



## NANOBIOSACCHARIDES

NANOtechnologies for BIO-inspired polySACCHARIDES:  
biological 'decoys' designed as knowledge-based,  
multifunctional biomaterials

[www.nanobiosaccharides.org](http://www.nanobiosaccharides.org)

### 1. PROJECT OBJECTIVES

The NANOBIOSACCHARIDES project convenes an interdisciplinary consortium of scientists from **academia and industry** to develop and exploit nanotechnologies for the generation of knowledge-based, multi-functional, **bio-inspired polysaccharides** to be used as intelligent, sustainable, environment-friendly, consumer- and patient-safe bio-materials. The project is based on the **novel concept of biological decoys** acting as functional bio-medical materials. This concept which was recently developed by core partners of the NANOBIOSACCHARIDES project causally links the physico-chemical properties of polysaccharides, in particular of chitosans, to their biological activities. The NANOBIOSACCHARIDES project aims at validating this concept which upon verification would generate **breakthrough knowledge** in the highly promising field of nanobiotechnologies, and which upon implementation would potentially lead to **significant transformation** in the field of medical bio-materials, drug and gene delivery, and cell and tissue engineering.

### 2. CONTRACTORS INVOLVED

- Westfaelische Wilhelms-Universitaet Muenster (WWU), Germany
- EZUS Lyon 1 (EZUS), France
- Care Sense Consulting (Care), Germany
- Université Claude Bernard Lyon 1 (UCBL), France
- DANISCO A/S (Danisco), Denmark
- Gillet Chitosan EURL (Gillet), France
- Universidade de Santiago de Compostela (USDC), Spain
- BioMérieux (BioMérieux), France
- Advanced In Vitro Cell Technologies S.L. (Advancell), Spain
- Universitaetsklinik Muenster (UKM), Germany
- Cotech Srl (Cotech), Italy
- University of Hyderabad (UH), India
- Mahidol University (Mahidol), Thailand
- Prince of Songkla University (Songkla), Thailand

### 3. COORDINATOR CONTACT DETAILS

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### 4. WORK PERFORMED, RESULTS ACHIEVED SO FAR, AND EXPECTED END RESULTS

The NANOBIO-SACCHARIDES project aims at a molecular, nano-scale understanding of structure/function relationships in bioactive polysaccharides. These will be used as the scientific basis for the development of nano-scale modified bio-inspired polysaccharides for applications in medical cell and tissue engineering, and in drug and gene delivery. The first year of the project was mainly devoted to the generation and characterisation of the raw materials to be used in the project, and in the establishment and optimisation of experimental protocols. The second year of the project was mainly devoted to the generation of the first nano-scale modified speciality polysaccharides and the further optimisation of techniques, particularly by aiming at a molecular or nano-scale understanding of the processes of nanoparticle and hydrogel formation.

The polysaccharides to be studied in this project are mainly chitosans, pectins, alginates, and hyaluronates. The main emphasis lies on chitosan, and the production of a large enough sample of a reference chitosan to be used by all partners throughout the project was one of the first goals defined during the kick-off meeting. One of the commercial partners in the consortium, Dr. Dominique Gillet from Gillet Chitosan, generated this chitosan from a unique and precious source, namely squid pen. The chitin present in squid pen is  $\beta$ -chitin which is less crystalline than the usually available  $\alpha$ -chitin from crab or shrimp shells and, therefore, has superior solution properties. Moreover, the chitin in squid pen is not associated with calcium carbonate so that the extraction procedure is less drastic. Consequently, a much higher quality chitin can be isolated from squid pen than from shrimp or crab shells. Importantly, our partner has then used comparatively mild processes for the de-N-acetylation reaction in order to avoid excessive breakdown of the polymers. The material obtained was sent to Prof. Dr. Alain Domard in Lyon, France, the physico-chemist in our consortium. There, the expected very high degree of polymerisation and very low degree of acetylation of the reference chitosan, which is far superior to any material commercially available in the market, was experimentally verified.

Using the reference chitosan, Prof. Domard has optimised the procedures required for the partial re-N-acetylation and for the partial depolymerisation of the fully de-N-acetylated polymer. Re-N-acetylation was performed using fresh acetic anhydride as a reactive. The following precipitation and purification steps were adjusted according to the specific physico-chemical properties of the chitosans with different degrees of acetylation (DA). The result of these reactions is a homogeneous series of chitosan chains having the same distribution of degrees of polymerisation (DP) but different DAs. Depolymerisation of chitosan was either achieved by the traditional way of oxidative deamination using  $\text{HNO}_2$ , or using an alternative, physical way which leads to products with a very narrow distribution of molecular weight, i.e. with a polydispersity index (Ip) approaching unity. This method is in the process of being patented.

During concomitant exchange visits to the lab of Prof. Domard, Dr. Goycoolea and Dr. El Gueddari from the labs of Prof. Alonso and Prof. Moerschbacher, respectively, have learned these protocols of carefully controlled depolymerisation and re-N-acetylation which are now available as standard operation procedures (SOP). A first set of speciality chitosans with precisely defined degrees of polymerisation and acetylation covering the three domains of Domard's general law of behaviour of chitosans in aqueous solutions has been prepared and is now being used in all partner labs for reference work.

All of the procedures described above used in the generation of the chitosans are purely chemical or physical in nature, and they consequently yield samples with random patterns of acetylation (PA). As the basic concept of our project involves the assumption that the PA of chitosans influences their biological activities, we have proposed to use enzymes for the depolymerisation and de-N-acetylation steps. Such enzymes are being identified and characterised by the enzymologist of our consortium, Prof. Dr. Bruno Moerschbacher in Münster, Germany. Currently, a large number of bacterial, fungal, and plant chitinases, chitosanases, and chitin-de-N-acetylases have been identified and the corresponding genes have been cloned. Selected first enzymes have now been heterologously expressed in the fission yeast *Schizosaccharomyces pombe* to obtain reasonable quantities of highly pure enzymes. For this purpose, a new vector has been developed which allows the expression and secretion of any enzyme without addition of potentially disturbing extra amino acids at the N-terminus of the protein. Two patents describing the vector and a fungal chitin de-N-acetylase are in preparation. Alternatively, one enzyme has been purified from the spent medium of a fungus. These pure enzymes have been or are in the process of being characterised concerning their substrate specificities and product patterns. One of these enzymes turned out to have a novel cleavage specificity, defining a new class of chitinolytic/chitosanolytic enzymes. We have begun to use these enzymes in the generation of partially acetylated chitosan polymers and oligomers with a non-random PA. Of paramount importance will be the analysis of PA of the enzymatically produced/modified chitosan polymers and oligomers, and appropriate methods, e.g. using mass spectrometry, are currently being developed. The specificities of the enzymes analysed in some detail so far seem to allow the hope that they might become valuable tools in the tailor-made engineering of designer chitosans with e.g. inbuilt digestibility in the human body and, therefore, improved clearing rates from tissues. It should also become feasible to engineer a designer chitosan polymer which upon cleavage in the human body sets free bio-active breakdown products.

In parallel to this work on enzymatic production or modification of chitosans, Prof. Domard's group has continued to chemically synthesize such oligomers with fully defined architecture. Currently, the synthesis of deprotected disaccharides has been achieved in mg-quantities, and larger oligomers will become available during the project. These will be precious reference samples for the analysis of PAs of partially acetylated chitosans. Our progress in this field has allowed us to start adding PA as a third dimension to the previous two dimensions of DP and DA of the chitosan matrix.

Dr. Hans-Christian Buchholt from the second company involved in the production of polysaccharides, Danisco from Copenhagen, Denmark, has generated pectin and alginate samples as defined by the necessities of the other partners. Pectins are a group of highly complex, branched plant heteropolysaccharides so that generation of a defined sample is much more demanding than with the linear and rather simple chitosans. Work has first concentrated on generating high molecular weight samples of a linear homogalacturonan with a very high content of galacturonic acid, then on generating three oligomeric samples differing in their average DP. In parallel, a series of pectins differing in their degrees of methylesterification (DE) was produced and characterised. These samples have been distributed to Prof. Domard's and Prof. Moerschbacher's group, for use in the generation of hydrogels and nanoparticles, and for further characterisation and analysing their bio-compatibilities. A sample of low molecular weight pectins to be included in nanoparticles for drug delivery has been supplied to Prof. Maria José Alonso from Santiago de Compostela in Spain, the pharmaceutical engineer of our consortium. A commercial pectin-based product named Modified Citrus Pectin (MCP) has been purified, and the presumed active component has been sent to Münster for biochemical characterisation in the lab of Prof. Moerschbacher, and for analysis of its presumed anti-metastatic activity in the lab of PD Dr. Stefan Schneider, the dermatologist and biomedical physiologist in our consortium.

Similarly, alginates are complex polysaccharides containing two different monomeric building blocks, mannuronic acid (ManA) and guluronic acid (GuA). As GuA is known to induce the complement reaction in humans, samples with high ManA content are required for medical and pharmaceutical purposes. Such alginates have been produced by Dr. Buchholt to be used by the other partners. Interestingly, a new source for an alginate with an unusually high ManA content has been found recently in Chile

by partners of the Danisco group, and this allowed the first isolation of a high MW alginate with a high ManA/GulA ratio of above 7. Eventually, a series of high ManA alginate samples with DP ranging from 20 to 2100 has been prepared and distributed to the partners for further use. This is a potentially highly interesting biomaterial which has previously not been available for testing.

While work on preparing and characterising first the raw materials and then the first speciality polysaccharides was underway during the first and second year of the project, work on generating nanoparticles has been carried out in parallel in the labs of Prof. Alonso and Prof. Domard. The two groups follow different approaches at preparing nanoparticles, both by playing on the balance between hydrophobic and hydrophilic interactions. The first route to generate polysaccharide nanoparticles, developed and employed by Prof. Alonso's group, consists in associating chitosan with other anionic compounds such as cyclodextrins or alginates using a mild ionotropic gelification technique. Such nano-co-particles can synergistically combine the physico-chemical properties and biological activities of the two types of polysaccharides they contain. As the first nano-scale modified polysaccharides have only recently become available, the reference chitosan and commercially available chitosans were used to optimise the currently employed protocols. During the first year, nanoparticles consisting of chitosan and carboxymethyl- $\beta$ -cyclodextrin (CS/CD), were prepared and characterized with respect to stability, ability to associate, and delivery of insulin and transport across the nasal mucosa. Conditions for nanoparticle formation yielding products with sufficient stability in simulated intestinal fluids have now been defined. Insulin and heparin were incorporated into the nanocarriers with high association efficiencies, and their release profiles were shown to be highly dependent on the characteristics of the incorporated macromolecule and its interaction with the nanocarrier: insulin was released quickly while heparin remained highly associated to the nanoparticles for extended periods. As cytotoxicity is a key problem in developing gene therapy carriers, the effect of chitosan and CS/CD nanoparticles on the viability of proliferating human cells was evaluated. Interestingly, it turned out that the CS/CD nanoparticles were less cytotoxic than those composed only of chitosan. In addition to the above described nanoparticles containing cyclodextrins as a second polysaccharide, new chitosan/alginate (CS/ALG) nanoparticles were developed for macromolecular drug and gene delivery, using the novel alginate samples provided by Dr. Buchholt. First insulin loading and release studies using these CS/ALG nanoparticles have been initiated. Also, first experiments were already initiated to analyse the transport of selected model nanoparticles across the nasal mucosa of rats. The nanoparticles were found to closely interact with the mucus layer, and even penetrate into the epithelium. However, the underlying mechanisms need to be further investigated. The capability of these nanoparticles to encapsulate genetic material will be investigated next.

Dr. Thierry Delair from the company BioMérieux based in Marcy l'Etoile, France, our partner for the use of nanoparticles in gene delivery, uses polyelectrolyte complex formation of chitosan with dextran sulfate at various molar charge ratios to obtain either positively or negatively charged colloids according to the polymer in excess. DNA binding experiments were carried out with both positively charged and negatively charged particles and the level of complexation was quite satisfactory, but an aggregation was obtained with the positively charged colloids. Six different cell lines were used for transfection assays but so far, no positive result was obtained probably due to a lack of stability of both types of colloids in the cell culture media. Nonetheless, using chitosan alone, complexed with DNA, transfection was observed. Experiments are going on aiming at improving the colloidal stability of the particles in cell culture media, protein adsorption, particle cell internalisation, and particle/dendritic cell interactions, varying intrinsic parameters such as DP and DA of the chitosan used.

In a complementary route, developed and employed by Prof. Domard's group, the nanoparticle formation is carried out from solutions of chitosan salts only. Here, we take advantage of the possibility to synthesize tailor-made low  $I_p$  and random PA chitosans with various DP and various DA in order to play again on the balance between hydrophobic/hydrophilic interactions. We study the nanoparticle formation within the solutions, as a result of a physico-chemical perturbation (e.g. change of pH, concentration of chitosan, solvent). Consequently, a nano-scale understanding of the solution properties of chitosans is required that will enable us to tailor-make pure chitosan nanoparticles with known physico-chemical properties. This only will allow us to study in detail the relationship between physico-chemical properties and biological functionalities of the nanoparticles. We understand chitosan solutions as multi-scale organised systems whose morphology depends on various internal and external parameters, including but not limited to the DP and DA of the chitosans used. We have exploited ultrasound treatments as a means to decrease the DP of the reference chitosan, as detailed above. This method was first used on chitosans of low DA, then on those of high DA. Especially in the latter case, we observed the depolymerization of sol-

vated chains and the persistence of longer chains that are hidden in nanoparticles or submicrometric heterogeneities. We begin to understand that chitosans exist in aqueous solution in an equilibrium between solvated single molecules and nanoparticles. As we view nanoparticles as nano-scale hydrogels, this insight begins to reveal a continuum between solvation, nanoparticles, and hydrogels. Firstly, thus, the physico-chemical conditions under which nanoparticle formation occurs in chitosan solutions need to be further studied, namely the critical values of DA and DP above which nanoparticles form, the pH of the solution, the polymer concentration etc. Secondly, we need to understand the transition from nanoparticles to larger scale aggregates and formation of a multi-scaled hydrogel.

From a general point of view, the gel state is obtained by playing on the hydrophilic/hydrophobic balance of a polysaccharide solution maintaining a polymer concentration above the critical concentration for chain entanglement. Two main gelation routes can thus be considered, namely controlled neutralisation of a polyelectrolyte solution and slow solvent exchange to a poorer solvent and subsequent neutralization. Thus, chitosan gels can be formed from aqueous solutions via neutralization by ammoniac gas (route 1), and via evaporation of the water present in chitosan solutions in a water/propanediol mixture (route 2). We found that the neutralization step is the key physico-chemical treatment defining the structure of the gels obtained. Our realisation that the polysaccharide (chitosan) solutions already contain the basic structural elements constituting the gels, at the different length scales, leads us to the conclusion that gelation of aqueous solutions can be viewed as a redistribution of these heterogeneities during a phase separation. This basic knowledge offering a nano-scale understanding of gel formation allowed us to optimise the alcohol route for hydrogel preparation to yield a variety of gel morphologies, with amorphous/crystalline structures, isotropic or oriented gels, that should of course exhibit different physical and biological properties.

In parallel to the generation and physico-chemical characterisation of the polysaccharides, nanoparticles, and hydrogels, we have started to analyse their antimicrobial activities against bacteria and fungi, and their biocompatibilities towards different types of human cells. Prof. Moerschbacher's group was able to extend the previously made observations concerning the antimicrobial activities of chitosan polymers to include oligomers. We corroborated our earlier finding that the DA influences the antimicrobial activities of chitosans more strongly than their DP. We are currently trying to understand the mechanism underlying the anti-bacterial and anti-fungal activity of chitosan. Dr. Schneider's group is testing the effects of different chitosans on different types of human cells, such as endothelial and epithelial cells, macrophages and cancer cells. Chitosans with different DA and DP were used in these experiments, and we found that the DA greatly influences the bioactivities of chitosans, while the DP does so to a much lesser extent. Importantly, none of the chitosan preparations tested so far exhibited any toxicity in the tests used. As purified and well characterised, partially acetylated chitosan oligomers exhibited interesting biological activities, the focus of our work has somewhat shifted during the first year. Working with oligomers circumvents some of the problems encountered due to the low solubility of some chitosans in physiological fluids with a near neutral pH. Moreover, studying the biological activities of oligomers will allow us to draw detailed conclusions concerning structure-function relationships of defined polysaccharide epitopes, and first insights into the possible mechanisms of chitosan action towards melanoma cells have been achieved. As a consequence of our shift towards analysing the biological activities of oligomers, we have also initiated studies on human enzymes capable of degrading partially acetylated chitosan polymers. Knowledge of the cleavage specificities of such enzymes will be paramount for the development of the concept of engineered designer chitosans with inbuilt digestibility or release capacity for bio-active oligomer breakdown products.

The basic assumption underlying the **NANOBIOSACCHARIDES** project states that the biological activities of polysaccharides are governed by their physico-chemical properties and by their nano-scale physical and chemical characteristics. Thus, a detailed, molecular and nano-scale understanding of structure-function relationships is required to enable us to tailor-make bio-inspired polysaccharides, polysaccharide nanoparticles, and polysaccharide physical hydrogels, and possibly even to enzymatically engineer designer polysaccharides. While the activities of the first year of our project were mainly devoted to establish and optimise the required tools and protocols for the generation and characterisation of these polysaccharide preparations, and the second year focused on a detailed understanding of the underlying mechanisms, two fundamental break-throughs were already achieved. Firstly, we begin to understand that chitosan solutions are organised at multiple levels, with an equilibrium between solvated polymers, nanoparticles, and aggregates, and this basic knowledge led to the realisation that there is a continuum of morphology from the solutions through nanoparticles to hydrogels. We are convinced that it is such a multi-scale un-

derstanding of the complex physico-chemical properties of polysaccharides, and perhaps even the definition of a unifying theory of polysaccharide solutions, nanoparticles, and hydrogels, that will eventually allow us to develop tailor-made polysaccharide nanoparticles and hydrogels with known, desired biological functionalities. Secondly, we were able to clearly demonstrate that the biological activities of chitosans towards human cells greatly depend on their DA but are to a much lesser extent influenced by their DP. This result resembles the previously made observation concerning the biological activities of chitosans towards plant cells, where this realisation has been used successfully in the design of reliable, environment-friendly and consumer-safe plant disease protectants. A third break-through is beginning to appear on the horizon of our project, namely the possibility to control a third and potentially highly critical parameter of partially N-acetylated chitosans, namely the pattern of acetylation PA. The use of pure and well characterised polysaccharide modifying enzymes with known substrate specificities and product patterns may eventually allow us to engineer designer polysaccharides with inbuilt bio-functionalities.

While all the details and implications of our observations and results still need to be determined, we feel that our project, thus, promises to lead to real innovation in the field of biomedical and pharmaceutical applications of bioactive polysaccharides. Their commercial use has so far been hindered by the lack of reliability of their biological effects. We have argued that this poor reproducibility of results is most likely due to the use of poorly characterised polysaccharides combined with a lack of understanding the structural requirements underlying specific biological activities. We have proposed that the use of physico-chemically well characterised polysaccharides with defined, known biological activities may overcome this bottleneck, allowing us to exploit the vast potential of these precious natural resources. The first steps towards this goal have now been taken, further steps have been identified and appear to be in attainable reach, and we are confident that we will be able to successfully proceed on this way.