Master Thesis

The structure and function of the lipopolysaccharide transport system in gram negative bacteria

Universiteit Utrecht

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The maximum length of a proposal is 11 pages.

1a. Details of proposal

Title: The structure and function of the lipopolysaccharide transport system in gram negative bacteria.
Area: O Geo and Biosphere x from Molecule to Organism
Summary (scientific summary in English, max. 250 words):

Gram-negative bacteria contain lipopolysaccharide (LPS), also known as endotoxin, in the outer leaflet of their outer membrane (OM). LPS causes a strong immune response in humans and protects the bacterium against hydrophobic molecules, including many antibiotics. LPS is synthesised in separate parts in the cytoplasm and assembled at the inner and outer leaflet of the IM. The mature LPS is transported through the periplasm and across the OM. The synthesis pathway and transport across the IM have been described. The subsequent removal of LPS from the IM and transport to the outside of the OM have not been elucidated entirely. A complex of seven proteins, named LPS transport system (Lpt) A-G, is responsible for LPS transport to the OM. The complex is thought to span the entire bacterial envelope, likely at contact points between IM and OM as seen by Bayer in electron microscopy (EM) studies. This research aims to identify the localisation and interaction partners in the complex for each protein and resolve the structure of four proteins that together span the envelope. For these proteins the functional interaction domains for complex partners or LPS will also be determined. *Neisseria meningitidis* will serve as model organism. Its Lpt proteins will be analysed by EM, Förster resonance energy transfer, Nuclear Magnetic Resonance imaging and by reconstructing an artificial double membrane system with two different types of vesicles. This will provide fundamental insight into the process of OM synthesis of gram-negative bacteria and aid in the development of novel antibiotics.

1b. Details of applicant

Name: Nienke van Beek
Gender: O Male x Female
Date of birth: 31-08-1989
Institution: Utrecht University
Position: O Professor O Associate professor (UHD) O Assistant professor (UD) x
Other: Master student
Permanent position: O Yes x No, end date contract: -
E-mail: N. vanBeek1@students.uu.nl
Research School: Bacterial microbiology
Name and address of the responsible person at your institution (e.g. scientific director of the institute or dean of the faculty): Prof. Dr. H.A.B. Wösten

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1c. Alternative contact – not applicable

Name:
Tel:
Email:

1d. Renewed application? O Yes x No
In case of a renewed application please indicate the file number of the previous application and summarize the main changes

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1e. Applying for: x PhD student O Post Doc O Ship time

1f. Composition of the research group
List all staff members involved in the proposed research: provide name, initials, titles and type of involvement, e.g. daily guidance, technician, thesis supervisor, advisor.

Name and title	Specialization	Institution	Involvement
Prof. Dr. J.P.M. Tommassen	Microbiology	Utrecht University	Promotor
Prof. Dr. M. Baldus	ssNMR	Utrecht University	Advisor
Ing. F.H.R. Beckers	Technician	Utrecht University	Technician
Dr. Ing. J. Grijpstra	Microbiology	Utrecht University	Daily guidance co-promotor
N. van Beek, BsC		Utrecht University	

2. Summary for the general public

(please provide in 100 words a title and summary for the general public, preferably in Dutch)

Transport van bacterieel lipopolysacharide naar het buitenmembraan

Gram-negatieve bacteriën hebben een binnen- en buitenmembraan gescheiden door het periplasma. Ze bevatten lipopolysachariden (LPS) in hun buitenmembraan. LPS veroorzaakt een sterke immuunrespons in mensen en biedt de bacterie bescherming, bijvoorbeeld tegen vele antibiotica. Hoe LPS in het buitenmembraan terecht komt, is nog grotendeels onduidelijk. Hiervoor moet het als hydrofobe structuur door het hydrofiele periplasma. Een complex van zeven eiwitten, LptA-G, is hiervoor verantwoordelijk. Met dit onderzoek wordt uitgezocht hoe de componenten van dit complex interacties aangaan met elkaar en met LPS. Met de verkregen kennis kunnen nieuwe antibiotica ontwikkeld worden.

3. Top 5 publications of the applicant and research group related to the proposed research

1. Bos, M. P., & Tommassen, J. (2011). The LptD chaperone LptE is not directly involved in lipopolysaccharide transport in *Neisseria meningitidis*. The Journal of Biological Chemistry, 286, 28688-28696.

2. Bos, M. P., Tefsen, B., Geurtsen, J., & Tommassen, J. (2004). Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. Proceedings of the National Academy of Sciences of the United States of America, 101, 9417-9422.

3. Bos, M. P., & Tommassen, J. (2005). Viability of a capsule- and lipopolysaccharide-deficient mutant of *Neisseria meningitidis*. Infection and Immunity, 73, 6194-6197.

4. Renault, M., Bos, M. P., Tommassen, J., & Baldus, M. (2011). Solid-state NMR on a large multidomain integral membrane protein: the outer membrane protein assembly factor BamA. Journal of the American Chemical Society, 133, 4175-4177.

5. Bos, M. P., Robert, V., & Tommassen, J. (2007). Biogenesis of the gram-negative bacterial outer membrane. Annual Review of Microbiology, 61, 191-214.

4. Description of the proposed research

Max. 4 pages (and max. 3600 words), including figures, excluding literature references). Include details of objectives, innovative aspects, scientific approach, preliminary data, impact, and literature references (include full bibliographical details)

Introduction

Gram-negative bacteria have an asymmetric outer membrane (OM) that, apart from phospholipids in the inner leaflet, contains a large amount of lipopolysaccharide (LPS, also known as endotoxin) in the outer leaflet. LPS is an essential component of the gram-negative membrane in most gram-negative bacteria and helps defend against large hydrophobic molecules, like many antibiotics[1]. Transport of LPS to the OM requires machinery that enables crossing of the periplasm and the OM. This system needs energy from

the cytoplasm to operate, requiring a connection to the cytoplasm. A system composed of seven proteins has been found to be involved in this transport, i.e. the lipopolysaccharide transport (Lpt) system consisting of LptA to G (Figure 1)[2]. Deletion mutants of these proteins all have similar deleterious phenotypes, indicating that the proteins are all involved in the same essential process. The proteins together are thought to span the entire cell envelope of the bacteria[2]. It is currently believed this complex creates or is present at contact points between IM and OM. Bayer has previously described membrane contact points as areas of adhesion between the outer and inner membrane visible upon osmotic shrinkage of the protoplast (bacterium and just the IM)[3]. We propose these Bayer's patches to be the location of LPS transport by the Lpt system.

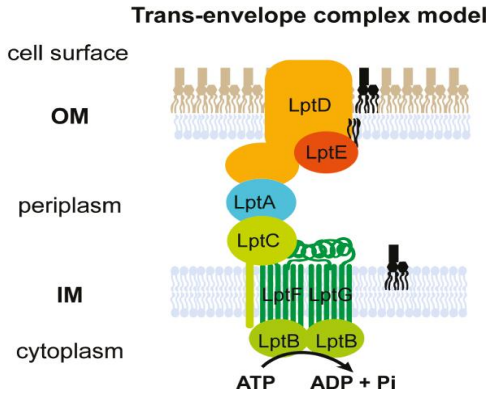

Figure 1 LPS transport system components and possible transport option. LptB is a cytoplasmic protein bound to LptF and G, IM proteins, and together forms an ABC transporter. LptC is anchored to the IM on the periplasmic side. LptA is an unanchored periplasmic protein. LptE and LptD are located at the OM. The most likely mode of transport, with contact points between IM and OM made by the LptA protein, is shown here[2, 10].

This idea is supported in several ways:

i. In spheroplasts LPS is still transported to the remaining patches of OM[4].
ii. When the membranes are fractioned on a sucrose gradient all Lpt proteins can be found together in a fraction containing, but not enriched for, OM[5].
iii. Several Lpt proteins form complexes with each other[2, 6-9]. LptB, C, F and G have been found in a complex that could link ATP hydrolysis to the release of LPS from the IM[6-8]. LptD and LptE have also been shown to interact in vivo[9, 10]. LptA could also be stacked head-to-tail with a hydrophobic groove in the stack's centre when one considers the protein structure[11]. Finally, LptA, C and LptD were recently shown to interact with each other to couple the IM and OM[12].

Thus, all seven Lpt proteins likely interact and this could be at specific IM and OM contact points. Yet, which components are essential and directly interacting is not clearly known. LPS in *Neisseria meningitidis* is not essential; therefore deletion mutants of the Lpt system can be generated in this bacterium[13]. To see how conserved the Lpt system is and also to find possible essential components, putative homologues of the *N. meningitidis* system were sought based on sequence homology or based on their similar genomic localisation[10]. The LptC, LptE and LptA proteins are not very conserved and homologues are thus hard to find via sequence homology searches[14, 15], yet putative functional homologues of these proteins could be found based on the genomic context. Genes coding for proteins with a similar secondary structure as the *N. meningitidis* Lpt protein and found in a genomic location similar to the one in *N. meningitidis* were selected as putative functional homologues. The putative homologues were used to complement *N. meningitidis* deletion mutants. The homologue for LptC from *Escherichia coli* and the putative homologue from *Campylobacter jejuni* could not restore the phenotype of a ΔLptC in *N. meningitides* (van Beek, unpublished data). This indicates they may not be sufficiently homologous to replace the function of a deleted gene in another organism. Perhaps, but unlikely, the inability to replace the function was due to differences in the sequences required for subcellular localisation. More likely explanations for the lack of functional homology are differences in interaction sites between species or that perhaps the genomic location, in the case of the *C. jejuni* gene, is no indication of homology. As the proteins do not seem to be functionally exchangeable between species, this project's aim is to discover the interactions within the complex and its subcellular localisation using the model organism *N. meningitidis.*

Objectives

The objectives of the research are summarised below:

i. Determining the *in vivo* interactions between members of the Lpt system, focusing on microscopy to visualise the proteins at the aforementioned Bayer's patches.
ii. Finding interaction partners that span the entire membrane, and as such might form the Bayer's patches, by using an Lpt system like protein from the thermophilic bacterium *Thermotoga petrophila*. *T. petrophila* has a cell envelope similar to gram-negative bacteria and one of its proteins, Tpet_1030, has sequence similarity to *N. meningitidis*' LptF/G in its N-terminal half and to and LptD in its C-terminal half, suggesting that it is a fusion protein that spans the entire cell envelope.
iii. Resolve the structure of the *N. meningitidis* Lpt proteins and their conformational changes in response to interaction partners, starting with LptA, C, D and F.

Scientific approach

Cloning and expression

LptA-G from *N. meningitidis* will be cloned in an *E. coli* expression vector that adds a histidine tag on either the C-terminus or N-terminus of the protein, depending on which terminus contains its signal sequence for membrane localisation. Each of the proteins will also have another version where a small tetracysteine motif for biarsenical fluorescent dye labelling will be added to either the N-terminus or C-terminus of the protein. The suspected interaction partners will be cloned, into *N. meningitidis* in a pEN21 vector, with different integrated motifs to allow for sequential binding to the following dyes: Red arsenical Hairpin binder (ReAsH) or fluorescein arsenical hairpin binder (FlAsH)[16]. This will facilitate the visualisation of two differentially labelled proteins within the same bacterium. *Tpet_1030* will also be ligated into a pEN21 vector and cloned into a *N. meningitidis* strain where the LptD and F/G gene will be deleted by recombination with an erythromycin- and kanamycin-resistance cassette, respectively (F and G are next to each other and can be deleted with one cassette). To this end, the genes are amplified from genomic DNA of *N. meningitidis* and *T. petrophila* by PCR with Hi-fi polymerase with primers designed to add restriction sites. After cloning into appropriate plasmid vectors, they will be maintained in the *E. coli* DH5α strain. The *N. meningitidis* strain HB-1 will mainly be used to express the proteins of interest. Expression conditions will be optimised by trying different levels of IPTG and a range of temperatures.

Research and analysis

There will be two distinct lines of research, the first focusing on *in vivo* interactions between system members within the cell, mainly using FRET and pull down assays, and the second focusing on the structure and interaction sites of the Lpt system proteins, mainly using Nuclear Magnetic Resonance (NMR). The results will be validated by means of mutagenesis and subsequently repeating the experiments and by creating an artificial system. The techniques for these two research lines and the validation will be described in more detail below.

In vivo interactions

Pull down assay: The proteins will be His-tagged to enable their purification from a cell lysate. They will be analysed by SDS-PAGE and western blotted for co-purification of other members of the complex.

Förster Resonance Energy Transfer (FRET): A protein's subcellular location can be visualised by fluorescence microscopy. If two proteins with a distinct colour fluorescent tag (FlAsH and ReAsH in this case) are present in the same cell then, if they are in close (1-10 nm) proximity, one fluorophore can excite the other, allowing detection of the emission spectrum of the second fluorophore. If they are not in close proximity, and thus not likely to interact, only the emission of the first fluophore will be visible.

Rescuing the N. meningitidis *Lpt deletion mutant phenotype with Tpet_1030:* Tpet_1030 will be expressed from the pEN21 vector by inducing the lac promoter with IPTG. Neisseria is naturally competent to take

up DNA, yet because Lpt system deletion mutants are less competent to take up foreign DNA[10], the wild-type strain will be transformed by natural transformation with the pEN21 vector with Tpet_1030 first. The ΔlptD and ΔlptF/G mutations will subsequently be introduced on the chromosome by means of electroporation[17]. The growth of this complemented strain will be compared with the wild type growth and the double deletion mutant's growth. *N. meningitidis* has a feedback system that downregulates LPS when transport fails[10]; therefore the presence of LPS will be analysed by SDS-PAGE of proteinase K-treated and silver staining the gel, which visualises LPS. The LPS levels will be compared to the levels in the wild type and the mutant. Accessibility of LPS in intact cells will be determined with a neuraminidase assay[18]. Neuraminidase cuts exposed sialic acid and will thus indicate whether the sialic acid of LPS is on the outside of the OM in the wild type and mutants.

Electron Microscopy (EM): Antibodies can help visualise the proteins in EM microscopy, showing the localisation of the proteins in the cell and their localisation in, and the existence of, Bayer's patches. *N. meningitidis* transformed with the Tpet_1030 protein will be used extensively in visualising Bayer's patches as Tpet_1030 might be sufficient to fixate the contact sites, thus demonstrating whether the contacts between LptD and LptF/G are sufficient to create the contact points. If Tpet_1030 is present at Bayer's patches while it can replace the function of *N. meningitidis'* LptD and F/G, then *N. meningitidis'* own Lpt proteins are likely present in similar structures with interactions between proteins where Tpet_1030's domains are connected. This would prove the existence of membrane contact points and the role of these specific contact points in transporting LPS. EM will also be used to visualise the purified Lpt proteins to provide preliminary structural information that might aid in determining the structure by NMR.

Structure and interaction sites

NMR: This work will be done in cooperation with the NMR group of Utrecht University. The first proteins to have their structure resolved by NMR will be LptA, C, D and F, as together these proteins span the envelope and could thus already provide an image of the steps required to transport LPS across the periplasm and OM. In time the structure of all seven proteins in the Lpt system will be resolved. Proteins will be metabolically triple labelled with ^{15}N,^{13}C and ^{2}H. The membrane proteins (LptC, D and F) will be reconstituted in liposomes after which the structure will be determined via solid-state NMR. LptA is not a membrane protein and will be analysed by solution NMR. Structure determination is easier for LptA and LptC and LptE, as their structure has already been determined by crystallography in *E. coli* (LptA and C) [11, 19] or *N. meningitidis* itself (LptE: PDB ID 3BF2). The availability of crystal structures facilitates resolving the structures by NMR, especially if some interaction partners cannot be purified without the other (LptD and LptE for example). The structure of LptE is not a priority in this project. Once the structures of LptA, C, D and F have been resolved, the structure in the presence of LPS and other proteins of the pathway will be determined. The change between the normal spectrum and the one when a possible interacting structure is added will enable identification of the interacting domains.

Validation and proof

Validation:

These data will be validated *in vivo* by mutating the found interfaces (Quickchange) and then testing both wild type and mutant with FRET and by transforming (by means of electroporation) *N. meningitidis* deletion mutants of the protein of interest with the mutated versions of these proteins. The complemented strain's LPS levels, membrane protein localisation and the LPS content of the membrane will all be analysed by SDS-PAGE of proteinase K-treated samples followed by silver staining. Whether or not LPS is still transported to the outside of the outer membrane will be determined by a neuraminidase assay, where neuraminidase cleaves sialic acid off LPS only when it is on the outside of the OM.

Artificial system:

Lastly, the minimal system required for transport of LPS will be reconstituted in an artificial reconstituted system. This will require two distinct types of vesicles. The first type will be a protoplast and thus only

have the IM, it will synthesise ATP and radiolabeled LPS and contain the IM Lpt proteins (LptB, F, G and C) in its membrane. The other type of vesicle will be small unilamellar vesicles (SUV) that will contain the outer membrane Lpt proteins (LptD and E) in the lipid layer. LptA will be added separately. The radiolabelled LPS from the protoplasts should be transported to the SUVs containing the outer membrane proteins. The protoplasts can then be pelleted to isolate the SUVs, the presence of radiolabelled LPS in these SUVs can then be analysed. To see whether the LPS is present inside the SUV or on the outside of the membrane a neuraminidase assay will be performed on the SUV's. If LPS is present inside the SUV it would mean the LPS reached the second vesicle and is thus effectively transported across the two separated membranes by the Lpt proteins.

Preliminary data

LptC from *N. meningitidis* has already been cloned in *E. coli* overexpression strains. LptD from *N. meningitidis* has already been purified and antibodies against this protein are already present. Staining with these antibodies for EM is currently being optimised.

Innovative aspects

This research is new in the use of NMR to identify both the structure and interaction domains in the Lpt system. Using NMR instead of crystallography to determine structures has the added benefit of being able to visualise ligand binding sites; when a ligand is added to the protein the structural change in the binding site will be visible. This research is also unique in its use of the *T. petrophila* protein (Tpet_1030) that appears to be a fusion of two polypeptides of the Lpt system. These bacteria are among the oldest branches in the domain Bacteria. If the fusion protein can replace the function of LptD and F/G in *N. meningitidis* this would indicate that these *N. meningitidis* proteins also span the IM and OM together and form contact points and that these contact points are possibly evolutionary conserved. Lastly, the construction of the artificial system, in two separate vesicles to simulate a double membrane, is new and would prove the functioning of the Lpt system in creating a contact between two separate membranes and transporting LPS across the membranes. This artificial system could be used for further experiments, for example in testing Lpt system targeted pharmaceuticals with an easy read-out.

Impact

Because the Lpt system is essential for most gram-negative bacteria and protects against large hydrophobic molecules, like many antibiotics, it is an important target for drug development and health care. Knowing the functioning of the Lpt system will give valuable fundamental information on the transport of LPS and the creation of the gram-negative non-symmetrical outer membrane, but will also give the knowledge needed for interfering with this system. Novel antibiotic agents could be developed that interfere with this system, and this also makes the bacteria susceptible to other antibiotics. In this time of increasing bacterial resistance to existent antibiotics, novel antibiotic targets will have great impact for human healthcare. If no new antibiotics are made we risk untreatable infections with potentially fatal consequences. The identification of the functional domains of the Lpt system can lead to the design of novel antibiotics. When conserved residues in the interacting domains can be found, antibiotics could be designed to target these residues specifically. These antibiotics will affect many gram negative bacteria and the bacteria are not likely to develop resistance. Antibiotics targeted against the transport of LPS will be especially useful in treating infections by for example multiple resistant *E. coli*, *Salmonella* or *Klebsiella*. Antibiotics can be designed based on the found structure of the system and perhaps even tested in the artificial system. Targeting these at the transport system does not require them to pass the whole membrane system, only the OM, which might facilitate their design.

References

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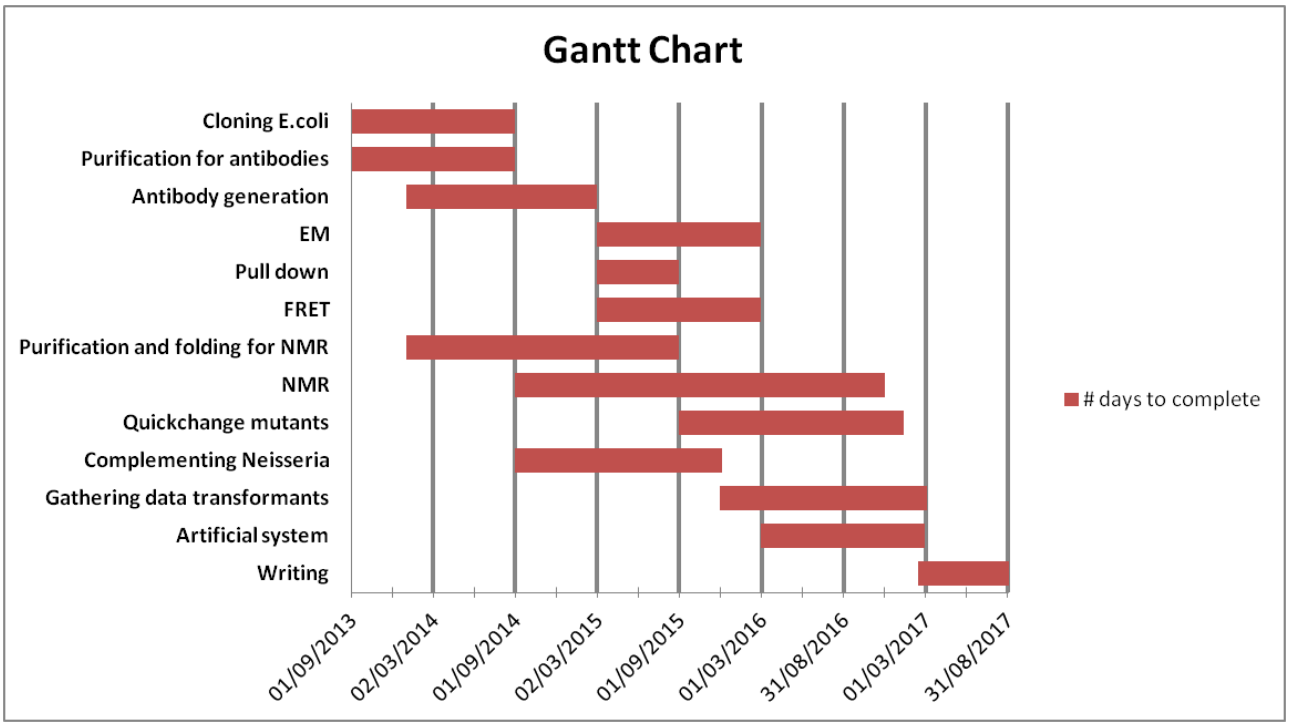
5. Timetable of the project



The start date is 01/09/2013, the project will take four years, ending at 01/09/2017. The active research will take three-and-a-half years starting with the cloning procedures and purification, after which the experiments can start for the already purified proteins. The PhD student will have half a year to write his thesis full-time.

6. Scientific embedding of the proposed research

Affiliation with national and international research programmes, national and international collaborations

This project will have collaborations between the Utrecht University Microbiology group from the Biology department and solid state NMR group from the Chemistry department led by Prof. Dr. Marc Baldus.

7. Societal significance

Many pathogenic gram negative bacteria cause additional symptoms due to their LPS.
LPS also protect the bacterium from a number of antibiotics. Antibiotic resistance is a growing issue around the world. Two projects, R-gnosis and EvoTAR, are collaborations to combat spread of resistant bacteria between seven and nine European countries, respectively. However, these mainly focus on the prevention and understanding of spread and infection. Elucidating the structure and interaction of the proteins responsible for its LPS transport could lead to the production of novel antibiotics or other drugs that disable the transport of LPS to the outer membrane, having the additional benefit that this is lethal to most bacteria. This means new drugs will be developed to which developing resistance seems unlikely as the targets are conserved. Targeting the transport also helps to increase the effects of existing antibiotics,

as the bacterial defence will be decreased when LPS is reduced. Therefore, the impacts to society, especially in a time when antibiotic resistance is a common and growing problem, are significant.

8. Budget

		Year 1	Year 2	Year 3	Year 4
Personnel (mm)		12	12	12	12
Research costs (k€)					
	Equipment	-	-	-	-
	Consumables*	13	14	14,5	5,5
	Fieldwork/Travel*	-	-	-	3

* The sums requested for consumables and fieldwork/travel expenses combined should not exceed 50,000 euro for the entire grant period.

Specification of the requested funds:
Equipment: -
Consumables: Enzymes, sequencing, primers, fluorescent dyes and labels, protein purification kits, isotopic labelled compounds for NMR, antibody generation, lipids, extruder and filters for vesicle formation, secondary antibodies, western blotting staining kit, DNA purification kits, disposables.
Fieldwork/Travel: Congresses and conferences; Gordon conference (West Dover, USA).

9. Financial assistance from other sources

Not applicable.

10. Statements by the applicant

YES I endorse and follow the Code Openness Animal Experiments (if applicable).

YES I endorse and follow the Code Biosecurity (if applicable)

YES I have completed this form truthfully

Name: Nienke van Beek
Place: Utrecht
Date: 09-11-2012

Please submit the application to NWO in electronic form (pdf format is required!) using the Iris system, which can be accessed via the NWO website (www.iris.nwo.nl). The application must be submitted from the account of the main applicant. For any technical questions regarding submission, please contact the IRIS helpdesk (iris@nwo.nl).