Executive Summary:
New nanoparticles were formulated and the relationship between polymer structure, ability to form nanoparticles and their potential activity to cross the mucus barrier was studied. All the mucopermeable strategies planned were addressed, 315 candidates were developed. A web-managed database including all the polymers, nanocarriers and characterization analysis was designed and implemented.
A battery of assays was developed to assess the effectiveness of nanocarriers crossing both the mucus and epithelial barriers without destroying the protective function of the mucus layer. In conjunction with the database of nanocarriers it was possible to screen and select the best performing candidate nanocarriers available through the consortium. Some promising candidates with self-nanoemulsifying drug delivery systems (SNEDDS) performed well in all measurements. There were also potential candidates in other strategies, such as the slippery surface and resorcinol/bisphenol diphosphates (RDPB) nanoparticles. The best candidates were chosen for in vivo studies.

For toxicological evaluation, a screening strategy including several in vitro assays was designed in order to evaluate the cytotoxicity, immunogenicity and genotoxicity of nanoparticles. This information was integrated with the evaluation of the capability to penetrate the mucus barrier and with information regarding the nanoparticles’ biocompatibility and degradation. The nanoparticles with the best in vitro profile were selected and tested in vivo for biodistribution. Finally, in vivo nanoparticle toxicity and in vivo genotoxicity were also assessed.

The potential of mucus permeating nanocarriers as superior oral delivery systems for macromolecular drugs was shown via numerous in vivo studies both in rodent and non-rodent animal models. Utilizing various nanocarriers designed within this project, the oral bioavailability of different peptide drugs was at least 5-fold improved in comparison to state-of-the-art formulations.

The toxicity of 18 nanoparticles and gene delivery efficiency of 12 different polymer combinations were evaluated in human conjunctival epithelial cells. The most promising nano-formulations were tested (in vivo tolerability studies with rats). An ex vivo porcine conjunctiva model and cassette-dosing methods were set up for the conjunctival drug permeability studies.

Project results were disseminated to the scientific community at various scientific events, 116 oral contributions and 55 posters were presented. 31 peer-reviewed publications were published, including a special issue of the European Journal of Pharmaceutics and Biopharmaceutics exclusively featuring the results by ALEXANDER partners.

European projects ALEXANDER, TRANS-INT and COMPACT jointly organized a scientific conference with DECHEMA as organizer. The Crossing Biological Barriers took place in Dresden on 9-11 November 2015 with 139 participants, 47 talks and 46 posters.

Three patents on new methods or technologies were filed by ALEXANDER partners.

Project Context and Objectives:
Numerous papers have been published dealing with nano-delivery systems for mucosal administration of macromolecular drugs (e.g. proteins, peptides, DNA-based drugs, etc.). A nano-delivery system should ideally exhibit an enhanced permeation rate through the mucus gel layer, a sustained drug release profile and sufficient protection towards enzymatic degradation of the drug, thus, resulting in an increased bioavailability of macromolecular drugs.

Today, none of the available nano-delivery systems is capable of permeating the various mucus gel layers in significant quantities because of present limitation in the nanocarrier cut-off size of 55-100 nm. In most cases, the current nano-delivery systems do not exhibit sufficiently high drug bioavailabilities, therefore, their commercialization cannot be justified. Thus, present research efforts are directed to the solution of
The multidisciplinary ALEXANDER project comprising 14 partners from 8 European countries aimed at the development of novel nano-delivery systems to overcome the mucus gel barrier without destroying it. Within this frame, ALEXANDER focused on oral and ocular delivery. It included novel strategies to overcome present limitations in enhanced nanocarrier permeation rates. These strategies included the immobilization of proteolytic enzymes on the nanocarriers surface, thiomer based nanoparticles exhibiting pH reactivity and, thus, ability to diffuse into the mucus without reacting with the mucus disulfide bonds, nanoparticles capable of changing their zeta potential from negative to positive once they have permeated the mucus gel barrier and reached the epithelium, self-nanoemulsifying-drug-delivery-systems (SNEDDS) and spontaneously forming nanoemulsions upon contact with aqueous media such as the intestinal fluid. Moreover, existing strategies, e.g. nanoparticles releasing low amounts of mucolytic agents during their transport through the mucus and nanoparticles exhibiting a densely charged but net neutral surface, have been further optimized.

R&D activities focused on the synthesis of functionalized nanocarriers capable of permeating the mucus gel layer and delivering their therapeutic payload to the epithelium or to the systemic circulation. The nanocarriers were characterised with respect to their physicochemical properties, their ability to cross the mucus gel layer as well as for their in vitro and in vivo cytotoxicity. The potential of the developed nanocarriers as delivery systems for mucosal administration of macromolecules were demonstrated via the oral delivery of polypeptides and the ocular delivery of oligonucleotide. The potential of a therapeutic gene delivery for the treatment of diseases (e.g. diabetes, tumor anemia and cystic fibrosis) was demonstrated in in vitro uptake studies successfully. The nicely improved transfection rates of pDNA in a self-nanoemulsifying drug delivery system and the CTFR gene in a zeta potential changing nanocomplex showed the great capability of these systems for gene delivery.

Project Results:
A short summary of the main S & T results is given below. However, all the different approaches and achievements of the ALEXANDER project are best described in the special issue of the European Journal of Pharmaceutics and Biopharmaceutics exclusively featuring the results by ALEXANDER partners. This special issue is called Mucus permeating Nano-carrier systems.

For an efficient scientific and administrative management of ALEXANDER, various structures and communication tools were established, ranging from regular phone conferences for close monitoring of the project progress, definition and implementation of scientific strategies and procedures.

New nanoparticles were formulated and the relationship between polymer structure, ability to form nanoparticles and their potential activity to cross the mucus barrier was studied.

The partners successfully prepared nanoparticles with the polymers designed and synthesized. These nanoparticles were characterized in terms of mucin and mucus permeation ability and toxicity. All the mucopermeable strategies planned were addressed. Altogether, more than 315 candidates were developed.

A web-managed database including the physico-chemical data of all the polymers and nanocarriers as
well as the permeation and toxicity data was designed and implemented.

A battery of complementary in vitro assays to model the epithelial mucosa has been developed. These assays were used to screen and assess the effectiveness of nanocarrier movement through (or adherence to) the mucus layers for effective drug delivery. As well as a single phase mucus permeation model, a more complete model of permeation through small intestinal epithelial mucosa was utilised, which includes modelling of the digestive phase and the epithelial cell barrier. Methods for assessment of retention time in the mucus as well mucosa have been developed. Using this enhanced battery in conjunction with the database of nanocarriers, it was possible to screen and select the best performing candidate nanocarriers available through the consortium. Some promising candidates were identified, with self-nanoemulsifying drug delivery systems (SNEDDS) from the University of Navarra, the Aristotle University of Thessaloniki and the University of Innsbruck performing well in all measurements. There were also potential candidates in other strategies, such as the slippery surface and resorcinol/bisphenol diphosphates (RDPB) nanoparticles (SAGETIS) that also performed well. The best candidates were taken into in vivo studies. Thorough toxicological evaluation of the nanocarriers synthesized in the project was pursued. A screening strategy including several in vitro assays was designed in order to evaluate the cytotoxicity, immunogenicity and genotoxicity of nanoparticles (NPs). This information was integrated with the evaluation of the capability to penetrate the mucus barrier, assessed in WP2, and with information regarding the nanoparticles’ biocompatibility and degradation. Nanoparticles with the best in vitro profile were selected and tested in vivo for biodistribution. To this aim, several techniques of nanoparticle radiolabelling were designed and applied for the monitoring of NPs biodistribution, retention and elimination. This was accomplished by magnetic resonance imaging, positron emission tomography (PET) or by luminescence detecting methods. Finally, nanoparticle toxicity was assessed in vivo after single dose and dose-repeated treatment (28 days) in animal models. Moreover, in vivo genotoxicity was also assessed at the level of the digestive tract by the application of the comet assay, that permits the detection of oxidative DNA damage. Some NPs produced a very light oxidative DNA damage in vitro, but this result has not been confirmed in vivo.

The potential of mucus permeating nanocarriers as superior oral delivery systems for macromolecular drugs was shown via numerous in vivo studies both in rodent and non-rodent animal models. Utilizing various nanocarriers designed within this project, the oral bioavailability of different peptide drugs was at least 5-fold improved in comparison to state-of-the-art formulations.

Topical drug administration via ocular surface including cornea and conjunctiva is non-invasive and the most widely used drug administration route in ophthalmology. The cornea is a tight barrier that allows only the treatment of anterior eye segment with small molecular drugs. The conjunctiva is believed to be leakier than the cornea and may, thus, offer a pathway even for macromolecules for the treatment of posterior eye segment. However, the detailed properties of the conjunctiva as a barrier for drug delivery are poorly understood. The ocular bioavailability i.e. the fraction of dose absorbed through ocular surface after topical administration is always less than 10%, often below 1%. One reason for poor bioavailability is the short contact time on ocular surface. The contact time may be increased with mucoadhesive and mucus penetrating polymers that can interact with the mucus layer covering ocular surface. Mucoadhesive and/or permeation enhancing nanoparticles were tested for their suitability for ocular delivery by using two approaches. In the first approach the drug absorption from the ocular surface was
increased by the epithelia loosening effects of mucoadhesive and permeation enhancing nanoparticles. In the second approach mucoadhesive nanoparticles suitable for gene delivery were transported to conjunctival epithelial cells for transfecting and triggering the secretion of therapeutic protein to the posterior eye segment. The clinical use of the nanoparticles is possible only after adequate safety and efficacy features have been identified.

In the absorption enhancement approach a total of 18 nanoparticles were screened at first stage for toxicity in cells modelling human ocular conjunctiva. Based on this screening 11 nanoparticles with low or moderate toxicity proceeded to tolerability studies performed with rats. All 11 nanoparticles were well tolerated after topical administration in a 2-week study with rats and continued to next step in which the ocular surface retention time of nanoparticles was evaluated in rats. One nanoparticle (nanoemulsion) showing highest ocular surface retention time after topical administration was selected for further studies in rabbits after topical administration. For these studies selected nanoemulsion was loaded with dexamethasone which is used in the treatment of ocular diseases. In rabbit studies the amount of dexamethasone was determined from tear fluid samples collected from ocular surface and from ocular tissues at different timepoints. The obtained results were compared to commercially available dexamethasone suspension formulation Maxidex®. The studied nanoemulsion showed almost equal but not improved ocular bioavailability compared to Maxidex®.

In the second approach a total of 39 gene delivery systems were tested first for efficiency in cells modelling human conjunctiva and the results were compared with commercially available gene delivery agent, Lipofectamine. Based on these results the six most promising gene delivery systems were selected for studies with rats. All tested systems were well tolerated after topical administration on ocular surface but did not show any efficacy.

In order to clarify the barrier function of ocular conjunctiva the permeability of a large set (termed as cassette) of drug molecules through porcine conjunctiva was evaluated. Cassette enables analysis of 35 different drug molecules with wide structural properties simultaneously from the same sample. The results showed that conjunctiva forms a strong barrier for drug permeation and the permeability of large molecules, such as proteins, is inefficient. The obtained data was used to generate a quantitative structure-properties (QSPR) model. This model defines which structural properties of drug molecules are important in defining the permeability across porcine conjunctiva. In case of the conjunctiva polar surface area (PSA) and hydrogen bound donor (HD) are two main descriptors for conjunctival permeability.

Potential Impact:
The primary goal of ALEXANDER was to address and successfully solve the fundamental problem of overcoming the mucus gel barrier in order to open the door for numerous radical improvements in therapy. Moreover, even first very simple discoveries made within the project can comparatively easily be incorporated in running product developments likely resulting in significant improvements in the performance of such products and in this way strengthening the competitiveness of the European health care industry.

ALEXANDER aimed at the explicit exploitation of the generated technologies for the development of effective and safe oral and ocular delivery systems and by this reaching the following goals:

1. Radical Improvements in Therapy

Generally, for all types of therapies with macromolecular drugs that are administered via nano-delivery systems on mucosal membranes, it is the mucus gel barrier that has to be overcome first. Being capable of bringing macromolecular drugs in comparatively high concentration in intimate contact with the underlying
epithelium offers the potential for radical improvements in the treatment of numerous diseases. ALEXANDER envisaged the following aims:

- Injectable-to-noninvasive-conversions of macromolecular drugs
- Injectable-to-noninvasive-conversions of anticancer drugs
- Radical improvement in the treatment of ocular diseases
- Radical improvement in non-viral gene therapy to mucosal tissues

2. Improvement of the Competitiveness of the European Health Care Industry

The pharmaceutical sector has in recent years suffered from a perceived lack of successful new drugs, especially blockbusters. Generic companies are predicted to have ~90% of the global market. Faced with unprecedented competition, pharmaceutical companies tackle new and innovative therapeutic targets that challenge their current science base. More efficient and convenient drug delivery systems are becoming one of the key issues for competitiveness of the European health care industry.

Strategic development of drug delivery technologies and quality partnerships will be the key to maintaining a competitive edge. Macromolecular drugs, for which more potent delivery systems are crucial to success, are (I) therapeutic peptides, peptide mimics and proteins, (II) DNA-based drugs and (III) oligo- and polysaccharides.

Presently, Europe has a strong position in the emerging field of nanotechnology for drug delivery which has a high potential for technological and conceptual breakthroughs, innovation and creation of employment. Nanotechnology for drug delivery is an area that would benefit from coordination at European level. Thus, close cooperation between industry, research centers, academia, hospitals, regulatory bodies, funding agencies, patient organisations, investors and other stakeholders can dramatically boost this promising field.

Within ALEXANDER, fourteen European partners from eight different countries have combined their efforts and expertise in complementary scientific fields to ensure accomplishment of the project objectives and transfer of the developed technology to a wider European basis.

3. Increase the Application of Nanotechnology in Medicine

In the framework of ALEXANDER, novel nanotechnology strategies were developed and existing ones were optimized for the efficient transport of nanocarriers through the mucus gel layer.

Finding ways to overcome this barrier is expected to render numerous already established particulate mucosal delivery systems and to open the door for their therapeutic use. Increasing the bioavailability of orally administered therapeutic polypeptides will likely lead to a much broader use of these macromolecules in treatment of various diseases. Furthermore, due to an improved uptake of DNA-based drugs, this project will increase the application of nanotechnology in treatment of various mucosal diseases by all kinds of therapeutic nucleic acids in the future.

Project results were disseminated to the scientific community at various scientific events, altogether 116 oral contributions and 55 posters were presented. A public ALEXANDER website is available as well as a specifically designed ALEXANDER overview poster. 31 peer-reviewed publications were published, including a special issue of the European Journal of Pharmaceutics and Biopharmaceutics exclusively featuring the results by ALEXANDER partners. This special issue called Mucus permeating Nano-carrier systems was published in November 2015 and is expected to generate a great impact on the research community. It reflects well the achievements and cooperation of the project. European Journal of Pharmaceutics and Biopharmaceutics, virtual special issue: Mucus permeating nano-carrier systems,
FP7 projects ALEXANDER, TRANS-INT and IMI project COMPACT jointly organized a scientific conference with DECHEMA as organizer. The Crossing Biological Barriers took place in Dresden on 9-11 November 2015 with 139 participants, 47 talks and 46 posters.

During the project lifetime, three patents on new methods or technologies were filed by ALEXANDER Partners LEK, SAGETIS and UNAV. One more patent is currently in preparation by Partner UNAV. Partner UoG has acquired national funding for further cooperation with SME SAGETIS, a joint patent is planned.

The ALEXANDER Consortium comprised of a number of groups involved in the synthesis and the characterization of nanoparticles, and the quantification of their interaction with mucus. A number of project partners focused on the synthesis of novel nanoparticles, whilst others focused entirely on the comparative physicochemical characterization of mucus in the absence and presence of these nanoparticles. To maximize the exploitability of this research, all of this information was captured and made available to every member within the Consortium. A searchable, encyclopaedic database has been created to collate and share this wealth. It now includes data of more than 500 NP systems with physical characterization of the size, zeta-potential, as well as toxicity data from many of these systems and information on their mucus behavior. As one of the last implementation the possibility to present the Technical Readiness Levels of the systems was included. This makes the system also valuable for further project cooperation.

In general, new knowledge and a deeper understanding of nanoparticle properties that enhance mucus permeation has been gained within ALEXANDER. New analytic techniques have been developed or existing ones have been expanded or optimized. New carrier systems with new qualities have been developed and optimized. All these tools have a great potential for further development and later application in industry or in the clinic. They may as well serve as the basis for new cooperations or projects.

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Last update: 9 December 2016

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