Akademie věd
České republiky

Teze disertace k získání vědeckého titulu "doktor věd" ve skupině molekulárně-biologických a lékařských věd

Lipid-based nanoparticles for construction of drug delivery systems, vaccines and theranostics

Komise pro obhajoby doktorských disertací v oboru Molekulární biologie a genetika

doc. RNDr. Jaroslav Turánek, CSc.
Veterinary Research Institute
Brno, 2017

Bibliographic entry

Author:	Ass. Prof. RNDr. Jaroslav Turánek, CSc. Veterinary Research Institute, Brno Department of Pharmacology and Immunotherapy

Title:	Lipid-based nanoparticles for construction of drug delivery systems, vaccines and theranostics
Year:	2017
Number of pages:	46
Key words:	Liposomes; recombinant vaccines; immunotherapy; nanotechnology; pharmacology; adjuvants; drug delivery systems; anticancer drugs; antiviral drugs

Summary of D.Sc. thesis

In this thesis I summarise more then 25 years of my work in the field of drug delivery systems and vaccines. The research focused on that topic I have started at Veterinary Research Institute in 1988 and in fact it was necessary to build an appropriate infrastructure and put together the team of scientist, who were my postgradual students. This part of my work has been compleeted by founding the Department of Pharmacology and Immunotherapy in 2007 and preparing the project OP VVV "FIT" (Pharmacology, Immunotherapy, nanoToxicology). The project started in 2017 and it will enable us purchase all necessary advanced instruments to set up world-class laboratory for development of recombinant vaccines and targeted drug delivery systems for cancer and antiviral drugs.

My interest in drug delivery systems is focused presumably on liposomes, which are one of the most effective structures for the delivery of biologically active compounds directly to the cells and also one of the major biocompatible carriers of adjuvants for construction of modern safe and effective recombinant vaccines. Thanks to the biological similarity of biomembranes and possibilities of targeted distribution of encapsulated substances, liposomes are attractive to a wide range of medicinal disciplines (pharmacology, immunopharmacology, immunology and genetic engineering).

Several methods for preparation of liposomes were introduced to my lab and also I have designed and constructed some devices for liposomal techniques based on extrusion, proliposome-liposome method and detergent removal method. All these methods were published in respected international journals. Recently a new method based on microfluidic mixing has been introduced in my lab in collaboration with the company Precission Nanosystems (Canada)

Lipophilic anticancer drugs like paclitaxel and several derivatives of vitamine E were studied and efficient liposomal formulations were developed in collaboration with industrial partners (e.g. Lachema. Brno) and academic institutions (Griffinth University, University of Utah, Institute of Biotechnology, Czech Academy of Sciences, Prague). Patents and publications based on this research are included in this thesis. The research directed to the field of delivery of antiviral drugs represents the field where we belongs to pioneers and in collaboration with prof. Holy and Dr. Ledvina (Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague) we developed cationic lipids for construction of liposomal carriers and proved the cocnept of liposomal antiviral drugs based on compound developed by prof. Holy. Interesting field of liposomal theranostic application is diagnostic and treatment of brain ictus. The development of theranostic preparations for this purpose is done within the join project with ICRC centre at FNUSA Brno (Ass. Prof. Mikulik) and Institute of Biotechnology, BIOCEV, Prague (Dr. Maly).

The main interest is devoted to recombinant vaccines, molecular adjuvants and noninvasive systems for mucosal and transdermal. This is the

most succesful part of my collaborative research with Institute of Organic Chemistry and Biochemistry (Dr. Ledvina) crowned by new apyrogenic molecular adjuvants based on on norAbuMDP (EU and USA patents), which are in the process of commercialisation (Czech company Apigenex). These adjuvants and developed metallochelating liposomes were used to develop nanoliposomal adjuvanted carriers for construction of recombinant vaccines (collaboration with Palacky University in Olomouc, prof. Raška). Finally, the mucoadhesive systems based on nanofibres has bee developed recently and PCT were applied to proctect quite new approach to noninvasive application of vaccines. New technology of "printed vaccines" is under development and a start-up company is to be set up in collaboration with foreign company KPT Therapeutics. This collaborative research is running with Technical University Liberec (ing. Lubasova) and UP Olomouc (prof. Raška) and is one of main scientific topic in the OPVVV project FIT.

All these topics are a part of my D.Sc. habilitation thesis and are briefly introduced in these thesis.

Contents

INTRODUCTION

Application of biocompatible nanocarriers for targeting of drugs and construction of theranostics and vaccines is one of main areas in modern pharmacology. Ideal nanocarriers can improve therapeutic index of drugs and with respect to construction of modern recombinant vaccines there is a trend in development of nanocarriers which allow formulation of antigens and adjuvants in selfassembling vaccination nanoparticles. Such a formulation can stabilise antigen, target it to immune cells, induce well focused immune response and make the vaccination safe because of eliminating all potentialy dangerous vaccine components. Also economic aspects of vaccine production, storage, distribution and application in vaccination campagne must be taken in account, because this is the key parameter for a succes of any vaccine. Therefore new biocompatible nanomaterials are of interest to use them for development of drug and vaccine nanocarriers.

The research in this field represents very complex approach including a lot of disciplines including medicine, immunology, pharmacology, pharmaceutical technologies, organic and physical-chemical chemistry to mention only some of them.

The exclusive place among nanocarriers belongs to liposomes, which are one of the most effective structures for the delivery of biologically active compounds directly to the cells and also one of the major biocompatible carriers of adjuvants for construction of modern safe and effective recombinant vaccines. Thanks to the biological similarity of biomembranes and possibilities of targeted distribution of encapsulated substances, liposomes are attractive to a wide range of medicinal disciplines (pharmacology, immunopharmacology, immunology and genetic engineering).

1. LIPOSOMES

Nanoparticle-mediated delivery of drugs, vaccines, and diagnostics is a rapidly developing area of nanomedicine that brings together the scientific disciplines of nanotechnology, pharmacology, immunology, and chemistry. This huge potential of nanoparticles and nanomaterials in modern medicine is reflected by intensive basic and oriented research. Well focused scientific programmes supporting the research in nanotechnology and nanomedicine are proposed in EU, USA as well as in technically developed Asian states like Japan, Korea and China.

Liposomes (phospholipid bilayer vesicles) represent an almost ideal carrier system for the preparation of synthetic vaccines due to their biodegradability and capacity to protect and transport molecules of different physicochemical properties (including size, hydrophilicity, hydrophobicity, and charge). Liposomes are attractive to a wide range of medicinal disciplines

(pharmacology, immunopharmacology, immunology and genetic engineering)[1–3]. Liposomal carriers can be applied by invasive (e.g. i.m., s.c., i.d.) as well as non-invasive (transdermal and mucosal) routes. In the last 15 years, liposome vaccine technology has matured and several vaccines containing liposome-based adjuvants have been approved for human and veterinary use or have reached late stages of clinical evaluation.

Given the intensifying interest in liposome-based vaccines, it is important to understand precisely how liposomes interact with the immune system and how they stimulate immunity. It has become clear that the physicochemical properties of liposomal vaccines – method of antigen attachment, lipid composition, bilayer fluidity, particle charge, and other properties – exert strong effects on the resulting immune response. In this chapter we will discuss some aspects of liposomal vaccines including the effect of novel and emerging immunomodulator incorporation. The application of metallochelating nanoliposomes for development of recombinant vaccine against Lyme disease will be presented as a suitable example.

1.1. LIPOSOMES AS BIOCOMPATIBLE DRUG CARRIERS

Many agents with different physico-chemical properties can be encapsulated into liposomes. Lipophilic substances are incorporated into the lipid bilayer and hydrophilic substances are encapsulated within the internal aqueous environment. Depending on the method used to prepare the liposomes, final liposomal product can vary in size, number of lamellae (unilamellar, oligo- or multilamellar vesicles) and have different physical and chemical characteristics that may greatly affect the amount of the encapsulated substance.

For the pharmaceutical field, liposomes as a carrier are very attractive for the following reasons:

1. Easy encapsulation of hydrophilic and hydrophobic drugs
2. Preparation of drugs from natural lipids, which are readily biodegradable and of low toxicity
3. Preparation of liposomes of varying size and morphology
4. Reduction in adverse effects of drugs
5. Specific drug delivery to the target organ or tissue
6. Targeting of drug and gradual drug release

1.2. METHODS FOR PREPARATION AND CHARACTERISATION OF LIPOSOMES

Various techniques have been developed during the last fifty years for preparation of liposomes and other lipid based particles. Selection of appropriate method is dictated by factors such as administration route (e.g., i.v. injection, transdermal/mucosal administration), lipid composition (saturated versus unsaturated lipids), stability of substances which are to be encapsulated (e.g. proteins, DNA, viruses, low molecular drugs), conditions for preparation (reconstitution of membrane proteins), morphology and size of final liposomal preparation, postforming modification of liposomes (binding of various ligands

– e.g. antibody, saccharide ligands, protective polymers – PEG) and scaling-up ability of the method (application of technology for industrial production) etc.
The size, morphology, lipid composition and surface modification are the most important parameters affecting interaction of liposomes with cells, their biodistribution and stability in the body, stability during technological processes (e.g. sterilisation by microfiltration, γ-irradiation or thermal sterilisation; fluid drying or lyophilisation), stability during transportation and storage in dry or liquid formulation[4–7].

1.3. THE SIZE AND MORPHOLOGY OF LIPOSOMES

The most commonly used parameter for the distribution of liposomes is classification according to their size and number of lamellae. Liposomes can have different sizes ranging from 20 nm to 100 nm with the lipid bilayer approximately 4 nm thick. They can be divided into small (SUVs; 10-100 nm) and large (LUVs; 100-1000 nm) unilamellar vesicles, which are of the order of only a few units of bilayers, and the large multilamellar vesicles MLV (100 nm - 20 nm) containing dozens concentrically arranged bilayers as multivesicular MVV vesicles (100 nm - 20 μm) which are arranged as non-concentric lamellae (Fig. 1).

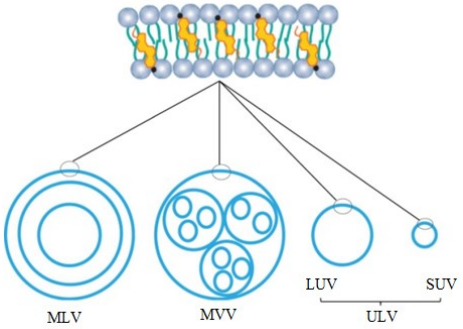


Fig. 1 Classification of liposomes. *SUV – small unilamellar vesicles, LUV – large unilamellar vesicles, MLV – multilamellar vesicles, MVV – multivesicular vesicles*.

1.4. CLASSIFICATION OF LIPOSOMES ACCORDING TO THE FUNCTION AND NATURE OF LIPID MEMBRANES

Liposomes can be classified according to the structure of the membrane and the surface modification into several basic types that have different uses in the design and preparation of systems for targeting various molecules to cells (Fig. 2)[8].
Conventional liposomes - non-specific interactions with the environment, instability in serum

Sterically hindered liposomes (Stealth liposomes) - long-term circulation in the bloodstream

Targeted liposomes – targeting is mediated via specific interaction with surface bound ligand structure on cell membranes (ligand-receptor interaction, antibody - epitope)

Cationic liposomes - the ability to interact with negatively charged DNA/RNA and induction of its condensation; they electrostatically interact with the negatively charged DNA to form complexes (lipoplexes), protect the DNA from degradation by nucleases, promotes entering into the cells via endocytosis or phagocytosis, after injection may be received by APC cells infiltrating the injection site.

The membrane of the liposomes may be modified to vary in its structure, depending on temperature or pH. This can allow a release of the encapsulated substances, e.g. in a tumour, where the temperatures are slightly elevated and pH is lower due to restricted blood flow and insufficient lymphatic drainage.

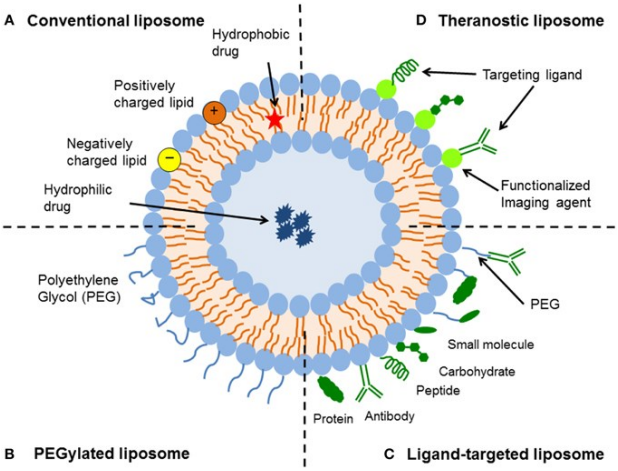


Fig. 2A Classification of liposomes according to membrane structure and surface modification.

Many synthetic, self-assembly nanoparticle technologies have already shown great promise in vivo. These nanoparticle technologies in question typically conform to the ABCD nanoparticle paradigm first invoked by Prof Miller's team approx 10 years ago to define nanoparticle structures most appropriate for in vivo use[9] (Kostarelos K, Miller AD. Adv. Genet. 2005, 53, 71-118**).**

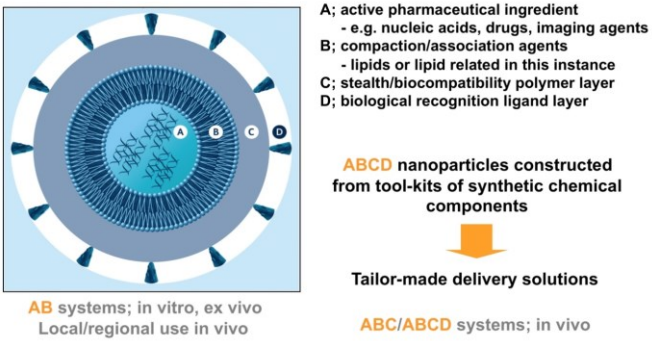


Fig. 2B Schematic presentation of functional synthetic nanoparticle based drug delivery systems. *In these nanoparticles, (active pharmaceutical ingredients APIs) of the type indicated (A) are condensed within functional concentric layers of chemical components designed for delivery into cells and intracellular trafficking (B components), biological stability (C) stealth/biocompatibility components – typically Polyethylene Glycol [PEG] and biological targeting to target cells (D components biological receptor-specific targeting ligand).*

According to this general paradigm, nanoparticles appropriate for functional delivery of activepharmaceutical ingredients (APIs) in vivo should typically comprise APIs (A-components) surrounded initially by compaction/association agents (B-components - typically lipids, amphiphiles, proteins or even synthetic polymers etc.) designed to help sequester, carry and promote functional cellular delivery of the A-components. Such core AB nanoparticles may have some utility in vivo, but more typically they require coating with a stealth/ biocompatibility polymer layer (C-layer; primary C-component – most often polyethylene glycol [PEG]) designed to render resulting ABC nanoparticles with colloidal stability in biological fluids. Finally, an optional biological targeting layer (D-layer; primary Dcomponents – bona fide biological receptor specific ligands) might be added to confer the resulting ABCD nanoparticle with cell target specificity. Importantly, much recent research work with ABC an ABCD nanoparticles has underscored a design weakness that we have called the C-layer paradox. According to this paradox, while C-layers variously assist with nanoparticle stability in biological fluids, nanoparticle storage, and with the avoidance of immune surveillance from cells of the immune system, these same C-layers are inhibitory toefficient API delivery once target cells are reached. (Miller A.D. Exp. Rev. Med. Devices, 2013, 10(6), 781-811; Miller A.D. Ther. Del., 2014, 5(5), 569–589).

This paradox now brings us to the state of the art and the whole concept is used to develop modern drug delivery systems within the OP VVV project FIT (Pharmacology, Immunotherapy and nanoToxicology; CZ.02.1.01/0.0/0.0/15_003/0000495; principal investigator Ass. Prof. RNDr. Jaroslav Turánek, CSc.)

1.5. METHODS OF LIPOSOME PREPARATION

Liposome preparation methods are based on the natural properties of phospholipids to form a lipid bilayer in an aqueous medium spontaneously. During interaction of water with the phospholipid film, phospholipid bilayer fragments are formed first. These intermediates are then converted into various stable vesicular structures. Membrane fragments formed in the process of hydration are the consequence of minimization of the interaction between water molecules and the hydrophobic parts of the phospholipids. The distance between the individual lamellae formed in multilamellar vesicles is a result of steric repulsive and attractive van der Waals forces.

General procedures for preparing liposomes include the preparation of lipid and aqueous phases, followed by hydration of the lipids in an aqueous medium, which finally leads to the desired final formation of liposomes[10].

Classification of liposome preparation methods:

a) Mechanical dispersion method (vortexing, sonication, high pressure homogenization)

b) Detergent dispersion method (dispersion of phosphatidylcholine solubilized with detergent to form micelles)

c) Two-phase dispersion methods (ethanol injection and ether injection, reverse-phase evaporation, and the recently introduced method of nanofluidic mixing)

Basic stages of liposome preparation:

1. Biphasic physical dispersion of lipids in the aqueous phase to form an emulsion of proliposomes and solubilization of lipids in a detergent to form micelles

2. Removal of the organic solvent or detergent to form liposome vesicles

3. Secondary processes to alter the morphology, size and stability of the primarily formed liposomes (extrusion through a membrane filter, the process of freezing and thawing, lyophilisation with cryoprotectants)

4. Chemical modification of liposomes (ligand binding)

1.5.1. Detergent removal method

Another primary method for preparation of liposomes is based on transformation of mixed lipid-detergent micelles into liposomes during the process of detergent removal. This can be achieved for example by dialysis using various devices (flow dialyzer ultrafiltration), removal of detergent by sorbents, or simple dilution of the mixed micelle solution). For the controlled process we designed and constructed a device for production of liposomes on a laboratory scale. Liposomes prepared by this method are unilamellar and extremely homogeneous in size distribution. The preparation is particularly suitable for reconstitution of membrane proteins. The principle of the method consists in converting small mixed micelles formed by phospholipids using a

suitable detergent (e.g. octylglucoside, deoxycholate) into disc shaped micelles, their coalescence and finally disc micelles of critical size vesiculate to form liposomes (Fig. 3). This process is driven by reducing the concentration of detergent removed by dialysis.

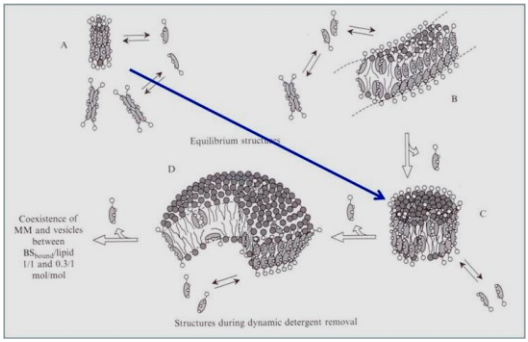


Fig. 3 Principle of detergent removal method and the formation of lipid supramolecular structures. *(Different types of micelles can be formed); A) mixed micelles; B) tubular fibre micelles (unwanted products); C) disc micelles – converting spontaneously into liposomes after reaching critical size (D) (figure adapted from Methods in Enzymology, 2003,367, 46-705).*

The following picture (Fig. 4,5) shows homogeneous liposomes (electron micrograph) and a comparison of the size of the micelles and liposomes (determined by dynamic light scattering). The laboratory equipment of my own design for controlled dialysis is an exemplified embodiment of the method. This allows us to prepare 10 ml liposomal suspension of lipid concentrations of 10 to 100 mg/ml.

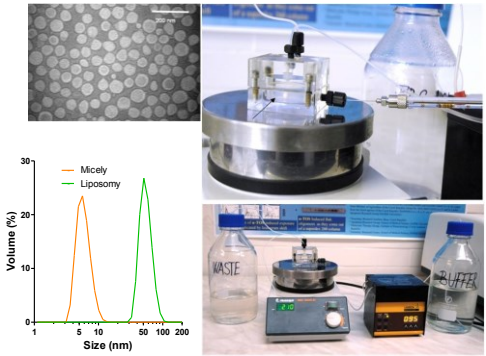


Fig. 4 The equipment developed and constructed by myself for the preparation of liposomes by a detergent removal method. *The stirred double-*

chamber flow-through dialisator enables well controlled preparation of liposomes. Small samples can be collected and analyzed during the process of liposome formation. Illustrative examples of size distribution of micelles and liposomes measured by dynamic light scattering and electron microscopy.

Fig. 5 System for preparation of liposomes by removal of detergent using ultrafiltration. *A) photo of the system linked with FPLC; B) photo of the ultrafiltration cell in detail (pink: LR-PE-labeled liposomes inside the cell). (C) monodisperse liposomes prepared by detergent removal method. (Analytical Biochemistry 408 (2011) 95–104).*

1.5.2 Proliposome-liposome method

The proliposome-liposome method is based on proliposomal preparation of hydrated stacked bilayer sheets in a water-ethanol solution. Generally, the organisation of a lipid/ethanol/water mixture can be described in terms of a three-phase diagram which can be divided into the following principal areas: lipid dissolved in aqueous ethanol, hydrated bilayers suspended in aqueous ethanol, and a liposomal area. Spontaneous formation of liposomal suspensions is accomplished by addition of excess aqueous phase to a lipid mixture (Fig.6).

This technique is simple and practical and is characterized by an extremely high entrapment efficiency, when compared with other methods based on passive entrapment. The technique is suitable for encapsulation of a wide range of drugs with various water and alcohol solubilities.

We have used this technique for encapsulation of antibiotics, anticancer drugs and molecular adjuvants. A stirred and thermostated cell for sterile preparation of liposomes was designed and constructed as a prototype. This cell is in use in our laboratory for research and teaching purposes (Fig. 7).

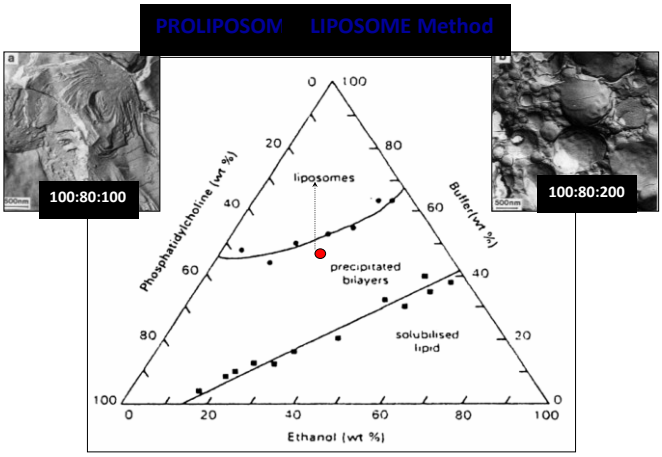


Fig. 6 Three-phase diagram and SEM pictures of proliposomes (left) and liposomes (right). *Three-phase diagram can be divided into the following principal areas: lipid dissolved in aqueous ethanol, hydrated bilayers suspended in aqueous ethanol and a liposomal area. Proliposome-liposome method is based on preparation of precipitated bilayers and their transition into liposomes in a stirred-thermostated cell by controlled dilution under sterile conditions (Methods in Enzymology 2003, 367, 110-125).*

Fig. 7 Photograph of whole system with stirred and thermostated cell for sterile production of liposomes by proliposome-liposome method. (*Methods in Enzymology 2003, 367, 110-125*).

1.5.3. Secondary processing methods

The most common secondary processing method is extrusion of the liposomes through polycarbonate filters. The principle of the method is shown in Fig. 8 as well as the available instrumentation in our laboratory (Fig.9). Extrusion of liposomes (in the case of saturated lipids, it is necessary to work at a temperature above the lipid transition temperature of 30-60 C) through the well-defined pores of polycarbonate filter leads to their deformation and stripping off the outer layers, which leads to size reduction.

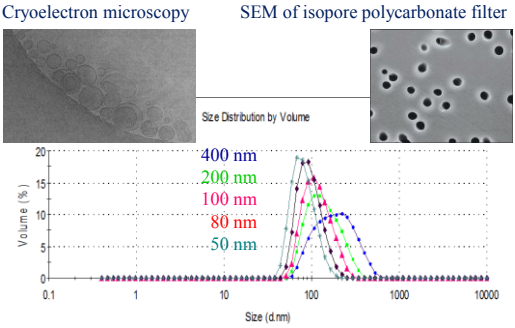


Fig. 8. *Structure of polycarbonate filter (TEM) with defined pores and picture of liposomes (Cryo-TEM) prepared by extrusion through polycarbonate filters of 100 nm pore size. The size distribution of the liposomes prepared by extrusion through polycarbonate filters of different pore sizes. Reduction in the size of the liposomes and their polydispersity is clearly noticeable (size measurements made using the dynamic light scattering method).*

A popular secondary processing method for preparing SUV liposomes on a laboratory scale is the use of ultrasonic homogenization. In this case it is necessary to cool the sample and possibly work in an inert atmosphere to prevent lipid oxidation. The advantage is the possibility to work with small sample volumes of liposomes. The sample may be immersed in the container in an ultrasonic homogenizer (preparation of larger volumes) or homogenized by special titanium or crystal glass probes for processing small volumes.

Recently, we have introduced to our NanoPharm laboratory (a joint laboratory of VRI and ICRC research centre) a new technology for preparation of liposomes by nanofluidic mixing using the instrument NanoAssemblr®. A memorandum of collaboration was signed between the VRI and the company Precision Nanosystems (Canada) (Fig. 10). This advanced new technology was used by us for the development of preparation of various functionalised liposomes, e.g. gadolinium labelled nanoliposomes for MRI (manuscript in preparation). Ultra-resolution picture from a cryo-electron microscope demonstrates the lipidised Ga-complex embedded in a lipid bilayer (Fig. 11).

High pressure homogenization and microfluidization represent secondary processing methods suitable for laboratory and industrial preparation of liposomes.

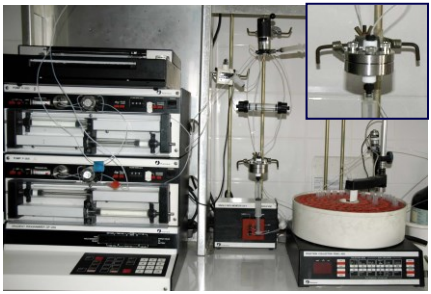


Fig. 9 Device for extruding liposomes by application of preparative FPLC system and using a high pressure extrusion cell. *The system can be automated with the use of automatic metering valves and using two 50 ml "Superloop". Insert - detail of high-pressure extrusion cell (designed by author and made in workshops at VRI). (Analytical Biochemistry. 1994, 218, 352-357).*

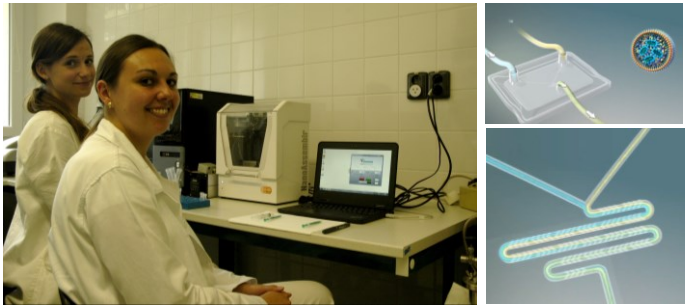


Fig.10 The instrument NanoAssemblr® in our laboratory NanoPharm. *NanoAssemblr® (left), nanofluidic mixing chamber (middle); scheme of nanofluidic mixing (right).*

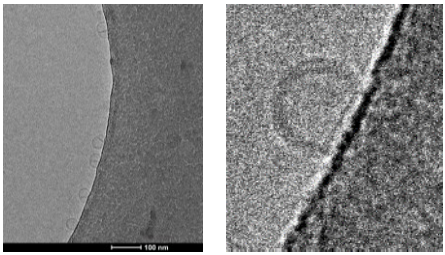


Fig. 11 Liposomes with Gadolinium-lipid for MRI contrast imaging. *Double layer is clearly visible owing to high contrast produced by Gadolinium atoms (manuscript in preparation).*

1.6. CONCLUSION

Preparation of liposomes and complex physico-chemical characterisation of liposomal formulations represent multidisciplinary nature of modern pharmacology and pharmacological technology. During the last two decades, a very complex set of methods has been introduced to my laboratory. At the same time, it requires highly qualified experimenters in a wide range of chemical disciplines. For preparation of liposomes, I have designed and constructed numerous equipments that have been brought to the stage of experimental samples and prototypes. Detailed description of the methods and devices can be found in the accompanying selected publications.

Within postgraduate studies, as supervisor I trained young scientists who are now able to use all these methods for complex research in drug delivery systems. The methodological background was used for the development of experimental therapeutics and vaccines based on liposomes as shown in the next sections.

The Department of Pharmacology and Immunotherapy is now a respected laboratory at the international level (collaboration with renowned laboratories, e.g. King´s College London, Institut de René Descartes Paris) and is on the list of liposome research oriented laboratories that are registered with the International Liposome Society.

2. HYDROPHILIC AND HYDROPHOBIC DRUGS AND SURFACTANTS: FORMULATION AND INTERACTIONS AND MEMBRANES

Pharmacologically active substances elicit their effects in the body by a number of mechanisms. I will mention here the mechanisms that are relevant to substances studied in the accompanying publications. To reach the targeted site of its action, the molecule of a drug must overcome various barriers in the body, in particular the barrier formed by lipid membranes of cells and cellular organelles. Patterns of distribution processes of pharmaceuticals in the organism can be derived from basic physico-chemical properties of molecules of a particular drug. For the distribution in the body, but also for resorption and excretion, solubility of the substance in aqueous medium is critical. This basic parameter also affects the dosage formulation of pharmaceuticals, which is critical for optimizing the biodistribution and thus the therapeutic effect. From the viewpoint of solubility, the drugs may thus be divided into three groups.

2.1. WATER SOLUBLE (HYDROPHILIC) SUBSTANCES

If a transport system (carrier protein) does not participate in their kinetics, hydrophilic drugs are then poorly absorbed after oral administration; after parenteral administration, they are distributed presumably in the extracellular space. Hydrophilic substances may enter into cells selectively, by either active or passive transport, pinocytosis or diffusion through the

membrane. The latter two mechanisms require a high local concentration of drug and a longer time to induce a physiological effect[11,12].

Encapsulation of these hydrophilic drugs into liposomes leads to their distribution in the aqueous phase of liposome vesicles and their concentration corresponds to the concentration in the hydration solution used in the preparation of liposomes.

The antiviral drug cidofovir (Fig. 12) developed by Prof. Antonin Holy is a good example of substances that have been studied in my laboratory[13,14]. This drug has a methylphosphonate group and corresponding salts thereof are very soluble in water. The main obstacle for its effect on an intracellular target - viral DNA polymerase - is the transition through the cell membrane. Cidofovir ((S) -1- (3-hydroxy-2-phosphonylmethoxypropyl) - cytosin (HPMPC) represents a new class of broad-spectrum antiviral drugs and is active against a broad spectrum of herpes viruses. Viral DNA polymerase is from 8 to 600 times more sensitive to inhibition by cidofovir than human DNA polymerases alpha, beta and gamma. A significant and prolonged antiviral effect of cidofovir is attributed to intracellular persistence of its derivatives which, after further phosphorylation, are retained in the cell and can in the long term inhibit viral DNA polymerase[15].

Due to the mechanism of action of cidofovir (intracellular viral DNA polymerase) liposomes which are readily internalized into cells are suitable carriers for encapsulation of this drug to improve its activity. Targeting of liposomes to certain types of cells (particularly hepatocytes) can be achieved by cationic liposomes prepared from cationic lipids with low cytotoxicity or liposomes surface-modified with certain ligands such as fragments of hyaluronic acid, for which there are specific receptors on hepatocytes. In collaboration with IOCB, new cationic lipids were designed and synthesized (see the joint Czech patent and US patent based on PCT). Antiviral activity of cidofovir encapsulated in cationic liposomes has been demonstrated against BHV-1 virus (bovine herpesvirus) on *in vitro* models. General scheme of the principle of antivirals targeting the cytoplasm of cells is shown in the figure. Active lipids have been prepared with an aminooxy group to enable orthogonal (selectively oriented) binding of carbohydrate onto liposomes, which allowed preparation of original surface-modified liposomes using various fragments of hyaluronic acid (patent application in preparation). Targeting of liposomes to hepatocytes, cells of the immune system and tumour lines is currently being intensively studied.

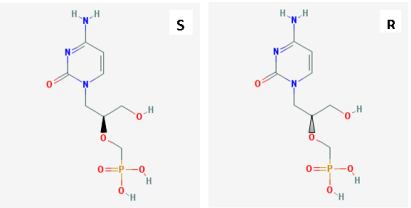


Fig. 12 Formulae of Cidofovir (S and R optical isomers, R-isomer is inactive).

General scheme demonstrating the principle of targeting antiviral drugs into the cytoplasm of cells is shown in Fig. 13. In collaboration with Prof. Miller (King´s College, London) and Dr. Ledvina (Institute of Organic Chemistry and Biochemistry, Prague) we designed and synthesized new active lipids with an aminooxy group. These lipids were applied for surface modification of liposomes by various carbohydrates via orthogonal N-oxi ligation. Polysaccharides like mannan or hyaluronic acid were bound onto liposomal surface to demonstrate feasibility of this approach. Fig. 14, 15 demonstrates the selective oriented binding of polysaccharides to liposomes, which allowed preparation of original surface-modified liposomes (*joint VRI and VSCHT CZ patent application*). Targeting of carbohydrate-modified liposomes to hepatocytes, immune cells (denritic cells, macrophages) and cancer cells is currently being intensively studied.

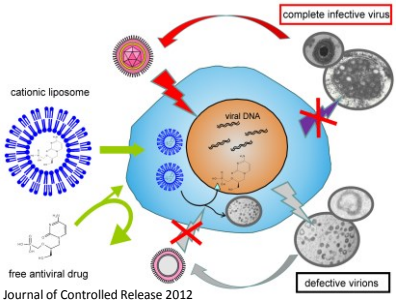


Fig. 13 Targeting of antiviral drugs into the cytoplasm of cells using cationic liposomes. *The liposomes enable to achieve a sufficient concentration of antivirals in the cell due to the transport of drug through a barrier formed by a cytoplasmic membrane. Drug is released and penetrates through nuclear pores into nucleus. DNA polymerase is inhibited and defective virions are formed. This system is particularly suitable for targeting of antiviral drugs to organs such as the liver (potential applications in therapy of hepatitis).*

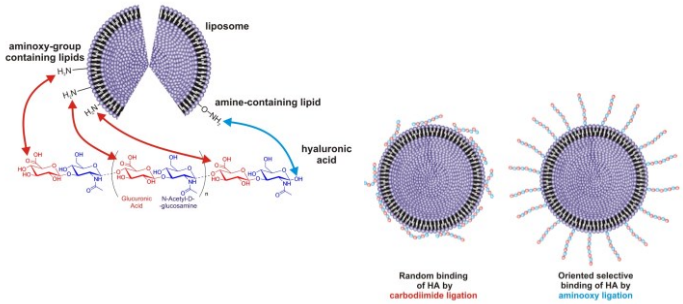


Fig. 17 Schematic presentation of various means for binding of HA onto liposomal surface.

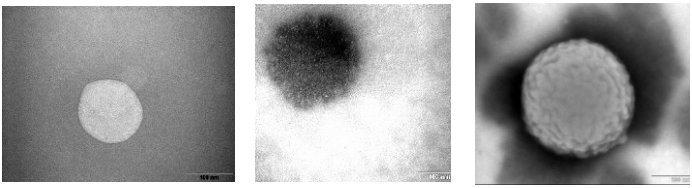


Fig. 18 TEM and SEM of liposomes with surface modified by mannan. *TEM of plain liposome (left), TEM of mannan modified liposome (middle), SEM of mannan modified liposome (right).*

2.2. LIPOSOMES AS CARRIERS FOR HYDROPHOBIC DRUGS

These compounds, depending on their octanol/water coefficient of solubility (partition coefficient) bind other hydrophobic structures (hydrophobic domain transport proteins, lipid structures in cells - membranes, fat globules). Generally, in the fatty tissues, they are not rapidly metabolized and excreted from the body. From the pharmacological point of view, the problem is their low solubility and tendency to form aggregates or crystals. In terms of dosage, forms for parenteral administration, it is necessary to follow two main objectives: a) solubilisation of the drug in stable system, which prevents the formation of crystals or aggregates; b) targeting drugs to the place of action - in the case of tumours - passive or active targeting of nanoparticulate formulation. Surface modification of the particles significantly improves a long-term circulation in the blood stream and their penetration through fenestrations present in the endothelial lining of capillaries in the tumour vasculature. Here again liposomes play a substantial role in the development of a convenient system for targeting and delivery of lipophilic drugs. My research has been focused on three different types of lipophilic compounds with antitumour activity.

2.2.1. Paclitaxel

Over the past three decades, taxanes represent one of the most important new classes of drugs approved in oncology. Paclitaxel (PTX), the prototype of this class, is an anti-cancer drug approved for the treatment of breast and ovarian cancerPTX was found to be also effective in treating a broad spectrum of advanced human cancer types including breast and ovarian cancer as well as non-small cell lung carcinoma (NSCLC), melanoma and head and neck cancer (Fig. 19)[16–18].

The commercial PTX preparation (Taxol®) is formulated in a vehicle composed of Cremophor EL® (polyethoxylated castor oil used as a solubilizing surfactant) and dehydrated ethanol, which provides a homogeneous preparation. However, the present-day chemotherapy employing Taxol® is accompanied by serious problems. One of the major problems associated with this formulation is the fact that the diluted Cremophor EL®/ethanol vehicle is

toxic. The negative side effects include serious hypersensitivity reactions, nephrotoxicity and neurotoxicity. PTX solubilized in Cremophor EL® (Cr-P) shows also an incompatibility with the plastic components of the administration sets. Furthermore, the short-term stability of PTX upon dilution with aqueous media can result in possible drug precipitation]. However, notwithstanding a suitable premedication, present-day chemotherapy employing a commercial preparation of PTX (Taxol®) is associated with serious side effects and hypersensitivity reactions. Liposomes represent advanced and versatile delivery systems for drugs. Generally, both *in vivo* mice tumour models and human clinical trials demonstrated that liposomal PTX formulations[19] significantly increase a maximum tolerated dose (MTD) of PTX which outperforms that of Taxol®. Liposomal PTX formulations are in various stages of clinical trials. LEP-ETU (NeoPharm) and EndoTAG®-1 (Medigene) have reached the phase II of the clinical trials[20–22]; Lipusu® (Luye Pharma Group) has already been commercialized.

We have introduced application of negatively charged pocket-forming lipids which can significantly increase the encapsulation capacity of liposomes for PTX. Low encapsulation capacity of classical liposomes is the main drawback in their application as drug carriers for PTX. The examples of pocket-forming lipids are lysophospholipids whose asymmetric molecules form cavities - pockets in the liposomal membrane bilayer. These hydrophobic pockets can accommodate the bulky hydrophobic molecule of PTX and the encapsulation capacity could be increased up to 15 mol%.

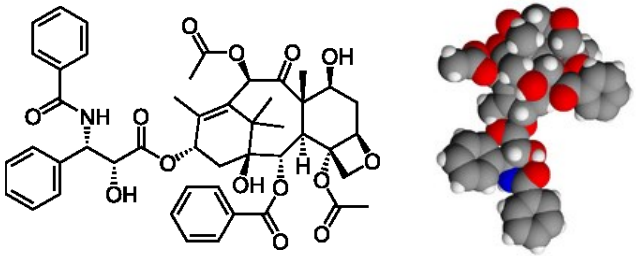


Fig. 19 The structural formula of paclitaxel and spatial models of molecules of paclitaxel. *PTX represents a bulky hydrophobic molecule which is difficult to accommodate into the lipid bilayer.*

We investigated the possibility of preparing a stable liposomal formulation of PTX using lysolipids. In this case, we have achieved more than twice the encapsulation efficiency in comparison with conventional liposomes. This system is suitable for the preparation of both passive and active targeted liposomes. The antitumour activity was demonstrated in a mouse model of lung melanoma. It was possible to administer an extremely high dose of paclitaxel into liposomes because this formulation was non-toxic compared with paclitaxel solubilized in Cremophor®.

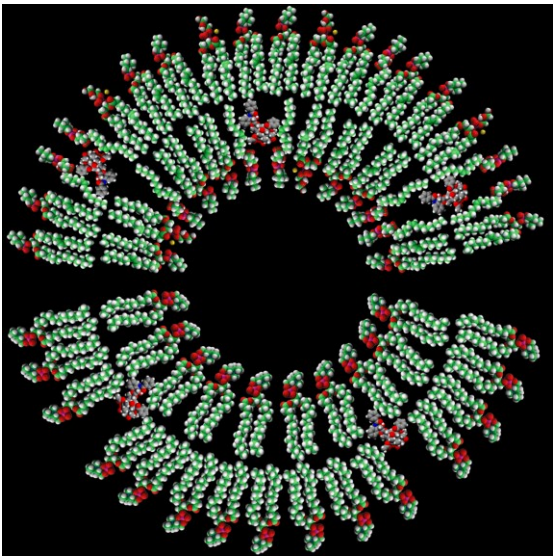


Fig. 20 Arrangement of the encapsulated PTX in different lipid membrane bilayers of the liposomal carrier. *The upper part shows the molecule of PTX encapsulated in the liposomal bilayer which is composed of unsaturated- and lyso-lipids. These lipids increase the bilayer fluidity and create bilayer "pockets" in which the bulky hydrophobic molecule of PTX is embedded. Such a liposomal composition enables to encapsulate up to 15 mol% of PTX. The lower part demonstrates the molecule of PTX which is encapsulated in the liposomal bilayer composed of saturated DPPC-based lipids. The incorporation of PTX into the saturated membranes disturbs the structure of the membrane bilayer. At higher drug concentrations, the molecule of PTX is forced out of the bilayer and needle-like crystals of PTX are formed in the liposome formulation. This effect results in a low encapsulation capacity of PTX (3-4 mol%) and in a short stability of the liposomes.*

2.2.1.1. Conclusion

It was demonstrated that liposomal carriers are superior to Taxol® as regards the delivery of PTX. Presently, two liposomal formulations are in clinical trials and close to commercialization. The preparations based on conventional liposomes represent the first generation of LEP. There is no doubt that patients will benefit from the improved therapeutic effect of these modern drug delivery systems. It seems that conventional liposomes represent a non-toxic solubilization system rather than a real targeted drug delivery system. The second generation of LEP is based on functionalized liposomes (cationic or ligand-targeted). They are under the development in various stages of preclinical and clinical trials. This great progress in the development of LEP preparations demonstrates the versatile and unique properties of liposomes as drug carriers. The impact of both the therapeutic effectiveness and the price of LEP suggests a near-future success on the drug delivery market.

2.2.2. Derivatives of vitamin E with antitumour activity

α-Tocopheryl acid succinate (α-TOS) is a semisynthetic analogue of vitamin E with a relatively selective effect on tumour cells and antitumour activity *in vivo*. A number of its analogues have been synthesized. These are characterized by resistance to esterases, since they contain hardly cleavable ether or amide bonds. α-Tocopheryl maleamid (α-TAM) represents one of these new substances and is characterized by much higher *in vitro* cytotoxicity and antitumour activity than *in vivo* in mouse tumour models[23–25].

A serious limitation of the use of these substances for the treatment of tumours is their low solubility in aqueous solutions. Their hydrophobic character thus determines the dosage formulation. Experimental formulations for i.v. or i.p. administration (solubilized in ethanol, DMSO or oily emulsions) can be used with limitation in the study of anti-tumour effect in mouse models, but are completely unacceptable for human intravenous administration. Vesicular forms or different micellar forms using suitable surfactants (e.g. polyethylene glycol) were prepared and tested in mice as possible formulations suitable for use in human medicine[26].

Especially, the preparation of vesicular structures by spontaneous vesiculation appears to be a suitable method for preparing a suitable dosage form. Problems with this approach arise with long-term stability of the preparation and toxicity of some derivatives after *i.v.* application (e.g. α-tokoferyl oxalate). This toxicity may be associated with and extremely high negative surface charge of these particles and their instability in the blood. Obviously, these nanoparticles can cause complement activation, formation of microembolism and other unwanted phenomena[27–29].

Even in the case of tocopherol derivatives, it has been proved that the application of liposomes is a suitable alternative for the development of a safe drug delivery form which also meets the other criteria imposed on pharmaceutical formulation for *i.v.* application.

In this area, we managed to develop a technology for preparation of stable liposomal formulations α-TOS and α-TAM. Liposomal preparations of both drugs have also been tested with excellent results in mouse tumour models. We managed to maintain a significant antitumour effect and completely eliminate the side effects that were found in previous formulations of α-TAM. Liposomal form eliminated immunotoxic effects (especially to bone marrow), which opens the possibility to use these compounds for combined immuno- and chemo-therapy of tumours. Practical applications in veterinary medicine are directed to the treatment of mammary tumours in dogs. The first therapeutic applications have been focused on intratumour administrations. Results in two bitches showed destruction of the vascular supply to the tumour, stagnation of tumour growth and clear localisation of tumour borders (*unpublished study*). The course of therapy was similar to the situation in a mouse model of spontaneous mammary tumours. Clear localisation of residual tumour tissue after treatment by liposomal vitamin E derivative allowed easy surgical removal of tumour tissue.

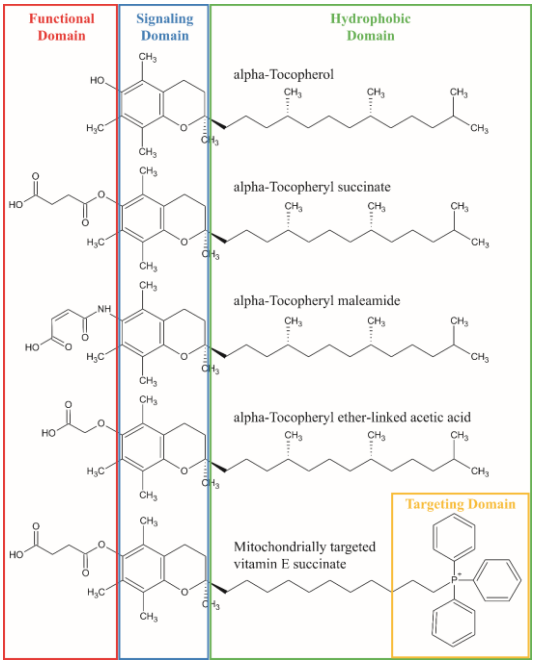


Fig. 21 The structural formulas of some derivatives of vitamin E with antitumour activity.

The combination of vitamin E derivatives with molecular adjuvant opens the way for immunotherapy of tumours using intratumoural applications. Liposomes play a role of therapeutic depot-forming carriers. Massive apoptosis of tumour cells and release of tumour antigens in the presence of molecular adjuvant is a prerequisite for inducing anti tumour immunity, which eliminates the metastatic deposits normally inaccessible to surgical removal or intratumour application of therapeutics. Intratumour application via direct tumour injection or transdermal transfer to subcutaneous tumours with dermatological cream allows to reach high concentrations of chemotherapeutic agents in a tumour, while eliminating reaching toxic doses in other organs. For this type od therapy I coin the term "chemotherapy-induced anti-tumour immunity." Liposomal derivatives were used for experimental treatment of spontaneous breast cancer in mice. Inhibition of tumour growth has been achieved without side-effects in mice after *i.v.* injection. The course of treatment and visualisation of tumours is documented in Fig. 22. The general overview of application routes is shown in Fig. 23.

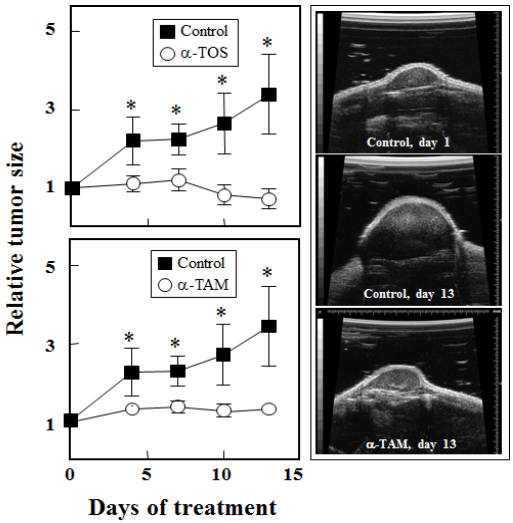


Fig. 22 ***Liposomal VE analogues inhibit proliferation of cancer cells in vivo and suppress breast carcinomas.*** *Transgenic FVB/N c-neu mice with spontaneous breast carcinomas were treated by injection of liposomal α-TOS and α -TAM at doses corresponding to 15 and 1.5 μmol of α -TOS and α -TAM, respectively, administered on days 0, 4, 7 and 13, as detailed in Materials and Methods. The tumour volume was quantified non-invasively by USI. Panel D shows representative images of a control mouse at the onset of the experiment and on day 13, and a mouse treated with liposomal α-TAM on day 13.The data shown are mean values ± SD (n=5), the symbol "*" denotes statistically significant differences with p<0.05.*

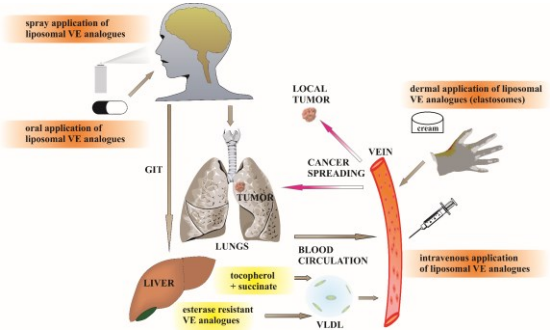


Fig. 23 ***The administration routes for liposomal delivery of VE analogues.*** *The various routes of administration can be used to reach the targeted tissue or tumour.*

Spraying of liposomal VE analogues is a suitable route for the direct targeting of lung cancer as well as for the delivery of VE analogues into the circulation. The bypassing of hepato-pancreatic port and the directing of VE analogues into the brain circulation is the advantage of this administration route. Oral application of acidic-resistant capsules filled by liposomal VE analogues represents a simple administration route. The oral application may be feasible in the case of VE analogues having ether or amide bonds linking dicarboxylic acid to the tocopherol. These analogues of VE are not hydrolysed after absorption in the intestines. Transdermal local application of liposomal formulation of VE analogues can be used for the treatment of skin tumours or subcutaneous tumours. Elastosomes (elastic liposomes) are of interest for these purposes owing to their improved penetration through stratum corneum. After parenteral administration (i.v.), the liposomal VE analogues can enter various pathways. These pathways and the biodistribution of VE analogues are determined by various factors, e.g. the character of liposomes (steric stabilization), morphology (size and lamellarity) or lipid composition. In the blood stream, VE analogues are redistributed between liposomes and the blood components (cellular and protein elements). Liposomal VE analogues can penetrate through fenestrations of the tumour vasculature. In the tumour, liposomal drug is accumulated and subsequently released in the vicinity of the tumours and the tumour endothelial cells. (Adapted from our work published in Journal of Controlled Release., 2015 Volume: 207 Pages: 59-69)

2.2.2.1. Conclusion

α-TOS and the other VE analogues with a potent pro-apoptogenic activity demonstrated efficient cytotoxic activity in vitro and anti-cancer effect in vivo on various experimental animal models. The lack of immunotoxicity of liposomal formulations and the possibility to be used with other anti-cancer drugs offers interesting opportunity for the combined chemo- and immuno-therapy. More data from the experimental, epidemiological and finally clinical studies are necessary for further active investigation of these highly promising agents that may be established as routine anti-cancer drugs. Our preliminary data from dogs demonstrate safety and efficacy of mammary gland adenocarcinoma treatment after intratumour application of liposomal α-TEA (*unpublished results*). Development of safe formulations which can be applicable for industrial scale production is the assumption for future clinical trials.

3. LIPOSOMES FOR CONSTRUCTION OF RECOMBINANT VACCINES

Vaccinology is a complex multidisciplinary field that is based on both rational theoretical foundations and the empirical approach[30]. Notable advances in immunology and genetics contribute to the understanding of the immune system functions at the molecular and cellular levels. These findings together with the development of recombinant technology, biotechnology and the availability of new biocompatible materials, particularly micro- and nanomaterials, lead to significant advances in the development and production of modern recombinant vaccines[31].

Subunit vaccines and recombinant vaccines, such as the special branch of the modern trends in the development of vaccines and vaccinology, significantly contributed to the efficient and safe treatment of infectious,

autoimmune and malignant diseases[32–36]. The development of these new vaccines is based on the identification of suitable antigens and their epitopes. The fundamental question is how to present antigen to the immune system[37]. The key factors that are addressed in the research are a) search for antigens, their modification and preparation of recombinant technologies; b) a suitable formulation of the antigen; c) the selection and development of safe and effective adjuvant; d) the method of vaccine administration with an emphasis on minimally invasive applications and the need for specially trained personnel. Factors that drive the research in the field of recombinant vaccines are safety, cost and the short time for the development of new vaccines against pathogens. The factor of time is of importance for pathoges like influenza virus because of rapid seasonal antigen variations due to antigenic drift and ability of influenza viruses to produce reassortants (antigenic shift).

In the development of modern vaccines, the liposomes represent one of the most successful micro and nanoparticulate systems because of their versatility and biocompatibility There are already virosomal (Inflexal® V, Crucell Company) and hepatitis A (Epaxal®, Crucell company) vaccines on the market[38,39]. Many liposomal vaccines against infectious diseases and cancer are in various stages of clinical testing.

We have dedicated research in this area to the development of original fully synthetic adjuvants based on normuramylglycopeptides. This research was done in collaboration with the Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague (Dr. M. Ledvina) and resulted in the filing of national and international patents that protect these new adjuvants. As a spin-off company, Mendel Therapeutics, Ltd. has been founded to push forward commercialization of these compounds. Finally, the compounds were licensed to Apigenex, s.r.o. Fig. 24 represents a series of synthetic adjuvants that are currently tested for use in recombinant vaccines in cooperation with Bioveta, a.s. and certain foreign institutions. These substances are characterized by suppressed pyrogenicity and were safety-tested on a wide variety of animal species (mice, guinea pigs, cattle, pigs, dogs, cats, rabbits). Mechanism of action on molecular level has been proved experimentally on THP1 cell line. Receptor NOD2 and NLRP3 (inflammasome) were found to be activated by derivatives of norAbuMDP.

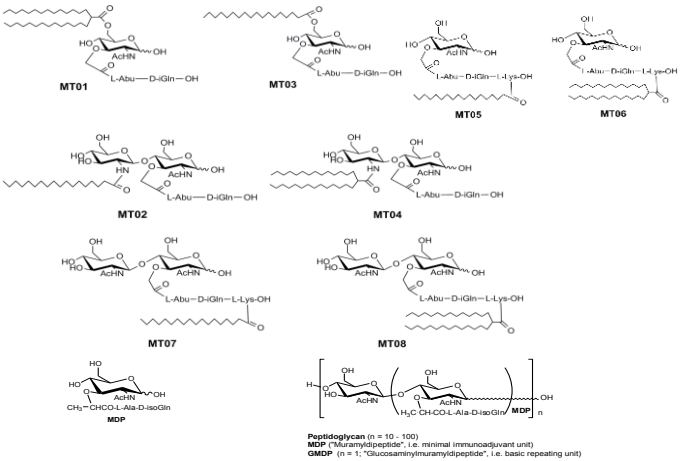


Fig. 24 Synthetic analogues of muramyldipeptide (MDP).

Structural changes in the norAbu MDP preserved specific interaciton with these receptors but interaction with thermoregulatory receptors in brain has been supressed as can be deduced from significant suppresion of pyrogenicity. (Turanek et al, Journal of Medicinal Chemistry, submitted 2017, in revision) The mechanism of action on melecular and whole body levels is discribed at Fig. 25.

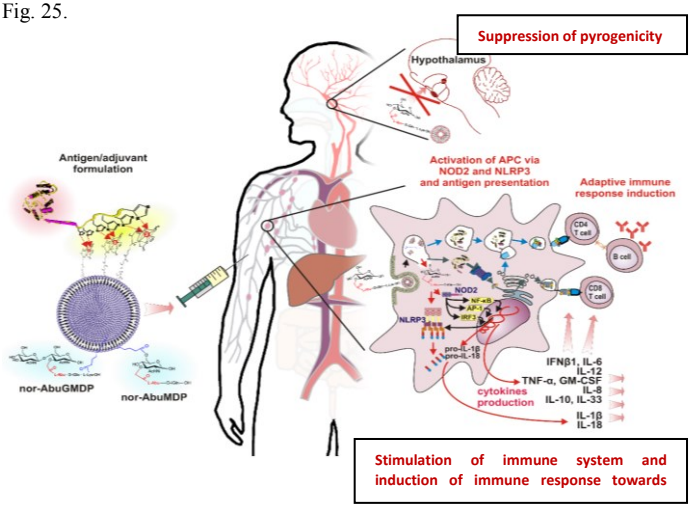


Fig. 25 Schematic presentation of immune reaction towards norAbuMDP-based adjuvants formulated in liposomes. *NorAbuMDP/GMDP-based molecular*

adjuvants, preferably formulated in biocompatible nanoparticles like liposomes, can be targeted towards immune cells (e.g. dendritic cells) at the site of application or in draining lymph nodes. After being internalised by endocytosis or phagocytosis, molecular adjuvants are released from their formulation and interacts with intracellular receptors such as NOD2 and NLRP3. Activation of NOD2 and NLRP3 induces production of various cytokines, including proinflammatory cytokine IL-1β and IL-18. Locally produced cytokines promotes immune reaction towards antigens co-applied with adjuvants formulated in liposome-based vaccination nanoparticles, but the local production of proinflammatory cytokines did not cause systemic pyrogenic reaction. Contrary to NorAbuMDP/GMDP-based molecular adjuvants MDP- and MDP-based analogues can induce strong pyrogenic reaction owing to activation of receptor(s) in thermoregulation centre in hypothalamus. Suppression of pyrogenicity and preservation of immunostimulatory activity via stimulation of NOD2 and NLRP3 is related to structural changes in the molecule of NorAbuMDP/GMDP prepared by total synthesis. This explanation is in a good accordance with experimental data based on in vitro (NOD2 and NALP3 activation test) and in vivo (rabbit pyrogenicity model, induction of costimulatory activity of mice sera).

Inflammasome, toll-like receptors and NOD1/2 receptors were found as components of signalling cascade responsible for stimulatory activities of varioust adjuvants[40–45]. We studied liposomes fthe construction of experimental vaccines, and we have developed a special metallochellating nanoliposomal carrier incorporating lipophilicderivatives based on norAbuMDP and norAbuGMDP. The technology of preparation is described in the section "Methods for preparing liposomes." The proposed structure of proteoliposomes that have been validated by modern microscopy methods (TEM, SEM and AFM) in combination with gel permeation chromatography, PAGE and immunoblotting (Fig. 26).

These metallochelating liposomal carriers are suitable for oriented non-covalent binding of recombinant antigens with a His Tag anchor. If necessary, carbodiimide condensation reaction can be used to convert the metallochelating noncovalent bond to a covalent bond with the preservation of the precise orientation of the protein on the surface of the liposomal lipid bilayer. The system based on metallochelating nanoliposomal carriers is currently tested for the development of veterinary and human recombinant chimeric vaccine against borreliosis (collaboration with Bioveta, a.s.).

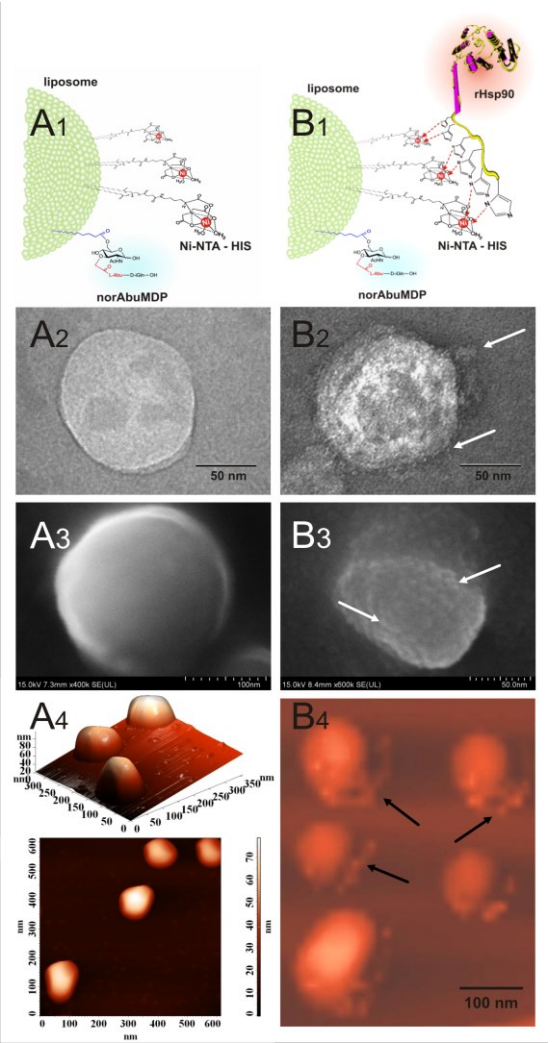


Fig. 26 Structures of proteoliposomes visualised by modern microscopic methods. *Schematic view of a metallochelating nanoliposomal carrier to construct recombinant vaccines. Free carrier (A1) and a carrier-bound recombinant protein (B1). Specific metallochelating binding allows us to achieve accurate orientation of the molecules of recombinant protein on the surface of liposomes.The structure of the plain liposome carrier (A2-4) and corresponding proteoliposomes (B2-4) visualised by*

microscopic methods (TEM, SEM and AFM). Arrows indicate individual molecules of the protein (rHSP 90) bound on liposomal surface.

The mechanism(s) of action of liposome-based molecular ajduvants based on lipophilised analogues of normuramylglycopeptides is/are described in Figure 27. The key event is targeting of molecular adjuvants to intracellular receptors NOD 2 and activation of signalling molecular pathways including activation of the inflammasome NLRP 3 (NALP3). This area is currently being intensively studied at our department.

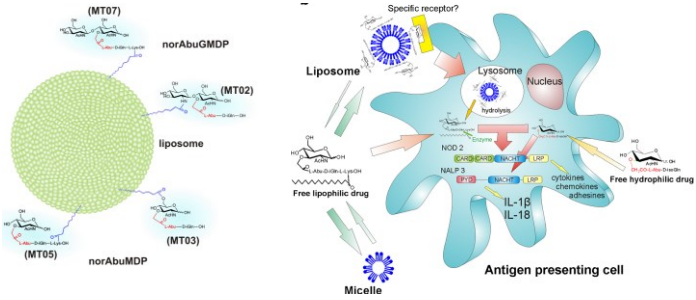


Fig. 27 Schematic structure of norAbuMDP/GMDP nanoliposomes and their pharmacokinetic pathways. *Schematic structure of liposomes with hydrophobic analogues of normuramyl glycopeptides located on the liposomal surface (left). Gylcopeptide part of the molecule is exposed on the surface of nanoliposome and can form molecular patterns recognised by various receptors on immune cells. Various modifications of the norAbuMDP/GMDP molecule by lipidic residues affect the exposition of sugar- and peptide-moieties on the liposomal surface. Depending on the concentration of particular analogues or their mixture in the bilayer, new molecular patterns can be formed on the liposomal surface. These molecular patterns can be recognized by cell-membrane receptors. Schematic presentation of pharmacokinetic pathways of liposomal and free MDP analogues at cellular and intracellular levels (right).*

A free hydrophilic drug (e.g., MDP) has difficulties to cross the cell membrane and thus, it is difficult to reach an efficient intracellular concentration in vivo. The hydrophobized analogues of MDP form micelles whose pathways are difficult to be predicted owing to the complexity of the interactions with the components of biological milieu. Direct interaction with the cell membrane and penetration into the cytoplasm is supposed to occur. The liposomal formulation of both hydrophilic and lipophilic derivatives of MDP is relatively stable in biological milieu and endocytozed by dendritic cells. In comparison to the free drug, liposomal formulations significantly increase the intracellular concentration of MDP analogues. Lysozomal and cytoplasmatic enzymes cleave the ester-bond-linking glycopeptide part to a hydrophobic residue. The importance of this pathway has not yet been recognized and fully understood. Nevertheless, it is reasonable to suppose that various derivatives will differ in their sensitivity to the enzymatic hydrolysis. Moreover, the mechanism of MDP-based activation of NOD and NALP3 receptors has not been precisely described and it is a hot issue. A question of a special relevance is, whether the hydrophobic residue of some derivatives has to be cleaved off to release the active glycopeptide molecule. The complexity of these processes is reflected in the variances in

the effects of particular analogues having the same glycopeptide core but different position and structure of the hydrophobic residue.

Liposomal formulations of synthetic adjuvants were tested as stimulators of innate immunity in various *in vivo* models, and have been proven effective in the suppression of experimental cryptosporidiosis in infected newborn kids, or regeneration of bone marrow in sublethally irradiated mice. A murine model has also demonstrated stimulation of the immune system and stimulation of hemopoiesis and survival after lethal irradiation.

Safety of liposomal vaccines and new synthetic adjuvants were tested in rabbits and pigs after i.d. or s.c. applications and compared with oil-based adjuvants which can cause necrotic lesions and inflammations. Liposomal experimental vaccines did not induce any undesirable side-effects. This is especially important for the intended future applications in human medicine. Comparison of side-effects of oil-based vaccines and liposomal formulation after i.d. application in pigs is demonstrated in Fig. 28. Safety of liposomal formulation of recombinant vaccine against Borreliosis was also demonstrated for puppies and kittens (collaboration with Bioveta, a.s. under the project of TAČR focused on the recombinant multi-epitope vaccine against Borreliosis for veterinary applications).

Fig. 28 Necrotic area after *i.d.* application of an oil-based vaccine (left) and a liposomal vaccine (right) to a pig.

3.1. APPLICATION OF ADVANCED NANOMATERIALS FOR DEVELOPMENT OF NONINVASIVE MUCOSAL AND TRASDERMAL VACCINES

At present, our research in the field of modern recombinant vaccines is focused on noninvasive mucosal vaccination utilizing the technology of biocompatible nanotextiles based on nanofibers[46–48].

This is an entirely new area in which we are pioneers. The factors of technology, nonivnasiveness of application and safety favour this type of vaccines for future use both in veterinary and human medicine. Clear advantages of sublingual vaccines are their non-invasiveness and ease of administration, application security, the ability to induce mucosal and systemic immune responses[49,50], which is the basis for successful vaccination in a wide range of infections (e.g. influenza). Another major feature of the formulation is

technological feasibility of producing and easy storage (important for vaccine distribution in hard to reach areas of the Third World). It is a priority of our research at the Department of Pharmacology and Immunotherapy and we prepared and submitted projects of basic and applied research in cooperation with domestic (Palacky University in Olomouc, IBT, Institute of Physics, Technical University Liberec) and foreign institutions (King's College, University of Kent, Institute of René Descartes, Pasteur Insitute).

The key steps for achieving success in sublingual immunization are: a) a system for sublingual administration of antigens; b) antigen formulation and delivery system; c) appropriate adjuvants and their formulation. Our contribution applies to all three parts. Liposomal molecular adjuvant and antigen formulations are described in previous chapters. We developed and applied a PCT protecting the original mucoadhesive system for sublingual application. The main purpose of the mucoadhesive system is to maintain the antigen on sublingual mucosa for a longer time and allow its penetration into the sublingual tissue, where it is recognized by dendritic cells and transported to the draining lymph nodes. The mucoadhesive system maintains a high concentration gradient of the antigen and defends its removal by mucosal secretions (saliva), which can wash vaccination particles away from the application site. The principle is schematically shown in Figure 29, and the appearance of mucoadhesive films and their application to the sublingual mucosa is in Figure 30. An example of the structure of nanofibres with immobilized liposomes is shown in Figure 31.

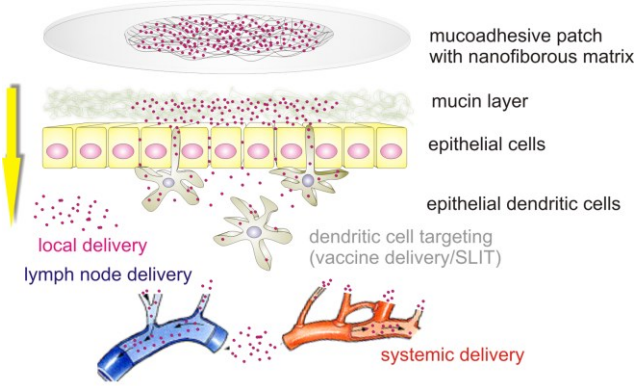


Fig. 29. Schematic presentation of the principle for improving delivery of drug-delivery and vaccination nanoparticles by means of a nanofibrous mucoadhesive film. *High adsorption loading capacity of nanofibrous materials ensures a high concentration of nanoparticles to be reached after the rapid release from the reservoir layer to the limited volume of the fluids at the application site. Protective backing layer prevents removal of nanoparticles from the site of administration by flow of mucosal secretions and saliva. A concentration gradient is formed, which then exerts "pressure" on the mucosal layer, thus rapidly enabling the formation of a nanoparticle diffusion potential across the mucosal surface into the submucosa. The different fate of nanoparticles (local/systemic delivery) is based on their physicochemical properties and*

presence of targeting moieties. Dendritic cells (DCs) present in the submucosa are then free to capture vaccination nanoparticles for delivery to the local lymph nodes that drain the submucosal zone of application. Vaccination nanoparticles not captured by DCs, are otherwise free to diffuse through the submucosa reaching lymphatic capillaries by means of which they drift to the local lymph nodes for capture by professional antigen-presenting cells.

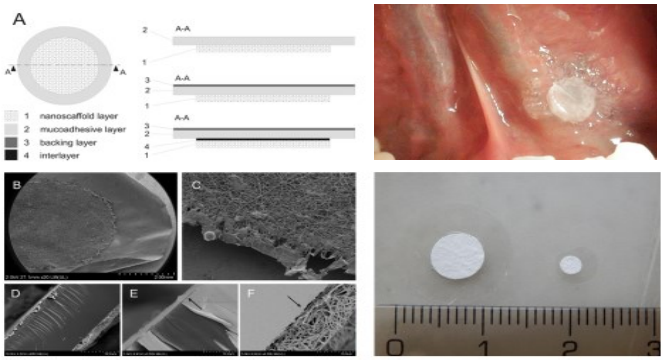


Fig. 30. Schematic structure and real appearance of nanofibrous mucoadhesive film. *A: The scheme shows the bottom view (left) and cross-section (right) of possible variants for construction of nanofibrous mucoadhesive film; B: Photograph of nanofibrous mucoadhesive film, left – design for large animal experiments (pig), and right – design for small animal experiments (mice) (centimetre scale). Nanofibrous reservoir layer (asterisk), mucoadhesive layer (arrow); C: Scanning electron microscopy (SEM) picture showing individual layers of a three-layered nanofibrous mucoadhesive film. The mucoadhesive layer creates a peripheral adhesive ring surrounding the central part on which the electrospun nanofibrous reservoir layer is fixed. Nanofibrous reservoir layer (asterisk), mucoadhesive layer (arrow); D: Cross-section of mucoadhesive layer observed in its native state after freezing (cryo-SEM). Nanofibrous reservoir layer (asterisk), mucoadhesive layer (square) and backing layer (arrow); E: Detail of cross-section of nanofibrous mucoadhesive film, arrow indicates the Eudragit® L 100-55 backing layer (arrow); F: Detail of cross-section of nanofibrous mucoadhesive film, arrow indicates the connection of the mucoadhesive layer and the nanofibrous reservoir layer.*

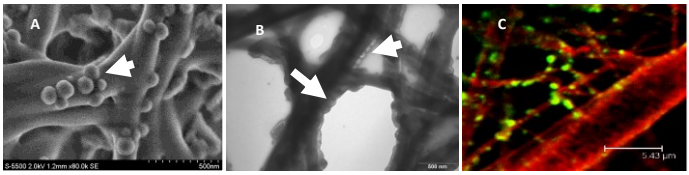
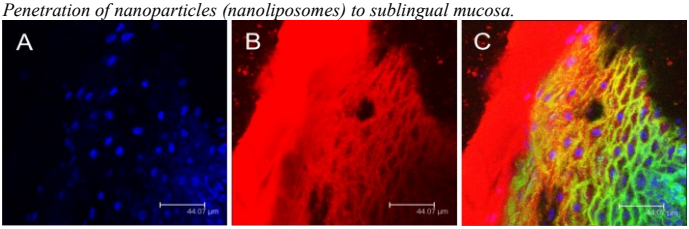


Fig. 31 Structure of nanofibres with adsorbed liposomes. *The images were taken by scanning and transmission electron microscopy (A, B) and confocal microscopy (C). Liposomes can be clearly seen adsorbed onto the surface of nanofibers. For confocal microscopy, metallochelating proteoliposomes with bound rGFP (green fluorescence) were*

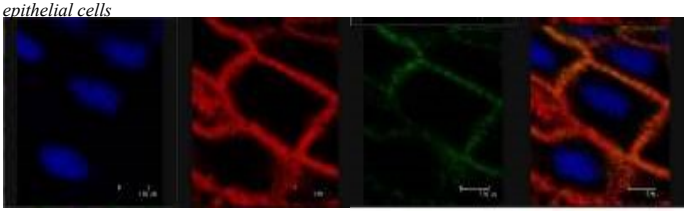
used and nanofibres were labelled with fluorescent lipid (red fluorescence). Arrows indicate the liposomes on the surface of nanofibers.

Application of fluorescently labelled liposomes or polymer nanoparticles to the sublingual mucosa using mucoadhesive films showed rapid paracellular penetration, absorption and transport by dendritic cells to draining lymph nodes, which is the basic prerequisite for inducing immune responses against antigens. Experimental confirmation of our assumptions were achieved using a porcine model. The histological slides were examined by confocal microscopy and confirmed paracellular penetration of nanoparticles into deeper layers of stratified sublingual mucosa and delivering the nanoparticles into the draining lymph nodes by dendritic cells. Fluorescent nanoparticles have been found in T and B areas of the lymph nodes, where they were transported by dendritic cells (Fig. 32).

Penetration of nanoparticles (nanoliposomes) to sublingual mucosa.



Detailed view of paracellular penetration of nanoparticles to the submucosal layer of epithelial cells



Proof of delivery of nanoparticles to the T and B zones of draining lymph nodes

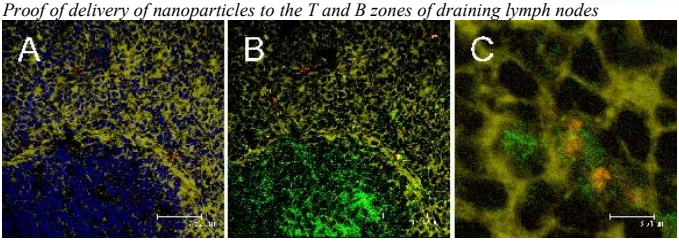


Fig. 32 Proof of penetration of fluorescent nanoparticles into the sublingual tissue and lymph nodes after sublingual administration using mucoadhesive film. *(images were taken using a confocal microscope).*

Penetration of nanoparticles (nanoliposomes) to sublingual mucosa.
A) histological section of lymph nodes showing the T and B zones. Red fluorescent particles are apparent in both zones. B) histological section of a lymph node showing lymphocytes in T and B zones (SLA II antigen) C) a detailed view of dendritic cells internalized in fluorescent liposomes (T zone)
Blue - cell nuclei; Red - nanoparticles; yellow-green - actin; Green - SLA II antigen (porcine lymphocytes)

3.2. CONCLUSION

A complex system for sublingual vaccination is a modern application of nanotechnology for medical applications. The developed system has the potential of breakthrough technologies for which we coin the name "Printed Vaccines Technology". In cooperation with Czech (UP Olomouc and TU Liberec) and foreign partners (Global Acorn and King´s College Precision Therapeutics, UK) we have filed an international patent (Turánek J., J. Masek, M. Raska, R. Lukac, P. Knötigová, D. Lubasová, A.D. Miller mucoadhesive carriers of particles, method of preparation and uses thereof PCT / GB2015 / 052833) and in the Journal of Controlled Release we published the paper entitled "Multi-layered nanofibrous films mucoadhesive buccal and sublingual for administration of drug-delivery nanoparticles and vaccination - Important step towards effective mucosal vaccines". Commercialisation of printed vaccines technology is moving forward with King´s College Precision Therapeutics, UK and with the support of foreign investors.

4. CONCLUSION OF THE DISERTATION THESIS

Liposomes are the oldest and most studied nanosystem for use in medicine. It serves as a model for the study of biological membranes and distribution of nanoparticles in the organism. Preparation of the liposomes is handled on both laboratory and industrial scale. Liposomal formulations are authorized by the FDA for the treatment of infectious and neoplastic diseases. On the market there are already dermatological preparations based on liposomes as well as liposomal diagnostics.

Biocompatible carriers and new safe adjuvants are a prerequisite for the development of modern vaccinology directed to recombinant vaccines. Research on vaccines based on liposomes has already thirty years of history and new modern recombinant vaccines have been introduced to the market.

We have published papers and chapters in monographies in the field of technology for the preparation of liposomes, the development of targeted anticancer drugs, safe adjuvants for construction of recombinant vaccines and study of new application procedures. Patents on adjuvants and vaccine delivery systems are valuable base for founding a start-up company to speed up intended commercialisation of modern vaccines.

Some results are also being prepared for implementation in Czech companies. A number of students and young scientists in postgraduate studies have been involved in this modern research. I believe that this work will contribute to their professional growth and enrich them with new knowledge in the area of modern pharmacology, nanotechnology and immunotherapy.

5. SCIENTIFIC PUBLICATIONS AND PATENT APPLICATIONS THAT FORM THE BASIS OF THE DISSERTATION (sum of citations/sum of citations without self-citation)

1. **Turánek, J**. Fast-protein liquid chromatography system as a tool for liposome preparation by extrusion procedure**.** ***Anal. Biochem.,*** 1994, vol. 218, p. 352-357; IF=2.334; cited: 14/1
2. **Turánek, J**., Toman, M., Novák, J., Krchňák, V., Hořavová, P.: Adjuvant effect of liposomes and adamantylamide dipeptide on antigenicity of entrapped synthetic peptide derived from HIV-1 transmembrane region glycoprotein GP41. ***Immunol. Lett.,*** 1994, vol. 39, p. 157-161; IF=2.860; cited: 10/10
3. **Turánek, J**., Záluská, D., Hofer, M., Vacek, A., Ledvina, M., Ježek, J.: Stimulation of haematopoesis and protection of mice against radiation injury synthetic analogues of muramyldipeptide incorporated in liposomes. ***Int. J. Immunopharmacol.,*** 1997, vol. 19, p. 611-617; IF=2.551; cited: 11/3
4. **Turánek, J**., Záluská, D., Neča, J. Linkup of a fast protein liquid chromatography system with a stirred thermostated cell for sterile preparation of liposomes by the proliposome-liposome method: Application to encapsulation of antibiotics, synthetic peptide immunomodulators, and a photosensitizer. ***Anal. Biochem.,*** 1997, vol. 249, p. 131-139; IF=2.334; cited: 21/15
5. **Turánek, J**., Záluská, D., Vacek, A., Borkovcova, P., Thurnvaldova, J., Blaha, l., Mašek, K.: Stimulation of nonspecific imunity, haematopoesis and protection of mice against radiation injury by 1-adamantylamide-L-alanyl-D-isoglutamine incorporated in liposomes. ***Int. Immunopharmacol.,*** 2001, vol. 1, p. 167-175; IF=1.559; cited: 7/6
6. **Turánek, J**., Kašná, A., Záluská, D., Neča, J. Preparation of sterile liposomes by the proliposome-liposome method. ***Methods Enzymol.,*** 2003, vol. 367, p. 111-125; IF=2.088; cited: 9/2
7. Žák, F., **Turánek, J**., Kroutil, A., Sova, P., Mistr, A., Poulova, A., Mikolin, P, Žák, Z., Kašná, A., Záluská, D., Neča, J., Šindelová, L., Kozubík, A.: Platinum(IV) complex with adamantylamine as nonleaving amine group: Synthesis, characterization, and in vitro antitumour activity against a panel of cisplatin-resistant cancer cell lines. ***J. Med. Chem.,*** 2004, vol. 47, p. 761-763; IF=6.259; cited: 49/49
8. **Turánek, J**., Kašná, A., Záluská, D., Neča, J., Kvardová, V., Knotigová, P., Horváth, V., Šindlerová, L., Kozubík, A., Sova, P., Kroutil, A., Žák, F., Mistr, A.: New platinum(IV) complex with adamantylamine ligand as a promising anti-cancer drug: comparison of in vitro cytotoxic potential towards A2780/cisR cisplatin-resistant cell line within homologous series of platinum(IV) complexes. ***Anti-Cancer Drugs,*** 2004, vol. 15, p. 537-543; IF=2.320; cited: 41/38
9. Kašná, A., **Turánek, J.**, Vacek, A., Záluská, D., Knotigová, P., Mašek, K.: Restoration of femoral GM-CFC progenitors in sublethally irradiated

mice of various agens treated with liposomal adamantylamide dipeptide. ***Int. Immunopharmacol.,*** 2004, vol. 4, p. 1099-1106; IF=2.956; cited: 2/0

10. **Turánek, J.,** Kašná, A., Koudela, B., Ledvina, M., Miller, A.D.: Stimulation of innate imunity in newborn kids against Cryptosporidium parvum infection-challenge by intranasal/per-oral administration of liposomal formulation of N-L-18-norAbu-GMDP adjutant. ***Parasitology,*** 2005, vol. 131, p. 601-608; IF=2.713; cited: 4/2
11. Kozubík, A., Horváth, V., Švihálková-Šindlerová, L., Souček, K., Hofmanová, J., Sova, P., Kroutil, A., Žák, F., Mistr, A., **Turánek, J**.: High effectiveness of platinum(IV) complex with adamantylamine in overcoming resistance to cisplatin and suppressing proliferation of ovarian cancer cells *in vitro*. ***Biochem. Pharmacol***. 2005, vol. 69, p. 373-383; IF=4.581; cited: 45/45
12. Vyas, S.M., **Turánek, J**., Knotigová, P., Kašná, A., Kvardová, V., Koganti, V., Rankin, S.E., Knutson, B.L., Lehmler, H.J.: Synthesis and biocompatibility evaluation of partially fluorinated pyridinium bromides. ***New J. Chem.,*** 2006, vol. 30, p. 944-951; IF=3.269; cited: 30/28
13. Procházka, L., **Turánek, J.,** Tesařík, R., Knotigová, P., Polášková, P., Andrysík, Z., Kozubík, A., Žák, F., Sova, P., Neužil, J., Machala, M.: Apoptosis and inhibition of gap-junctional intercellular communication induced by LA-12, a novel hydrophobic platinum(IV) komplex. ***Arch. Biochem. Biophys***. 2007, vol. 462, p. 54-61; IF=3.165; cited: 12/12
14. Dong, L.F., Swettenham, E., Eliasson, J., Wang, X.F., Gold, M., Medunic, Y., Stantic, M., Low, P., Procházka, L., Witting, P.K., **Turánek, J.**, Akporiaye, E.T., Ralph, S.J., Neužil, J.: Vitamin E analogues inhibit angiogenesis by selective induction of apoptosis in proliferating endothelial cells: the role of oxidative stress. ***Cancer Res.,*** 2007, vol. 67, p. 11906-11913; IF=9.122; cited: 69/64
15. Neužil, J., Dong, L.F., Ramanathapuram, L., Hahn, T., Chladová, M., Wang, X.F., Zobalová, R., Procházka, L., Gold, M., Freeman, R., **Turánek, J.,** Akporiaye, E.T., Dyason, J.C., Ralph, S.J.: Vitamin E analogues as a novel group of mitocans: Anti-cancer agents that act by targeting mitochondria. ***Mol. Aspects Med***., 2007, vol. 28, p. 607-645; IF=5.686; cited: 62/62
16. Dong, L.F., Low, P., Dyason, J.C., Wang, X.F., Procházka, L., Witting, P.K., Freeman, R., Swettenham, E., Vališ, K., Liu, J., Zobalová, R., **Turánek, J.**, Spitz, D.R., Domann, F.E., Scheffler, I.E., Ralph, S.J., Neužil, J.: Alpha-tocopheryl succinate induces apoptosis by targeting ubiquinone-binding sites in mitochondrial respiratory complex II. ***Oncogene***, 2008, vol. 27, p. 4324-4335; IF=7.519; cited: 142/127
17. Li, X., **Turánek, J**., Knotigová, P., Kudláčková, H., Mašek, J., Pennington, D.B., Rankin, S.E., Knutson, B.L., Lehmler, H.J.: Synthesis and biocompatibility evaluation of partially fluorinated, single-tailed glucopyranoside surfactants. ***New J. Chem***., 2008, vol. 32, p. 2169-2179; IF=3.269; cited: 20/17
18. Li, X., **Turánek, J**., Knotigová, P., Kudláčková, H., Mašek, J., Parkin, S., Rankin, S.E., Knutson, B.L., Lehmler, H.J.: Hydrophobic tail length, degree of fluorination and headgroup stereochemistry are determinants of

the biocompatibility of (fluorinated) carbohydrate surfactants. ***Colloid. Surface. B***, 2009, vol. 73, p. 65-74; IF=3.883; cited: 22/21

19. **Turánek, J.,** Wang, X.F., Knötigová, P., Koudelka, Š., Dong, L.F., Vrublová, E., Mahdavian, E., Procházka, L., Sangsura, S., Vacek, A., Salvatore, B.A., Neuzil, J. Liposomal formulation of α-tocopheryl maleamide: *In vitro* and *in vivo* toxicological profile and anticancer effect against spontaneous breast carcinomas in mice. ***Toxicol. Appl. Pharmacol.,*** 2009, vol. 237, p. 249-257; 3.791; cited: 33/29
20. Koudelka, Š., Turánek-Knötigová, P., Mašek, J., Korvasová, Z., Škrabalová, M., Plocková, J., Bartheldyová, E., **Turánek, J**.: Liposomes with high encapsulation capacity for paclitaxel: preparation, characterisation and *in vivo* anticancer effect. ***J. Pharm .Sci.,*** 2010, vol. 99, p. 2309-2319; IF=2.713; cited: 14/11
21. Procházka, L., Dong, L.F., Vališ, K., Freeman, R., Ralph, S.J., **Turánek, J.,** Neužil, J.: alpha-Tocopheryl succinate causes mitochondrial permeabilization by preferential formation of Bak channels. ***Apoptosis,*** 2010, vol. 15, p. 782-794; IF=3.833; cited: 37/35
22. Koudelka, Š., Mašek, J., Neuzil, J., **Turánek, J.:** Lyophilised liposome-based formulations of α-tocopheryl succinate: preparation and physico-chemical characterisation. ***J. Pharm. Sci.,*** 2010, vol. 99, p. 2434-2443; IF=2.713; cited: 10/6
23. Dong, L.F., Jameson, V.J., Tilly, D., Procházka, L., Rohlena, J., Vališ, K., Truksa, J., Zobalová, R., Mahdavian, E., Klučková, K., Stantic, M., Štursa, J., Freeman, R., Witting, P.K., Norberg, E., Goodwin, J., Salvatore, B.A., Novotná, J., **Turánek, J.,** Ledvina, M., Hozák, P., Zhivotovský, B., Coster, M.J., Ralph, S.J., Smith, R.A., Neužil, J.: Mitochondrial targeting of α-tocopheryl succinate enhances its pro-apoptotic efficacy: a new paradigm for effective cancer therapy. ***Free Radic. Biol. Med.***, 2011, vol. 50, p. 1546-1555; IF=5.606; cited: 51/47
24. Mašek, J., Bartheldyová, E., Korvasová, Z., Škrabalová, M., Koudelka, Š., Kulich, P., Kratochvílová, I., Miller, A.D., Ledvina, M., Raška, M., **Turánek, J**.: Immobilization of histidine-tagged proteins on monodisperse metallochelation liposomes: Preparation and study of their structure. ***Anal. Biochem.,*** 2011, vol. 408, p. 95-104; IF=2.334; cited: 11/4
25. Lukáč, R., Kauerová Z., Mašek, J., Bartheldyová E., Kulich, P., Koudelka, Š., Korvasová, Z., Plocková, J., Papoušek, F., Kolář, F., Schmidt, R., **Turánek, J**.: Preparation of metallochelating microbubbles and study on their site-specific interaction with rGFP-HisTag as a model protein. ***Langmuir,*** 2011, vol. 27, p. 4829-4837; IF=3.833; cited: 4/2
26. Mašek, J., Bartheldyová, E., Turánek-Knötigová, P., Škrabalová, M., Korvasová, Z., Plocková, J., Koudelka, Š., Škodová, P., Kulich, P., Křupka, M., Zachová, K., Czerneková, L., Horynová, M., Kratochvílová, I., Miller, A.D., Zýka, D., Michálek, J., Vrbková, J., Šebela, M., Ledvina, M., Raška, M., **Turánek, J**.: Metallochelating liposomes with associated lipophilised norAbuMDP as biocompatible platform for construction of vaccines with recombinant His-tagged antigens: Preparation, structural study and immune response towards rHsp90. ***J. Control.Release***, 2011, vol. 191, p. 193-201; IF=7.786; cited: 13/9

27. Křupka, M., Mašek, J., Bartheldyová, E., Turánek Knotigová, P., Plocková, J., Korvasová, Z., Škrabalová, M., Koudelka, Š., Kulich, P., Zachová, K., Czerneková, L., Strouhal, O., Horynová M., Šebela, M., Miller, A.D., Ledvina, M., Raška, M., **Turánek, J**.: Enhancement of immune response towards non-lipidized *Borrelia burgdorferi* recombinant OspC antigen by binding onto the surface of metallochalating nanoliposomes ith entrapped lipophilic derivatives of norAbuMDP. ***J. Control. Release,*** 2012, vol. 160, p. 374-381; IF=7.786; cited: 6/3
28. Koudelka, Š., **Turánek, J**.: Liposomal paclitaxel formulations. ***J. Control. Release,*** 2012, vol. 163, p. 322-334; IF=7.786; cited: 89/86

29. Korvasová, Z., Drašar, L., Mašek, J., Turánek Knotigová, P., Kulich, P., Matiašovic, J., Kovarčík, K., Bartheldyová, E., Koudelka, Š., Škrabalová, M., Miller, A.D., Holý, A., Ledvina, M., **Turánek, J**.: Antiviral effect of HPMPC (Cidofovir®) entrapped in cationic liposomes. *In vitro* study on MDBK cell and BHV-1 virus. ***J. Control. Release,*** 2012, vol. 160, p. 330-338; IF=7.786; cited: 3/1
30. Koudelka, S; Knotigova, PT; Masek, J; Prochazka, L; Lukac, R; Miller, AD; Neuzil, J; **Turanek, J.** Liposomal delivery systems for anti-cancer analogues of vitamin E. ***Journal of Controlled Release.***, 2015 Volume: 207 Pages: 59-69; IF=7.786; cited: 10/10
31. Knotigova, Pavlina Turanek; Zyka, Daniel; Masek, Josef; Kovalova, A; Krupka, M; Bartheldyova, E; Kulich, P; Koudelka, S; Lukac, R; Kauerova, Z; Vacek, A; Horynova, MS; Kozubik, A; Miller, AD; Fekete, L; Kratochvilova, I; Jezek, J; Ledvina, M; Raska, M; **Turanek, J**. Molecular Adjuvants Based on Nonpyrogenic Lipophilic Derivatives of norAbuMDP/GMDP Formulated in Nanoliposomes: Stimulation of Innate and Adaptive Immunity. ***Pharmaceutical Research,*** 2015 Volume: 32 Issue: 4 Pages: 1186-1199; IF=3.314; cited: 2/0
32. Krupka, M; Masek, J; Barkocziova, L; Knotigova, PT; Kulich, P; Plockova,; Lukac, R; Bartheldyova, E; Koudelka, S; Chaloupkova, R; Sebela, M; Zyka, D; Droz, L; Effenberg, R; Ledvina, M; Miller, AD; **Turanek, J**; Raska, M. The Position of His-Tag in Recombinant OspC and Application of Various Adjuvants Affects the Intensity and Quality of Specific Antibody Response after Immunization of Experimental Mice. ***Plos One***, 2016 Volume: 11 Issue: 2 DOI: 10.1371/journal.pone.0148497; IF=2.806; cited: 0/0

33. Zouharova, Darina; Lipenska, Ivana; Fojtikova, Martina; Kulich, P; Neca, J; Slany, M; Kovarcik, K; Turanek-Knotigova, P; Hubatka, F; Celechovska, H; Masek, J; Koudelka, S; Prochazka, L; Eyer, L; Plockova, J; Bartheldyova, E; Miller, AD; Ruzek, D; Raska, M; Janeba, Z; **Turanek, J**. Antiviral activities of 2,6-diaminopurine-based acyclic nucleoside phosphonates against herpesviruses: In vitro study results with pseudorabies virus (PrV, SuHV-1) ***Veterinary Microbiology***, 2016, vol. 201; pages: 84-93; IF=2.628; cited: 1/1
34. Vacek, J; Zatloukalova, M; Geleticova, J; Kubala, M; Modriansky, M; Fekete, L; Masek, J; Hubatka, F; **Turanek, J**. Electrochemical Platform for the Detection of Transmembrane Proteins Reconstituted into Liposomes. ***Analytical Chemistry,*** 2016, vol. 88, p. 4548-45564; IF=6.32; cited: 4/4
35. Josef Mašek, Daniela Lubasová, Róbert Lukáč, Pavlína Turánek-Knotigová, Pavel Kulich, Jana Plocková, Eliška Mašková, Lubomír Procházka, Štěpán Koudelka, Nongnut Sasithorn, Jozsef Gombos, Eliška Bartheldyová, František Hubatka, Milan Raška, Andrew D. Miller and **Jaroslav Turánek**. Multi-layered nanofibrous mucoadhesive films for buccal and sublingual administration of drug-delivery and vaccination nanoparticles - important step towards effective mucosal vaccines. ***Journal of Controlled Release***, 2017, 249, 183-195; IF=7.786; cited: 0/0
36. Miroslav Ledvina, Pavlína Turánek-Knötigová, Helena Čelechovská, Daniel Zyka, Anna Kovalová, Josef Mašek, Eliška Bartheldyová, Štěpán Koudelka, Róbert Lukáč, Michal Křupka, Lucia Barkocziova, Petr Kosztyu, Ladislav Drož, Andrew D. Miller, Milan Raška, and **Jaroslav Turánek**. Nonpyrogenic molecular adjuvants based on norAbuMDP and norAbuGMDP: synthesis, molecular mechanism(s) of action, biological activities *in vitro* and *in vivo*. ***Journal of Medicinal Chemistry***, 2017 (*submitted*); IF= 6.259

- Zak, F.; Mistr, A.; Poulova, A.; Melka, M.; **Turanek, J**.; Zaluska, D. Platinum complex (II), its preparation and therapeutic application. PCT/CZ99/00014; US 6350737 B1, **2002**
- Zak, F.; Mistr, A.; Poulova, A.; Melka, M.; **Turanek, J**.; Zaluska, D. Platinum complex (II), its preparation and therapeutic application. PCT/CZ99/00014; EP 1082329 B1, **2003**
- Zak, F.; Mistr, A.; Poulova, A.; Melka, M.; **Turanek, J**.; Zaluska, D. Platinum complex (IV), its preparation and therapeutic application. PCT/CZ1999/000015; US 6503943 B1, **2003**
- Zak, F.; Mistr, A.; Poulova, A.; Melka, M.; **Turanek**, J.; Zaluska, D. Platinum complex (IV), its preparation and therapeutic application. PCT/CZ1999/000015; EP1082330B1, **2003**
- Ledvina M., Ježek J., Hříbalová V., **Turánek J.** Lipofilní ananlogy N-acetyl-mormuramyl-L-α-aminobutanoyl-D-isoglutaminu s imunostimulační aktivitou. Czech Pat CZ 296 720, **2006**
- Hipler K., Miller A.D., **Turánek J**., Ledvina M. (Compound (Adjuvants): PCT appl., WO 2009/11582 A2, **2009**

- **J. Turanek**, J. Neužil, B. Salvatore. Liposomal formulations of tocopheryl amides Application number US20100260830 A1, 2009901523 PTC, **2011**
- Hipler K., Miller A.D., **Turánek J**., Ledvina M., (Derivatives of muramyl peptide, WO 2009/11582 A2, EP 2271 661 B1, **2013**
- Hipler K., Miller A.D., **Turánek J**., Ledvina M., (Derivatives of muramyl peptide, EP13150255.1, **2013**
- Drašar, L.; Ledvina, M.; Korvasová, Z.; **Turanek, J**., Lipopolyaminy sperminového typu pro konstrukci liposomálních transfekčních systémů. Czech. Pat. 303963, **2013**
- Ledvina, M., Drašar, L., **Turánek, J**., Korvasová, Z. Lipopolyamines of spermine type for construction of liposomal transfection systems. PCT/CZ2013/000004; EP2802556 A1, **2014**
- Hipler K., Miller A., **Turanek J**., Ledvina M. (Normuramyl glycopeptide compounds, US 08653049, **2014**
- **Turánek J**., Mašek J., Raška M., Weigl E., Křupka M., Bittner L., Nepeřený J., Vrzal V., Ledvina M., Kratochvílová I. (Peptidový antigen, vakcinační konstrukt a vakcína pro prevenci lymeské boreliózy, PV 2014-320, **2014**
- **Turanek J** et al. Mukoadhesivní nosiče části, způsob přípravy a použití CZ 2014.665 A3, **2014**
- **Turánek J**aroslav, Mašek Josef, Raška Milan, Weigl Evžen, Křupka Michal, Bittner Libor, Nepeřený Jiří, Vrzal Vladimír, Ledvina Miroslav, Kratochvílová Irena.Vaccine for prevention of Lyme diseas. WO 2015/169271 A1, **2015**
- **Turanek J** et al. Mucoadhesive carriers of particles, method of preparation and uses thereof, PCT/GB2015/052833, **2015**
- Drašar, L.; Ledvina, M.; Korvasová, Z.; **Turanek, J**., Lipopolyamines of spermine type for construction of liposomal transfection systems. US Pat. US 9,393,200 B2, **2016**

6. REFERENCES

1. Grimaldi N, Andrade F, Segovia N, Ferrer-Tasies L, Sala S, Veciana J, et al. Lipid-based nanovesicles for nanomedicine. Chem Soc Rev. 2016;45(23):6520–45.
2. Kapoor M, Lee SL, Tyner KM. Liposomal Drug Product Development and Quality: Current US Experience and Perspective. AAPS J. 2017 May;19(3):632–41.
3. Li Q, Cai T, Huang Y, Xia X, Cole SPC, Cai Y. A Review of the Structure, Preparation, and Application of NLCs, PNPs, and PLNs. Nanomaterials. 2017 Jun;7(6):122.
4. Chen C, Zhu S, Huang T, Wang S, Yan X. Analytical techniques for single-liposome characterization. Anal Methods. 2013;5(9):2150–7.
5. Eloy JO, de Souza MC, Petrilli R, Abriata Barcellos JP, Lee RJ, Marchetti JM. Liposomes as carriers of hydrophilic small molecule drugs: Strategies to enhance encapsulation and delivery. Colloid Surf B-Biointerfaces. 2014 Nov 1;123:345–63.

6. Mallick S, Choi JS. Liposomes: Versatile and Biocompatible Nanovesicles for Efficient Biomolecules Delivery. J Nanosci Nanotechnol. 2014 Jan;14(1):755–65.
7. Patil YP, Jadhav S. Novel methods for liposome preparation. Chem Phys Lipids. 2014 Jan;177:8–18.
8. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, Joo SW, Zarghami N, Hanifehpour Y, et al. Liposome: classification, preparation, and applications. Nanoscale Res Lett. 2013 Feb 22;8:102.
9. Kostarelos K, Miller AD. Synthetic, self-assembly ABCD nanoparticles; a structural paradigm for viable synthetic non-viral vectors. Chem Soc Rev. 2005 Nov;34(11):970–94.
10. Shmeeda H, Even-Chen S, Honen R, Cohen R, Weintraub C, Barenholz Y. Enzymatic assays for quality control and pharmacokinetics of liposome formulations: Comparison with nonenzymatic conventional methodologies. In: Duzgunes N, editor. Liposomes, Pt A. San Diego: Elsevier Academic Press Inc; 2003. p. 272–92.
11. Duzgunes N, Simoes S, Slepushkin V, Pretzer E, Flasher D, Salem II, et al. Delivery of antiviral agents in liposomes. In: Duzgunes N, editor. Liposomes, Pt E. San Diego: Elsevier Academic Press Inc; 2005. p. 351–73.
12. Ruozi B, Riva G, Belletti D, Tosi G, Forni F, Mucci A, et al. Cidofovir-loaded liposomes: an intro-study using BCBL-1 cell line as a model for primary effusion lymphoma. Eur J Pharm Sci. 2010 Oct 9;41(2):254–64.
13. Holy A, Rosenberg I, Dvorakova H. Acyclic Nucleotide Analogs - Synthesis of (3-Hydroxy-2-Phosphonylmethoxypropyl) Derivatives of Heterocyclic Bases. Collect Czech Chem Commun. 1989 Sep;54(9):2470–501.
14. Declercq E. Therapeutic Potential of Hpmpc as an Antiviral Drug. Rev Med Virol. 1993 Jun;3(2):85–96.
15. Declercq E. Therapeutic Potential of Hpmpc as an Antiviral Drug. Rev Med Virol. 1993 Jun;3(2):85–96.
16. Michaud LB, Valero V, Hortobagyi G. Risks and benefits of taxanes in breast and ovarian cancer. Drug Saf. 2000 Nov;23(5):401–28.
17. Wani M, Taylor H, Wall M, Coggon P, Mcphail A. Plant Antitumor Agents .6. Isolation and Structure of Taxol, a Novel Antileukemic and Antitumor Agent from Taxus-Brevifolia. J Am Chem Soc. 1971;93(9):2325-.
18. Rowinsky E, Donehower R. Drug-Therapy - Paclitaxel (taxol). N Engl J Med. 1995 Apr 13;332(15):1004–14.
19. Cabanes A, Briggs KE, Gokhale PC, Treat JA, Rahman A. Comparative in vivo studies with paclitaxel and liposome-encapsulated paclitaxel. Int J Oncol. 1998 May;12(5):1035–40.
20. Fishman MN, Elsayed YA, Damjanov N, Steinberg JL, Mahany JJ, Nieves JA, et al. Safety and tolerability of liposome entrapped paclitaxel (LEP-ETU) administered with and without premedication in patients with advanced cancer. Ann Oncol. 2004;15:108–108.
21. Fishman MN, Elsayed Y, Damjanov N, Steinberg JL, Mahany JJ, Nieves JA, et al. Phase I study of liposome entrapped paclitaxel (LEP-ETU) in

patients with advanced cancer. J Clin Oncol. 2004 Jul 15;22(14):154S–154S.
22. Damjanov N, Fishman MN, Steinberg JL, Fetterly GJ, Haas A, Grahn A, et al. Final results of a Phase I study of Liposome Entrapped Paclitaxel (LEP-ETU) in patients with advanced cancer. J Clin Oncol. 2005 Jun 1;23(16):147S–147S.
23. Neuzil J, Weber T, Gellert N, Weber C. Selective cancer cell killing by alpha-tocopheryl succinate. Br J Cancer. 2001 Jan 5;84(1):87–9.
24. Neuzil J, Tomasetti M, Mellick AS, Alleva R, Salvatore BA, Birringer M, et al. Vitamin E analogues: A new class of inducers of apoptosis with selective anti-cancer effects. Curr Cancer Drug Targets. 2004 Jun;4(4):355–72.
25. Mahdavian E, Sangsura S, Landry G, Eytina J, Salvatore BA. A novel synthesis of tocopheryl amines and amides. Tetrahedron Lett. 2009 Jan 7;50(1):19–21.
26. Ireland DJ, Kissick HT, Bellharz MW. Alpha-Tocopheryl succinate: Toxicity and lack of anti-tumour activity in immuno-competent mice. Food Chem Toxicol. 2008 Feb;46(2):508–12.
27. Ireland DJ, Kissick HT, Bellharz MW. Alpha-Tocopheryl succinate: Toxicity and lack of anti-tumour activity in immuno-competent mice. Food Chem Toxicol. 2008 Feb;46(2):508–12.
28. Turanek J, Wang X-F, Knotigova P, Koudelka S, Dong L-F, Vrublova E, et al. Liposomal formulation of alpha-tocopheryl maleamide: In vitro and in vivo toxicological profile and anticancer effect against spontaneous breast carcinomas in mice. Toxicol Appl Pharmacol. 2009 Jun 15;237(3):249–57.
29. Koudelka S, Masek J, Neuzil J, Turanek J. Lyophilised Liposome-Based Formulations of alpha-Tocopheryl Succinate: Preparation and Physico-Chemical Characterisation. J Pharm Sci. 2010 May;99(5):2434–43.
30. Donati C, Rappuoli R. Reverse vaccinology in the 21st century: improvements over the original design. In: Rose NR, editor. Year in Immunology. Oxford: Blackwell Science Publ; 2013. p. 115–32.
31. Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: Influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens (vol 30, pg 2256, 2012). Vaccine. 2012 Aug 24;30(39):5799–5799.
32. Gregoriadis G. Engineering liposomes for drug delivery: Progress and problems. Trends Biotechnol. 1995 Dec;13(12):527–37.
33. O'Hagan DT, Valiante NM. Recent advances in the discovery and delivery of vaccine adjuvants. Nat Rev Drug Discov. 2003 Sep;2(9):727–35.
34. Perrie Y, Mohammed AR, Kirby DJ, McNeil SE, Bramwell VW. Vaccine adjuvant systems: Enhancing the efficacy of sub-unit protein antigens. Int J Pharm. 2008 Dec 8;364(2):272–80.
35. Zanetti AR, Van Damme P, Shouval D. The global impact of vaccination against hepatitis B: A historical overview. Vaccine. 2008 Nov 18;26(49):6266–73.
36. Munoz N, Manalastas R, Pitisuttithum P, Tresukosol D, Monsonego J, Ault K, et al. Safety, immunogenicity, and efficacy of quadrivalent human

papillomavirus (types 6, 11, 16, 18) recombinant vaccine in women aged 24-45 years: a randomised, double-blind trial. Lancet. 2009 Jun 6;373(9679):1949–57.
37. Storni T, Kundig TM, Senti G, Johansen P. Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev. 2005 Jan 10;57(3):333–55.
38. Bovier PA. Epaxal (R): a virosomal vaccine to prevent hepatitis A infection. Expert Rev Vaccines. 2008 Oct;7(8):1141–50.
39. Herzog C, Hartmann K, Kuenzi V, Kuersteiner O, Mischler R, Lazar H, et al. Eleven years of Inflexal (R) V-a virosomal adjuvanted influenza vaccine. Vaccine. 2009 Jul 16;27(33):4381–7.
40. Magalhaes JG, Fritz JH, Le Bourhis L, Sellge G, Travassos LH, Selvanantham T, et al. Nod2-Dependent Th2 Polarization of Antigen-Specific Immunity. J Immunol. 2008 Dec 1;181(11):7925–35.
41. Geddes K, Magalhaes JG, Girardin SE. Unleashing the therapeutic potential of NOD-like receptors. Nat Rev Drug Discov. 2009 Jun;8(6):465–79.
42. Harris J, Sharp FA, Lavelle EC. The role of inflammasomes in the immunostimulatory effects of particulate vaccine adjuvants. Eur J Immunol. 2010 Mar;40(3):634–8.
43. Ting JPY, Duncan JA, Lei Y. How the Noninflammasome NLRs Function in the Innate Immune System. Science. 2010 Jan 15;327(5963):286–90.
44. Steinhagen F, Kinjo T, Bode C, Klinman DM. TLR-based immune adjuvants. Vaccine. 2011 Apr 12;29(17):3341–55.
45. Rathinam VAK, Vanaja SK, Fitzgerald KA. Regulation of inflammasome signaling. Nat Immunol. 2012 Apr;13(4):333–42.
46. Lu X, Wang C, Wei Y. One-Dimensional Composite Nanomaterials: Synthesis by Electrospinning and Their Applications. Small. 2009 Nov 2;5(21):2349–70.
47. Bhardwaj N, Kundu SC. Electrospinning: A fascinating fiber fabrication technique. Biotechnol Adv. 2010 Jun;28(3):325–47.
48. Malinova L, Stolinova M, Lubasova D, Martinova L, Brozek J. Electrospinning of polyesteramides based on epsilon-caprolactam and epsilon-caprolactone from solution. Eur Polym J. 2013 Oct;49(10):3135–43.
49. Lawson LB, Norton EB, Clements JD. Defending the mucosa: adjuvant and carrier formulations for mucosal immunity. Curr Opin Immunol. 2011 Jun;23(3):414–20.
50. Kraan H, Vrieling H, Czerkinsky C, Jiskoot W, Kersten G, Amorij J-P. Buccal and sublingual vaccine delivery. J Control Release. 2014 Sep 28;190:580–92.

7. SCIENTOMETRIC DATA FOR J. TURANEK

Citation Report: WOS, July 2017
Results found: 74
Sum of the Times Cited: 1109
Sum of Times Cited without self-citations: 1000
Citing Articles: 832
Citing Articles without self-citations : 790
Average Citations per Item: 14.99
h-index:18