code for amino acids is shown. Fmoc-protected amino acids were used, unless otherwise stated (e.g. Boc). Small letters were used for D-amino acids. (Acronyms: DB – denatonium, A(N3) – azidoalanine, Cf-Carboxyfluorescein, PEG – polyethylene glycol)*

Nr	Internal number	Amino acid code
1	28DB	DB-PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-A(N3)-Q
2		G-P-Q-I-G-A-G-Q
3	19_2	G-P-Q-G-I-A-G-Q-A(N3)-Q
4		G-P-Q-G-I-A-G-Q-A(N3(Cy5))-Q
5	24	G-p-q-G-I-A-G-q-Q.
6	25	A(N3)-G-P-Q-G-I-A-G-Q.PEG
7	26	G-P-Q-G-I-A-G-Q-A(N3)-Q
8	26Cf	Cf-G-P-Q-G-I-A-G-Q-A(N3)-Q
9	26DB	DB-G-P-Q-G-I-A-G-Q-A(N3)-Q
10	27	PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-Q
11	27Cf 1. Koppl	Cf-PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-Q
12	28	PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-A(N3)-Q
13	28Cf 1. Koppl	Cf-PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-A(N3)-Q
14	28Cf 2. Koppl	Cf-PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-A(N3)-Q
15	28DenCf	Cf-DB-PEG-K(Boc)-G-P-Q-G-I-A-G-Q-PEG-A(N3)-Q
16	31	G-G-G-G-P-Q-G-I-A-G-Q-G-G-G-PEG(3)-A(N3)-Q
17	31Cf	Cf-G-G-G-G-P-Q-G-I-A-G-Q-G-G-G-PEG(3)-A(N3)-Q
18	32	G-G-G-G-P-Q-G-I-A-G-Q-G-G-G-PEG(6)-A(N3)-Q
19	32Cf	Cf-G-G-G-G-P-Q-G-I-A-G-Q-G-G-G-PEG(6)-A(N3)-Q
20	33	G-G-G-G-P-Q-G-Y-A-G-Q-G-G-G-PEG(3)-A(N3)-Q
21	33Cf	Cf-G-G-G-G-P-Q-G-Y-A-G-Q-G-G-G-PEG(3)-A(N3)-Q
22	34	G-G-G-G-P-Q-G-Y-A-G-Q-G-G-G-PEG(6)-A(N3)-Q
23	34Cf	Cf-G-G-G-G-P-Q-G-Y-A-G-Q-G-G-G-PEG(6)-A(N3)-Q
24	36	G-P-Q-G-I-A-G-Q-A(N3)-Q
25	36Cf	Cf-G-P-Q-G-I-A-G-Q-A(N3)-Q
26	43	G-P-Q-G-Y-A-G-Q-A(N3)-Q
27	43Cf	Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q
28	44	G-P-L-G-I-A-G-Q-A(N3)-Q

29	44Cf	Cf-G-P-L-G-I-A-G-Q-A(N3)-Q
30	45	G-G-G-G-P-L-G-I-A-G-Q-PEG(6)-A(N3)-Q
31	45Cf	Cf-G-G-G-G-P-L-G-I-A-G-Q-PEG(6)-A(N3)-Q
32	46	G-G-G-G-P-L-G-I-A-G-Q-G-G-G-PEG(3)-A(N3)-Q
33	46cf	Cf-G-G-G-G-P-L-G-I-A-G-Q-G-G-G-PEG(3)-A(N3)-Q
34	47	G-G-G-G-P-L-G-I-A-G-Q-G-G-G-PEG(6)-A(N3)-Q
35	47cf	Cf-G-G-G-G-P-L-G-I-A-G-Q-G-G-G-PEG(6)-A(N3)-Q
36	50	G-P-Q-G--I-A-G-Q-A(N3)-Q
37	51	G-P-Q-G-I-A-G-Q-A(N3)-Q
38	51DB	DB- G-P-Q-G-I-A-G-Q-A(N3)-Q
39	52	G-P-Q-G-I-A-G-Q-A-Q

Different methods were used to discover how to optimize the peptide sequences in respect to MMP-cleavage and explained in the next points here afterwards.

MMP-activity - unmodified sequences

First we studied the cleavage of 248 different unmodified peptide sequences with MMP-8 and analyzed the cleavage-products by LC-MS. Deliverable D1.1. describes the analytics. Thirty MMP-8 cleavable sequences were selected and additionally tested for MMP-1 and MMP-9 sensitivity.

Based on the results three peptide sequences were selected for further experiments: G-P-Q-G-I-A-G-Q; G-P-Q-G-Y-A-G-Q, G-P-L-G-I-A-G-Q.

MMP-activity in solution

MMP-activity in solution was analyzed using carboxyfluorescein or denatonium-decorated peptides with azido groups (**Figure 13**).

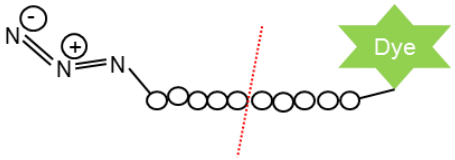

Figure 13: *Example of a peptide-structure containing an azido-group and modified with a dye*

MMP cleavage of the peptide sequence Cf-G-P-Q-G-I-A-G-Q-A(N3)-Q revealed an increasing cleavage rate with increasing MMP-8 concentrations and increasing incubation times, MMP-8 was observed to show better cleavage rates in solution than MMP-1 or MMP-9. N-terminal modifications with dye or denatonium resulted in equal MMP-8 cleavage and lead to better cleavage rates in comparison to the unmodified sequence carrying no azido group. Both peptide sequences including substituted amino acids: Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q, Cf-G-P-L-G-I-A-G-Q-A(N3)-Q were not cleaved after MMP-exposition. The adding of glycine spacers or PEGylation into the sequence Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q resulted in cleavability of the sequences.

MMP-activity in spherical systems/ on a surface

To modify the alkyne functionalized PMMA-beads (**Figure 14 A**) provided by PolyAn Berlin, functionalized peptides with azido groups were used (**Table 5**). To obtain 1 to 2 mg of beads (dry weight) approx. 40 µl of the bead suspension was transferred to a 2 ml Eppendorf centrifuge tube. The sample was centrifuged and the supernatant (water) was pipetted off. The beads were washed in 100 µl Tris-buffer (50mM, pH 7.5).

Afterwards a mixture of Cu_2SO_4 / TCEP was added (1 µl Cu_2SO_4 (1mM) and 1 µl TCEP (1mM)), followed by 0,4 µl TBTA (20µM) and 10 µl peptide (0,5 mg/ml). The beads were washed several times. Controls were performed without adding Cu_2SO_4. The beads were re-suspended in water and analyzed by fluorescence microscopy (**Figure 14**) and flow cytometry.

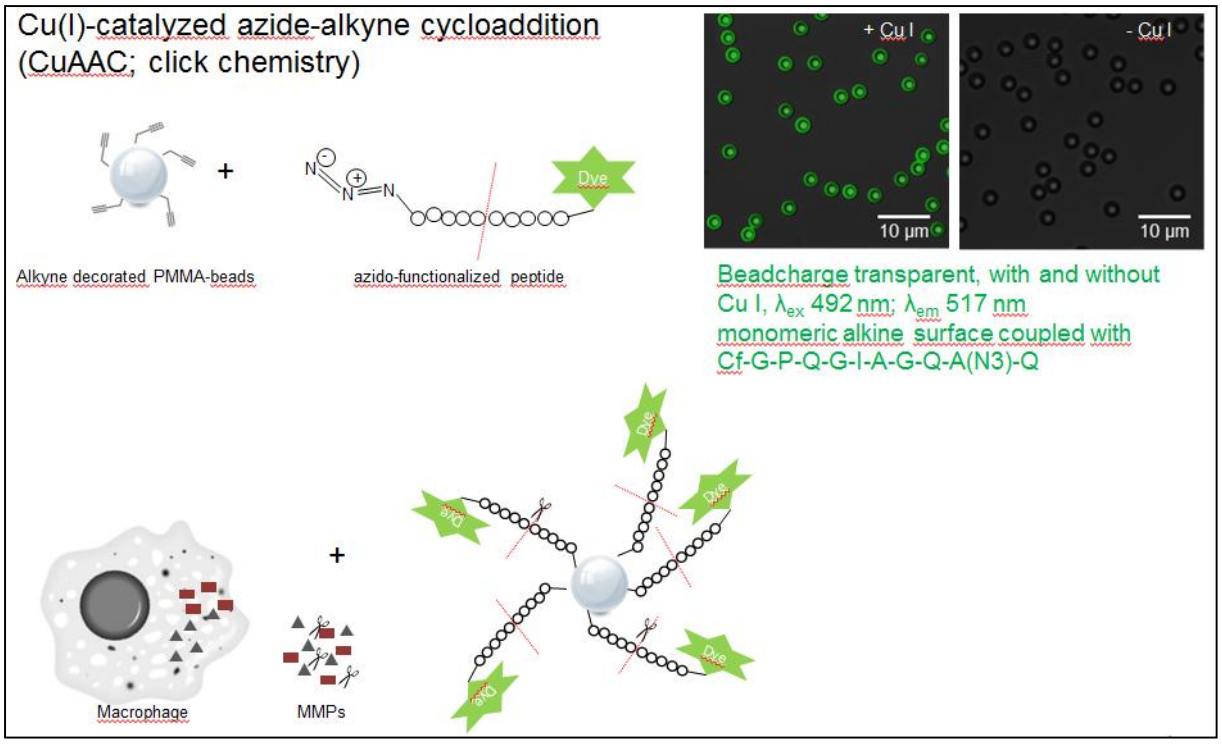

Figure 14: *(A) Schematic view of Cu(I)-catalyzed azide-alkyne click chemistry. Alkyne decorated PMMA beads were decorated with azido functionalized peptides. (B) Schematic view: In response to bacterial infiltration, host macrophages secrete collagenases, like active matrix metalloproteinase 8 (aMMP-8), a marker in predicting the progression of periodontal disease, cleave the sequence placed between the nanoparticle and the flavor/fluorescence molecule.*

Using flow cytometry the azido-alkyne coupling was studied, as well as the cleavage experiments performed afterwards. PMMA beads which pass through the beam scattered light and were detected by forward scatter (FSC) and side scatter (SSC). FSC correlates with the cell size and SSC depends on the density of the particle. PMMA-particles were distinguished based on differences in their size and density. The combination of scattered and fluorescent light was detected and analyzed by gating PMMA-beads at a laser wavelength (FL1-H) of 488 nm (Excitation Peak 500 nm; Emission Peak 520 nm). It was possible to clearly distinguish between plain particles and decorated PMMA. After exposure to MMP-8, less fluorescent signals were monitored. This decrement was used for calculation of the cleavage rate in percentage to the uncleaved systems.

Coupling of peptides to PMMA beads was performed with incubation times of 20 minutes and 60 minutes as described above.

Cleavage of the CL as a function of increasing MMP-8 concentrations followed a simple exponential pattern. The maximum cleavage observed within the concentration range selected in this study was approximately 60 %, and extrapolation suggested a maximal cleavage as of about 30 % at higher protease concentrations. Control experiments storing the CL in absence of MMP-8 for 30 days did not demonstrate any disintegration (data not shown). MMP-1 cleavage at a concentration of 24 nM results in a higher cleavage rate of approximately 8 % in comparison to MMP-8 cleavage. MMP-9 cleavage was not appreciable. Adding glycine-spacer and PEGylation at the N- and C-termini of the sequences did not hardly affect the cleavage rate of the sequence.

Both peptide sequences including substituted amino acids: Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q, Cf-G-P-L-G-I-A-G-Q-A(N3)-Q were not cleaved after MMP-exposition. Adding glycine spacers and PEGylation into the sequence Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q resulted in better cleavability of the sequences.

Coupling times (PMMA-beads to peptide) of 20 or 60 minutes had no difference in the MMP-8 cleavage rate. MMP-1 incubation leads to slightly better cleavage rates than incubation with MMP-8. MMP-9 expose does not lead to a cleavage of the system. (B) Modification of a glycine-spacer and/or by PEGylation at the N- and C-termini of the sequences did not affect the cleavage rate of the system. (C) Both peptide sequences including substituted amino acids: Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q, Cf-G-P-L-G-I-A-G-Q-A(N3)-Q were not cleaved after MMP-expose Modification of a glycine spacer or by PEGylation of the sequence Cf-G-P-Q-G-Y-A-G-Q-A(N3)-Q resulted in cleavability of the sequences.

D 5.1. Proof of principle that the flavoring substance will be separated in an early stage of peri-implant

Six mini-pigs were used for the proof-of-principle study. Animals were randomly allocated to an experimental silk ligature-induced peri-implantitis group (n=4 animals) and a reference healthy group (n=2 animals). After extraction of the premolars and first molar and a healing period of 8 weeks, each n= 4 dental implants were placed in each mandible. In the four animals of the experimental group, a single silk ligature (4.0) was placed around the cover screw and slightly pushed downwards into the pocket. This finally led to a monolayer application at soft tissue level. Ligatures were checked and maintained after two and four weeks. The two control animals got no silk ligatures. After two weeks (time point 1), four weeks (time point 2) and six weeks (time point 3), sulcus fluid was collected from the peri-implant zone in all three groups (n=6) using standardized MMP-8 collection strips. The strips were being placed into the pockets for 30 seconds. Additionally, unstimulated saliva was collected using an absorbent device (Salimetric Oral Swab). The swabs were put on the floor of the mouth for 5 mins.

Immediately after removal, the MMP-8 collection strips were further processed. Stripes were put into Eppendorf-tubes filled up with a suspension of 100 µl MMP-8 buffer (200 mM NaCl, 50 mM Tris-HCl, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, 0.05% BRIJ® 35 Detergent, 0.05% NaN_3, pH 7.0)and the beads that were coupled to the peptide sequence (40 µl). Saliva samples (50 µl) were mixed with 40 µl bead suspension and 50 µl MMP-8 buffer. The coupled beads had been vortexed to guarantee an equal distribution of the beads. Specimens were incubated (37 °C) for one hour. Sulcus fluid stripes were incubated (37 °C) in 100 µl MMP-8 buffer with 40 µl bead suspension also for one hour. Controls were performed using 40 µl bead suspension in 50 µl MMP-8 buffer and 50 µl water. Finally, the reaction was stopped by addition of 4 µl 250 mM EDTA. Cooled samples (4°C) were sent to Wurzburg and analyzed by flow cytometry.

Overall, it turned out that auto-fluorescence of certain natural contents in the saliva and sulcus fluid influenced the measurement and analysis of the samples. With increasing inflammatory reaction, also the amount of auto-fluorescent particles accumulated. Therefore a reasonable and concluding evaluation was not possible.

A possible solution to this issue could be an initial centrifugation and filtration of the saliva and sulcus fluid before incubation of the beads. However, in the present experiments this was not possible anymore. Yet in further experiments this has to be carefully considered.

D 5.2. Analysis of peri-implant hard and soft tissue structures at transmucosal implants in healthy and periimplantits minipigs

Six minipigs were used for the analysis of peri-implant hard and soft tissue structures. Animals were randomly allocated to an experimental silk ligature-induced peri-implantitis group (n=4 animals) and a reference healthy group (n=2 animals). After six weeks animals were sacrificed. All experiments were conducted according to the Swiss laws of animal protection and welfare and were authorized by the local federal authorities (authorization #73/2013).

Anaesthetized minipigs were placed in lateral recumbency and the mouth was kept open with a mouth gag. After careful elevation of a full thickness flap, teeth were extracted using standard dental instruments (forceps, elevators). Extraction sockets were cleaned. Afterwards, the alveolar bone crest was down-leveled for 1 to 2 mm and sharp bony edges were smoothened. Following eight weeks of healing anaesthetized minipigs were placed analogous to the procedure of the tooth extraction. The alveolar ridge was accessed through a full-thickness flap. Implant sites were prepared according to a standard and approved drilling protocol using rotating pilot and twist drills in ascending order (diameter). Each n= 4 dental implants were placed in each mandible. Mucoperiosteal flaps were repositioned and closed tension-free with single sutures. In the four animals of the experimental group, a single silk ligature (4.0) was placed around the cover screw and slightly pushed downwards into the pocket. This finally led

to a monolayer application at soft tissue level. A willful neglect of any plaque control supported the chosen approach. Ligatures were checked and maintained after two and four weeks. The two control animals got no silk ligatures.

Overall, ligature-induced peri-implantitis provoked a local inflammation around the experimental implants. Additionally, a loss of crestal bone surrounding the implants could be detected. In contrast reference control implants revealed no severe signs of inflammatory reactions and merely minimal loss of bone volume.

In the experimental peri-implantitis group, probing depth of implant pockets, and the presence of bleeding characterized a soft tissue breakdown, whereas microradiographs and histological sections confirmed a peri-implant bone loss with vestibular dehiscence defects. Mostly, loss of vertical bone height was more prominent on the buccal aspect than on the lingual side. These findings were in accordance with the known behavior in humans. Histologically, the measurable loss was up to two implant threads. In some cases even more. Lingually, the bony atrophy in the vertical direction was less distinctive. In several cases a pronounced bony pocket formation respectively crater was obvious. Generally, however, resorption was not limited to the outer diameter of the silk ligature, but was more comprehensive. In the apical part of the test as well as control implants histological assessment of osseointegration processes revealed a close bone-to-implant-contact-line. Thereby, implant threads and pits were covered with new bone and the former osteotomy site was not visible anymore.

In accordance to the clinical situation in humans soft tissue structures showed a coronal detachment in the peri-implantitis group. In contrast implants of healthy minipigs revealed a tight sealing along the whole cover screw. Interestingly, in the control group the seam epithelium around the implants was almost reaching the bone level. A dense and intertwined network of collagen fibers could be seen at the supracrestal aspect. Nuclei of fibroblasts were cut in the long and transversal axis. Thus collagen fibers were growing in different directions. In the experimental group some remnants of the silk ligature were visible. In cases of severe buccal bone loss the implant threads were covered with connective tissue structures. Orientation of collagen fibers was less obvious.

Clinically, bleeding on probing and a pocket depth of more than 5 mm demonstrated a diseased status after 6 weeks in the ligature-induced peri-implantitis animals. Pus formation could not be detected. Yet, the peri-implant gingiva was slightly swollen and displayed a remarkable reddening. Additional impact and accumulation of food debris around the implants supported the soft tissue breakdown. Removal of food debris around implants induced a local bleeding.

In contrast healthy animals showed some minor plaque formation and food remnants around the implants respectively the cover screw. Yet bleeding was less conspicuous and peri-implant

gingiva was less affected. Overall, marginal gingiva was much more “attached” than in comparison to the experimental animals.

In conclusion, clinical, radiological and histological findings of the present animal experiment supported the overall applicability of the ligature-induced peri-implantitis minipig model. The animals showed a progressive inflammatory soft tissue reaction with bleeding and reddening. A rapid breakdown of peri-implant hard tissues could be detected mainly on the buccal side. Soft tissue structures revealed a status of severe mucositis with subsequent transition to peri-implantitis.

D 5.3/D 5.4 Ethic commission request and clearance

A detailed study plan regarding the ethic commission request for the animal study was written according to Good Laboratory Practice (GLP) guidelines, including the purpose of the study and the materials and methods used. Furthermore, the study was performed under GLP-like conditions based on the Swiss Ordinance relating to Good Laboratory Practice adopted May 18th, 2005 [SR 813.112.1] and in compliance with the OECD Principles of Good Laboratory Practice, as revised in 1997 and adopted November 26th, 1997 by decision of the OECD Council [C (97) 186/Final].

These principles are compatible with Good Laboratory Practice regulation specified by regulatory authorities throughout the European Community, the United Stated (EPA and FDA), and Japan (MHLW, MAFF, and METI).

<u>The following guidelines were applied:</u>

Council Directive 93/42/EEC of June 1993, version 11.2.2007 concerning medical devices.

ISO 10993-1: 2009/COR 1: 2010 Biological evaluation of medical devices – Part 1: Evaluation and Testing.

ISO 10993-2: 2006(E): Biological evaluation of medical devices - Part 2: Animal welfare requirements.

ISO 10993-6: 2007 Biological evaluation of medical devices – Part 6: Tests for local effects after implantation.

ISO 10993-12 Biological evaluation of medical devices – Part 12: Sample preparation and reference materials (2002).

The ethic commission clearance was granted with the obligation that only the defined persons in the study plan were allowed to perform the animal trials. Experiments were authorized by the local federal authorities (authorization #73/2013).

D 6.1 Providing bitter value for cleaved peptide sequence-flavoring substance fragments for optimization

For taste testing of the synthesized bitter substances an electronic tongue was used. The measurements were performed on the system TS-5000Z (Insent). The electronic tongue has different sensor types for bitterness, sourness, saltiness and umami. The sensors consist of an artificial lipid membrane. Electrostatic or hydrophobic interactions between different substances and the membrane were determined compared to a reference solution. After a defined washing procedure an after taste can be determined.

The measurement of the new synthesized carboxylated bitter-tasting derivatives was compared to the known bitter compound denatonium benzoate (**Figure 15**).

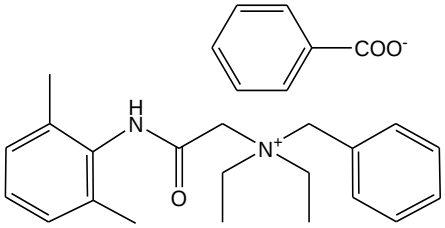

Figure 15: *Denatonium benzoate*

Three sensors for bitter taste of cationic compounds (SB2AC0, SB2AN0 and SB2BT0) are available for the determination of bitterness.

A change in the sensor signal dependent on the concentration of the denatonium benzoate as well as the synthesized new compound was shown. The behavior of the methyl esters of the carboxylated derivatives was very similar to the denatonium benzoate. This finding might indicate that the electronic tongue identified the substance as bitter, comparable to the denatonium benzoate.

Because of the fact that after cleavage of the sensitive system by MMP-8 still amino acids bonded to the flavoring substance we decided to synthesize a denatonium derivative coupled with glycine ethyl ester and determine the bitterness with electronic tongue.

All three sensors detect differences in concentration of the glycine-derivative of denatonium salt. At low concentrations of 0.05 and 0.1 mol/L the signals of the glycine-derivative are in the near of the buffer signal at the sensor SB2AC0. The derivative is not as bitter as denatonium benzoate at low concentrations but show suitable bitter taste enabling a diagnostic system.

D 6.2. Sphere loaded chewing gum performance in patients with peri-implant disease

In upcoming studies, the system must be optimized for human use. This included a thorough risk assessment. In an effort to minimize exposure to humans, we deployed the electronic tongue as an established and well documented platform for the testing of tastes. The system is less prone to individual sensitivity for bitterness as compared to humans and excellently suited to compare similar molecules for *relative* changes in bitterness. The human tongue among individuals is better suited to test for *absolute* bitterness. As the question at hand was relative changes, the electronic tongue is the better instrument as compared to testing in humans.

Potential impact

General

The STEP consortium is opening an exciting new approach to monitor dental health by means of a full mouth profile, particularly for those who were subject to tooth replacement using implants. Each individual SME partner can expand from one's own expertise and mutually develop into new applications including (i) exploitation of the tongue / gustatory system as a new detector, (ii) advanced surface functionalization to expand into biomedical applications and a large market expanding from niche application in diagnostics (iii) expanding the biotechnological expertise to a mass market product and lastly (iv) providing advanced, stimuli responsive implants with 7/24 monitoring for peri-implant disease.

Clearly, if successful the impact may be huge. In fact, this self-monitoring approach may enrich current treatment modalities by early on diagnosis (diagnosis must be made by the dentist once the patient has experienced a gustatory change) and treatment accordingly. It is this potential which excited the consortium and motivated to address these ambitious plans by means of this proposal.

In Europe, the loss of implants due to implant disease is enormous. In spite of the fact that only few studies robustly assessed the prevalence of peri-implant diseases, this cross-sectional data on implant-treated subjects indicated the enormous prevalence. Peri-implantitis was identified in 20% of subjects and in 10% of implant sites [17].

Market size and market share

One of the largest markets that are still under-developed by medical device and diagnostic companies is the diagnosis and treatment of device related complications. Demographic

changes in western countries are significantly increasing implantation procedures and therefore the incidence of device related complications. Treatment costs of infected devices are in the rate of 15'000 - 50'000 US Dollar [18]. According to Bryers and Darouiche medical devices are responsible for about 60–70% of hospital-acquired infections [19, 20]. Bacterial colonisation of a device, followed by biofilm formation, can be the start point of an infection and result in serious illness and death [21]. It is well known for years that device- associated infections are difficult to treat with antimicrobial agents because the organisms are encased within a protected microenvironment hampering the prevention and treatment of established biofilm [22]. Nevertheless, the efforts to reduce device related infection using specially developed materials, local drug delivery systems respective local antibiotic therapies have had only modest clinical success [22, 23].

Therefore, early infection diagnosis and the judicious implementation of simple and optimal diagnosis tools would reduce potential device related infections and reduce the treatment related costs. In case of dental implant, infections resulting from surface colonization may result in local tissue destruction in a manner analogous to periodontitis [24] respective peri-implant mucositis. It is obvious that a diagnostic kit developed for the detection of periimplantitis can be used for the diagnosis of periodontitis respective peri-implant mucositis.

Effect on competitiveness of members of the SMEs

Based on the proposed research and manufacturing development, PolyAn and Biovendor will enhance their technology pipeline. A successful product launch and sales will result in increased production capacities and facilities. The developed sensing technology will be used as platform technology for sensing other diseases. Based on this platform further developments and cooperation can be initiated by PolyAn and Biovendor. There is a high potential for stepping in new diagnostic markets afterwards. PolyAn and Biovendor competitiveness will be strongly enhanced. The companies' market position will be enforced for the next product generation in the diagnostic supplier markets. Companies like Thommen Medical are becoming a complete new customer segment for PolyAn and Biovendor.

The partnership of PolyAn and Biovendor with InDent is essential for being successful in product development and especially in clinical product testing. InDent has a strong interest to be part of a product development driven scientific network. Thommen Medical can strongly profit from the various SME's experiences. The sensing implant coating platform product will become a real unique selling proposition (USP) and strengthen the competitiveness and the scientific reputation of the company in a highly competitive market. The cooperation with InDent, the well-known opinion leader in the international dental medical community, will add another important piece to the USP puzzle.

On the other hand it might be possible that Thommen Medical runs a Blue Ocean strategy based on the chewing gum development. This will create a new market for a dental implant company. The product will be launched within the existing customers (dental surgeons) base. Different product packages can be offered in combination with dental implants, including training and opens a new window of opportunity to develop new customer groups. Shortly after the customer base will be extended to non-surgical dentists. Another strategic opportunity for Thommen Medical is to act as licensor for a start-up company. Background are new market research figures (Euromonitor 2014) demonstrating only a minor distribution channel proportion of 1% for dentists respectively 4% for pharmacies in the Oral Health market. Therefore drugstores and supermarkets will become a part of the distribution strategy (e.g. mouth washing market in drugstores and supermarkets is 750 Mill Euro). This company is focusing on the final product development and the sales & marketing of the product. Main market will be the Oral Care market (7.7. Bill Euro in Europe, growing rate 3%/y) focusing on the part of the population who cares about their health and appearance. Foreseen distribution partners are dentists, pharmacies but also drugstores and supermarkets. Thommen will profit from sales related license payments. The opinion leaders, like InDent, will introduce the technology into the international dental medical community. In summary, Thommen Medical will become more competitive by improving profit margins and enhancing sales volume, furthermore a new market segment will be formed.

Thommen Medical and InDent's industrial and clinical network will be used to open the platform technology to other applications in the health science business. The business of PolyAn and Biovendor will profit extremely from these future applications.

Contributions towards community societal objectives

Europe is the major revenue generator for dental implant manufacturers. At the same time among European population there is a remarkably high density of patients possessing one or more dental implants. Furthermore, peri-implantitis caused by infected dental implants or mucositis is a frequent burden in chemo- or radiotherapy treated patients. Therefore, peri-implant disease poses a major threat for the affected individual as well as a high economic burden for the European Society as a result of implant loss, long-lasting therapies and sequelae with associated health care recovery costs. Having in mind that there is no universal agreement on the most effective therapy for peri-implantitis, it appears particularly important to detect first signs of implant infection at the earliest possible time point and to allow the dentist to stop its progression and to attempt implant-saving treatments at a stage at which the entire disease course can be halted and reversed by means of relatively benign treatment regimens. Furthermore, peri-implant diseases are a risk factor to develop other diseases. Recent

research has indicated that the oral health status is predictive for the overall risk to develop diseases affecting the heart and brain. Zu-Yin Chen reported at the 2011 American Heart Association conference in Orlando, FL a study in 100'000 participants and demonstrated, that professional cleaning of the oral cavity reduced the risk for stroke by 13 % and risk for cardiac infarct by 245%. Thus, the successful conclusion of this project would have positive effects on a range of Community societal objectives including improved health, long-term performance, and safety of medical devices, and increasing living conditions.

Contribution to standards

Success with the project will contribute to the realization of EC directive 93/42/EEC concerning medical devices which came into force in 1994 and was amended by the directive 2007/47/EC of the European Parliament and of the Council of 2007. Following the guidelines of these standards the project will provide an important tool to assess and to improve dental implant longevity and safety.

Dissemination of foreground

In order to promote the dissemination of the STEP project, a number of activities was conducted, mainly in the second half of the project (list of dissemination activities below).

LIST OF DISSEMINATION ACTIVITIES

No.	Type of activities	Main leader	Title	Date	Place	Type of audience	Countries addressed
1	Press releases	THOMMEN MEDICAL AG	EU-Forschungsförderung für Thommen Medical	27/11/2012	Bundesverband der implantologisch tätigen Ärzte in Europa e.V.	Scientific community (higher education, Research) - Industry	Europe
2	Press releases	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Diagnose-Kaugummi für Implantate	25/02/2013	POnline press site University of Würzburg	Scientific community (higher education, Research) - Industry - Civil society	Germany
3	Oral presentation to a wider public	THOMMEN MEDICAL AG	Chancen für Unternehmen in Horizon 2020 und Enterprise Europe Network (EEN)	29/10/2013	Fachhochschule Nordwestschweiz Basel	Scientific community (higher education, Research) - Industry	Switzerland
4	Press releases	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Diagnose-Kaugummi für Implantate	25/02/2014	Universitätsklinikum Würzburg website	Civil society	Germany
5	Articles published in the popular press	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Diagnose-Kaugummi für Implantate	26/02/2013	zm Zahnärztliche Mitteilungen	Industry	Germany
6	Web sites/Applications	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Infektion bei Zahn-Implantaten	26/02/2014	www.apotheken.de	Civil society	Germany
7	Articles published in the popular press	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Could chewing gum prevent implant failure in the future?	27/02/2014	Dental Tribune - the World`s Dental Newspaper	Scientific community (higher education, Research) - Industry - Civil society	Europe
8	Articles published in the popular press	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Kaugummi zur Früherkennung von Entzündungen im Mundraum	05/03/2014	Die Zahnarzt Woche DZW	Industry	Germany

9	Articles published in the popular press	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Periimplantitis: Diagnose-Kaugummi statt Gentest für sichere Implantate?	04/04/2014	Praxis Implantologie	Industry	Germany
10	Press releases	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Diagnose-Kaugummi für Implantate	08/05/2014	recall - das Praxisteam Magazin	Industry	Germany
11	Articles published in the popular press	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Could chewing gum prevent implant failure in the future?	16/05/2014	Dental Tribune - U.S. Edition	Scientific community (higher education, Research) - Industry - Civil society	USA
12	Web sites/Applications	THOMMEN MEDICAL AG	Chewing chewing gum for science	03/07/2014	Thommen Medical Website	Industry - Civil society	international
13	Articles published in the popular press	JULIUS-MAXIMILIANS UNIVERSITAET WUERZBURG	Kaugummi kauen für die Wissenschaft	17/09/2014	Dentale Implantologie und Parodontologie	Scientific community (higher education, Research) - Industry	Germany
14	Videos	UNIVERSITAET ZUERICH	STEP Sensing peri-implant disease	20/11/2014	https://cast.switch.ch/vod/clips/wosrg2ob/link_box	Scientific community (higher education, Research)	international

The goal of these activities was to attract potential business partners to drive marketability of the end product. These general dissemination activities are going to be followed by peer reviewed journal article publication and scientific conference contributions to address not only industry but also the scientific community. Due to business protection according to the Grant Agreement, detailed publication of the R&D results is delayed. The main topics covered in the publications are:

- Animal model “Ligature-induced peri-implantitis in minipigs revisited” submitted to Clinical Oral Implants Research
- Whole data set to be published in PNAS, Angewandte Chemie or Nature Materials
- Clinical correlation MMP-8 levels and clinical diagnosis of Peri-implantitis
- Synthesis of the Bittern

The following statement will be mentioned on each presentation and where appropriate but for sure on any publication: “The research leading to these results has received funding from the European Union's Seventh Framework Programme managed by REA-Research Executive Agency http://ec.europa.eu/research/rea ([FP7/2007-2013] [FP7/2007-2011]) under grant agreement n° [314911]”.

Exploitable foreground

For reasons of business protection, all data connected to IPR is confidential. Nonetheless, the SME partners in the project were able to generate exploitable foreground directly applicable to their product portfolio on a non-confidential basis (table below).

OVERVIEW TABLE WITH EXPLOITABLE FOREGROUND							
Type of Exploitable Foreground	**Description of Exploitable Foreground**	**Confidential**	**Exploitable product(s) or measure(s)**	**Sector(s) of application**	**Timetable for commercial use or any other use**	**Patents or other IPR exploitation (licences)**	**Owner and Other Beneficiary(s) involved**
Commercial exploitation of R&D results	assay for determination MMP-8 in body fluids	No	Human MMP-8 (total) ELISA	medical research	6 months	no	Biovendor
Commercial exploitation of R&D results	Advancement of coupling chemistry for bead technology	No	new coupling chemistry protocols established	new products for bead technology	6 months	no	PolyAn

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Public website address

http://www.step.uni-wuerzburg.de/

Contact

Dr. Falko Schlottig
Thommen Medical AG
Neckarsulmstrasse 28
2540 Grenchen
Switzerland
falko.schlottig@thommenmedical.com

Prof. Dr. Dr. Lorenz Meinel
Lehrstuhl für Pharmazeutische Technologie und Biopharmazie
Universität Würzburg
Am Hubland
97074 Würzburg
Germany
l.meinel@pharmazie.uni-wuerzburg.de