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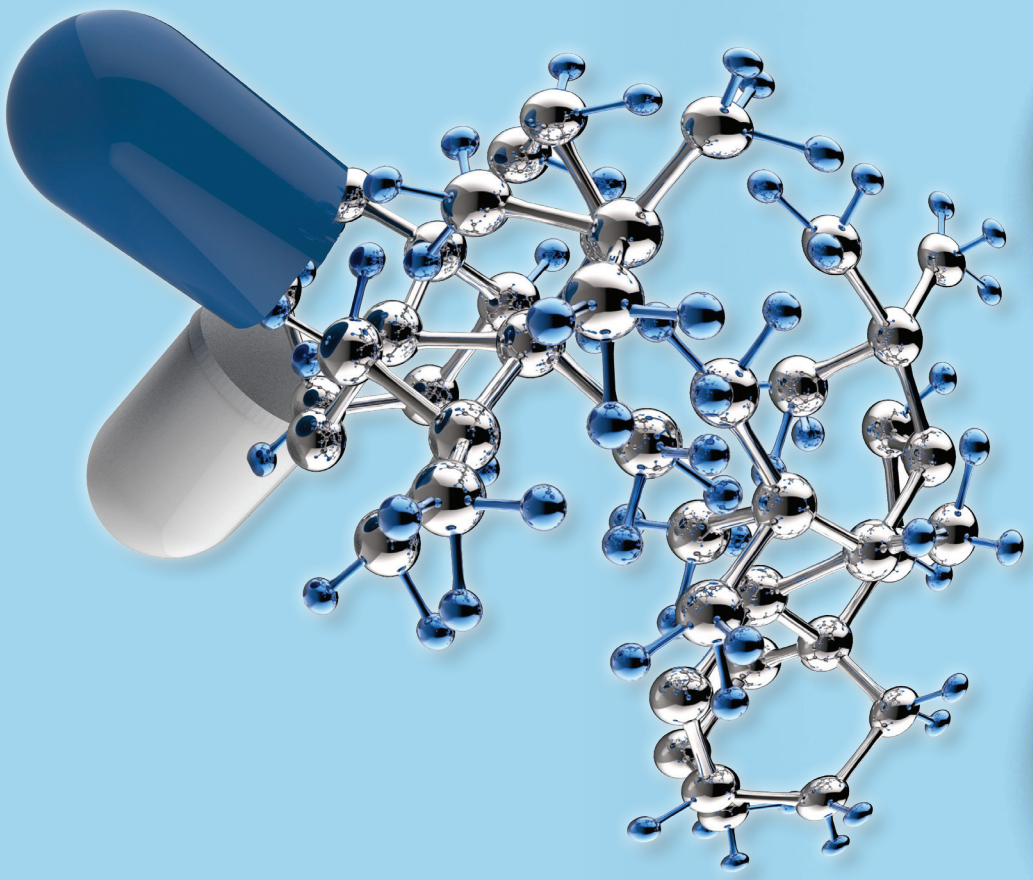
A QUARTERLY EDITION ON PHARMACEUTICAL SCIENCES' TOPICS



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ΦΑΡΜΑΚΕΥΤΙΚΗ

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About the Editors

Costas Demetzos is Professor of Pharmaceutical Nanotechnology in the National and Kapodistrian University of Athens. He serves as Director of the Laboratory of Pharmaceutical Technology and since 2008 he serves as President of the Hellenic Pharmaceutical Society (HPS). He has published more than 270 research papers [(h index 49), i10-index 151 (google scholar)], three monographs, he has co-authored one book and edited nine books. He has gained awards and honors for his contribution to the science of biomaterials and of pharmaceutical nanotechnology. In 2018, was honored with an award by the Order of Sciences of the Academy of Athens for his scientific achievements in Pharmaceutical Nanotechnology and for his monograph '*Pharmaceutical Nanotechnology. Fundamentals and Practical Applications*', Springer, 2016. In 2021, Prof. Demetzos has been elected as Ordinary member in Class IV-Natural Sciences of the *European Academy of Sciences and Arts* (*Academia Scientiarium et Artium Europaea*).

Efstathios P. Efstathopoulos, is Professor of Medical Physics at the Medical School of the National and Kapodistrian University of Athens (NKUA), Head of the Medical Radiation Physics Unit at Attikon General University Hospital in Athens. He is currently serving as Vice President of Administrative Affairs, Academic Affairs and Student Care, Hellenic Open University (HOU). He provides clinical work at "Attikon" University General Hospital where he is the Radiation Protection Expert for Radiology and Nuclear Medicine. He teaches Medical Physics, Radiation Protection and Principles of Nanomedicine, at undergraduate and postgraduate level. His research focuses on x-ray dosimetry of patients and staff and quantification of carcinogenic risk, imaging of the cardiovascular system, detection of vulnerable atherosclerotic plaque, and applications of nanotechnology to health. He has published (with his collaborators) more than 130 original papers in international scientific journals, which have received more than 3300 citations in international literature (https://scholar.google.com/citations?hl=en&user=xt9Uy7gAAAAJ). He has authored four chapters in scientific books. He has presented more than 100 papers in scientific conferences and delivered more than 60 invited lectures in Greek and international scientific conferences. In 2018 he designed and organized in collaboration with Professor C. Demetzos from the Department of Pharmacy and Professor M. Gazouli from School of Medicine, a new Postgraduate Program in "Nanomedicine", in which he is the Director and Chairman of the Special Interdepartmental Committee. He is a founding member and President of the Hellenic Society of Nanotechnology in Health Sciences (www.hsnanohs.eu). He is a member of the Radiation Protection Committee of the European Society of Cardiovascular and Interventional Radiology (CIRSE). He is also a member of the following scientific societies: European Society of Cardiovascular and Interventional Radiology (CIRSE, www.cirse.org), European Radiological Society (ECR), Hellenic Society of Medical Physicists (www.efie.gr), Hellenic Society of Interventional Radiology, Hellenic Society of Physicists. He has been a chess athlete since 1979. He is the President of the Hellenic Chess Federation.
Maria Gazouli is Professor of Biology - Nanomedicine, at the National and Kapodistrian University of Athens, School of Medicine. Prof M. Gazouli work refers mainly to genetic and molecular basis of diseases mainly autoimmune, inflammatory diseases and cancer, to molecular detection of pathogens and the investigation of the pathogenesis of the diseases they cause to humans and animals. Prof Gazouli was also involved in the incorporation of nanotechnology to targeted cancer detection, imaging and drug delivery. These activities have produced more than 288 publications in peer reviewed journals [(h

index 56), i10-index 201 (google scholar)], she has authored and editing several books. She honored with Fulbright Scholarship for the Development of Nanotechnology-based Biosensor Arrays for the Detection of Circulating Colorectal Cancer Cells at University of Maryland, College Park, MD, USA. The research has been recognized by distinguished awards and funded by national and international (EU) competitive research grants. Maria Gazouli has been actively involved in undergraduate and post graduate training, as well as ERASMUS program and her laboratory has trained a significant number of young scientists. She is coordinator of National and European Research programs. Currently, Prof Gazouli was appointed as the National Representative at the European Medicine Association in the Committee of Advanced Therapies- (CAT).

Preface

Nanotechnology is the engineering and manufacturing of materials at the atomic and molecular scale and refers to structures in the 1–100 nm size in at least one dimension. The applications of nanotechnology for treatment, diagnosis, monitoring, and control of biological systems is referred to as **Nanomedicine**. This special issue of the Journal FARMAKEFTIKI, aims to highlight the recent advances of Nanomedicine and of pharmaceutical nanotechnology and addressed to all those involved in recent advances in innovative diagnosis and therapeutics in the frame of nanomedicine. The students of the interdisciplinary master course NANOMEDICINE aimed to publish in this special issue their review articles in special fields of nanomedicine and promote new aspects in therapeutics and diagnosis of diseases. This special issue we expect to shed light on the new and advanced nanotechnological properties of innovative nanomedicines.

This special issue includes seven review articles. The first one entitled '**Advanced Drug Delivery Systems for Doxorubicin** ', discusses the importance of using liposomal nanoparticles as *trojan horse* to deliver toxic anticancer agents such as doxorubicin to the target tissue. The main concern is to review the most promising pre-clinical formulations of doxorubicin and the utilization of nanoparticles (NPs) and nanomaterials as biodegradable, bioactive and "smart" carriers for its delivery. Moreover, the authors discuss the polymers and polymeric conjugates, micelles, super paramagnetic iron oxide nanoparticles, exosomes and alternative carriers towards the most promising nanoparticles, used as drug delivery platforms. In the chapter '**Novel nanoparticle-based adjuvants: a review**', the authors discuss the importance of effective vaccine development as the only way to eradicate infectious diseases has been particularly designated during the ongoing pandemic of SARS-CoV-2. The aim of this article is to present the main categories of nanoparticle-based adjuvants, their advantages, and their useful physicochemical characteristics, after briefly describing the general role of adjuvants. The article '**Lipid -based nanovaccines'**, provides information about lipid-based nanoparticles in which nucleic acids such as DNA (as plasmids) and RNA (as mRNA) are encapsulated in order to be used for vaccination. Subsequently, there is presented a short overview according to the first lipid-based marketed products, Inflexal V and Epaxal, and their correlation with today's lipid-based nanovaccines. This review also focuses on the research efforts for the development of lipid-based vaccines against SARS, MERS and of the recent developments in nanotechnology-based approaches in view of the ongoing pandemic of COVID-19. The liquid crystalline state of matter is discussed in terms of cosmeceutical applications. The article titled '**Liquid Crystals in Cosmetics: Technological Forms, Challenges and Opportunities',** deals with opportunities that the Liquid crystals provide to cosmetic products becoming popular in local and international markets. The article aims to examine the technological forms, difficulties, and opportunities of liquid crystal applications in cosmetics objectively. The future perspectives of nanogels as 3D nanoparticulate systems in the article '**Nanogels: biomedical applications and future perspectives**' provide unique swelling capabilities, while their porous structure enables loading with other substances and they can be controlled and act as useful tools in drug-deliv-

ery, imaging or theranostics. Finally, the article provides information regarding nanogel-based vaccines which are shaping up as promising alternatives to conventional vaccines, paving the way for faster and more effective prevention of various diseases. The recent advances in neurodegenerative disease present in the article titled '**Recent advances in the application of Nanomedicine for the diagnosis and treatment of Alzheimer's and Parkinson's disease',** which provides chronic neurodegenerative disorders that eventually lead to neuronal death and severe impairment. Nanomedicine research aspires to provide a novel and promising approach aiming to overcome the limitations of existing diagnostic and treatment drawbacks, such as those related to the limited penetration of blood-brain barrier (BBB) by therapeutic and diagnostic agents. This brief review was based on a literature search spanning the last decade, and used the PubMed, Scopus, and Science Direct search engines, introducing relevant keywords. Finally, the article titled '**Simvastatin in the light of nanotechnology and its therapeutic potential',** aims to demonstrate the therapeutic potential of semisynthetic simvastatin by using novel nanotechnology applications for improved treatment of such diseases. In particular, the formulation and characterization of simvastatin nanoparticles and their potential novel therapeutic applications are discussed.

We would like to express our gratitude to all authors for their important contributions. This special issue is a comprehensive and valuable issue for all those willing to have a quick look in recent nanotechnological advances.

Athens, Costas Demetzos
Stathis Efstathopoulos
Maria Gazouli

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ΦΑΡΜΑΚΕΥΤΙΚΗ, 33, III, 2021 | 128-135 ΑΡΘΡΟ ΕΠΙΣΚΟΠΗΣΗΣ

PHARMAKEFTIKI, 33, III, 2021 | 128-135 REVIEW ARTICLE

Advanced Drug Delivery Systems for Doxorubicin

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KEYWORDS:
Doxorubicin(DOX), Nanoparticles (NPs) Polymers & polymeric conjugates, Dendrimers, Micelles, Exosomes

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ABSTRACT

Doxorubicin (DOX) (anthracycline) is a widely used chemotherapy medication approved for medical use in 1974 in the United States to treat various forms of cancer. While its mechanism of action is still under investigation (disrupting gene expression, generating ROS, inhibition of topoisomerase II) it is used synergistically with chemotherapy and radiotherapy as a first line of defense in multiple clinical cases. Despite the great antitumor effects it presents, the existence of several adverse effects, while as a general realization already existing products combining liposomal technologies, polymers, micelles and other advanced excipients can be further improved. The aim of this paper is to review the most promising pre-clinical formulations of doxorubicin and the utilization of nanoparticles (NPs) and nanomaterials as biodegradable, bioactive and "smart" carriers for its delivery (1,2). Polymers and Polymeric conjugates, Micelles, Super paramagnetic iron oxide nanoparticles, Exosomes and alternative carriers will be examined towards the most promising nanoparticles, used as drug delivery platforms.

1. Introduction

Originally made from the bacterium Streptomyces peucetius, doxorubicin (DOX) causes G0/S as well as G2/M arrest, increases oxygen radicals while also having p53 dependent and independent mechanisms. The method of delivery (mechanism as well as route of administration) may influence which pathway of action is activated while in certain cases it can lead to an increased number of target-cell apoptosis.[1,2]

The repetitive administration of free doxorubicin presents several toxicity issues with the most serious long-term side effect being irreversible cardiomyopathy (based on dosage and length of treatment). In the scope of meditating these effects and also enhancing its therapeutic properties, during the last decades, new formulations have been clinically tested and approved including drugs such as Caelyx, Evacet, Lipodox, Doxil, Myocet and several others. The main advantage of these drugs is the utilization of nanoparticles and nanomaterials as biodegradable, bioactive and "smart", stimuli - responsive carriers of the active substance. During the last years, while these products present many advantages over the conventional administration of doxorubicin, new formulations are being developed and tested, by utilizing new technologies in the area of nanomedicine, that show even more

promising results.[3] In this review, we will examine the most promising nanoparticles used as drug delivery platforms in today's pre-clinical applications divided into four major classes. These will include Polymers and polymeric conjugates, Micelles, Exosomes and lastly some alternative carriers. In the examination of each class, we will discuss the type of particles used as well as the mechanism of their action.

2. Polymeric NPs & Polymeric conjugates

2.1 Chitosan

Chitosan is a linear biodegradable polysaccharide in which NPs have been formulated to deliver chemotherapy and nucleic acids. Chitosan NPs have shown great potential as drug delivery carriers for multiple drugs presenting many advantages. Chitosan is considered as a harmless material and it is a natural polymer that possesses biodegradable and biocompatible properties since it can be digested by lysozyme enzymes present in mucosal surfaces under physiological condition. Its flexible molecular structure enables chemical modifications while its structure presents the ability for controlled drug release.[1,3,4]

A dextran-Dox conjugate can be incorporated into a chitosan nanoparticle, showing a reduced tumor size (subcutaneously implanted J774A.1 macrophage tumor) and prolonging survival in Balb/c mice compared to "free" (DOX) or the dextran conjugate alone. Treatment with the chitosan nanoparticle can result (16 mg/kg/dose) in up to 50% regression in tumor size at day 90 compared to a maximum size at day 45. Moreover, the group treated with the nanoparticle had prolonged survival with 50% of the mice alive at day 90, while all mice in the free Dox group died by day 60 and only 25% of mice in the dextran-conjugate group survived by day 90. These results were replicated by a follow-up study where Dox-loaded chitosan NPs inhibited tumor growth and prolonged survival of mice compared to "free" Dox.[1] Each time there was little or no evidence that the mice treated with the chitosan conjugate presented any adverse effects related to (DOX) toxicity. The nanoparticles enter the cells through endocytic mechanisms and release DOX intracellularly, while enhanced in vitro anti-tumour activity against human melanoma cells and murine colorectal carcinoma cells was observed.[3]

Other chitosan-DOX formulations, currently in pre-clinical trials, include glycol-chitosan DOX nanoaggregates conjugated with florecinisothiocynate FITC as well as Succinoyl chitosan-loaded DOX nanoparticles with size varying from 120-200nm presenting significant advantages due to the existence of metastable phases and "smartness" (ability to change morphology depending on the microenvironment) resulting in improved pharmacokinetics and advantageous release profiles.[3]

3. Polymersomes

A polymersome is one type of artificial architecture of polymeric self-assembly and its occurrence is closely related to the molecular structure of amphiphilic copolymers[5]. The main reason behind their development in conjunction with chemotherapeutic drugs is to overcome the fact that in the recent years by the use of conventional core-shell micelles hydrophilic, water-soluble molecules such as doxorubicin can't be encapsulated. Polymersomes, having doth hydrophilic and hydrophobic chambers, have the potential to be used as delivery platforms for both water-soluble and lipophilic drugs while possessing many advantages over liposomes including enhanced membrane stability, lower drug leakage and better pharmacokinetic profiles[2,5,6].

Hydrophilic doxorubicin hydrochloride (DOX·HCl) or hydrophobic doxorubicin base (DOX) could be encapsulated into PEP polymersomes (amphiphilic graft polyphosphazenes) with high payload and encapsulation efficiency due to strong intermolecular interactions. PEPs have shown encouraging results in resistance reversal at breast cancer stem cells, which exhibit resistance to traditional chemotherapeutic agents[5].

It is notable that other formulations including phospholipid polymeric hybrid systems have been formulated showing great results against other types of MultiDrug Resistant tumours like NK/Ly lymphoma and L1210 leukemia cells resulting in

100% cured animals even at low concentrations (0,1mg/kg). The enhanced accumulation of (DOX) due to active targeting resulted in the resistance levels dropping from 6fold to 2fold while the active substance was rapidly (10minutes) transported into both resistant and sensitive cancer cells without distinction[7].

4. PLA & PLGA along with Polyethylene Glycol

From the synthesis process all the way to clinical practice we can understand why having formulations that are effective but also easily scalable and relatively cost-effective is of grave importance. Here we study poly (lactic-*co*-glycolic acid) (PLGA), polylactic acid (PLA), and PEGylated PLGA (PLGA-PEG) nanoparticles which can easily be prepared, and well tolerated delivery systems with the ABCB1 substrate doxorubicin[8,9]. The results were compared with the effectiveness of doxorubicin solution administration in neuroblastoma cells. The produced NPs had a size ranging from 73 to 246 nm with the PLGA-PEG nanoparticle preparation resulting in the smallest size, while PLGA NPs had a preferable controlled release of (DOX) compared to the other formulations and the doxorubicin solution. The use of PEG "masks" the cytotoxic active compound until the in situ release. Even though the above formulations exhibited stronger anticancer effects due to their small size and superior pharmacokinetics they didn't result in an increased efficiency in ABCB1-expressing cell lines in comparison to doxorubicin [8,10].

5. Dendrimers

Dendrimers are synthetic polymeric macromolecules, able to be synthesized from simple monomer units that present many advantages in drug delivery. Due to the easy manipulation of the number of generations and the vast amount of terminal active groups, the main advantage that polymeric-doxorubicin conjugates present is the ability to attach many active compounds, varying from targeting moieties and ligands to drugs to genes, while exhibiting the same ability of phospholipid-polymeric formulations to carry both hydrophilic (outer part) and hydrophobic (inner core) compounds[1,11]. DOX-dendrimer conjugates have been demonstrated to cure with a single dose SCID mice with subcutaneously implanted colon cancer (C-26 cell lines) that previously showed to be (DOX) resistant. These 8nm carriers werecomposed of a polyester dendrimer-PEG conjugate with a topologically globular structure[1].

6. Micelles

In general, micelles have no particular properties since they can have special behaviors on drug release at the same conditions, depending on different degrees of protonation at the same pH[12]. Stimuli-responsive polymers took place in the field of nanomedicine, so apart from these novel nanoparticle drug delivery systems, new beneficial polymeric micelles have also rapidly evolved during the last decades[13]. These kinds of micelles are promising drug delivery vehicles, since they allow the controlled release of anticancer drugs. Their inner core consists of hydrophobic moieties which can encapsulate a large variety of lipophilic drugs[14]. There is another category of polymeric micelles, formed by amphiphilic copolymers, which are one of the most preferable candidates for enhanced anticancer drug release, localized at the site of action. The most principal factors about drug release at the site of action are pH and temperature (stimuli-responsiveness). Functional copolymers can act like surfactants and can create micelles because of phase separation in the solution state[13].

It has been found that by using encapsulation techniques in polymeric micelles, all the above issues can be addressed, by incorporating both chemical and biological factors. Super paramagnetic iron oxide nanoparticles (SPIONs) show some interesting properties in plenty of biomedical applications. However, the agglomeration of SPIONs, since they have large surface to volume ratio, as well as their low efficiency of internalization, are serious problems that should be addressed. Polymeric micelles offer a solution to those problems by having great colloidal and thermodynamic stability[15]. Co-capsu-

lation of the chemotherapeutic drugs and contrast agents into PMs has shown a combination of diagnosis and treatment of the tumor in a single modality. A multi-functional micelle using SPIONs is ST-OA-SPIONs/DOX. DOX behaves as a model drug, while ST-OA-SPIONs consist of a contrast agent, which is coated by hydrophilic starch (ST) and hydrophobic octanoic acid (OA). ST-OA is used in this part because it acts as a nanocarrier for the co-encapsulation of the antitumor drug DOX and SPIONs. The final formulation is developed through the self-assembly process at the critical micelles concentration (CMC). These multi-functional vehicles can be used as contrast agents for magnetic resonance imaging and simultaneous tumor therapy.[15]

By using chemotherapy, it is crucial to be able to have an effect in multidrug resistant (MDR) tumors. Up to date, there are plenty of mechanisms, in order to provide cytotoxic chemicals to these MDR cell lines. To reach this statement the pH-sensitive micelles carrying DOX poly(L-histidine)-b-PEG-folate and poly (lactic acid) PLLA-b-PEG-folate were used. The stability of these micelles was tested by using different pH environments. All the searches conclude that micelles offer a great efficacy in DOX delivery against sensitive cells. To defeat MDR cells the micelles must consist of an active cellular entry tool that loses its stability only at a pH lower than the extracellular conditions of most solid tumors. It is important to keep the micelle stable before its entrance to the cell, or else drug leakage will take place and thus undesirable toxicity to healthy tissue[16].

The measurement of pH function is dependent on the CMC, size, and transmittance of micelle solutions. When pH is up to 7 or 8, then stabilization takes place since the conversion of no ionized histidine residues to hydrophilic ones by the protonation of the imidazole groups. This function takes advantage of achieving an acceleration of DOX release from L-his based polymeric micelles (endosomal ph 6). Using this method along with active targeting, via folate receptor-mediated endocytosis at the same time, this nano platform is able to efficiently kill drug-sensitive ovarian cells just like drug-resistant counterpart cells[16].

As a result of research, flaunt that co delivery, which provide therapeutic efficacy and overcome drug resistance, depends on pH-sensitive Poly prodrug might be an important nanomedicine for combination with cancer chemotherapy. Additionally, the structure based on poly prodrug and chemical drug is also possible to be a useful preparation method for multifunctional nanovehicles[17].

Specific polymeric micelle PTX/DOX-PMs, was created consisting of DOX-conjugated copolymer molecules and hydrophobic PTX in aqueous (self-assembly process). Concerning PTX/DOX-PMs, the DOX molecules were chemically linked on the pH-sensitive diblock copolymers, and PTX molecules were physically loaded in the core of the micelle. The PTX/DOX-PMs presented increased stability for prolonged circulation in the body at normal pH (7.4). After deposition at the tumor sites, part of tertiary amine residues in the PAE block were protonated, which resulted in the increase of surface charge of PTX/DOX-PMs, facilitating the cellular uptake by tumor cells. Thereafter, the pH-sensitive bonds between polymer and DOX molecules would be broken as a response to intracellular acidity (low pH)[17].

7. Exosomes

Exosomes are natural and shelf produced nano-sized extracellular vesicles, which consist of a lipid membrane that encloses cell-related substances, such as proteins, mRNAs, microRNA, etc.Exosomes can transport many types of molecules with good targeting ability. Ranging from 40−200 nm in size, exosomes are formed by intraluminal vesicles (ILV), although their formation mechanism in the endosome is still unclear. Most of the traditional membrane sprouting processes entail the deformation of the membrane to encapsulate the organelles into the cytoplasm. During the formation of the ILV, the membrane sprouts away from the cytoplasm and enters the endosome. Exosomes gain more and more attention due to many factors such as their ability to 'allow' intracellular macromolecular transport and their ability for excellent 'cell communication''. Exosomes play an important role in transmission

of proteins, lipids, mRNAs etc. Clinical trials have shown that exosomes are biocompatible materials that could be safely used in humans. Phase I studies showed no severe adverse effects and set up new clinical grade protocols for the preparation of exosomes[18]. In fact, due to exosomes' lipid bilayer membrane structure, they can act as natural drug carriers, compared to artificially synthesized polymers. Exosomes are thought to contribute to homeostasis and disease development (including cancer and neurodegenerative disorders). More specifically, it has been proven that exosomes can increase the therapeutic index of doxorubicin (DOX) and ExoDOX is safer and more effective than free DOX[19].

Exosomes can be used as unique nano - platforms for drug delivery, due to their physical properties (stability, biocompatibility, permeability, low toxicity, and low immunogenicity). For the treatment of breast, pancreatic, lung, prostate cancers and glioblastoma, exosomes have been used to encapsulate natural products as well as mRNA. They act like "radars" that confirm that the organs are ready to receive the tumor cells. This happens because exosomes trigger the necessary molecular reactions in the receiving organs - inflammation, angiogenesis, etc. to accept the tumor cells so that they can survive and proliferate.

Many well-known nanoparticles have been used in combination with exosomes and pharmaceutical agents, such as doxorubicin for the treatment of cancer. For example, one recent study refers to the combination of porous silicon nanoparticles (PSiNPs) with doxorubicin. More specifically, (PSiNPs) with doxorubicin, are firstly endocytosed into cancer cells after incubation. Then, these particles can be found in multivesicular bodies and auto-phagosomes in the cell. After the MVBs fusion with the cell membrane, the nanoparticles are exocytosed into extracellular space as exosomes. When these exomes with the loaded nanoparticles are injected into a tumor bearing mice, they 1) accumulate in tumor tissues, 2) penetrate deeply into the tumor, 3) are efficiently internalized into bulk cancer cells and cancer stem cells (CSCs) to produce anticancer efficacy[20].

These particles were tested in H22 tumor-bearing mice after intravenous injection of of PBS, E-PSiNPs, free DOX, DOX@PSiNPs, DOX@E PSiNPsexocytosed from H22 cells at DOX dosage of 0.5mg kg−1, or free DOX at high dosage of 4mg kg−1. The result showed a reduced tumor volume in the case of DOX@E PSiNPs, compared with all the other cases, as shown in Figure 3, where the arrows in the x-axis indicate the drug injection time.

It has been proven that co-delivering genes and chemotherapeutic drugs can produce synergistic effects against cancer. Apart from that, scientists recently developed a strategy to produce nanoscale targeted specific exosomes to deliver both cholesterol modified miRNA and chemotherapeutic drugs, such as doxorubicin[4.] The main goal for this study was to evaluate their therapeutic ability for the Triple breast cancer (TNBC), which comprises approximately 15% of all breast cancers and shows a higher morbidity because of its aggressive behaviour, poor prognosis, and lack of targeted treatments.

In this study, target- specific exosomes were generated at a high yield by stimulating THP-1 cells with PMA* which effectively increased exosomal release. Doxorubicin packaging into exosomes was achieved by mixing an appropriate concentration of the above in a trimethylamine solution overnight. Then, the Cho-miR159 with Exo/Dox, were co-incubated to form a co-delivery system (Co-A15-Exo). The maximal loading was that of ~ 160 ng Dox in 1 μg of exosomes. These extracellular vesicles were evaluated in terms of their targeting and therapeutic effects, both in vitro and in vivo[21].

One more study was recently conducted, with the objective to understand if exosomes may be used to increase the efficacy of doxorubicin (DOX) in breast and ovarian cancers, in particular. The methods that were used include the isolation of exosomes from specific breast and STOSE ovarian cancer cell lines, and loaded with DOX via electroporation. The next step was the conduct of in vitro experiments in order to measure the ability of exosomal doxorubicin (exoDOX) to cross the myocardial endothelial cells. At the same time the toxicity exoDOX was evaluated in mice by histopathology analysis. Finally, biodistribution in mouse tumor tissues of exoDOX was

obtained by MS analysis. The results indicated that ExoDOX partially limits myocardial endothelial cells crossing of DOX. Moreover, DOX encapsulated in exosomes is less toxic and allows to treat mice at a higher concentration. An important outcome was that when treated with exoDOX compared with free DOX, the volume of breast and ovarian mouse tumors is reduced and the concentration of exoDOX in the tumor is higher than that of free DOX. Finally, it was proven that exosomes increase the therapeutic potential of DOX. ExoDOX is a novel alternative therapy for breast and ovarian cancers, both highly lethal diseases[22].

In Figure 4 the Biodistribution and antitumor efficacy of Co-A15-Exo in vivo, is presented. An in vivo imaging of Cy5-Cho-miRNA loaded Exo in MDA-MB-231 tumor-bearing nude mice after tail vein injection of free Cy5-Cho-miRNA, Exo-Cy5-Cho-miRNA, or A15-Exo-Cy5-Cho-miRNA. (a) Images were taken 1 h, 2 h, 4 h, or 8 h after the administration of free Cy5-Cho-miRNA, Exo-Cy5-Cho-miRNA, or A15-Exo-Cy5-Cho-miRNA[22].

8. Conclusions

In spite of the great antitumor properties that DOX offers to the treatment of cancer, the existence of several adverse effects is one of the most important setbacks. A safer and efficacious providing of antitumor drugs necessitates new advanced drug delivery systems. During the last years, formulations and products such as Caelyx, Evacet, Lipodox, DoxilMyocet have been clinically tested and approved. In addition to the usage of already existing products that combine liposomal (nano) technologies, polymers and micelles, other advanced excipients should be further investigated. The aim of this paper was to enlighten the most recent research studies, with respect to the most promising pre-clinical formulations of doxorubicin along with the utilization of nanoparticles (NPs) and nanomaterials as biodegradable, bioactive and "smart" carriers for its delivery. According to a plethora of publications and to this review, polymeric NPs and conjugates have shown great potential as drug delivery carriers for multiple drugs.

For example, chitosan NPs - as natural polymers, can encapsulate Dextran-Dox conjugates and by entering the cancer cell with endocytic mechanisms, they can offer up to 50% regression in tumor size. Moreover, polymersomes as artificial architectures by the self-assembly process, can encapsulate DOX showing encouraging results in resistance reversal at breast cancer stem cells which exhibit resistance to traditional chemotherapeutic agents. Furthermore, DOX-dendrimer conjugates have been demonstrated to cure (with a single dose) mice with subcutaneously implanted colon cancer that previously was DOX resistant. Apart from dendrimers, by using encapsulation techniques in polymeric micelles, or super paramagnetic iron oxide nanoparticles (SPIONs) appear to have interesting properties in plenty of biomedical applications. Nano-sized extracellular vesicles – Exosomes - have also been investigated as a way to increase the therapeutic index of DOX. It should be mentioned that exosomes have been investigated for the treatment of breast, pancreatic, lung, prostate cancers and glioblastoma by encapsulating natural products as well as mRNA. Specifically, ExoDOX is a novel alternative therapy for breast and ovarian cancers, both highly lethal diseases.

Taking into account the pre-mentioned formulations, it seems that nanotechnology can assist not only to rapid and sensitive detection of **cancer**-related molecules, but also carries the potential to generate entirely novel and highly effective therapeutic agents. □

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PHARMAKEFTIKI, 33, III, 2021 | 136-159 REVIEW ARTICLE

Novel nanoparticle-based adjuvants: a review

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ABSTRACT

The importance of effective vaccine development as the only way to eradicate infectious diseases has been particularly designated during the ongoing pandemic of SARS-CoV-2. Sub-unit antigens alone are usually unable to achieve the required immunity, rendering the use of adjuvants imperative. Difficulties related with the manufacturing process of adjuvants, as well as the possible induction of local or systemic adverse effects comprise the main challenges of their use. For this reason, the development of novel adjuvants is needed. The role of nanotechnology in medicine is significant, especially in vaccine development, as nanoparticles play a crucial role due to their inherent adjuvanticity and their ability to act as vaccine carriers. The aim of this article is to present the main categories of nanoparticle-based adjuvants, their advantages and their useful physicochemical characteristics, after briefly describing the general role of adjuvants. Furthermore, the gradually increased use of nanoparticulate adjuvants in vaccine development, especially against the viruses SARS, MERS, Ebola and Zika, as well as the important role of recombination technology, are commented. Finally, the main challenges and future perspectives are discussed.

1. Introduction

Vaccines are considered the safest, most cost-effective, and trustworthy tool to fight or even eradicate infectious diseases. Nevertheless, antigens alone are often unable to provide the required immune- potency. Therefore, adjuvants have been used in vaccine-formulations for more than 70 years to promote long-term and sterilizing immunity. However, until now their exact mechanism remains empirical[1]. Among the various adjuvants that were developed, aluminum-containing ones were the first vaccine-adjuvants approved for clinical-use. Nonetheless, conventional-adjuvants cannot be considered as ideal components of vaccine-formulations due to several weak points they present. Hence, there is a need for developing novel, safer, and improved adjuvants for more effective vaccines.[2] Nowadays, numerous studies are applying in the field of nano-vaccines, which gained tremendous popularity, especially after the COVID-19 vaccine arrival.

The scope of this review is to describe the role, mechanisms, and advantages of novel-adjuvants, and present the most important types, their physicochemical-characteristics, as well as their

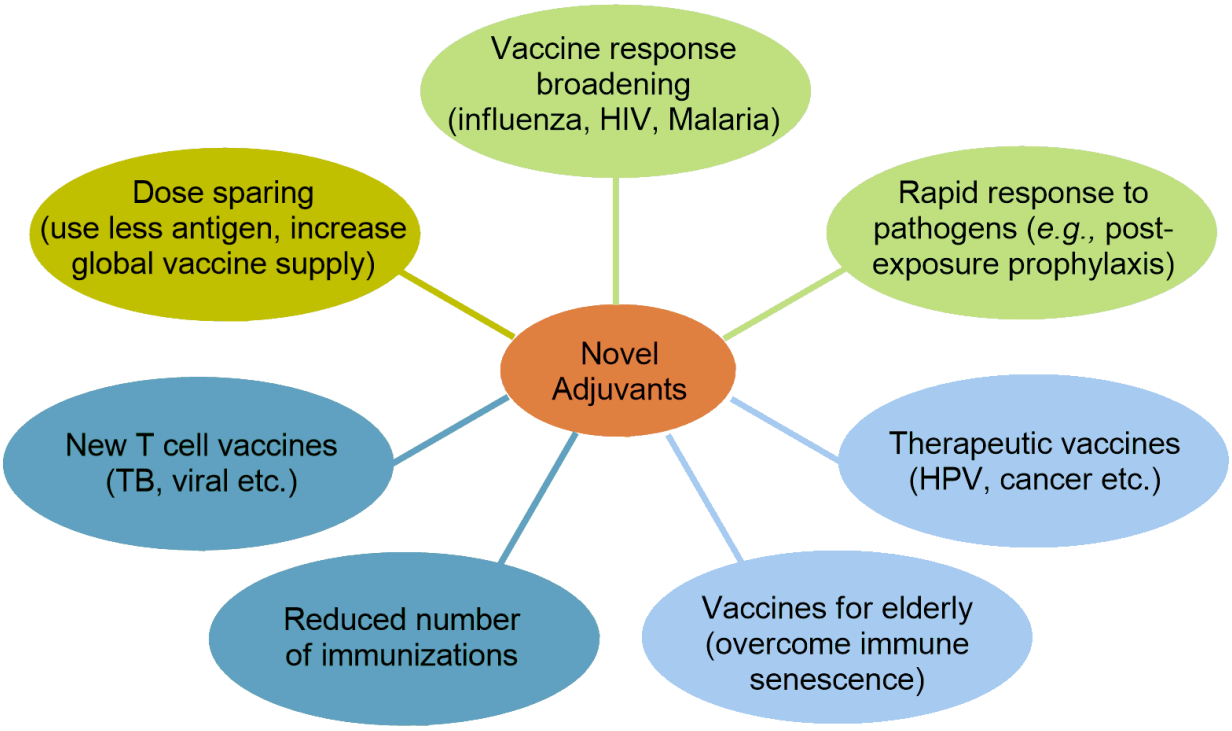

Figure 1: *Potential benefits from the development of novel-adjuvants (HPV: human papillomavirus, HIV: Human Immunodeficiency Virus, TB: Tuberculosis). Adapted from Ref.*[10]

challenges and future-perspectives. Indicative examples of nano-vaccines development against several viruses are also reported.

2. Role of Adjuvants

An adjuvant is defined as any substance that increases the immune-response against an antigen. It consists of a large group of structurally heterogeneous compounds that potentiate the immune-response in many ways via a variety of functions within vaccine-formulations[3]. Adjuvants' overall target is to induce an immune-response capable of providing long-term protection against a subsequent infection. Moreover, adjuvant-vehicles may increase vaccine-stability and shelf-life, allowing for a cost-effective vaccine to be widely developed. Thus, several parameters should be taken into account for the selection of the most appropriate adjuvant(s) corresponding to the specific vaccine-application every time.[4]

Nowadays, a wide-range of compounds with adjuvant-properties exist and seem to exert their functions through different modes of action. Mineral-salts, emulsions, microparticles, cytokines, saponins, microbial-components and liposomes have all been assessed as adjuvants[5]. However, few adjuvants are licensed for human-use and several formulations are currently being evaluated in clinical-trials (*Table 1).*

3. Mechanism of action

Although adjuvants have been used in many vaccine-formulations and a lot of efforts have been made to investigate their mode of action, until now, it remains poorly understood [1. Within the past decade, studies have slowly started to reveal their unknown mechanisms. In the following part, the possible proposed mechanisms are briefly presented.

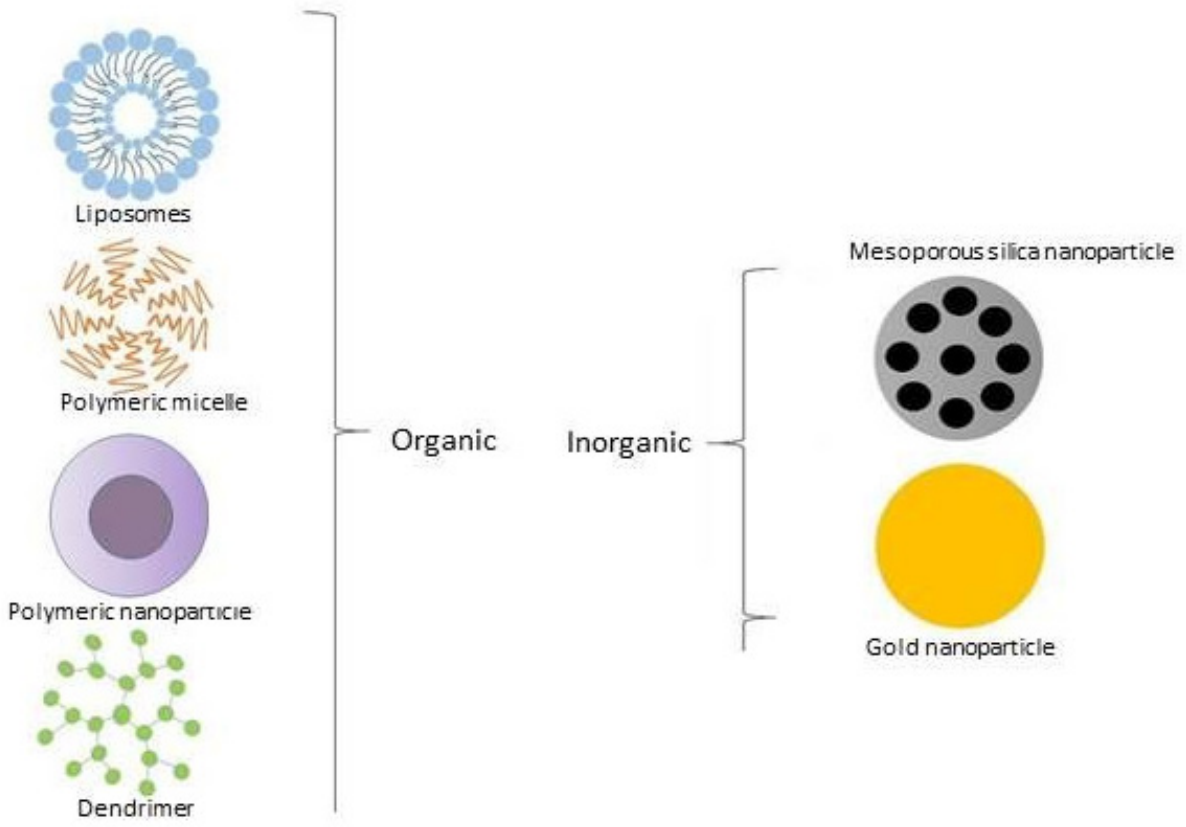

***Figure 2. Frequently** used types of organic and inorganic nanocarriers. Adapted from Ref.[11]*

3.1. Depot-Formation

To sustain an antibody-response, antigen-supply is essential. An adjuvant can help the immune- response through the formation of a depot of antigens at the injection-site, resulting in the sustained-release of small quantities of antigen over a long-period of time. Despite depot-effect, the quantity of antigens will eventually be reduced to a great extent. At that time, a second antigen-injection (booster-dose) may be given. Antigenic-depot-provision can finally lead to reduced doses or number of immunizations required, enhancing thus patient-compliance[7].

3.2. Immune-Cell Recruitment at the Injection-Site

Adjuvants are, also, able to activate the local immune-environment to secrete various chemoattractants and pro-inflammatory cytokines that activate and sustain the immune-response after immunization. Particulate or crystalline adjuvants can induce production of inflammatory cytokines at the injection-site, leading to immune-cell recruitment[1].

3.3. Triggering of Immune-System by Dendritic-Cells

Adaptive immune-response is characterized by complete maturation and activation of DCs. Recent studies have shown that antigen-absorption by adjuvants can lead to enhanced antigen-uptake and antigen-presentation by APCs leading to increased period of antigen-presentation[1].

Table 1: FDA-approved vaccines containing novel-adjuvants. Adapted from Ref[6]

Adjuvants	Composition	Formulation	Vaccines	Year
Licensed adjuvanted pediatric vaccines				
Aluminum	One or more of the following: Aluminum hydroxide, luminum phosphate, potassium, Alum, AAHS	Various/ Aqueous (PBS-based)	Anthrax (BioThrax), DT, DTaP (Daptacel), DTaP (Infanrix), DTaP-IPV (Kinrix), DTaP-IPV (Quadracel), DTaP-HepB-IPV (Pediarix), DTaP –IPV/Hib (Pentacel), Hep A (Havrix), Hep A (Vaqta), Hep B (Engerix-B), Hep B (Recombivax), HepA/Hep B (Twinrix), HIB (PedvaxHIB), HPV (Gardasil 9), Japanese encephalitis (Ixiaro), MenB (Bexsero, Trumenba), Pneumococcal (Prevnar 13), Td (Tenivac), Td (Mass Biologics), Tdap (Adacel), Tdap (Boostrix)	1930 – present
Licensed adjuvanted pediatric vaccines				
MF59	Oil in water emulsion, squalene-based	Emulsion-based	TIV (Fluad) (for adults aged 65 or older)	2015
AS01B	MPL and QS-21, natural extract from the Chilean soapbark tree	Liposome-based	RVL (Shingrix) (for adults aged 50 or older)	2017
CpG-1018	CpG, synthetic form of DNA mimicking bacterial oligodeoxynucleotide and viral genetic material	PBS-based	Hep B (Heplisav-B) (for adults aged 18 or older)	2017
Approved – not commercially available in U.S.				
AS04	MPL and aluminum salt	VLP and MPL adsorbed onto Alum, PBS-based	HPV (Cervarix)	2009
AS03	a-Tocopherol, squalene and polysorbate 80	Emulsion-based	Monovalent Pandemic H5N1 Swine Influenza A (Q- Pan H5N1) (for adults aged 18 or older)	2013

AAHS: *aluminum hydroxyphosphate sulfate; Alum: aluminum sulfate;* ***CpG:*** *cytosine phosphoguanine;* ***DT:*** *diphtheria and tetanus toxoids;* ***DTaP:*** *diphtheria and tetanus toxoids with acellular pertussis;* ***HPV:*** *human papillomavirus;* ***IPV:*** *inactivated polio vaccine;* ***MPL:*** *Monophosphoryl lipid A;* ***RZV:*** *recombinant zoster vaccine;* ***Td:*** *tetanus and diphtheria toxoids; Tdap: tetanus and diphtheria toxoids with acellular pertussis;* ***TIV:*** *trivalent inactivated influenza vaccine;* ***VLP:*** *virus-like particle*

4. Conventional Adjuvants and need for Novel Adjuvants

Despite the usage of adjuvants in the preparation of most inactivated vaccines, their progress has been particularly slow. So far, very few adjuvants have been approved. Since the time, aluminum- salts (alum) begun to be utilized in the preparation of tetanus and diphtheria toxoids in 1920s, the approval of the first vaccine containing a ''novel'' adjuvant (the oil-in-water MF59/influenza-vaccine) was given in 1990's. Another wave

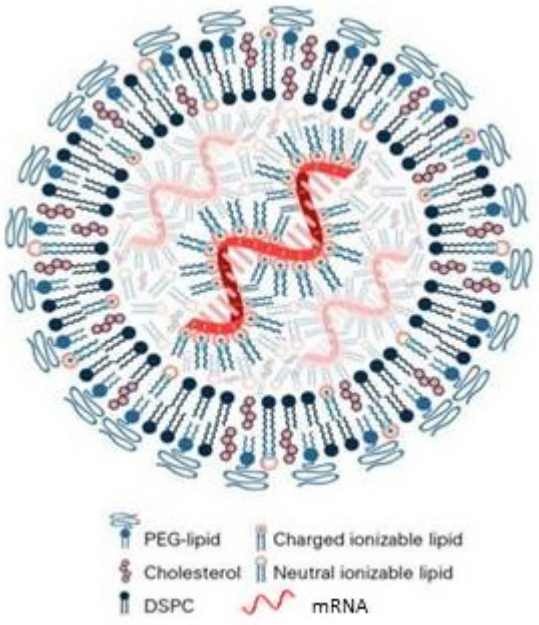

Figure 3. *Unilamellar-liposome combines the incorporation of mRNA in the core and the surface attachment of ligand PEG. Adapted from Ref. [19]*

of adjuvanted vaccines was approved in the beginning of the 21st century, with the consecutive approval of vaccines against avian-influenza-virus (AS03), HBV and HPV (AS04), and finally herpes-zoster-virus (AS01) and HBV (CpG) (*Table 1).*[9]

This quite slow progress of new adjuvanted vaccines, as well as the failure to develop promising adjuvants from preclinical-phases to approval, is mostly associated with the manufacturing process (need for reproducible formulations, limited antigen-stability) or with local and systemic adverse-effects. Consequently, there is a great medical necessity for the development of novel, safer and more effective adjuvants, since preventive-vaccines are administered often to healthy children in the first years of their life and therefore must be extremely safe and well tolerated [3. Potential benefits from novel-adjuvants-development are depicted in Figure 1[10].

The categories of novel nanoparticle-based adjuvants are presented in the section 5. The structures of the main types of nanoparticles are schematically represented in Fig. 2.

5. Types of nano-adjuvants

5.1. Lipid-based Nanoparticles (LNPs)

Liposomes are biocompatible and biodegradable vesicular structures composed of an aqueous core and phospholipid bilayers. Their shell consists of neutral, anionic or cationic phospholipids and cholesterol, which is necessary for the self-assembling process and the bilayer's integrity. Key-advantages are their enhanced self-adjuvanticity and versatility as they combine the entrapment of molecules either in the core or the lipid bilayer as well as the surface-attachment of ligands (Fig. 3).[12,13,14] Conjugation with antibodies, immunomodulators or recognition-receptor-ligands (i.e., TLR)[15] offers increased targeting, while PEG enhances stability[12]. Cationic liposomes, although being more toxic, enhance the immunogenicity via stronger interactions with the negatively charged cell-membrane.[12] pH-sensitive liposomes are a great advance in mRNA-vaccine development, especially ionizable-lipids which are charged only in acidic environments. Lipo-Merit, an mRNA-vaccine, against melanoma in Phase-I, is such an example[16,17]. The fluidity and lamellarity of the membrane are crucial for the efficacy of liposomal adjuvants.[18] A limitation, though, is their short shelf-life, which can be increased with lyophilization[12].

Liposomes were reported for the first-time as immunological-adjuvants for diphtheria-toxoid antigen, by Gregoriadis (1974)[20]. They can carry subunit-antigens for induction of both humoral and cellular immunity[21]. Up-to-date, typical marketed liposomal-vaccines include Mosquirix®[12,14,22], against malaria and Shingrix®[22], against shingles, both considered also as virosome- based vaccines. Indicative RNA-vaccines are Onpattro®, siRNA-vaccine against polyneuropathy[22] and mRNA-vaccine against COVID-19 (BNT162b2). Vaccines under clinical-trials comprise CAF01 (cationic-lipids included) and AS01

liposomal-adjuvants, against tuberculosis[14].

The addition of non-ionic surfactants (niosomes) or bile-salts (bilosomes) in lipid-bilayer, enhances the stability and permeation across cell-membranes compared to conventional-liposomes[12]. Bilosomes are promising in oral-vaccine delivery due to their resistance to decomposition by gastric-acid. Several attempts have been made with bilosomes against influenza, HBV, diphtheria, tetanus[23] and with niosomes against influenza[24].

Archaeosomes, composed of polar ether lipids, offer enhanced stability in extreme thermal and pH conditions, as well as pH-dependent properties, holding a great promise for needle-free vaccines[25]. A limitation, however, is Archaea's challenging purification [12].

Another category of lipid-based adjuvants are virosomes, with incorporated viral-envelope proteins (haemagglutinin, neuraminidase) in the lipid-bilayer. Their advantages are increased immunogenicity and stability, although the extraction of proteins from influenza-particles is needed.[12] The first marketed liposomal-vaccines were: anti-Influenza Inflexal V® *and* anti-HAV Epaxal®[14]. Invivac®[21] is also a virosome-based flu-vaccine. Lastly, a promising gp41-virosome against HIV is under clinical-trials[26].

Remarkably, Vaxi-Patch against Influenza[27], which is considered the first vaccination-system for point-of-care applications, is based on a microneedle skin-patch for virosomes' delivery,

5.2. Emulsions

Emulsions, composed of two immiscible liquid phases forming a dispersion, are easily manufactured and with good self-adjuvanticity. However, thermodynamic instability leads to limited antigen-protection and inability of modification with target-molecules.[12,21,22] Contact interactions between nanoemulsion-droplet and the mucosal-surface that promote the targeted- antigen delivery to APCs, permit intranasal-vaccination[28]. Licensed vaccines include Pandemrix®- AS03, Fluad®-MF59, Humenza®-AF03, against Influenza. MF59 against malaria, HIV, HCV, AS02 against malaria and generally oil-in-water emulsions against HAV are in clinical-research.[29,2] MF59 adjuvant with self-amplifying-mRNA was also reported to be a potent-tool for HIV-vaccine[30]. Montanide is a large group of both water-in-oil and oil-in-water emulsions[21]. Montanide ISA- 51 was used in CIMAvax-EGF® vaccine against non-small cell lung cancer[31].

5.3. Virus-like Particles (VLPs)

VLPs are composed of self-assembling proteins (mainly capsid) lacking genome. They carry the same structural-natural properties, but they are non-replicating and non-infective as viruses. They can also be chemically-modified. They possess enhanced stability in extreme environments, particulate nature and strong immune-response (natural affinity to DCs).[18,32] Recombinant-VLPs, the first- class of nano-vaccines, have the strongest evidence for safety up-to-date. However, their manufacturing needs an in-vivo step of proteins' expression and self-assembly in a vector, followed by purification.[33] Licensed VLP-based vaccines are anti-HBV EngerixTM-B, Recombivax-HB® and Fendrix®, anti-HPV GardasilTM and CervarixTM. More anti-HBV nano-vaccines were also approved and VLPs-vaccines against malaria, Ebola, HIV, SARS are under clinical-trials.[32,18,22]

5.4. Peptide-based Nanoparticles

Self-assembling property into specific nanostructures, renders peptides promising in nano-vaccines. Their high-order molecular architectures exhibit also great importance. (nanofibers / micelles / nanotubes / nanoribbons / hydrogels).[34] Other advantages are, the biocompatibility, self-adjuvanticity and resemblance to pathogens, important for triggering immune-responses (danger-signals)[34]. For mRNA-vaccines, peptides should be cationic to interact electrostatically with nucleic-acids. Arginine-rich RALA-peptide was synthesized to deliver OVA-mRNA, inducing T-cell immu-

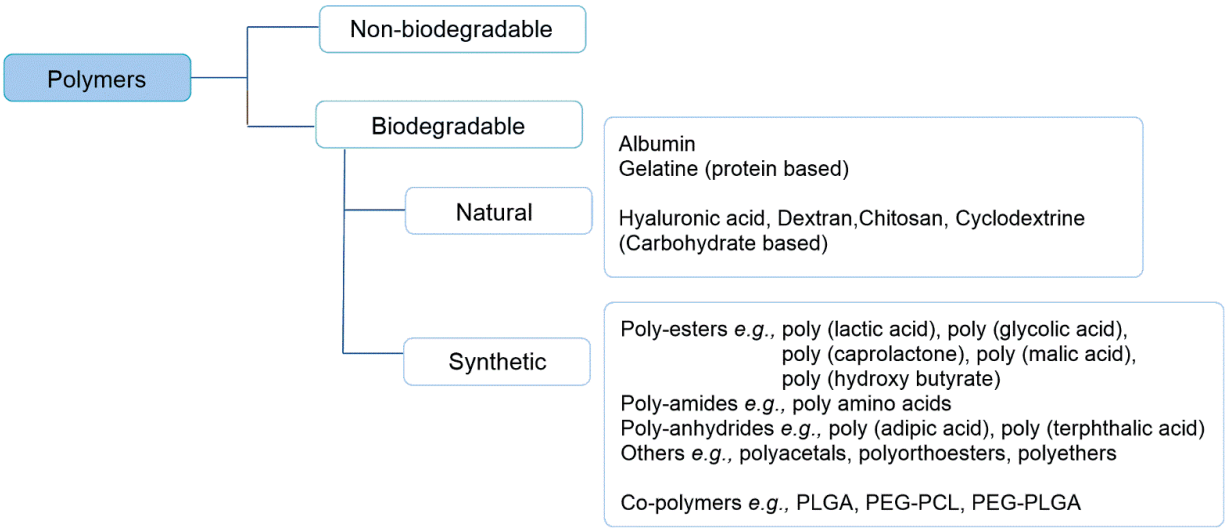

Figure 4. *Composition of polymeric-NPs used as vaccine-carriers. Adapted by Ref.* [43]

nity in mice[35]. A great challenge, though, remains the need of precise control of the self- assembling and the final architecture[34,36].

5.5. Immunostimulating complexes (ISCOMs)

ISCOMs are self-assembled pentagonal-dodecahedron structures[37] promising for vaccines against HPV, Tuberculosis and Newcastle-disease[21]. However, up-to-date they are approved only in veterinary-vaccines[2]. These negatively-charged, cage-like structures are composed of cholesterol, phospholipids, Quil-A and hydrophobic antigen. Quil-A is a purified saponin-triterpenoid extracted from the plant Quillaja Saponaria Molina and acts as surfactant. Hydrophobic interactions between saponin and lipids stabilize micelles, that spontaneously aggregate forming complex structures.[12,37,38] ISCOMATRIX, approved for human-vaccines, has the same composition with ISCOMs without including antigen and thus, can be mixed with hydrophilic antigens, overpassing ISCOMs' limitation for hydrophobic ones [21,2,33]. ISCOMs induce lymphocyte and long-lasting antibody- production as well as balanced cell-mediated and humoral immune-responses, due to inherent immune-modulating properties of saponin, while ISCOMATRIX induce mainly humoral immune- responses[2,39]. Due to the fact that Quil-A is quite toxic and with hemolytic activity, several vaccines, i.e., Mosquirix®, are based in a more purified saponin fraction, QS-21, with less toxicity and strong adjuvanticity, although being less stable than Quil-A. [12,39,40] Lastly, QB-90 saponin extracted from Quillaja Brasiliensis was investigated, resulting in stronger adjuvanticity, but further studies are needed for its toxicity[39,40].

5.6. Polymeric Nanoparticles

Humoral and cellular immunity provoked by polymeric-NPs against viral-infections has been investigated the last decade[41], following VLPs and liposome-based-nanovaccines[42]. This potential adjuvanticity derives from their tunable physicochemical properties and the sensible selection of polymer chemistry[4]. Polymers as particle-based-adjuvants are biocompatible, non-toxic[4,43], natural or synthetic[4]. Their possible structures include nanocapsules, nanoparticles, polymeric micelles, hydrogels and dendrimers[42], some of which are presented on Figure 3.

Natural polymeric-adjuvants include the polysaccharides chitosan, inulin, alginate[4,33] dextran,

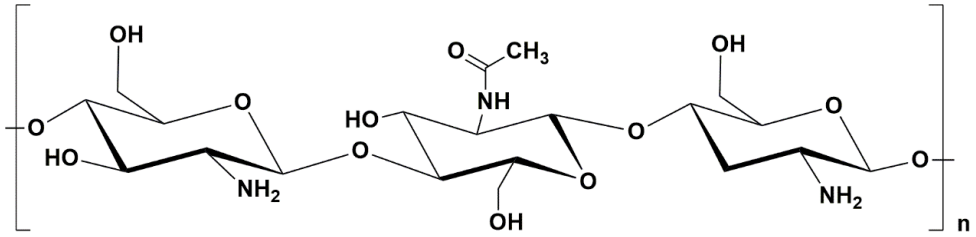

Figure 5. *Structure of chitosan polymer-chain. Adapted from Ref.* [46]

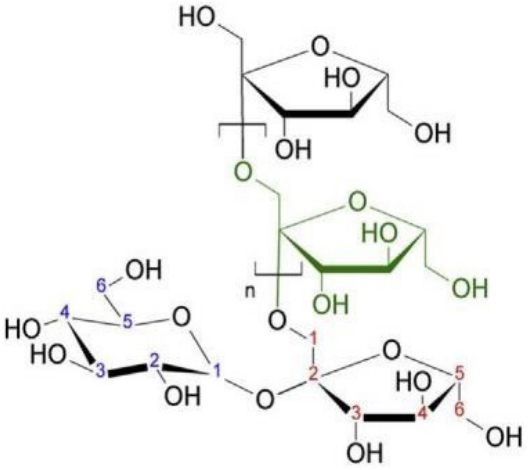

Figure 6: *Structure of a single insulin polymer-chain.* [47]

hyaluronic-acid, and cellulose[4]. Natural polymers permit chemical/physical modifications, via functional groups and anionic/cationic nature, permitting antigen and nucleic-acid loading[4]. Polysaccharides trigger the innate-immune-system through interaction with PRRs, acting as PAMPs[42]. Chitosan and its derivatives are characterized by intrinsic adjuvanticity, as they interact with the APCs-receptors[4]. In the FDA-approved nano-vaccine FluMist have been used chitosan-based- nanocomplexes[44]. *Chitosan-NPs are* promising adjuvants for HBV[33,45], influenza[45], Newcastle-disease and DNA-vaccine-development[33]. Low solubility[4,46], precipitation at physiological pH[4], inefficient targeting and residuals of organic solvents/heavy metals at chemically-modified chitosan[46], though, comprise clinical challenges. Chitosan-modification improves low solubility[46].

Delta-inulin spherulite-like discoid[47] microparticles[48] (Fig. 5), AdvaxTM, that exhibit adjuvanticity, have been examined in preclinical and clinical studies[42] for HBV, influenza and JEV[42,45,46,49].

The adjuvanticity of synthetic polymeric NPs based on polymers such as polyesters, polyanhydrides[4] and polyphosphazenes[42] has also been investigated. PLGA, the most studied polyester[2,4] (FDA[4,42] / EMA[42] approved), is biodegradable and biocompatible, while inherent adjuvanticity is ascribed to PLGA-particles[42], due to uptake by macrophages and DCs[50]. Preclinical-efforts related to

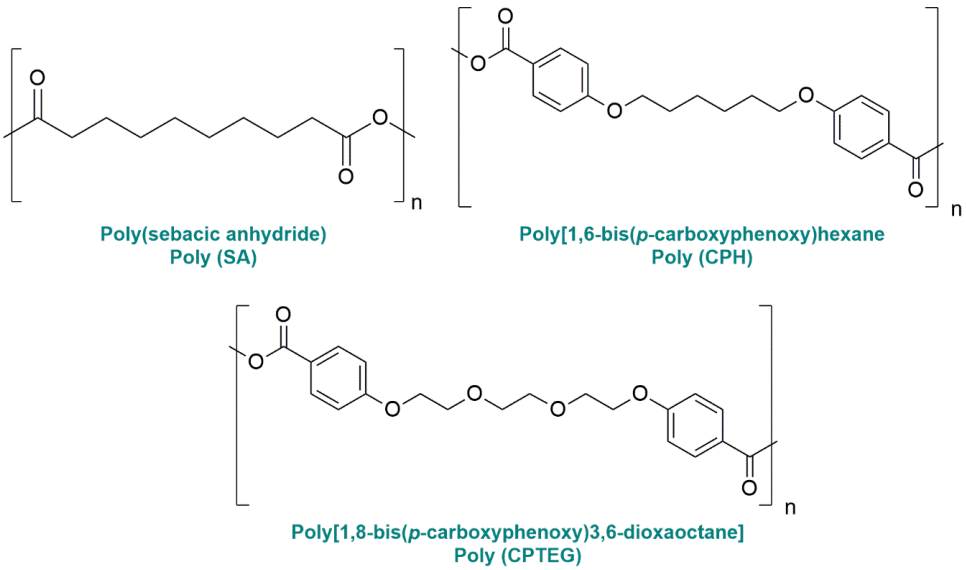

Figure 7: *Structure of the polyanhydrides Poly(SA), Poly(CPH) and Poly(CPTEG).*

PLGA-micro/NPs developed as antigen-carriers were conducted[42], while humoral and cellular immunity is reported[4,51]. PLGA-microparticulate-adjuvants were studied in clinical-trial against HIV[2,42], while PLGA-NPs in vaccines against HBV, mycobacterium tuberculosis[2], malaria[52], as well as for OVA-antigen-delivery[2,53,54]. Denaturation of protein-antigens[2], bulk erosion[4], acidic degradation products[2,4] and weak transfection efficacy[55] comprise the main problems.

Polyanhydrides are biocompatible and biodegradable polymers, already used in marketed controlled- release products (specifically Gliadel[4] and Septacin[56])[4,57]. Polyanhydride-particles are easily internalized by APCs[4,57,58], permit antigen stability[4,58,59], sustained antigen release[4,60] and possess intrinsic adjuvanticity[4,57,58] via B-cells and T-cells activation, provoking strong humoral and cellular immunity[4,58]. Monomers studied for their adjuvanticity[4,55] are CPH, CPTEG[57–59] and SA[60,61]. Polyanhydride-NPs have been investigated against influenza[4,56] and for OVA-antigen delivery[56,57]. Short shelf-life and deficiency of synthetic methods permitting narrow dispersity of polymers comprise the main challenges[56].

PCPP and PCEP are the most investigated polyphosphazenes[63], enhance antibody responses and long-lasting immunity[64]. PCPP-microparticles have been investigated for vaccine development against HBV[55], HCV[64], tetanus, OVA-antigen delivery[63] and in clinical-trials for influenza and HIV[55], while PCEP, with greater adjuvanticity[63,64], has been used for HCV[64], HBV and influenza[63] vaccines. A more cost-effective, green synthetic method would facilitate the clinical translation, while the FDA-approval of polyphosphazene-coated stents diminishes the existing regulatory concerns[65].

Dendrimers consist multivalent, well-defined, reproducible carriers for immunostimulators/antigens to be bound through their surface functional-groups, exhibiting adjuvanticity[66,67], sustained antigen-release, and enhanced antigen-uptake by APCs[68]. Peptide-dendrimers formulated as MAPs were investigated for influenza-A, HBV, HCV, HIV and malaria vaccines. Major challenges for peptide-dendrimers are the expensive synthesis and low biostability.[69] PAMAM and PEI dendrimers are the most studied for vaccine-development[42]. PAMAM-based-dendrimers exhibited intrinsic adjuvanticity when used for OVA-antigen-delivery[66] and Ebola[68,70], *Toxoplasma-godgii*[68] and influenza[66,68] vaccine-formulation. PAMAM-den-

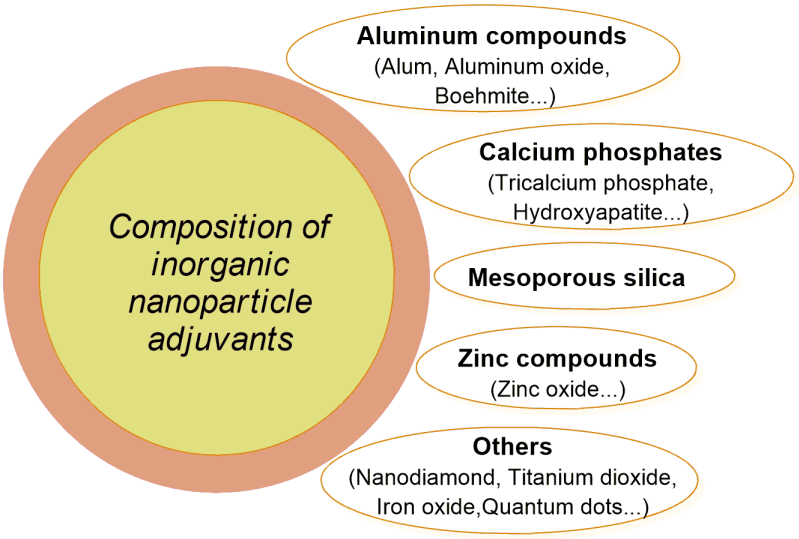

***Figure 8: Inorganic** particles possessing adjuvant-properties. Adapted from Ref.[73]*

drimer-generation is linearly-correlated with their adjuvanticity, however, cationic-dendrimers with G>G6 are potentially toxic[66]. Animal studies using PEI-based mRNA-vaccines against Ebola and influenza indicated effective immunization[42]. PAMAM and PLL-based-dendrimers have reached clinical-trials[71]. Recently, Phase I clinical trials for a PLL-dendrimer based vaccine against breast cancer indicated antibody response without adjuvant, significantly increased using the AS15-immunistimulant (MAG- Tn3/NCT02364492)[71,72].

5.7. Inorganic Nanoparticles

Even if inorganic NPs are generally non-biodegradable, their use in vaccine development is investigated, due to their controllable synthesis and stiff structure[33].

Alternative aluminum-based compounds exhibit intrinsic adjuvanticity. γ-AlOOH nanofibers and mesoporous-NPs (especially long-rod-shaped) show greater adjuvanticity than commercial alum, as well as *aluminum-oxide-NPs* (α-Al2O3), which enhance antigen cross-presentation and anti-tumor- immunity.[73] Calcium-phosphate, being biocompatible and resorbed by body[73], has been used as adjuvant in Europe, against diphtheria, tetanus,[73,74] pertussis, and poliomyelitis, in hydroxyapatite-form[74]. Calcium-phosphate-NPs are reported to be non-toxic[33], exhibit adjuvanticity[74] greater than alum, enhance antitumor-immunity[73], apart from mucosal, and were investigated for use in DNA-vaccines[33]. The clinical-translation of calcium-phosphate and aluminum-based nano-adjuvants is more feasible in comparison to the rest of inorganic-NPs, since alum and calcium-phosphate have already been used as adjuvants[73], but further research is required[74].

Metallic NPs are reported to enhance APCs-activation, cytokine-production, and humoral-immune- responses[75]. Due to the various shapes (spherical, cubic, rod), the size range of 2-150 nm and the easy surface-modification with carbohydrates, *AuNPs,* FDA-approved for phase-II clinical-trials[73], are the most commonly investigated as antigen-delivery-systems (i.e., influenza, RSV)[33].

AuNPs were used as adjuvants[73], in early-experimental level, in vaccines against HIV[33] and SARS-CoV [76] and for OVA-antigen-delivery[73]. IONPs, FDA-approved for clinical-use[73], are studied for their adjuvanticity[75], reported as greater than that of alum[73]. IONPs have enhanced immune- response against malaria[73] and, as DNA-vaccine-carriers, against malaria and melanoma[77]. AgNPs are reported to exhibit adjuvanticity and were used for influenza vaccine-development and OVA-antigen delivery, however, they are mainly investigated for antiviral-treatment[76]. Zinc-oxide- NPs exhibit strong adjuvanticity, however, high-toxicity is reported at high doses[73].

Mesoporous-SiNPs are biodegradable, have increased loading-capacity[33] and enhanced cellular-recognition and uptake by APCs, due to their easy modification through surface-silanol-groups[18,33] and their small size[73]. Major concern is the toxicity of reducing-agents and stabilizers used during synthesis[18], although they are FDA-approved for clinical-trials[73].

CNPs are reported to exhibit adjuvanticity[18,33]. CNTs, possessing large surface-area, allow antigen/adjuvant-delivery in large amounts to APCs 78 and amplify the IgG-response[33]. Mesoporous-CNPs are investigated as oral-vaccine adjuvants[33]. However, limited biocompatibility and biodegradability impede clinical-trials[78].

Thus, the understanding of the molecular mechanism of action[73,76] and the effects of composition and physicochemical characteristics of inorganic-NPs[73] is pivotal, for translation to clinical-trials.

6. Vaccine formulations under development and recombinant technology: The example of Novavax's vaccine

On Table 2, indicative examples of vaccine-development trials of different stages against the viruses MERS, SARS, Ebola and Zika are presented, denoting the gradually augmented use of novel adjuvants in vaccine research and development. The ongoing pandemic of SARS-CoV-2 designates the importance of the development of effective and safe vaccines, as vaccination is considered as an efficient way for the pandemic to come to an end. A number of vaccines have already been developed. Notably, a part of them, presented on Table 2, fall under nanovaccines.

Among SARS-CoV-2 nanovaccines, NVX-CoV2373 vaccine, developed by Novavax, is particularly interesting, as recombinant technology for nanoparticle formation and nano-adjuvants are combined[79,80]. Specifically, Novavax is a subunit vaccine based on VLPs[79] composed of the full-length (trans-membrane domain included) SARS-CoV-2 spike (S) glycoprotein and the adjuvant Matrix-MTM[80].

The S protein is the key for subunit COVID-19 vaccine development, as it renders feasible the binding of the virus on the human angiotensin-converting enzyme 2 (hACE2) receptor to enter host cells[80]. For the formation of the recombinant SARS-CoV-2 NPs, cultures of insect cells are infected by a genetically engineered baculovirus, to produce S protein trimers. After chromatographic purification, the timers are combined with polysorbate 80. Polysorbate 80 forms a micellar core, with which the trimers interact hydrophobically and, thus, manage to be held together. The assembled S trimers are organized in rosettes. The formulated recombinant protein NPs (VLPs) are combined with the adjuvant Matrix MTM81 right before the injection[79].

Matrix MTM comprises a proprietary adjuvant of Novavax, that is an ISCOM, based on saponin deriving from *Quillaja saponaria Molina tree. Matrix M*TM is composed of two different categories of 40 nm sized NPs, based on different saponin fractions, that complete each other's properties.[82] During the clinical trials of NVX-CoV2373 vaccine, antibody and CD4+ T-cell responses were increased with the use of the adjuvant, while induction of functional adjuvants was, also, observed[80]. It is deemed that enhanced dendritic cell uptake and APCs presentation is provoked by this adjuvant, leading to increased maturation of the immune cells and local transient pro-inflammatory response[79, 83].

Recombinant technology has been used for the

formation of other vaccines as well, with or without adjuvant addition, as it can be observed on Table 2. Apart from recombinant VLPs, recombinant viral vectors *e.g., for Pox and MVA and recombinant adenovirus vaccines have been extensively studied,* as well84. Apparently, recombinant NPs which are based on recombinant proteins, hold a great promise for vaccine development. The main advantage of recombinant NPs is the low toxicity and the low cost of production, in comparison with the conventional materials used, such as synthetic/natural polymers, metals or even lipids. The broad application of those NPs in nano- medical applications is attributed to their inherent homogeneity, as well as the ease of modification to form multifunctional molecules.

Site directed or random mutagenesis permit the introduction of desired functional groups or the desired hydrophilicity/hydrophobicity. Gene fusion, on the other hand, allows for the introduction of new functionalities (peptides, other proteins) that may, among others, enhance cellular uptake, in a less expensive way than the high-cost subsequent conjugation chemistry. Consequently, all those advantages justify the gradually increased production of recombinant nanoparticles, where recombinant technology and nanotechnology meat each other.[85]

7. Physicochemical characteristics

The numerous advantages of NPs as nano-carriers in vaccines are due to their unique physicochemical characteristics and pharmacokinetic behavior. Nano-vaccine's properties and in vivo effectiveness depend on adjuvants' physicochemical characteristics.

7.1. Size

Cytokine response induction, cellular-uptake, internalization, cellular-specificity and migration are determined by NPs' size[124,125]. Larger NPs (1, 7 & 17 μm) present much lower internalization capacity than smaller NPs (300 nm)[126], while NPs of maximum particles' size of 200nm are channeled to lymph-nodes[127]. Signaling-pathways were also proved to get activated more efficiently by small NPs[128].

7.2. Shape

Cellular-interactions, intracellular-circulation and antigen-release into host-cells are affected by the shape of NPs[129,130]. Studies have shown that spherical NPs are more effectively internalized compared to similar sized rod-shape NPs[131]. The latter type of NPs, as well as cubic NPs have also presented reduced ability in provoking immune-response compared to spherical[132].

7.3. Surface Charge

Immune response induced by nano-adjuvants is also influenced by their surface charge. Electrostatic binding to heparan sulfate proteoglycans on cell surface boosts antigen-response by APCs. Therefore, the interaction of APCs with cationic-NPs is more efficient than the interaction with neutral or anionic ones.[133,134] System's stability, transmembrane permeability and adsorption of NPs are strongly affected by surface charge as well.

7.4. Hydrophobicity

Hydrophobicity of NPs is crucial to immune-response. Soluble proteins and cells of immune-system are allowed or obstructed to interact with NPs depending on their hydrophobicity[135]. It was reported that hydrophobic moieties of NPs can induce stronger cell-signaling process by cytokines and increased rate of immune-cells activation than hydrophilic ones[136,137,138].

Hydrophobic parts also promote opsonization, by enhancing antibody-adsorption on the cell-surface[139]. On the other hand, different studies have presented that PEGylation on the surface of NPs decreased interaction rate and binding to receptors of immune-system[140,141].

Table 2: Examples of vaccine-formulations, marketed or in several development levels, for MERS, SARS, EBOLA and ZIKA viruses.

Vaccine Type / Name	Carrier Platform	Co-adjuvant	Antigen	Route of administration	Stage	Ref.
MERS (2012 - now)						
GLS-5300 (Plasmid-DNA pGX9101-based)	–	–	MERS-CoV S GP	I.M	Phase I/II(2020)	Inovio/ GeneOne LifeSciences [86],[87]
MVA-MERS-S	Recombinant MVA vector	–	MERS-CoV S GP	I.M	Phase I (2020)	University of Hamburg-Eppendorf[86],[88]
ChAdOx1 MERS (RNA-based)	Chimpanzee adenoviral vector	–	MERS-CoV S GP	I.M	Phase I (2020)	Oxford University[86],[89]
–	Spike nanoparticles	Matrix-M1	MERS-CoV S GP	I.M	Preclinical	[90],[91]
–	SAPNs	Alum	MERS-CoV S GP	I.M	Preclinical	[90],[92],[93]
–	VLPs	Alum or poly(I:C)	RBD of MERS-CoV S protein	I.M	Early stage	[92],[94]
–	Ferritin-based nanoassembly	Alum or MF59	RBD of MERS-CoV S protein	I.M	Early Stage	[95]
SARS (2003 - now)						
VRC-SRSDNA015-00-VP (plasmid-DNA-based)	–	–	SARS S GP (SΔCD)	I.M	Phase I (2017)	NIAID [96],[97]
–	Gold	–	SARS S GP	S.C	Early Stage	[98]
Plasmid-DNA-based	Biotinylated Chitosan	Anti-CD40 monoclonal antibody (aCD40 mAb)	SARS-CoV N protein	I.N/I.M	Early Stage	[99]
Plasmid-DNA-based	PEI	–	SARS-CoV S protein	I.N	Early Stage	[100]
–	Self-assembled polypeptide nanoparticle	–	SARS-CoV S GP	I.P	Early stage	[101]
SARS-CoV-2 (2019 - now)						
mRNA-1273 (mRNA-based)	LNPs	–	SARS-CoV-2 S protein	I.M	Phase III/Marketed	Moderna [79],[102]
BNT162b2 (mRNA-based)	LNPs	–	RBD of S1 protein	I.M	Phase III/ FDA EUA (2020)	Pfizer/BioNTech[79],[103]
NVX-CoV2373 (subunit vaccine)	VLPs	Saponin-based Matrix-M	SARS-CoV-2 recombinant S protein	I.M	Phase II/III (2020)	Novavax [79],[104]
COVAX-19/NCT04453852 (subunit vaccine)	–	Advax™	COVID-19 recombinant S protein	n.m	Phase I (2020)	Vaxine [105],[106]

Table 2. *(Continued)*

Vaccine Type / Name	Carrier Platform	Co-adjuvant	Antigen	Route of administration	Stage	Ref.
EBOLA VIRUS (1976 - now)						
rVSVΔG–ZEBOV-GP (Ervebo®)	Live attenuated virus	–	ZEBOV GP	I.M	Approved (2019)	Merck & Co [107],[108]
Ad26.ZEBOV/MVA-BN (Prime-boost Zabdeno®/ Mvabea®)	Recombinant Ad26 and MVA vectors	–	ZEBOV Mayinga variant GP	I.M	Approved (2020)	Johnson & Johnson [107],[109]
EBOV GP	Recombinant GP NPs	Saponin-based Matrix-M™	EBOV/Makona GP	I.M	Phase I (2016)	Novavax [107],[110]
mRNA-based	LNPs	–	EBOV GP	I.M	Early stage	[111]
–	VLPs	QS-21	SUDV/EBOV VP40, NP, GP	I.M	Preclinical	[112],[113]
–	VLPs	Ribi Adjuvant System® (o/w emulsion)	EBOV VP40, NP, GP	I.M	Preclinical	[113],[114]
–	VLPs	GLA-SE or CpG-ODN (o/w emulsion)	EBOV VP40, NP, GP	I.M	Preclinical	[113],[115]
KUN VLPs	Kunjin replicon VLPs	–	EBOV GP with a D637L mutation (GP/D637L)	I.M	Preclinical	[113],[116]
ZIKA VIRUS (1996 - now)						
BBV121	VLPs	Alum	Whole virion	I.M	Phase I (2018)	Bharat Biotech [117],[118],[119],[120]
ZIKV envelope dimers	Protein NPs	Alum or Saponin-based Matrix-M™	ZIKV E protein	n.m	Preclinical	Novavax [117],[119]
–	Biodegradable NPs	–	HLA-peptide	n.m	Early stage	Mayo Clinic [117],[121]
mRNA-1893 (mRNA-based)	VLPs	–	prM-E	n.m	Phase I (estimated 2021)	Moderna [118]
mRNA-based	LNPs	–	prM-E	n.m	Early stage	Moderna [117],[121]
Self-amplifying mRNA	Modified Dendrimer	–	prM-E	I.M	Preclinical	[122]
GLS-5700 (Plasmid-DNA-based)	–	–	prM-E	I.D	Phase I (2017)	Inovio/GeneOne Life Sciences [118],[119],[123]
TAK-426	Inactivated virus	Alum	Whole virion	I.M	Phase I (2020)	Takeda [118],[119],[123]

Ad26: *Adenovirus 26,* ***CoV:*** *Corona Virus,* ***COVID-19:*** *Corona Virus Disease 2019,* ***CpG-ODN:*** *CpG-oligonucleotide,* ***EBOV:*** *Ebola virus,* ***EUA:*** *Emergency Use Authorization,* ***GLA-SE:*** *Glucopyranosyl lipid A – stable emulsion,* ***GP:*** *Glycoprotein,* ***HLA:*** *Human Leukocyte Antigen,* ***I.D:*** *intradermal,* ***I.M:*** *intramuscular,* ***I.N:*** *intranasal,* ***I.P:*** *intraperitoneal,* ***LNPs:*** *lipid nanoparticles,* ***MERS:*** *Middle East Respiratory Syndrome,* ***MVA:*** *Modified Vaccinia Ankara,* ***n.m****: not mentioned,* ***NPs:*** *nanoparticles* ***NIAID:*** *National Institute of Allergy and Infectious Diseases,* ***NP:*** *Nucleoprotein,* ***o/w:*** *oil in water emulsion,* ***PEI****: Polyethyleneimine,* ***Poly(I:C):*** *Polyriboinosinic acid,* ***prM-E:*** *premembrane and envelope proteins,* ***RBD:*** *Receptor Binding Domain,* ***S.C:*** *subcutaneous,* ***SAPNs:*** *Self-assembling protein nanoparticles,* ***SARS:*** *Severe Acute Respiratory Syndrome,* ***SUDV:*** *Sudan virus,* ***SΔCD:*** *cytoplasmic domain truncated,* ***VLPs:*** *virus-like nanoparticles,* ***VP40:*** *Viral-matrix protein,* ***VSV:*** *Vesicular stomatitis virus,* ***ZEBOV:*** *Zaire Ebola virus,* ***ZIKV:*** *Zika virus*

7.5. Surface-Modification

Surface-modification of NPs changes their specificity and the interactions of their ligands with APCs[142]. For instance, it is reposted that CD47 proteins on the surface of NPs decreased internalization by immune cells[143]. Modification of NPs with TLR agonists, on the other hand, resulted to higher cytokine-levels and immune-regulatory genes expression[144]. Conjugation of some other TLR agonists (TLR2, TLR4) and galactose polymer to NPs were found to activate the complement-cascade, due to stable binding to C3b complement-factor[145]. The above findings indicate that physicochemical-characteristics of NPs are considered a critical tool for vaccine-adjuvants' targeting and induction of the desired immune-response.

8. Challenges and Future Perspectives

Despite the multiple benefits of nano-vaccines, some points need to be considered and further investigated. Several adjuvants have appeared to be locally and systemically toxic. For example, Aluminum-adjuvants have been reported to induce systemic-toxicity.[147] Moreover, excessive RES-uptake enhanced by the NPs' corona-coating needs to be surmounted[148]. Regulatory aspects requiring clinical trials to validate safety of nano-vaccines to humans appears to be another barrier. Stability of nano-vaccines is also an issue that should be considered. For instance, liposomal structure enables encapsulation of cargo which could lead to structural-instability.[149,150] Lastly, significant challenge is the cost of nano-vaccines' production as ligands'-loading and surface-functionalization in a single nano-carrier may be extremely difficult and expensive.

Research and development of nano-vaccines in the last few years comprise an exceptional evolution in the field of medicine. Due to their benefits and unique characteristics, they are more and more investigated with remarkable research outcomes that have already been reported. Strong and well- established understanding of all aspects of the diseases is the first step to design an effective vaccine where nano-adjuvants stand out.

Target-specific delivery, prolonged circulation, lack of major side-effects, biocompatibility, low toxicity and induced immune-response consist some of the advantages of nano-vaccines that renders them promising to treat several diseases.[151] Controlled release and stimulation of immune- response can be achieved with antigen-loading into NPs.

9. Conclusion

In this review, the role, mechanisms, types, and physicochemical characteristics of novel-adjuvants are summarized. Several attempts concerning the use of nano-vaccines for the fight against diseases such as Ebola, Zika, SARS and MERS, are also highlighted. Scientific research has evidenced the numerous advantages of nano-adjuvants. However, various challenges exist along with the future- perspectives of those promising adjuvants and are of main concern. Several innovative nano- adjuvants have already been approved and many other are being investigated. Hopefully, it is expected that nano-adjuvants will be introduced in even more clinical-products in future, offering unique possibilities and holding the promise for eradication of many challenging diseases, even pandemics. □

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PHARMAKEFTIKI, 33, III, 2021 | 160-169 REVIEW ARTICLE

Liquid Crystals in Cosmetics: Technological Forms, Challenges and Opportunities

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ABSTRACT

Nanotechnology in cosmetic formulations is perceived to be cutting edge and one of the most innovative technologies possible in recent years. Many researchers study nanoparticles' use to improve skin permeability, long-lasting effects, high moisture efficiency, skin barrier regeneration, formula stability, and more. Liquid crystals are one of the latest cosmetic product applications becoming popular in local and international markets. This study aims to examine the technological forms, difficulties, and opportunities of liquid crystal applications in cosmetics objectively.

1. Introduction

Liquid crystals (LC) comprise a distinct form of matter between a crystal and a liquid with multiple possible phases that can also be interchangeable depending on phase-specific factors such as temperature and the concentration of the LC in an aqueous (most often) solution[1].

1.1 Historic Review

The early observation of peculiar physicochemical properties such as the existence of two different melting temperatures in liquids containing large biomolecules by Reinitzer[2], led to the characterization of an intermediate 'crystalline' phase, using polarized light and crystallography methods, by Lehmann[3] who also coined the term LC. Later, in 1957 Alder notably reported the crystallization of colloidal fluids under high pressure[4]. The interest in LC kept growing, with many scientists and labs undertaking research that led to the synthesis of the majority of the currently known LC forms by the beginning of the 20th century and enabled further research on possible applications[5].

1.2 Main LC types

Anisotropy determines the behavior of LC. This can change their significant properties depending on their constituents' axial direction[6,7], called mesogens. LC ('continuum theory') characterization was initially based on mesogen shape, their ability to move and form ordered structures. These structures (mesophases) consist of a combination of flexible and inflexible parts resulting in various states of ordered arrangement and fluidity, responsible for specific properties such as elasticity and viscosity[1,5]. However, several initially developed LC forms lost their anisotropy and returned to their liquid phase at high temperatures because heat disrupted the ordered arrangement of mesogens. That led to the characterization of thermotropic LC[6]. Thermotropic LC mesophases are summarized in Table 1.

Lyotropic LCs comprise another main category, including mesogens, usually self-assembled amphiphilic molecules of specific concentration in the appropriate solvent and under specific pressure and temperature conditions. The solvent molecules are interposed between the 'flexible' mesogen segments and allow for their degrees of freedom and the 'elastic fluidity' of lyotropic LC[7]. The amphiphilic mesogens' concentration determines the potential shape of their self- assembly and the order of their organization[5,7]. An overview of the various phases and types is shown in Table 1.

2 LC and living tissue

Biological tissues behave like lyotropic LC (biological LC)[8]. Examples include the biphasic nature of the lipid bilayer in somatic cells, the metastability properties of the cell membrane, and the epithelium's functional LC properties when it discards 'unwanted' cells[9]. The phospholipidic bilayers have excellent self-assembly capabilities, forming various structures starting from the simple micellar spheres up to the more complex cubosomes and microemulsions[1,5,9]. Other biological macromolecular solutions containing proteins and polypeptides act like LC and form structural (cytoskeleton) and functional (enzymatic membranes) systems in living tissues[9,10].

LC in treatments implies that their interaction with living tissue must be safe, effective, reliable, repeatable and tissue/lesion-specific. Due to the amphiphilic properties of lyotropic LC (LLC).
They can incorporate hydrophilic and hydrophobic drugs and exhibit biocompatibility, bioadhesive, trans-tissue permeability with sustained-release delivery properties[11]. The release rate of an included drug depends primarily on the dimensions of the LLC system. Therefore, simpler systems such as lamellar, which allow the more effortless internal movement of the solvent, release the drug faster than more complex ones, such as the reverse cubic that is a closed system of tightly stacked inverse micelles that can sustain the drug release much longer[11,12]. By changing other conditions (pH, temperature, light, magnetic field), previous LLC phases can be switched into another (e.g., the hexagonal to cubic) to achieve the desired effect, such as the slower release of an active molecule. These conditions affect the Critical Packing Parameter (CPP), the degree of spatial stacking of the constituent amphiphilic mesogens that form the LLC system. CPP depends on the surface of the LLC molecules' hydrophobic tails and hydrophilic heads[13].

3. LC and cosmetics

In cosmetics, treatment goals include but are not limited to protection from sunlight/barrier function from the environment, hydration optimization, skin repair and nourishment, skin quality and appearance improvement (pigmentation, tightness, cellular turnover, etc.), safety/non-toxicity. Microemulsions have a central role in skin and cosmetic product development. They enable the stable dispersion of a water-soluble substance within multiple protected reversed micelles in an oil solvent. Skin application of microemulsion resulted in transient thickening of the epidermis, skin lightening, and increased cell turnover[14], initiating and promoting skin self-repair mechanisms. For example, the monoolein drug delivery system revolutionized microemulsion use in skin treatments because it is non-toxic, biocompat-

ible, and biodegradable via enzymatic lipolysis[15].

In cosmetics, the desired properties of an LLC system often include the ability to change mesophases at different temperatures, act as an emulsifying agent in hydrocarbon bases for ointments, control viscosity, and rheological behavior retain and release active substances depending on the environment[8,16]. LLC systems in cosmetics are up-and-coming but still under constant investigation and development. The real-time behavior of mesophases, longevity, drug release efficacy of the system, long-term effects following interactions with living tissues, availability and cost-effectiveness evaluation are only a few things we still do not know about them[16].

4. Drug delivery-skin permeability

LLC as drug delivery systems bears the advantage of localized drug administration within the stratum corneum (SC). Estracanholli et al.[17] incorporated celecoxib into the cubic phase of an LLC system that, when applied topically, the drug systemic circulation release is minimal. This is also supported by Lopes et al.[18], who used the hexagonal phase as a topical delivery system for vitamin K. That way, the active substance was localized in the SC, avoiding side effects. Furthermore, ringing gels (cubic LC-based system of optically transparent surfactant gels) have been used for topical administration of NSAIDs, such as ibuprofen, ethyl salicylate, and methyl nicotinate, and some formulations based on this technology are currently on the market. The cubic phase may interact with the SC structure, forming a cubosome depot that will lead to a sustained drug release[19]. In addition, due to the cubic phase's ability to mimic the SC lipids, greater bioadhesion to the skin can be achieved[20]. Finally, Nesseem et al.[21] developed an itraconazole LC formulation for topical delivery and observed a more potent inhibition against Candida albicans than a control formulation.

4.1 Drug delivery-cosmetic

Efficient, targeted drug delivery in cosmetic and skin treatments is of paramount importance. Therefore, the particular LLC mesophase is also critical. It was shown in vitro that cubosomes and hexosomes follow a specific pattern regarding drug release kinetics[22]. Initially, a high percentage of the incorporated active substance is released rapidly before a constant release is achieved. This process might be triggered by endogenous and exogenous stimuli that cause phase changes on the LLC[23,24]. Moreover, the hexagonal phase usually releases its content slower than the cubic phase[13,24].

The active molecule's effectiveness following its delivery to the target is strongly associated with LCC use in cosmetic and skin treatments. For example, Sherif et al.[25] found that alpha-lipoic acid used as a local skin rejuvenating and the anti-wrinkle agent had improved results when used in the form of cubosomes. Although this study's importance is highlighted due to including human volunteers and in vivo evaluations, the small sample affects its statistical significance. Musashi et al.[26] also obtained the same findings and limitations after using LLC gels to improve skin tone.

Kadhum et al.[27] reported ways to increase LLC delivery systems efficiency in passing active drugs through the skin, showing the comparative superiority of cubic LLC especially when the carried drugs are hydrophilic. Previously used in cosmetics, non-lamellar forms systems showed less efficient skin permeability due to their higher viscosity and larger active molecules. Similarly, the superiority of cubosomes over solid lipid nanoparticles in skin moisturization and repair was also reported by Esposito et al.[15].

LLC systems seemed to increase resveratrol's bioavailability to the skin when used in preloaded cubosomes[28] and quercetin when loaded in non-lamellar systems[29], justifying the growing importance of the LLC systems in the local skin application of antioxidants and anti-aging agents. However, such agents are usually sensitive to factors that alter their chemical status, resulting in low product stability, efficacy, and high skin irritancy. Therefore, they need unique delivery systems to drive them through the epidermis, maximizing their functionality.

The efficiency of phosphatidylcholine (PC) based

LLC systems for drug delivery was further investigated by Martiel et al.[30]. They investigated the possibility of creating non-lamellar mesophases with PC to observe materials with a natural profile. This is a challenge since PC tends to create lamellar structures, and in order to overcome this, the authors suggested the addition of Cyclopentasiloxane or (R)-(+)-limonene or vitamin E. Triacylglycerides and fatty esters are not suggested to overcome this.

On another note, Zasada et al.[31] suggested the improved effects of retinol on the skin using an LLC system other than lipid-based. They developed a novel LC formula containing retinol that had a comparative advantage in providing optimum retinol effectiveness, substantially more significant increases in EGF expression (role in wound healing), and less pro-inflammation response. However, additional studies using more conventional vehicles such as oil/water and water/oil must establish LLC's role on retinol.

Phytantriol based drug delivery systems are of particular interest in cosmetic applications. They seem to increase the deposition of active skin preparations and compounds such as panthenol and various peptides for skin repair and rejuvenation. The specific role of cubic and hexagonal such LLC systems, the challenge of customizing their internal water channels, and their drug delivery potential are further analyzed by Akbar et al.[32].

By increasing the bioadhesion of an active molecule on the skin, its desired action can be prolonged. In this way, it was possible to prolong mosquito repellents' action by formulating them in cubic LLC systems. More specifically, a tea tree oil-based cubic LC system was developed as an alternative to N, N-diethyl-meta-toluamide (DEET) lotion, a commercial mosquito repellent that can cause skin irritation and dermatitis[33]. The supramolecular assembly of cubic systems decreased the diffusion of volatile compounds from the supramolecular networks and exhibited higher viscosity and greater bioadhesion, resulting in a prolonged effect. Although the commercial DEET lotion exhibited a better repellency, the LC system is a promising alternative repellent.

Li et al.[34] compared both in vivo and in vitro LLC and standard o/w cream to deliver 3-Oethyl- ascorbic acid (EA) and potassium4-methoxysalicylate (4-MSK) in the skin to treat hyperpigmentation by stopping melanogenesis and promoting depigmentation. LLC delivery system allowed efficient bioadhesion of the two active ingredients with low toxicity. In addition, the cream delivered a higher amount of drug to the skin than to the muscle, with low systematic exposure, comprising a stable structure with greater thixotropy than the traditional formula.

4.2 Stability

The LLC system's stability as a drug delivery system is of great significance, especially when localized treatment is required. For example, Chu et al.[35] showed that the efficient transdermal release of simonene hydrochloride was achieved using an LLC delivery system, which posed difficulties in establishing the drug's stability and sustained release. Nevertheless, formulations that contained higher drug percentages increased the skin penetration rate but simultaneously altered the LLC structure and negatively affected the release process's kinetics. Consequently, the way to find the right concentration of the incorporated drug remains a challenge.

Jia B. et al.[36] also investigated the stability issue and reported significant improvement in multiemulsions stability by covering their droplets with LLC. These findings agreed with da Rocha-Filho P et al.[37] one year earlier, who found that sunflower oil can retain its antioxidants in emulsions in high temperatures during the emulsification process when used in the form of stable LLC microemulsion. Their findings emphasized the importance of the type of the used substance, its physicochemical properties, and molecular interaction potential, and the LLC's inherent stability. However, this study's limitations included that the emulsions' pH values had an acidic character, a trait that was not appropriate for skin use and should be corrected. Besides, the primary stability tests performed in this analysis are not meant to predict the product's lifespan but rather to aid in screening formulations.

Similarly, stability associated issues include LCC

Table 1: Brief description of every Liquid Crystal superategory, its phases and their mesogen molecular shape

Supercategory	Phase	Mesogen molecular shape	Description
Thermotropic	Nematic (low molar mass)	Rods	Multiple self-aligned rod-like mesogens along usually one and less often two axes. Standard rod form includes a 'flexible' molecular 'tail and rigid molecular 'core'. No ordered layers or position, just pointing in the same direction. Considered more fluid (isotropic and homogenous) than solid.
	Smectic A (low molar mass)	Rods	Multiple rod-like molecules are usually stratified into layers or planes and well oriented along a specific axis parallel to the elastic fluidic mobility of the LC system (long axis). Motion is possible within these layers
	Smectic C (low molar mass)	Rods	Like Smectic A but aligned along an axis that is at an angle to the long axis
	Columnar Discotic (low molar mass)	Discs (simple, rectangular, hexagonal)	Multiple disc-like molecules aligned along one axis, forming layers columns. Columns then remain either random or form rectangular or hexagonal shapes
	Nematic Discotic (low molar mass)	Discs	Multiple disc-like molecules aligned along one axis like nematic ones. Ordered layers
	Chiral nematic (low molar mass)	Rods (cholesteric)	Like nematic but showing a gradual twisting (spiral) of their alignment axis. It takes place incrementally along the axis, forming distinct layers of different orientation and depends on the temperature
	Conic (low molar mass)	Pyramidic, conical	They can form long columns or form as transitional mesophases in smectic and other systems
	Blue (low molar mass)	Cubic with internal defects (chiral nematic)	Intermediate phases forming, especially when cooling down LC. The actual mesogenic shape is chiral nematic rods stacked irregularly, forming cubes with internal gaps (lattices) in a very narrow temperature window.
	Other (low molar mass)	Originating mostly from nematic	Include forms like onion, donut, string bean, bowls

	Sidechain polymers (high molar mass)	Chain branch	Acrylic or siloxane polymers, parts of a larger backbone. Usually, copolymers that develop by grafting various side chains on a longer backbone.
	Main chain polymers (high molar mass)	Elongated chain	The macromolecular backbones of the previous type.
Metallotropic		Variable (lamellar, cubic, hexagonal tetrahedral) (aka metallomesogens)	Developed by the addition of inorganic (such as Zn, Ni, Cd, Co, Cu, Fe) molecules in the anisotropic assembly of lyotropic LC,
Lyotropic	Standard micellar	Single micellar sphere	Amphiphilic surfactant molecules (polar lipids, polymers) that self- assemble to produce the simplest, more thermodynamically stable structure
Increasing Amphiphile	Standard hexagonal	Cylindrical	Amphiphilic surfactant molecules aggregate and form micellar cylindrical structures packed in a hexagonal fashion
Concentration	Standard cubic	Discontinuous cubic	Separate micelles (discontinuous) stacked in cubic symmetry. Includes two continuous but not overlapping hydrophilic regions divided by lipid bilayer
	Lamellar	Sheet like, membranes	Amphiphilic surfactant molecules forming true bilayers organized into separate layers (lamellae)
	Microemulsion		Water and oil dispersions with the addition of the appropriate surfactant (and co-surfactant) produce thermodynamically stable optically transparent systems and of water- like viscosity (much more stable than macroemulsions and with much smaller parts)
	Reversed cubic	Bicontin uous cubic	Hydrophobic tail larger than hydrophilic head resulting in an inverted curvature of its bilayers toward the polar medium than that in standard cubic phases
	Reversed hexagonal	hexagonal	Like the non-reversed type but with inverted curvature of its bilayers toward the polar medium than that in standard hexagonal phases
	Reversed micellar	micellar	Micelle with the hydrophobic tails externally and the hydrophilic heads internally

emulsion production, application, and self-life. Zhang et al.[38] offer further insight as they found that LLC emulsions show adequate elastic properties to last for 18 months in storage. However, at the same time, they may lose some of their ability to transform into a more liquid-like state (needed for drug release) when applied to the skin.

To maintain its biochemical activity and prevent the aging process, vitamin C must be kept stable in its free form. In this study, the stabilization of vitamin C seems promising as it is stabilized for more than four months when encapsulated within droplets of the α-gel phase. This formulation is also very promising because of the well-known favorable properties of the α-gel process in cosmetic products[39].

4.3 Skin barrier

Alternatively, LLC interaction with the skin may not affect a carried substance's ability to enter through skin layers. However, it may create an effective barrier that would improve skin hydration, promote skin repair mechanisms, or protect deeper skin layers and systemic circulation by stopping an undesired agent. For this reason, Bialas et al.[40] developed a liposome/alcohol/water suspension resembling the stratum corneum layer of the epidermis and acting as an artificial membrane/barrier that limits hair dyes transdermal passage.

Wang et al.[41] showed the improved skin barrier effect of a wax-based LLC formulation, which alters friction on the LLC/skin interface depending on skin moisture level. The amount of LC content was proportional to its moisturizing effectiveness and skin barrier function. These long- lasting effects have been due to a reduction in trans-epidermal water depletion.

The development process and biomimetic efficiency of lamellar LLC in forming a skin barrier improving skin moisturization were evaluated in vivo by Ahn Y-H et al.[42]. The results showed a comparative superiority in retaining the skin moisturizing and promoting the damaged tissue regenerating ability without causing significant discomfort. This can be due to low cytotoxicity relative to other general surfactants. However, there is a need for more in vivo studies, including more participants.

Similarly, skin barrier properties in oil/water microemulsions were reported by Kim et al.[43], who developed an optimized method that ensured a formulation with skin-temperature stability. As a result, they found that the moisturization function of the skin and water depletion prevention effects are significantly enhanced simultaneously. Although, there is space for further determination of the right concentration of LC, preserving the total effect on skin without becoming greasy.

Additionally, Iwai et al.[44] showed the comparative superiority of lamellar LLC gel in preventing water loss and increasing epidermal hydration by creating a stable skin barrier over standard emulsions. They developed a new type of skincare product that contained a synthesized pseudo- ceramide, similar to the ceramides found among the SC lipids. It exhibited higher water holding capacity in vitro and a significant moisturizing effect in vivo (especially the periorbital area) than conventional oil/water and water/oil emulsions.

5. Special forms

LLC systems based on hair-specific agents that improve hair dye and hair repairing activity were evaluated by Nagasawa et al.[45]. They designed a new LLC-based additive for hair care products called HAIRCARECUBE (HCC). HCC increased the penetration of pigments and other active ingredients into the hair fibers' core, effective at low concentrations and stable under a wide pH range. Moreover, due to its solubility and stability in water, HCC can be easily blended into various hair products without affecting their stability. However, more studies need to be carried out about its compatibility with other active ingredients.

The use of LLC shampoo improved silicon deposition on dyed hair, according to Brown et al.[46], who developed an LC colloidal structure to increase silicon's conditioning effect. The resulting increase in the shampoo's hydrophobicity increased the deposition of silicone contained in the shampoo.

LLC cleansing formulations achieved improved removal of the organic and inorganic burden from the surface of the skin, according to Yeo et al.[47]. They presented an LC system consisting of nonionic surfactants and polyhydric alcohols as a potential makeup remover. Compared to the conventional oil/water and water/oil cleansers, the LC remover proved to have a stronger cleansing ability without causing skin irritation. In addition, it can dissolve the cosmetic residues immediately and be easily rinsed off due to the fine emulsion droplets formed because of the extremely low interfacial tension between the oil and the liquid crystalline phase.

6 Conclusions

Liquid crystals comprise valid and promising systems in cosmetic applications and treatments because of their biocompatibility and their superiority in the active ingredient delivery process, bioavailability, and stability compared with traditional compounds and agents. However, their safety, longevity, and ability to combine with several other active ingredients remain challenging and still under investigation. □

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Simvastatin in the light of nanotechnology and its therapeutic potential

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ABSTRACT

Though statins are mainly used as antihyperlipidemic drugs, several studies have reported that they could be of benefit for the treatment of various diseases including cancer, osteoporosis, cardiovascular diseases, ischemic stroke, diabetes, neurodegenerative diseases, as well as offering advantages in post organ transplantation. This short non-exhaustive literature review aims to demonstrate the therapeutic potential of semisynthetic simvastatin by using novel nanotechnology applications for improved treatment of such diseases. In particular, the formulation and characterization of simvastatin nanoparticles and their potential novel therapeutic applications are discussed. We present: 1) the use of Simvastatin-loaded Pluronic polymeric micelles for delivering vasoprotective drugs to the liver in order to increase the therapeutic window of simvastatin in chronic liver disease; 2) the application of Herceptin liposomes co-loaded with simvastatin and doxorubicin for controlling prostate cancer along with SMV/SPIONs-PLGA NPs, as bio-safe co-delivery systems; 3) the use of Simvastatin-loaded star- shaped CA-PLGA nanoparticles and immunoliposomes with simvastatin for breast cancer treatment; 4) the breakthrough application of SMV-QRC NP-loaded ISG treatment for tongue carcinoma; 5) an innovative method using nanoparticles loaded with both GM and SMV for pancreatic cancer; 6) the combined use of silver nanoparticles and/or biogenic silver nanoparticles and simvastatin as promising antimicrobials; 7) the use of Simvastatin loaded Chitosan-tripolyphosphate nanoparticles as a novel strategy treatment for bone regeneration in severe cases of osteoporosis and 8) an effective therapeutic method for osteoporosis, which

involves the incorporation of HANPs and SMV to PCL-PGS nanofibers. Existing in vitro and in vivo experimental and preclinical data highlight the promising perspectives of developed statin nanomedicines. Present knowledge is based mostly on theoretical grounds and therefore a better understanding is needed regarding the clinical benefits, the underlying mechanisms and the adverse effects of simvastatin either as monotherapy or in combination with other drug delivery systems.

1. Introduction

Statins are the most common drugs prescribed to lower plasma lipids and decrease the risk of developing cardiovascular disease which is the major cause of death worldwide[1]. They can be either fungal-derived or synthetically produced. Lovastatin, pravastatin, and simvastatin are fungal-derived statins, while atorvastatin, cerivastatin, fluvastatin, pravastatin, pitavastatin, and rosuvastatin are fully synthetic compounds[2]. The first FDA-approved statin was lovastatin, a natural statin produced by fermentation of Aspergillus terreus[3].

However, because statins have low oral bioavailability, mainly due to their limited aqueous solubility, their synthesis should focus on a stable formulation with enhanced bioavailability following oral administration; moreover, significant adverse effects may develop during chronic treatment. By improving statin bioavailability, minimization of adverse effects and toxicity associated with higher plasma concentrations could be achieved.

In the present paper we focus on semisynthetic Simvastatin (SMV) and innovative delivery systems to enhance its therapeutic utility. Chemically, SMV is produced by further treatment of Lovastatin usually involving direct alkylation[3], both having the lowest bioavailability compared with the other statins, as shown in Table 1[1].

Statins have been evaluated for the prevention and/or treatment of a variety of diseases including cardiovascular diseases, ischemic stroke, diabetes, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, cancer, osteoporosis, as well as offering benefit in post organ transplantation[1]. In this context, nanotechnology has a key role in the enhancement of statins' bioavailability. Advanced drug delivery systems including nanocrystals, pol-

Table 1: Pharmacokinetic properties of statins (taken from[1]).

Property	Statin							
	Atrovastatin	Cerivastatin	Fluvastatin	Lovastatin	Pitavastatin	Pravastatin	Rosuvastatin	Simvastatin
Absorption %	30	98	98	31	80	37	50	65-85
Tmax (h)	2-4	2.5-3.0	0.5-1.5	2-4	1.0-1.8	0.9-1.6	3-4	1.3-2.4
Biovailability %	12	60	10-35	<5	>60	18	20	<5
Solubility	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Lipophilic	Hydrphilic	Hydrphilic	Lipophilic

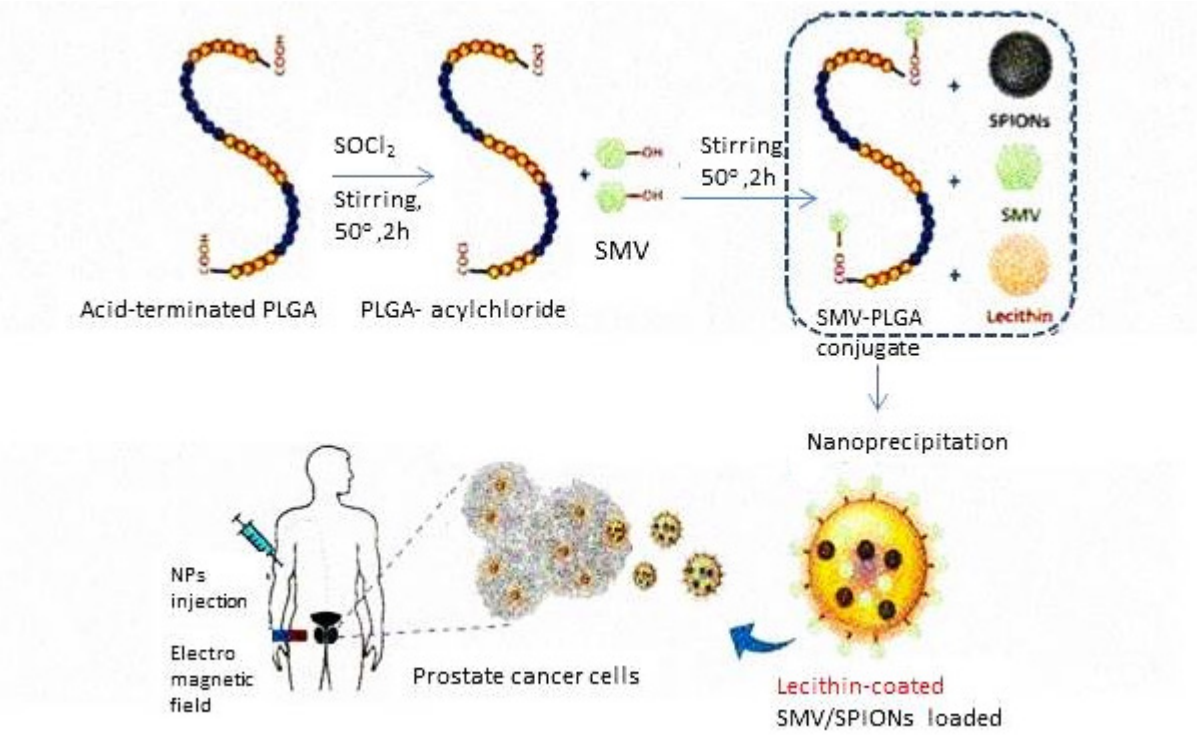

Figure 1. *Synthesis of Simvastatin-PLGA conjugate followed by fabricating of lecithin-coated SMV/SPIONs- loaded PLGA-conj-SMV NPs (adapted from[14]).*

ymeric nanoparticles (NPs), solid lipid NPs (SLNs), liposomes, and micelles used for statin delivery.

2. Formulation and characterization of simvastatin nanoparticles

Synthesis of SMV nanocrystals was obtained using a sonoprecipitation technique by injecting 0.50% (w/v) methanol solution of simvastatin into 0.20% (w/v) water solution of Pluronic F68 under sonication amplitude of 400 W and processing temperature of 3°C. The SMV nanocrystals formulation showed a fourfold increase in the dissolution rate, faster absorption (tmax reduced from 2.88 h to 1.99 h), and a 1.5-fold increase in bioavailability following oral administration in rats compared with unformulated SMV[4].

Formulation of SMV nanoparticles (NPs) using a novel evaporation technique in which volatile solvents were evaporated from an oil-in-water microemulsion was performed. Resulting NPs were used for tablet preparation by mixing them with excipients (lactose monohydrate, microcrystalline cellulose and crosspovidone). The comparison between these tablets and conventional tablets having the same excipients showed remarkable improvement in dissolution profile[5].

Another promising nanocarrier for delivering vasoprotective drugs to the liver alongside with increasing the therapeutic window of simvastatin in chronic liver disease has been reported. Simvastatin-loaded Pluronic polymeric micelles were synthesized with two different types of Pluronic (PM127 and PM108). It was found that the PM127 formulation was better as far as liver sinusoidal endothelial cells (LSECs) targeting was concerned. PM127 indicated a polydispersity of 0.27 ± 0.02 nm and Z potential of -9.10 ± 1.34mV while values for PM108 were 0.58 ± 0.01

nm and -4.03 ± 0.79mV respectively. A rat model of advanced chronic liver disease (CLD) was used for evaluation of the formulation. It was found that PM127-SMV exhibited increased efficacy compared with free simvastatin by significantly reducing portal hypertention, an important factor related to CLD progression; moreover, this was achieved without the toxicity caused by free Simvastatin[6].

Simvastatin had also been chosen in a study of polypill formulation. Four active substances (amlodipine (AML), hydrochlorothiazide (HTZ), losartan (LS), and simvastatin in a 2.5/12.5/25/40 weight ratio) were used for forming a polypill with mesoporous silica nanoparticles (MSN) as a carrier. MSN-41 with hexagonal pore arrangement was synthesized by sol-gel in the presence of structure directing agents with a modified *Stober et al. (1968) method* which is a chemical process to prepare silica (SiO2) particles[7]. This carrier allowed an effective controlled release of AML, LS and SMV and an immediate release of HTZ as it was designed. Thus, the controlled release of SMV could be suitable for long term treatment and the stability of the four active substances was increased, as well as their half life and metabolism time[7].

Nanostructured lipid carriers (NLCs) for the delivery of SMV in order to enhance its low oral bioavailability (<5%) have also been developed. SMV-NLCs were prepared with emulsification technique followed by ultrasonication. The inactive ingredients were stearic acid, oleic acid, lecithin and pluronic F-68. The in vivo pharmacodynamic study of the SMV-NLCs exhibited a sustained and increased lipid lowering activity in comparison with the SMV suspension. In addition, there was a 4-fold increase in oral bioavailability of SMV-NLCs compared to the SMV suspension[8].

Another research demonstrated that the formulation of two SMV carrier systems, i.e. polymeric drug inclusion complex (IC) and mixed micelles (MM) nanoparticles loaded onto mucoadhesive buccal films may improve SMV bioavailability. The inclusion complex of SMV with hydroxypropyl-beta-cyclodextrin (HP-β-CD) was more efficient than all other studied polymers in improving solubility of SMV in aqueous media. The formation of SMV-loaded mixed micellar system prepared using phosphatidylcholine (PC) and sodium deoxycholate (SDC) shaped spherical shape nanoparticles, and both systems succeeded in enhancement of drug permeation across oral epithelial cells. Thus, SMV-loaded mucoadhesive buccal film could be considered as an alternative to SMV oral tablets[9].

Simvastatin, due to its potential therapeutic use in neurodegenerative diseases, was also investigated for nasal administration of a lipophilic drug to obtain a faster route to the CNS. To this end, chitosan, lecithin and oil excipients were used for the preparation of nanocapsules loaded with simvastatin (SMV-loaded lecithin/chitosan nanoparticles (LCNs)). The addition of oil combination Maisine (Glycerol/Glyceryl monolinoleate) and Labrafac (consisting of medium- chain triglycerides of caprylic (C8) and capric (C10) acids) that was called MaiLab had a remarkably smaller particle size (204nm), increased positive surface charge (up to 50mV) and high drug loading capacity, encapsulating 98% of SMV. The blank nanoparticles did not show cytotoxicity, by using a suitable model of nasal mucosa, proving the safety for nasal administration. Preliminary in vivo gamma scintigraphy studies showed an enhanced nose to brain delivery compared with SMV suspension[10]. However, additional studies are required to elucidate whether the nanoparticles are taken up by the nasal epithelium or facilitate drug absorption without crossing the mucosa.

4. Simvastatin-loaded nanoparticles for cancer treatment

High doses of statins have been reported to destroy a rogue protein produced by a damaged gene associated with nearly half of all human cancers. In particular, the therapeutic potential of simvastatin using nanotechnology is considerable and might contribute to the development of more effective anti-cancer treatment strategies.

Some of the novel nanomedical platforms aim to prostate cancer therapy. Currently, the use of anth-

racycline doxorubicin (DOX) in its treatment is considerably limited due to side effects and its non-selectivity[11]. Simvastatin (SMV) in combination with DOX is a promising candidate for cancer therapeutics. Herceptin is a targeting monoclonal antibody ligand used to treat cancer. The use of Herceptin modified liposomes as target receptor for the delivery of DOX and SIM to prostate cancer cells seems to be a quite effective method[12]. Specifically, the targeting liposomes containing co-loaded DOX and SIM efficiently inhibit tumor growth and display significantly better antitumor activity than all other treatments. Treatment with Lip (non- targeting liposome with DOX/SMV) and H-Lip (Herceptin targeting liposome co-loaded with DOX/SMV) at a dose of DOX 1mg/kg and SIM 2mg/kg resulted in significant tumor shrinking with an inhibition rate of 68,77% and 80,36% compared with free DOX/SIM combination (43,82%). The H-Lip treatment was more effective in controlling tumor growth compared to the non-targeting ones due to the HER-2 receptor expression in the prostate tumors[13]. Furthermore, H-Lip may be a potential biosafe co-delivery nanomedicine system for controlling prostate cancer due to its easy preparation process, therapeutic efficacy, physiochemical properties, biodistribution, release behavior and its minimal toxicity (non-significant weight loss observed, no visible lesions in the major organs, no significant treatment toxic effects).

Another nanotechnology-based treatment strategy for prostate cancer is the chemical conjugation of simvastatin to acid-terminated poly (D, L-lactic-co-glycolic acid) PLGA chains followed by its conversion into nanoparticles with in situ incorporation of more simvastatin and superparamagnetic iron oxide nanoparticles (SPIONs) in the PLGA NPs. This PLGA-based hybrid nanocarrier significantly improved SIM anticancer activity against human prostate cancer cell line through both an apoptosis mechanism and retardation of G2-M phase of the cell cycle. The incorporation of SPIONs into the nanocarrier is beneficial in guiding the drug to the tumor cells avoiding side effects from other organs. Further in vivo and clinical studies are required to define the clinical usefulness of this nanosystem[14].

Moreover, novel simvastatin-loaded nanoparticles based on cholic acid (CA)-core star-shaped PLGA, show promising results for the treatment of breast cancer. Researchers developed a star- shaped polymer CA-PLGA with three branch arms for nanoparticle formulation of small molecular anti-tumor drugs and characterized the properties of the nanoparticle in vitro. This nanoformulation proved to be very stable and appropriate for drug delivery. Simvastatin was in an amorphous or disordered crystalline phase of a molecular dispersion or a solid solution after being encapsulated by NPs. SIM exhibited significantly high levels of cytotoxicity and exceptional antitumor activity against breast carcinoma through inhibiting tumor cell growth and proliferation. These 110 nm-sized biodegradable SIM-loaded star-shaped CA-PLGA nanoparticles can achieve a higher drug loading capacity, improved drug absorption, encapsulation efficacy and a faster drug release[15].

In addition, a targeted therapeutic liposomal carrier of simvastatin, characterized by high stability and specificity towards breast cancer cells has been developed. Long-circulating targeted liposomes with simvastatin combined with the commercially available antibody trastuzumab (humanized anti-HER2/neu antibody) as a specific agent defining the target of the drug, have been designed. Trastuzumab and breast cancer cell lines which are known to overexpress HER2 were chosen because these cell lines showed the highest sensitivity to liposomal simvastatin treatment among all other tested breast cancer cells of different molecular subtypes[16]. Furthermore, the liposomal and immunoliposomal formulations with simvastatin were stable in diameter for at least 12 months, but the drug content, although stable for 8 months, decreases afterwards to 75% of the initial value and remained stable for up to 12 months. In general, the immunoliposomal formulation of simvastatin is characterized by a satisfying drug to lipid ratio, high sensitivity, long-term stability and good anti-tumor potency in vitro[17]. This makes it a promising candidate for breast cancer treatment but the validity of using simvastatin in monotherapy or in combination for anticancer therapies should be further studied.

Another important nanomedical application of simvastatin regards treatment of tongue carcinoma. The poorly soluble simvastatin loaded to chitosan nanoparticles followed by in situ gel (ISG) preparation via a face-centered central composite design (FCCCD) was investigated for treatment. Coated simvastatin NPs were doped with quercetin (QRC) using a modified nanoprecipitation method. A formulation containing 0.24% Poloxamer 188 and 0.43% chitosan can fulfill the prerequisites of optimum formulation for preparing simvastatin-quercetin nanoparticle-loaded in situ gel[18]. These formulations show better stability under refrigerated conditions. SIM concentrations must be controllable because higher concentrations may cause adverse effects such as rhabdomyolysis and inflammatory myopathies[19]. ISG formulation showed a remarkable increase in apoptosis and enhanced the tumor suppressor protein levels. This novel localized treatment could be a breakthrough by overcoming the side effects of systemic chemotherapy for tongue carcinoma.

Finally, a significant therapeutic approach for the treatment of pancreatic cancer, involves the combination of delivery of anticancer drug gemcitabine (GM) and simvastatin (SMV) through PLGA polymeric nanoparticles. It was shown that dual drug-loaded NPs with GM and SIM exhibited higher intracellular uptake, longer duration of drug release and remarkable cytotoxicity to pancreatic cancer cells. Concurrently, these NPs were less toxic to healthy cells, showed better absorption and stay longer in the systemic circulation, due to reduced rate of elimination and longer T1/2. The encapsulation of both drugs in a nano-matrix is more effective than conventional chemotherapy and such innovation could prolong the survival of pancreatic cancer patients and provide a better quality of life[20].

5. Antimicrobial effects of simvastatin-loaded nanoparticles

Several studies have explored the pleiotropic effects of statins (well-characterized anti- inflammatory and immunomodulatory effects on host cells) in combating multisystem microbial infections, such as sepsis and pneumonia, and a growing number of studies are demonstrating that statins can directly influence the growth and virulence of bacterial pathogens. This is supported by: a) clinical evidence that prior use of statins may reduce the risk of morbidity and mortality of patients with microbial infections (such as bacteremia, pneumonia, sepsis, and some acute infections); b) direct antibacterial effects of statins on in vitro bacterial growth of both Gram-positive and Gram-negative bacterial pathogens, which may be statin specific and/or strain/species specific or both; c) effects of statins on intracellular growth of bacteria probably due to pleiotropic effects of modulating the mevalonate pathway in the host; d) effects of statins on (in vitro) bacterial virulence; e) co-prescription of statins with antibiotics may increase the efficacy of treatment. The potential mechanisms by which statins modulate bacterial growth and virulence are shown in Figure 8. Among the statins, simvastatin and atorvastatin have more antibacterial effects than other members of the statin family[21].

Multidrug-resistant bacteria such as extended-spectrum beta-lactamase, Enterobacteriaceae (Enterococcus faecalis), and methicillin-resistant Staphylococcus aureus, as well as emerging resistant fungi pose a major challenge to the health care system. In this context, the well- established antibacterial and antifungal activity of silver nanoparticles (AgNPs) and biogenic silver nanoparticles (biAgNPs; obtained by cell-free filtrate of Fusarium oxysporum) and semi- synthetic simvastatin, alone and in combination has been evaluated against multi-drug resistant bacteria and several toxigenic Aspergillus species. These compounds have shown synergistic and additive antimicrobial effects against resistant bacteria and toxicogenic species of Aspergillus that help control fungal growth as shown by the prevention of biofilm formation and compromised germination of spores (checked through scanning electronic microscopy analysis of morphological alterations)[22-24]. Also, optimized simvastatin-loaded niosomes (formed from non-ionic surfactants and cholesterol which create vesicular structures that can encapsulate both hydrophilic and hydrophobic drugs in the center or between the double layers of the vesicles) have been used to decrease the drug's releasing

rate and significantly enhance antibacterial activity against S. aureus and E. Coli[25].

6. Role of simvastatin in osteoporosis and therapeutic nano-drug delivery systems

Osteoporosis occurs when the balance between new bone formation and osteoclastic process is altered leading to an increased activity of osteoclasts in comparison to osteoblasts. Treatments have been designed to restore this balance, mostly by inhibiting osteoclast activity or by stimulating bone formation. Prescribed drugs include calcium and vitamin D supplementation, bisphosphonates, raloxifene and calcitonin. Simvastatin has been well investigated since the 90s for its osteopromotive properties[26, 27].

6.1 Mechanisms of Simvastatin action on the bone

The major mechanisms of Simvastatin on the increase of new bone formation include: a) promotion of osteoblast proliferation and differentiation, b) protection of osteoblasts from apoptosis, and c) suppression of osteoclastogenesis by inhibiting osteoclastic activity[28].

6.2 Role of Simvastatin in osteogenesis

Simvastatin promotes osteogenesis by increasing viability and differentiation of osteoblasts. Moreover, it up-regulates Bone Morphogenetic Protein type 2 (BMP-2) gene through the Ras (a family of related proteins which is expressed in all animal cell lineages and organs) signaling pathway and activates mitogen-activated protein kinases. Other beneficial actions of simvastatin on osteogenesis include: a) reversal of the suppressive effects of tumor necrosis factor (TNF), b) mediation of osteogenesis, at least in part by induction of ER-a and not by BMP-2 alone and c) an antinflammatory effect[28, 29].

6.3 Role of Simvastatin in osteoblastic apoptosis

Simvastatin protects osteoblasts from apoptosis via the TGF-β/Smad3 signaling pathway (Figure 9). TGF-β activates Smad3 by initiating multiple reactions leading to phosphorylation of a special kinase. Smad3 promotes new bone formation by synthesizing matrix proteins and increasing both ALP activity and mineralization. In addition, Smad3 reduces osteoblast apoptosis by inhibiting the conversion of osteoblasts to osteocytes and their apoptosis. Furthermore, SIM acts on the mevalonate pathway to reduce the prenylation of GTP-binding proteins (key regulators of receptor-mediated signaling pathways), which blocks osteoblast apoptosis[28-30].

6.4 Role of Simvastatin in osteoclastic differentiation and activity

The nuclear factor kappa b ligand (RANKL)/RANK signaling pathway and its receptor/activator osteoprotegerin (OPG) play major roles during osteoclastogenesis [30]. Simvastatin increases OPG mRNA expression, decreases RANKL mRNA expression and blocks RANKL-induced differentiation of osteoclasts. As a result of its increased expression, osteoprotegerin binds to RANKL and prevents its interaction with RANK. Estrogen receptor (ER) has also a considerable role in inhibition of osteoclastogenesis. Estrogens inhibit osteoclastogenesis by reducing RANKL and increasing OPG. Finally, SIM acts on the mevalonate pathway to reduce the prenylation of GTP-binding proteins which blocks the osteoclasts activity[28,30].

As far as nanotechnology is concerned, SIM loaded Chitosan - tripolyphosphate nanoparticles (CS-TPP), synthesized by the ionic gelation method, is a good strategy to achieve slow and controlled release of SIM. As a local drug delivery system, CS-TPP NPs possess several advantages, such as biocompatibility and biodegradability, controlled drug release due to diffusion and slow polymer degradation, high entrapment efficiency, enhanced permeability and high stability[31].

The SIM CS-TPP NPs had an optimum particle size of 106 nm, showed good storage stability over the first month, controlled and steady release over 2 weeks that effectively delivered SMV in a therapeutic dose needed for bone regeneration.

Another nanotechnology-based treatment of osteo-

porosis involves the incorporation of Hydroxyapatite nanoparticles (HANPs) and SMV to Poly(ε-Caprolactone)/Poly (Glycerol Sebacate) nanofibers using the electrospinning technique. Hydroxyapatite nanoparticles were synthesized by wet chemical precipitation technique. Moreover, HANPs were added to the PCL-PGS solution and ultra-sonicated to make PCL-PGS-HA composite. Finally, SMV was added to the composite solution immediately before electrospinning. The SIM release behavior was characterized by an initial burst around 20% until 24h, followed by a gradually slow and nearly linear release. As shown in Figure 12, during the 7 days, 79.5% of SIM was released from the PCL-PGS-HA-SIM[32].

This composite shows excellent biomineralization making a bonelike apatite layer on its surface. The incorporation of HANPs and SIM into the composite nanofiber exhibits enhanced osteoblast cell growth, offering great potential for bone tissue regeneration[32].

7. Conclusions

Nanotechnology provides advantages for the delivery of simvastatin, most importantly in oral bioavailability. At least two mechanisms should be considered to enhance the oral bioavailability of simvastatin: (i) increasing their dissolution in the gastrointestinal tract; and (ii) reducing or eliminating first-pass metabolism, following oral absorption, which can prevent from attaining desired systemic concentrations.

Given that oral administration of statins is preferable, improving dissolution and bioavailability is key to enabling lower effective doses. Polymeric micelles, liposomes, immunoliposomes, SPIONs, silver nanoparticles and chitosan- tripolyphosphate nanoparticles are drug delivery systems that have positive impacts on drug solubilization in the gastrointestinal tract because of the decreased particle size of the entrapped simvastatin. Liposomes, as polymeric micelles, should be considered for other-than-oral administration routes, especially when the goal involves site-specific drug delivery and sustained release. When selecting among polymeric micelles and liposomes, biocompatibility, formulation stability, and temporal release properties become important. Given that sustained and controlled drug release is crucial for the success of an injected nanocarrier formulation, polymeric micelles could be the appropriate candidates to achieve a depot effect.

Also, nanocarriers for the safe and effective delivery of simvastatin, can meet the biopharmaceutical challenges that sometimes deteriorate the full therapeutic potential of a drug. By cautious selection of a nanocarrier system, challenges such as poor solubility, inadequate dissolution, chemical instability, limited oral bioavailability, compromised site-specific drug delivery and inadequate sustained or controlled release of the active, can be overcome.

This non-exhaustive literature review aims to demonstrate the therapeutic potential of statins (simvastatin in particular) by using novel nanotechnology applications for the improved treatment of various diseases, such as hyperlipidemia, chronic liver disease, cancer, resistant microbial infections, and osteoporosis. Although in vitro and in vivo experimental and preclinical data highlight the future promising perspectives of developed nanomedicines, it should be born in mind that drawbacks and limitations of these formulations have not been extensively investigated so far. Present knowledge is based mostly on theoretical grounds and therefore a better understanding is needed regarding the clinical benefits, the underlying mechanisms and the adverse effects of simvastatin either as monotherapy or in combination with other drug delivery systems. □

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REVIEW ARTICLE

Nanogels: biomedical applications and future perspectives

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ABSTRACT

Nanogels are 3D nanoparticles that exhibit unique swelling capabilities, while their porous structure enables loading with other substances. Based on their synthesis process, they are divided into physically-fragile crosslinked and chemically-stable nanogels. Nanogels can be stimuli-responsive and release their cargo when certain circumstances are met. Thus, they can be controlled and act as useful tools in drug-delivery, imaging or theranostics. Their distinctive properties lead the way for the development of biosensors and tissue-engineering by means of wound healing, treatment of osteoporosis -supported by in -vivo studies - and heart-attack recovery using stem cells. Finally, nanogel-based vaccines are shaping up as promising alternatives to conventional vaccines, paving the way for faster and more effective prevention of various diseases.

1. Introduction

Ever since the revolution led by the creation of materials in the nanoscale, scientists have been on the search for the development of nanoparticles with ideal properties to act as drug-carriers, imaging-enhancement agents, scaffolds for tissue engineering, detectors in biosensors, and vaccines. Nanogels are multifunctional and can meet all these requirements. In this review, we will discuss the whole spectrum of nanogels, ranging from how they are synthesized and classified to breakthrough studies that demonstrate why nanogels may pave the way for extraordinary applications in the near future.

1.1 Definition

Nanogels were first described by Kabanov et al in 1999 and are defined as 3D nanoparticles composed of crosslinked hydrophilic or amphiphilic swellable polymer networks, either natural or synthetic. Nanogels keep the advantageous properties of hydrogels, eg. tunable hydrophilicity, while augmenting them with properties that are typical of materials in the nanoscale. Their structure includes pores that can be filled with other molecules. These nanoscale systems exhibit unique properties thus making them the target of numerous studies: the flexible nano-size, large surface area for multivalent conjugation, biocompatibility, colloidal stability, viscoelasticity, shape control and loading capacity. However, their unique characteristic is the ability to swell after absorbing water. This behavior can be controlled through structural parameters (cross-linking degree, presence of a functional group), as well as

by external stimuli (pH, temperature, specific molecules).[1]

1.2 Classification

There are two main types of classification of nanogels, depending on the synthesis process or their responsive action.

1.2.1 Synthesis Process

Nanogels can be categorized by the crosslinking method, to physically or chemically crosslinked nanogels. Physically crosslinked nanogels are formed under mild conditions, are stabilized by relatively weak interactions between polymer chains, such as hydrogen bonding, hydrophobic interactions or ionic interactions. This makes physically crosslinked systems more fragile than their crosslinked counterparts, since the latter are formed by covalent bonds between the polymer chains during either polymerization of low molecular weight monomers or crosslinking of polymer precursors [2].

1.2.2 Responsive Action

Nanogels can also be classified based on their responsive action to stimuli-responsive and non-responsive. [3] There are many kinds of stimuli such as: changes in temperature, pH, enzyme concentration, redox conditions, glucose levels or the application of external light, ultrasound waves, electric/magnetic field.

This stimulus, via a conformational or structural change in the nanogel that alters the hydrophilicity/hydrophobicity/extent of interaction of the system with the water molecules, causes the swelling or deswelling of the nanogel network, and the release of the entrapped cargo. Below, we discuss a subset of the possible stimuli, redox and light stimuli.

Redox reactions are oxidation-reduction reactions that play a key role for the cell's survival. An important molecule is glutathione (GSH) which alleviates oxidative stress through redox reactions, by acting as an electron donor and thus reducing disulfide bonds. GSH concentration inside normal tissues, is 1000-fold the extracellular concentration, whereas in tumor tissues it is 4 times higher. This creates a reducing intracellular environment in malignant tissues, which can be effectively targeted by redox-responsive nanogels comprised mainly by disulfide bonds.[3]

Photodynamic therapy, in general, uses photothermal transductors (PTs) that - when targeted by near-infrared irradiation (NIR) light- produce reactive oxygen species, causing tissue destruction, whereas photothermal therapy uses specific PTs that transform NIR light into local heat. PTs with absorption in the biological optical window can be incorporated within nanogels. Then, NIR light can be used to externally regulate the impact of the nanogels in the targeted tissues. These thermoresponsive nanogels have great potential in cancer treatment, combatting the cells using a combination of local hyperthermia and release of the embedded chemotherapeutic agent.

1.3 In Vivo Behavior

After the administration of the nanogels via different routes, nanogels can stay in the circulation for sufficient amount time in order to deliver their payload at the target tissue. The most common strategy for the nanogels to evade the immune system and acquire stealth properties, is the conjugation of PEG or CD47 self-peptides with the surface.[4] As for pharmacokinetics, it is known that solid tumors and inflamed tissues show significant leakage of the endothelium and impaired lymphatic drainage, because of the enhanced permeability and retention effect (EPR). Thus, nanogels tend to accumulate in these tissues, upon entering the blood stream.[5,6] Adding to EPR, ligands to specific receptors or molecules overexpressed on the diseased cells improve their retention at the targeted site and help their cellular uptake.[7,2]

2. Biomedical applications (drug delivery, imaging, theranostics, biosensors, tissue engineering, vaccines) and future perspectives

2.1 Drug Delivery

As mentioned before, nanogels are highly absorbent and can incorporate approximately 30% of the weight

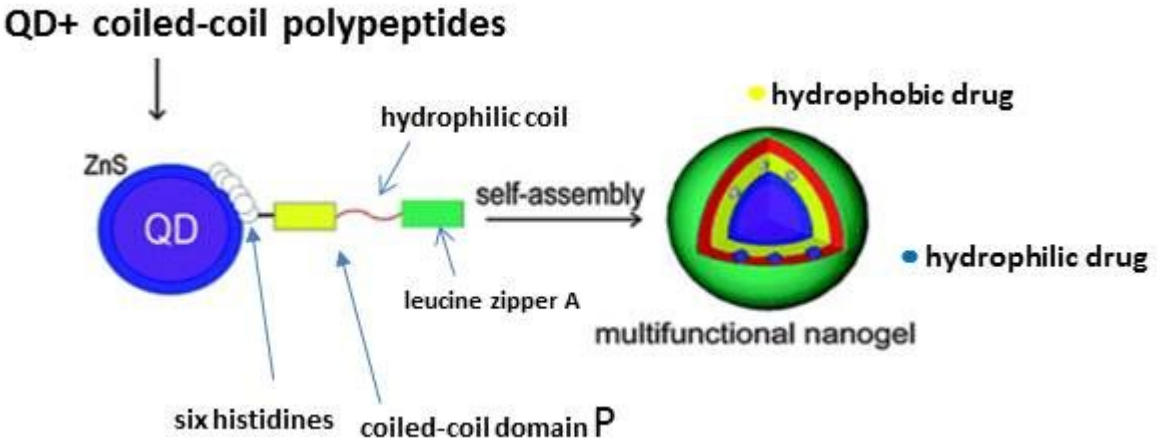

Figure 1. *Schematic representation of the QDs@polypeptide nanogel, adapted from*[12]

of active molecules, making them ideal carriers in drug delivery as their loading capacities exceed those of liposomes and polymeric micelles.[8]

Another property of paramount importance is the incorporation of molecules of the opposite charge. Cationic PEG-PEI (polyethyleneimine) nanogels can incorporate negatively charged active anti-cancer substances such as nucleoside analog- 5'-triphosphates (NATP). Vinogradov et. al published an in vivo study using these nanogels in human breast carcinoma MCF7 xenograft animal models and it showed faster cell accumulation at lower doses compared to parental drugs. Active NATPS are unstable when administered, so nanogels offer an alternative as drug-carriers.[9]

In 2014 Coll Ferrer et al. used antibodies like AntiI-CAM-1, conjugated with nanogels, against pulmonary endothelium in cases of acute pulmonary inflammation.[10] The researchers created Lysozyme dextran nanogels composed of a dexamethasone shell and a lysozyme core in an animal model of LPS-induced lung injury. The study showed a significant anti-inflammatory effect of ICAM-NG-DEX in mice.

Regarding stimuli-responsive nanogels, a group of nanoscientists from India designed locally injectable, biodegradable, and pH sensitive composite nanogels, for hepatocellular carcinoma (HCC). Those nanogels were composed of chitin-poly-lactic acid along with doxorubicin (Dox-chitin-PLA CNGs) that demonstrated controlled swelling, sustained release of Dox and degradation in acidic pH, as well as non-hemolytic properties in human liver cancer cell cultures.[11]

Apart from pH sensitive, there are also enzyme-responsive nanogels as presented by Kim et al who synthesized contact lenses by nano-diamond nanogels loaded with timolol, a drug prescribed against glaucoma. The aim of this study was two-fold; first, the avoidance of premature release of the drug and the prolongation of its action, while at the same time it is kept physically active for further release under degradation of the nanogel via lysozyme cleavage. The second one was the incorporation of a small number of nano-diamonds into the lens matrix in order to improve the tensile strength and the elastic modulus of polymers.[12]

The most intriguing part of drug delivery lies on the field of the release of multiple drugs. Fahmy and coworkers developed liposomal nanogels that can deliver small hydrophobic molecular TGF-β inhibitor and water-soluble protein cytokine (IL-2) to melanoma tumor-bearing mice. The simultaneous release of those substances resulted in a major activation of the immune system mechanisms and impeded the growth of cancer cells, when administered systemically.[13]

Similarly, Desale et al. demonstrated core-shell nanogels, consisting of triblock copolymers: PEG-poly(L-glutamic acid)- poly(L-phenylalanine) (PEG–PGlu–PPhe), which can entrap both hydrophilic drugs,

such as cisplatin and hydrophobic like paclitaxel. The scientific group found synergistic cytotoxicity against Human Ovarian A2780 Cancer Cells In Vitro And High Efficacy As Anti-Cancer Agents In Vivo.[14]

2.2 Imaging

Medical imaging is one of the most promising fields where the use of nanogels may play a key role in the near future. Plenty of inorganic molecules (i.e. metallic nanoparticles (NPs), magnetic, QDs) can be incorporated into the final structure depending on the technique, each of which adds special magnetic or optic properties to the nanogel. Alongside that, the high loading capacity of nanogels multiplies the number of those NPs and widens the amplitude of the upcoming magnetic/ electric fields, due to cluster effect.[15-18]

In MRI, magnetic NPs are part of the nanogel, accompanied by the proper outer coating (ie. PEG).18 In 2015, Wang et al. developed a pH-responsive chitosan nanogel with T1 and T2 relaxivity contrast agents, manganese oxide and SPIO nanoparticles (dual contrast agents). At this complex system, Mn^{2+} ions are released only in the presence of acidic pH, found mostly inside the cancer cells, leading to the release of T1 and T2 contrast agents. It is known that these agents cannot be mixed in a single system because this could result in mutual nullification of the signal.[19] Thus, nanogels lead the path towards multimodality.

Likewise, the use of QDs in nanogels has been studied thoroughly in the bibliography in many in vivo animal trials. The crucial thing about the study conducted by Yang et al. is the amphiphilicity of the encapsulated drugs, as described in figure 1.[12]

Another aspect of imaging is optical imaging, accompanied mostly by fluorescent agents that emit in the NIR region and the use of corresponding probes. Among the probes, only indocyanine green (ICG) has been approved for clinical imaging applications, but it still is quite inaccurate.[20] Several drawbacks have arisen, such as self-quenching, short half-life and degradation in aqueous media. This is why the use of nanogels is absolutely vital.

A plethora of studies have been conducted concerning the utility of nanogels in optical imaging. In 2014, a group led by Kim J. proposed the use of enzyme-sensitive shell-crosslinked hyaluronic acid nanogels (HA sc-nanogels) which incorporate ICG derivatives as a prognostic tool for cancer. An enzyme called hyaluronidase is associated with tumor metastasis and angiogenesis, so in its presence nanogels produce a strong fluorescence signal. This was demonstrated post intradermal injection of the nanogels in the forepaw of mice, both in vivo and in vitro. After the injection, fluorescence images were captured by IVIS imaging system.[21]

Furthermore, the application of nanogels in optical imaging has improved the detection of sentinel lymph node (SLN), the biopsy of which is crucial when staging malignancies. In this case, dextran-poly(acrylic acid) nanogels (DNG) were synthetized and 5-aminofluorescein was conjugated to make them fluorescent. When injected intradermally, the nanogels were detected in the SLN within one minute, via lymphatic drainage, while the signal peaked around 12hours and lasted for 60 hours. [22]

A study published one year later, in 2015, included nanogels composed of cholesterol-modified pullulan (NIR-CHP) with diameters of 30nm, tested in large animal models. It was demonstrated that the intensity of the signal was low at the injection site, but high at the SNL, without spreading to distal lymph nodes.

Consequently, those two characteristics; first the lack of rapid lymphatic clearance and second the restriction of the dye to the SNL, are a breakthrough in SNL navigation surgery.[23]

2.3 Theranostics

Theranostics is a term used to describe the combination of therapeutic and diagnostic effect of future drugs. Nanogels, due to their unique features, may play a part in that field, improving the prognosis of many diseases like cancer or diabetes.

One of the nanogels designed for this purpose is a redox-responsive fluorescent carbon nanogel (FCN) with paclitaxel. The presence of high levels of glutathione in cancer cells stimulates the nanogel and the fluorescence intensity of (PTX)-MnO2/FCN increases, owing to the reduction of MnO2 to Mn2+ and cleavage

Table 1: Examples of the role that nanogels loaded with active substances may play in wound healing

Main Component	Active substance	Dimensions	Outcome
Chitosan	Interleukin-2	1007 ± 459nm (PDI 0.763 ± 0.189)	Decrease of malondialdehyde levels and increase of GSH levels
Chitosan/alginate	Silver sulfadiarine	736.25nm	Decrease of the area of burn wounds
Lysine	Chlorhexidine diacetate	120nm	Decrease in inflammation markers in infected full-thickness wounds
Poly(acrylic acid)	Silver nanoparticles	200nm	Decrease in the area of full-thickness wounds
Methacrylic acid	Silver nanoparticles, Aloe vera and curcumin	10-200nm	Complete healing of full-thickness wounds
N-isopropylacrylamide	Silver nanoparticles	180-200nm	Inhibition of bacteria growth
Gellan-cholesterol	Baicalin	350nm (PDI<0.30)	Edema-inhibition in inflamed cutaneous injuries
Cholesterol-bearing pullulan	Prostaglandin B1	20-30nm	Decrease of the area of full-thickness wounds
Acryloyl group-modified cholesterol-bearing pullulan	Growth factors secreted by infiltrated cells	50nm	Tissue growth and andiogenesis in subcutaneous pockets
Collagen/hydroxypropyl methlcellulose	Curcumin	~120nm	Decrease of the area of full-thickness wounds
Poly(acrylic acid) coated with (poly-diallyldimethylammonium choloride)	Berberine	130nm	Improved antimicrobial efficacy
Poly(acrylid acid) coated with (poly-diallyldimethylammonium chloride)	Chlorhexidine	130m	Improved antimicrobial efficacy

of the disulfide bond. In this environment, the slow release of paclitaxel takes place too, acting as a theranostic tool.[24]

In another study by Weitai et al, glucose-sensitive zinc oxide@poly(NIPAM-AAm-FPBA) hybrid nanogels containing insulin cargo, produce fluorescent signals when levels of glucose are higher than the normal. The detection range is between 18 and 540mg/dl of glucose and the insulin release exhibits the slowest rate (~5% released in 76 h) at a normal glucose level (108.0 mg/dl) but becomes exponentially quicker as glucose levels are increased.[25]

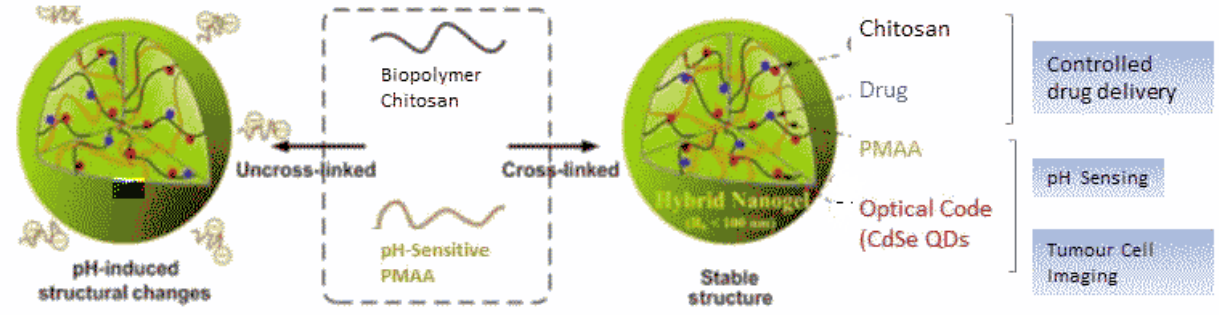

Figure 2. *Schematic representation of the concept for designing multifunctional chitosan-MAA-CdSe hybrid nanogel.*

The previous theranostic tools included only optical imaging for diagnosis. Apart from that, multifunctional core-shell hybrid nanogels have been developed that contain both fluorescent and magnetic properties, studied in mouse melanoma B16F10 cells. These nanogels are synthetized by coating bifunctional nanoparticles (BFNPs, fluorescent carbon dots embedded in the porous carbon shell and superparamagnetic iron oxide nanocrystals clustered in the core) with a thermo-responsive poly(N-isopropylacrylamide-co-acrylamide) [poly(NIPAM-AAm)]-based hydrogel as the shell. Thus, optical temperature sensing, MRI or PET imaging of cancer, and magnetic/NIR-thermally responsive release of chemotherapeutic agents, can be achieved all at the same time.[26]

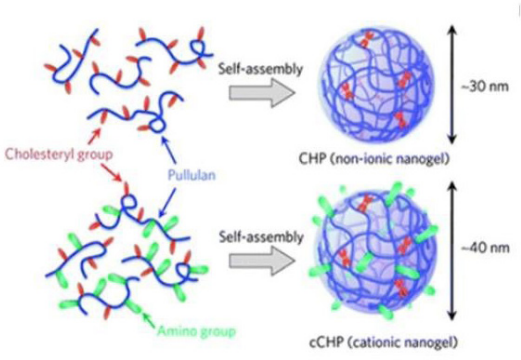

Figure 3. *Schematic representation of the hybrid nanogels, adapted from*[2].

2.4 Tissue engineering (wound healing, osteoporosis, mi)

A promising aspect of nanomedicine is tissue engineering accompanied by regenerative medicine. The aim is to restore damaged tissues or cells' functions. A human tissue engineered product has two main features; it consists of engineered cells and its properties correspond to the ones of the healthy tissue.

Wound healing is a major chapter in tissue remodeling and table 1 shows examples of nanogels with encapsulated active substances in order to accelerate the healing process, prevent local inflammation and achieve hemostasis. [27] [table 1]

Osteoporosis is another cause of tissue instability and a couple of in vivo studies have been conducted. One of them is based on temperature-sensitive p(N-isopropylacrylamide-co-butyl methylacrylate) nanogels (PIB) injected in ovariectomized rats. These nanogels are in a soluble gel-state in the syringes, but they become solid in body temperatures, delivering progenitor cells and growth factors. However, due to their low osteoconductivity, other substances are used to enhance osteoblast differentiation in vivo, such as mesoporous bioactive glass loaded with strontium (Sr-MBG). Thus, the combination of them resulted in significantly higher new bone formation.[27]

A truly promising application of nanogels concerns myocardial infarction, because cardiovascular disease is the leading cause of death worldwide as someone suffers a heart attack every 40 seconds, according to the CDC. Poly(NIPAM-AA) nanogels encapsulating

Table 2: Recent studies on immobilization of organic NPs on nanogels		
Nanogel	Organic Nanoparticles	Immobilization Technique
Poly(acrylic acid)-based nanogels	Glucose oxidase	Covalent
Polyacrylamide	Bovine carbonic anhydrase	Covalent
Polyacrylamide nanogel	Lipase	Encapsulation
Fe3O4/chitosan nanogel	Glucoamylase	Adsorption
Polyethyleneglycol	Lipase	Imprinted
Pluronic F127	Fluorescent probe (DMDP-M)	Adsorption
Polyurethane nanogel	Coumarin 6/Nile Red	Adsorption
Polyurethane nanogel	8-Hydroxypyrene-1-carbaldehyde	Adsorption
Poly(ethylene glycol) nanogel	2-Metbacryloyloxyethyl phosphorylcholine copolymer bearing oligonucleotides	Physical interaction

stem cells are used in vivo in mice and pigs, in MI models by the ligation of the left anterior descending artery. As shown in figure 8, injection of stem cells alone (blue bars) did not help to recover the damaged tissue, while the injections of nanogels alone (orange bars) showed comparable heart protection to the control group. The best results concerning the restoring of the ejection fraction were retained by administering stem cells encapsulated in nanogels (red bars).[27]

2.5 Biosensors

A biosensor is an analytical device which measures biological or chemical reactions, in order to detect the concentration of an analyte in the reaction.[28, 3]

There are three types of nanogels in a biosensor: nanogels as encapsulation vehicles for biosensor detectors, nanogels as multifunctional stimuli-responsive materials for biosensor detectors and nanogels as sensory membrane of biosensors.

Beginning with the first type, nanogels are used as scaffolds for other NPs. Inorganic NPs are frequently used due to their special properties. As discussed before, their main attribute is image-enhancement. On the other hand, organic NPs such as enzymes can also be used, but they must be thoroughly monitored, so that they operate in standard environmental variables. Several types of nanogels are mentioned in table 2.

The second type includes stimuli-responsive NGs like chitosan–poly(methacrylic acid) (PMAA)–CdSe (QDs) hybrid nanogel, which is pH responsive. CdSe QDs are mixed homogeneously in the nanogel system, can translate the volume phase transition into optical codes. These two parts make chitosan–PMAA–CdSe hybrid nanogel a good detector candidate for pH-biosensor.

A schematic representation of the concept for designing multifunctional chitosan-MAA-CdSe hybrid nanogel is illustrated in Figure 2. As for the final category, Lee et al. formed glucose sensitive artificial muscle via introducing Boronic acid (BA) nanogels into carbon multiwalled nanotube (MWNT) yarn. Given that the BA nanogels were sensitive to glucose, this artificial muscle rotated to different angles, responding to the glucose concentration accordingly.[29]

Table 3: Hybrid Nanogels and their current research phase [12]		
Phase	Hybrid nanogel	Application
Preclinical	Core-shell nanogels	Neurodegenerative disorders, Treatment of acute pulmonary inflammation
	CHP-W9-peptide	Bone loss disorder
	Plasmonic@NG	Cancer treatment
Clinical	CHP	Vaccines

pH responsive dendritic polyglycerol nanogels that measure pH along the hair follicle also belong to this category of nanogels and were studied ex vivo in a porcine ear model. The macromolecular precursors are labeled with a pH sensitive indodicarbocyanine dye (pH-IDCC) and a control dye (indocarbocyanine dye: ICC). It was demonstrated that these pH-nanosensors deeply penetrate the skin via the follicular pathway and the pH of the pig hair follicles increase from 6.5 at the surface of the skin to 7.4 in deeper areas of the HF.[30]

2.6 Vaccines

The most recent and novel application of nanogels are vaccines. Nowadays, in the COVID-19 pandemic, the development of vaccines has become more vital than ever. Nevertheless, scientists have been testing novel NPs and their potential applications for over a decade[31]. Granted, nanogel-based vaccines are still in their infancy, with a limited number of studies displaying noteworthy results. Namely, Nochi et al.[32] introduced CHP-Cholesterol-Bearing Pullulan nanogels as an intranasal vaccine-delivery system. A non-toxic subunit fragment of Clostridium botulinum type-A neurotoxin BoHc/A administered intranasally with cCHP nanogel was effectively taken up by mucosal dendritic cells after its release from the cCHP nanogel. This led to strong immune response, without the need of adjuvants. Furthermore, the olfactory bulbs or brain were spared of any accumulation.

3. Conclusions

Nanogels have been the focus in numerous studies, but only three clinical trials have been published as of yet. The lack of them seems like an insurmountable obstacle in the translation of nanogels in clinical practice. Still, as time goes by, data derived from preclinical trials may broaden our knowledge, along with the constant development of chemical engineering. The future is ahead for nanogels and hopefully they might become a part of everyday life of clinical physicians, in terms of drug administration, imaging, surgery navigation, tissue regeneration and biosensors.[12] □

Conflict Of Interest

The authors declare no conflict of interest, financial or otherwise.

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PHARMAKEFTIKI, 33, III, 2021 | 190-199 REVIEW ARTICLE

Lipid-based nanovaccines

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ABSTRACT

Vaccination has had a tremendous impact on global health and the quality of human life by reducing the mortality and morbidity caused by infectious diseases. However, effective and therapeutic vaccines have yet to be developed for completely carrying deadly diseases. In the past few decades, there has been increasing focus on the field of nanotechnology in the combination with vaccination. Nanovaccine formulations not only provide enhanced antigen stability and immunogenicity but also offer targeted delivery and prolonged release. A high number of NP vaccines with varied physicochemical characteristics and properties have been approved for clinical use. The initial part of this review provides information about lipid-based nanoparticles in which nucleic acids such as DNA (as plasmids) and RNA (as mRNA) are encapsulated in order to be used for vaccination. Subsequently, there is presented a short overview according to the first lipid-based marketed products, Inflexal V and Epaxal, and their correlation with today's lipid-based nanovaccines. This review also focuses on the research efforts for the development of lipid-based vaccines against SARS, MERS and of the recent developments in nanotechnology-based approaches in view of the ongoing pandemic of COVID-19. Finally, there are highlighted the promising new treatments and future perspectives of these nanovaccines.

1. Introduction

Nanotechnology has been found to play a unique role in the design of vaccines, providing them with increased potency and selectivity toward viral cells against the host cells. Nanovaccines can present enhanced and broad-spectrum immunity and at the same time include protection of the basic structure of the antigen and its prolonged presentation to immune cells. Nano-scaled materials, such as virus-like particles, liposomes, and lipid nanoparticles (LNPs) are carriers for the delivery

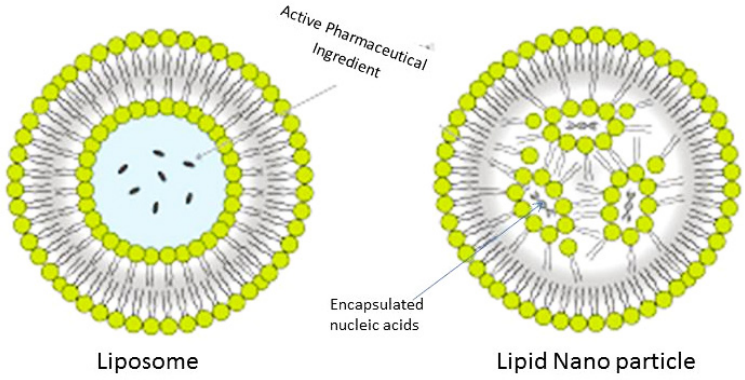

Figure 1: *Liposomes and lipid nanoparticles as delivery vehicles.*

of vaccine antigens and adjuvants, due to their beneficial advantages.

Lipid nanoparticles and liposomes are similar by design but different in composition and function. Lipid nanoparticles (LNPs) are composed primarily of cationic lipids along with other lipids such as cholesterol. Another common lipid ingredient is what is known as a PEGylated phospholipid—a polyethylene glycol (PEG) polymer covalently attached to the head-group of a phospholipid[1]. On the other hand, liposomes are spherical vesicles characterized by a bilayer of lipids with an internal aqueous cavity and composed of phospholipids. Charged phospholipids are used to impart a positive or negative charge to the liposome. They can be used to entrap hydrophilic molecules (e.g., drugs and vaccines) in the aqueous solution core or to load hydrophobic molecules in the lipid bilayer. Nevertheless, the interest in liposomal vaccines led to the development of the products of Epaxal and Inflexal V against hepatitis and influenza, respectively[3]. The founding-father of liposomes as immunological adjuvants in vaccines was Prof Gregoriadis who publishes his research in 1974s in the journal "Nature"[2]. This technology led to the development of vaccines for hepatitis A and flu in the 1990s and of vaccines for COVID-19 in the 2020s.

1.1 Nucleic acids in lipid-based vaccines

Nucleic acid-based vaccines, i.e., DNA [as plasmids (pDNA)] and RNA [as messenger RNA (mRNA)] vaccines, pave the way for safe and efficacious biologics to mimic inoculation with live organism-based vaccines. In many cases and according to its target, mRNA is easier to be delivered and much safer than DNA, because the mRNA does not interact with the genome in the nucleus and is only transiently expressed. Hence, in contrast to DNA vaccines, the FDA does not consider nonreplicating mRNA vaccines gene therapies[4].

Lipid nanoparticles (LNPs) are among the most frequently used vectors for in vivo RNA delivery. Cationic lipid nanoparticles spontaneously encapsulate negatively charged mRNA, according to Figure 2A, and provide protection from enzymatic degradation[5]. On the other hand, cationic lipid nanoparticles are recognized as one of the most promising delivery vectors for pDNA as they could condense it to form lipid/pDNA complexes, which protects pDNA from being digested by nucleases (*Figure 2B*). Once internalized, the nanovaccines follow the endosomal/lysosomal pathway where they must escape degradation[6].

The reasons why lipid nanoparticles (LNPs) are generally used as vectors are because they protect the mRNA against degradation and assist in endocytosis and endosomal escape. In addition,

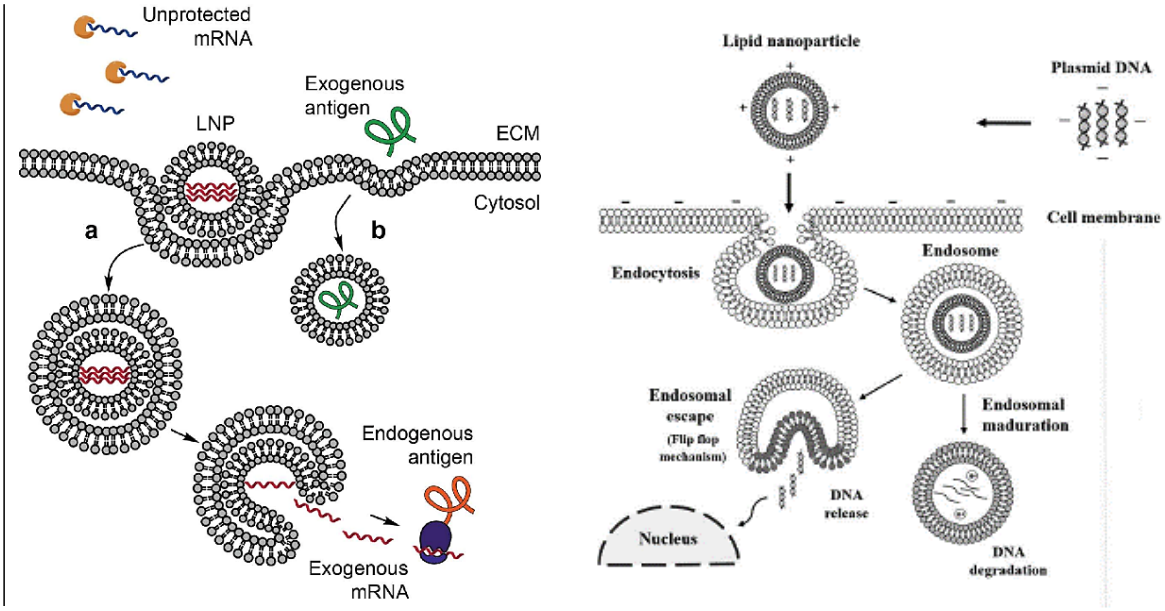

Figure 2: *From the left to the right: (A): Lipid Nanoparticles protect mRNA from degradation, and facilitate endocytosis and endosomal escape[5]. (B): Lipid nanovaccine transfection mechanisms of endocytosis and pDNA release[6].*

adjuvants can be incorporated in LNPs and assist in immune activation and potentially tailoring of the immune response. Another advantage of LNPs is that they can be targeted to specific cell types by decorating their surfaces with specific ligands[5].

2. Applications of liposomes in vaccine formulations

Lipidic adjuvants are used in the production of vaccines for their immunostimulatory action. Immunopotentiating Reconstituted Influenza Virosomes (IRIVs) are proteoliposomes of approximately 150nm in diameter with functional viral envelope glycoproteins, influenza virus hemagglutinin (HA) and neuraminidase (N) intercalated in the phospholipid bilayer[7]. They have been used since the 1990s against influenza (Inflexal V) and hepatitis A (Epaxal) and present a technology platform that can be adapted to many vaccines. Virosome-based vaccines are designed to maintain the immunogenicity of a live-attenuated virus but exhibit the safety of an inactivated virus. They are bereft of the nucleocapsid and the genetic information of the native virus which makes them unable to replicate and thus to infect[8,9].

Inflexal V is a strain-specific virosome vaccine used for the immunization of adults and children older than 6 months against the flu (influenza). It has an excellent safety profile and induces humoral responses even in immunocompromised patients. It is composed of tree monovalent virosomes, each containing specific surface Hemagglutinin and neuroamidase according to annual World Health Organization recommendations. Antibodies against HA provide immunity by killing the virus, while those against N limit the transmission of the virus from one cell to another[10-12].

Epaxal is a virosome-adjuvanted hepatitis A vaccine licensed in 1994.The virosome is composed from the haemagglutinin of the A/ Singapore/6/86 (H1N1) strain and the phospholipids lecithin and cephalin. Onto them, bound by electrostatic forces, are the hepatitis A antigens (strain RG-SB) that have been propagated in MRC-5 human diploid cells and inactivated with formaldehyde. Epaxal has been reported to induce the production of anti HAV antibodies within 10 days of vaccination and

after the second vaccination to induce an additional protection that lasts up to 20 years[13,14].

The functional characteristics of virosomes are at the basis for their enhanced immunogenicity. The superficial antigents allow the binding and endocytosis of the virosome. The interaction with receptors on B lymphocytes stimulates the production of antibodies while the interaction with Antigen Presenting Cells (APC) such as dendritic cells, results in the activation of T lymphocytes. Thus virosomal adjuvant system allows antigen presentation in the context of both Major Histocompability Complex (MHC) I and II and in this way, induces both B- and T-cell responses[7, 9, 13, 14].

The effectiveness of the virosomal-adjuvanted Influenza vaccine depends on the match between the vaccine and the circulating strains. While there is only one type of influenza B virus, influenza A has multiple subtypes. In order to characterize and differentiate the various strains of Influenza A, the World Health Organization established the use of the acronym HxNz. Up to date there are 16 known hemagglutinin and 9 neuraminidase genes that code for the viral envelope (surface proteins). The subtypes H1, H2, H3, H5, H7, H9 and N1-N2 are most common in virus strains that infect humans[7,9].

Influenza viruses have two major mechanisms of antigenic evolution: antigenic drift and antigenic shift. Antigenic drift occurs when the virus accumulates mutations at antigenic sites during replication producing variant viruses that can escape existing immunity. This phenomenon is common to both influenza A and B viruses. An antigenic shift occurs when a virus acquires an antigenically novel HA through reassortment. It involves large change in nucleotides of RNA, feature of influenza A. viruses only. The change is sudden and drastic. This results in the formation of a new subtype of virus to which everybody is susceptible leading, eventually to pandemics[12].

Both influenza A and B exist as genetically distinct lineages that co-circulate in humans, with the different influenza A strains varying sufficiently in the HA component to require different HA antigens to be present in the seasonal influenza vaccine to ensure the induction of adequate specific immunity[7, 13, 14].

In 2016, Blom et al studies the virosomes and liposomes mediated immune response on human respiratory tract triple culture model. Their results showed enhanced internalization of virosomes in epithelial cells of triple culture as compared to liposomes of similar sizes without inducing excessive inflammatory responses. At the moment, a number of virosomal formulations are in clinical trials as adjuvant for prophylactic as well as therapeutic vaccines against malaria, influenza, tuberculosis (TB), human immunodeficiency virus (HIV), and dengue fever[14-17].

3. Lipid Based Nanovaccines for COVID 19

Coronaviruses (CoVs) induced three major outbreaks of respiratory distress syndrome in the last decades, namely severe acute respiratory syndrome (SARS) in 2003 with the epicenter in Guangdong, China; Middle East respiratory syndrome (MERS) in 2012 in Saudi Arabia; and novel coronavirus disease (COVID-19), in Wuhan Province, China, in late 2019 .This virus was an unknown pathogen until January 10, 2020, when next-generation sequencing identified it as an RNA virus with genomic sequence similar to SARS-CoV of 2003 and was named as SARS-CoV2. It is a single- stranded positive sense enveloped RNA virus, approximately of 30 kb in length, the longest of the known RNA viruses. It is said to be zoonotic in origin, bats being the large reservoirs of this virus[18].

Following the publication of the genetic sequence of SARS-CoV-2 on January 11, 2020, intense research efforts have been devoted to developing a vaccine against COVID-19 and are mainly focused on the CoV transmembrane spike (S) glycoprotein, which extends from the viral surface and mediates host cell entry. SARS- CoV-2 S requires angiotensin-converting enzyme 2 (ACE2) to pass into cells. The receptor-binding areas of SARS-CoV S and SARS-CoV-2 S attach with similar

affinities to human ACE2, thus causing the effective spread of SARS-CoV-2 in large human populations. SARS-CoV-2 S glycoprotein shelters a furin cleavage site at the margin of S1/S2 subunits, which distinguishes this virus from SARS-related CoVs and SARS-CoV. Consequently, SARS-CoV-2 S ectodomain trimer was chosen to provide a blueprint for designing vaccines and inhibitors of viral entrance.

The structure of a coronavirus particle is depicted on the left, with the different viral proteins indicated. The S protein is the major target for vaccine development. The spike structure shown is based on the trimeric SARS-CoV-1 spike (PDB: 5XL3). One trimer is shown in dark blue, and the receptor binding domain, a main target of neutralizing antibodies, is highlighted in purple. The other two trimers are shown in light blue. SARS-CoV-2 vaccine candidates based on different vaccine platforms have been developed, and for some of them, pre-clinical experiments have been initiated. For one mRNA-based candidate, a clinical trial recently started to enroll volunteers shortly (ClinicalTrials.gov: NCT04283461).

Arcturus Therapeutics, a company leading into RNA therapeutics based in San Diego, and Duke-NUS Medical School, a research-intensive medical school at Singapore, have collaborated their efforts to develop an mRNA-based vaccine against COVID-19. They propose to use a LUNAR platform to develop lipid-based nanoparticles to encapsulate the mRNA to trigger rapid and prolonged antigen expression within host cells resulting in protective immunity against infectious pathogens. LUNAR is known to be composed of four lipid components. The amino groups remain unionized at physiological pH, preventing toxicity due to the otherwise used cationic lipid for RNA delivery. It gets positively ionized at acidic endosomal pH releasing the therapeutic RNA in the cytoplasm[18].

On March 16, 2020, Moderna, through a partnership with the Vaccine Research Center at the U.S. National Institutes of Health, enrolled the first participants into a Phase I clinical trial testing an mRNA vaccine (mRNA-1273) encapsulated in lipid NPs a record time of just 63 days following sequence selection (NCT04283461). The enrollment of the 1st cohort of participants (18-55 y.o healthy subjects) concluded on 4/2020. On May 18, Moderna announced that mRNA-1273 elicited antibody titers above the levels observed in convalescent individuals (and therefore considered potentially protective). On December of 2020, mRNA-1273 was granted FDA approval. CureVac and BioNTech (in partnership with Pfizer) are currently working on similar vaccines; Pfizer/ BioNTech, in particular, July 2020 started the recruitment in Phase III trials (NCT04368728, NCT04380701) and recently was granted FDA approval. The aforementioned vaccines present 95% efficacy after the second dose[19-24].

The use of plasmid DNA in a vaccine will allow the development of an advanced optimized payload that encodes various protein epitopes of the crucial immunogenic proteins of SARS-CoV-2. These protein epitopes will activate the natural production of antibodies in the body, as well as the protective immune response for the prevention of COVID-19[25,26]. A DNA plasmid vaccine by Inovio Pharmaceuticals (INO-4800) has showed promising results in mice and guinea pigs according to a recent article published in Nature Communications and has entered Phase I testing in humans (NCT04336410).

The University of Oxford and AstraZeneca (NCT04324606) has also produced another COVID-19 vaccine. The vaccine is based on a chimpanzee adenovirus vaccine vector (ChAdOx1) and the SARS-CoV-2 spike protein. On November of 2020 AZD 1222 vaccine met primary efficacy endpoint in preventing COVID-19 with 90% efficacy, FDA approval is pending. Another adenoviral vector vaccine developed by CanSino Biological Inc. and Beijing Institute of Biotechnology using a genetically engineered replication-defective adenovirus type 5 vector to express the SARS-CoV-2 spike protein (Ad5-nCoV) is currently being tested in Phase I/II trials (NCT04398147, NCT04341389, NCT0431312).

Inactivated vaccine developed by the China Na-

tional Pharmaceutical Group (Sinopharm), in collaboration with the Wuhan Institute of Biological Products, is currently tested in a Phase I/II trial (ChiCTR2000031809), and a second inactivated vaccine (in collaboration with the Beijing Institute of Biological Products) has been currently approved for clinical testing (ChiCTR2000032459).

Recombinant-protein vaccines and inactivated vaccines are safer but might require adjuvants to increase their immunogenicity. In the context of SARS-CoV-2, adjuvants are important for two reasons. First, adjuvants might increase the efficacy of the vaccine, especially in subjects with impaired immunological function, such as the elderly, or in subjects with comorbidities resulting in immune dysfunctions. Second, adjuvants can reduce the amount of vaccine protein(s) required per dose, which could facilitate scaling-up vaccine production in a reduced time frame. Alum is considered a typical adjuvant employed to increase the neutralizing antibodies for SARS-CoV in a VLP vaccine approach and responses to MERS-CoV S NPs and SARS-CoV S NPs in mice. Additionally, Matrix-M1 adjuvant increased the anti-MERS-CoV S neutralizing antibody reaction in vaccinated mice. Some licensed adjuvants developed specifically for COVID-19 vaccine are AS03 (GlaxoSmith- Kine), MF59 (Seqirus), and CpG 1018 (Dynavax).

Predominantly, two kinds of vaccines for MERS-CoV were investigated, which included spike protein NPs prepared with alum adjuvant and recombinant adenovirus serotype 5 encoding the MERS-CoV spike gene (Ad5/MERS). These vaccines stimulated precise immunoglobulin G against MERS-CoV. Consequently, a heterologous approach by priming with Ad5/MERS and boosting with spike protein NPs may be a promising and effective prophylactic approach against MERS-CoV infection[22-26].

SARS/MERS vaccine development research suggests spike protein S subunits, S1, whch contains the receptor binding domain (RBD) and S2 subunit[21]. Previous reports have suggested that both humoral and cell mediated immunity performs a protective role in the SARS-CoV infection. Novavax, Inc., a late-stage biotechnology company, had developed a proprietary virus-like particle vaccine for use against MERS-CoV that contained a minimum of one trimer of an S protein and their proprietary Matrix-M adjuvant aiming to enhance immune responses. They used the same technology to produce a vaccine candidate against SARS-CoV-2, NVX-CoV2373, and, at the end of May 2020, announced the enrolment of the first participants in a phase I/II clinical trial[19].

As of recently, the global COVID-19 vaccine R&D landscape includes 115 vaccine candidates, of which 78 are confirmed as active and 37 are unconfirmed (development status cannot be determined from publicly available or proprietary information sources). Of the 78 confirmed active projects, 73 are currently at exploratory or preclinical stages. The most advanced candidates have recently moved into clinical development, including mRNA-1273 from Moderna, Ad5-nCoV from CanSino Biologicals, INO-4800 from Inovio, and LV-SMENP-DC and pathogen-specific aAPC from Shenzhen Geno-Immune Medical Institute. Adjuvants could enhance immunogenicity and make lower doses viable, thereby enabling vaccination of more people without compromising protection. So far, at least 10 developers have indicated plans to develop adjuvanted vaccines against COVID-19, and vaccine developers including GlaxoSmithKline, Seqirus and Dynavax have committed to making licensed adjuvants (AS03, MF59 and CpG 1018, respectively) available for use with novel COVID-19 vaccines developed by others[27].

4. Future Perspectives

One of the main obstacles in the development of nanovaccines is considered the toxicity of lipid nanoparticles. As pre-clinical and clinical reports showed, the nano-scale sized LNPs dose-dependent acute and chronic toxicities with bioaccumulation that depends on the route of administration[28]. It is worthy to be mentioned that potentially the toxicity of LNPs derives from their components, including cationic lipids. The immunogenicity of

PEG and the decreased interaction of the LNPs with the endosomal membranes that hinders endosomal escape are also important issues for both siRNA and mRNA delivery. While LNP delivery of siRNA is already in clinical trials, and lessons from these experiences can be helpful for the translation of mRNA vaccines, there are no available commercial pDNA vaccines for human use. Nevertheless, plasmid DNA (pDNA) is currently being used to prepare a new generation of vaccines which are beginning to enter the marketplace and the same time it is already licensed animal vaccines increases the likelihood for the development of human pDNA vaccines in the near future. Human naked pDNA vaccines are under clinical investigation worldwide, including plasmids expressing malarial, HIV, influenza, tuberculosis, Zika, and Ebola virus antigens. Still, the demonstration of the clinic efficacy of this type of vaccines has only just begun[4-6].

Lipid nanoparticle (LNP) mRNA nanovaccines have shown interesting results against various infectious diseases including COVID-19[29]. However, their structural features should be further investigated[31]. Cholesterol, a major constituent within LNPs, contributes to their morphology that influences gene delivery[29]. Studies with the replacement of phospholipid, PEG-lipid, and ionizable lipids have led to the deconvolution of the size, shape, and internal structure of these clinically approved materials[31]. C24 alkyl derivatives of cholesterol show a polymorphic shape and various degrees of multilamellarity and lipid partitioning[29]. Furthermore, as a future prospect, in order to develop highly efficient systems, research should be focused on uncovering the structural modifications that occur at *in vivo* environments[29].

Recently, researchers are putting effort in the field of biomimetic nanotechnology in order to develop personalized medicines based on biomimetic nanovaccines[27]. Such nanovaccines can be inherently immuno-stimulatory and multi-antigenic[28]. In this direction, coating of bacterial outer membrane vesicles (OMVs) onto nanoparticles for anti-viral vaccination to deliver and neutralize bacterial toxins can displace the pathogen through its own survival mechanism[27]. As a result, the bacterial colonization is prevented effectively and consequently the direct selective pressure to develop antibiotic resistance can be reduced. These formulations can be developed by varying the outer membrane coating of these biomimetic nanovaccines[28].

Immunogenic responses generated against certain antigens can be used for the development of vaccines that can be divided in two categories: as prophylactic or as therapeutic[28]. Prophylactic nanovaccines confer protective immunity mostly against infectious conditions at low doses of immunostimulating antigen and reduced need for adjuvants, thus mitigating associated toxicities[28]. Therapeutic vaccines are administered after the onset of diseases to alter the course of disease by encouraging the immune system to fight harder against the prevailing conditions. As a future prospect, nanovaccines can be formulated to achieve both prophylactic and therapeutic responses[28].

Nanovaccines may provide an excellent future ahead if the following goals are attained: (i) stabilization at ambient temperature via encapsulation of vaccines in NPs, (ii) exploration of replaceable/interchangeable routes of administration, and (iii) facilitation of controlled/specific release at a particular location[20]. The development of optimal vaccine formulations is envisaged to contemplate novel materials combined with adjuvants and the generation of new knowledge on the processes behind the immune mechanisms elicited by liposomes[20].

Nanoparticles have shown their ability to target both adaptive (T cells, B cells) and innate immune systems (macrophages, monocytes, neutrophils) at the cellular level. Modulating APCs using nanoparticles could be very important, particularly for COVID-19 vaccine strategies. Entos Pharmaceuticals is developing a Fusogenix DNA vaccine to prevent COVID-19 infection. The goal is to promote the direct introduction of an antigen-encoding plasmid that stimulates the immune response by stimulating B and T cells. Entos' Fu-

sogenix platform is a proteolipid vehicle that uses a new fusogenix mechanism to provide its genetic load directly inside cells, allowing it to generate protection mechanisms against various structural components of SARS-CoV-2 (Nanotechnology Products Database 2020)[22].

Some neutralizing monoclonal antibodies isolated against SARS-CoV-1, like CR3022, can cross-react to the receptor binding domain of SARS-CoV-2. This suggests that SARS-CoV-1 vaccines might cross-protect against SARS-CoV-2. However, because these vaccines have not been developed further than phase I, they are currently not available for use[23].

GeoVax has used its experience with GV-MVA-VLPTM vaccines to design and produce vaccine candidates using genetic sequences of SARS-CoV-2, in which on this platform, the MVA, a large virus covers carrying various antigens of the vaccine, expresses proteins that group in the VLP immunogens of the person receiving the vaccine, where the production of VLPs in the vaccinated person mimics the production of viruses in a natural infection, with this stimulating the immune system (Nanotechnology Products Database 2020)[20].

A highly suitable nanotechnology platform is derived from plant viruses and bacteriophages that evolved as stable nanocontainers protecting their genome cargo under various environmental conditions. Vaccines could be produced in edible leaf tissue to enable vaccination of the human population but also livestock, since SARS-CoV-2 is a zoonotic virus that can infect humans and animals. Indeed, COVID-19 harbours the potential to become a seasonal disease; underscoring the need for continued investment in coronavirus vaccines. SARS and MERS vaccine candidates did not make it to market due to lack of financial incentive given the low infection numbers, and because the risk of a global pandemic from a newly emerged virus were largely ignored. Yet, because there is some conservation between the coronaviruses, continued research and product development is critical to tackle any new version of coronavirus that emerges in the future[24].

5. Conclusions

Nanovaccines are an alternate method for antigen delivery and activate different elements of the immune system, while having good biocompatibility. Due to their size, nanovaccines can elicit different immune responses, moving into cells by specific pathways. The limitations of nanovaccines comprise scale-up challenges for sterile production and toxicity effect concerns. Since the use of nanovaccines are relatively new, they do not have an enduring safety profile. Therefore, it is crucial to develop more research into nanovaccine toxicity. To conclude with, it is a matter of time for researchers to be able to use nanovaccine strategies that could enable safe and effective immune responses in humans, hopefully leading to a new generation of vaccines[31]. □

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PHARMAKEFTIKI, 33, III, 2021 | 200-209 REVIEW ARTICLE

Recent advances in the application of Nanomedicine for the diagnosis and treatment of Alzheimer's and Parkinson's disease

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ABSTRACT

Alzheimer's disease (AD) and Parkinson's disease (PD) are chronic neurodegenerative disorders that eventually lead to neuronal death and severe impairment. Nanomedicine research aspires to provide a novel and promising approach aiming to overcome the limitations of existing diagnostic and treatment drawbacks, such as those related to the limited penetration of blood-brain barrier (BBB) by therapeutic and diagnostic agents. This brief review was based on a literature search spanning the last decade, and used the PubMed, Scopus and Science Direct search engines, introducing relevant keywords (as shown below). Several representative review articles and selected original articles were included in order to highlight the recent advances regarding the application of nanotechnology in AD and PD. A multitude of nanotechniques are described in the literature and several have been experimentally implemented with promising results. In the present review, following a short informative presentation of the BBB, a non-all- inclusive focus is done on: USPIONs, aptasensors combined with fern-leaves gold nanostructures, chitosan nanocarriers for nasal delivery of anti-Alzheimer drugs, AuNps, SPCEs modified with gold nanoparticles-polyamidoamine (PAMAM) dendrimer nanocomposites, modified CNTs, GQDs, silver NPs, dendrimers, nanopolymers, nanoemulsions of mucoadhesive ibuprofen and schisantherin A, GQDs, encapsulation of curcumin in alginate nanoparticles, (BSA)-based nanocurcumin, nose- to-brain delivery

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of odorranalectin-conjugated NPs, and urocortin-loaded NPs. According to existing data, nanomaterials, due to their unique properties, appear to be particularly useful in the early diagnosis and more efficient treatment of neurodegenerative diseases. However, many more efficacy, safety and toxicity preclinical studies are required to confirm the usefulness of nanotechnological applications in neurodegenerative diseases. Furthermore, 'practical' obstacles (funding, lack of protocols, absence of regulatory framework, etc.) have to be overcome regarding the clinical translation of experimental nanomedical innovations to clinical practice.

1. Introduction

There are over 50 million people worldwide living with dementia in 2020, and more than 9.9 million new cases of dementia are recorded each year worldwide, implying one new case every 3.2 seconds. Considering the steadily increasing ageing population, this number will almost double every 20 years, reaching 82 million in 2030 and 152 million in 2050. The total estimated worldwide cost of dementia was US$ 818 billion in 2015, which represents 1.09% of global GDP[1].

Neurodegenerative diseases are a heterogeneous group of disorders characterized by the typically chronic and progressive degeneration of neurons in the central and/or peripheral nervous system leading to dysfunction and eventually neuronal death. Their underlying causes are diverse and both genetic and epigenetic factors are implicated. The pattern of neuronal loss is selective, affecting mainly the subcortical areas and the cerebral cortex or both, resulting in specific neurocognitive and motor symptoms depending on the lesion's localization. Thus, a useful clinical distinction has been drawn between patients with prominent cognitive deficits arising from pathology in the cerebral cortex (cortical dementias) and those with prominent basal ganglia, thalamic or brainstem pathology (subcortical dementias). Neurocognitive decline may be prominent in one or more cognitive domains (complex attention, executive function, learning and memory, language, perceptual-motor, or social cognition) leading to dementia and behavioral disturbances that interfere with independence in daily living activities; motor symptoms may be severely incapacitating as well[2-5].

The two most common neurodegenerative disorders are Alzheimer's disease (AD) and Parkinson's disease (PD), with Alzheimer's representing approximately 60-80% of dementia cases and 80% of PD patients eventually developing dementia with Lewy body pathology. Currently, medications for these disorders are limited and aim to palliative treatment, improvement of the quality of life and delay the progression of the disease and gradual loss of neurons. Acetylcholinesterase inhibitors (galanthamine rivastigmine, donepezil) and N-methyl-d-aspartate glutamate receptor antagonists (memantine) are approved for the treatment of AD, which is characterized by loss of cholinergic neurons in the hippocampus and cerebral cortex mainly affecting cholinergic neurotransmission; levodopa, monoamine oxidase B inhibitors, catechol- O- methyl transferase inhibitors, dopamine agonists, anticholinergics, and amantadine are the medications used to restore dopaminergic neurotransmission and treat PD movement disorders[2, 4, 5].

In this line of thought, novel diagnostic and treatment methods, i.e. theranostic methods, using nanotechnology may increase the sensitivity of the detection and early diagnosis of neurodegenerative processes (nanodiagnostics and nanoimaging), and improve treatment modalities. Significant work in the field of nanotherapeutics (dendrimers, gene

therapy, stem cell therapy, drug delivery and therapy) and nanoneuroprotection (fullerenols and other neuroprotective agents) is being done to characterize nanoparticles (NPs) with enhanced capacity to cross the blood–brain barrier (BBB) and carry therapeutic agents such as drugs or genes. Finally, nanoneuromodulation (carbon nanofibers, carbon nanotubes and nanowires) may be used to assess neuronal activity in vivo and provide feedback to modulate this activity[6].

2. Central Nervous System (CNS) barriers

In mammals, a variety of biological barriers isolate circulating blood from the surrounding interstitial fluid of the tissues[7]. The CNS has developed a series of barriers to protect itself from invading pathogens, neurotoxic molecules and circulating blood cells. These barrier layers, with diverse degrees of permeability, play an essential role in its optimal function and the required homeostasis[7-10].

There are three main sites in the brain and spinal cord where barriers are found: a) the endothelium of parenchymal microvessels, b) the epithelium of the choroid plexus (modified ependymal lining of the brain ventricles) secreting cerebrospinal fluid (CSF), and c) the arachnoid epithelium, the middle layer of the meninges forming the outer covering of the CNS[8].

At each of these sites, tight junctions significantly reduce the permeability of ions and other small hydrophilic molecules, thus forming a physical barrier[8].

2.1. Blood Brain Barrier (BBB)

The most important CNS barrier is the BBB[9]. The blood brain barrier is a highly complex and dynamic structure, which is composed mostly of brain microvascular endothelial cells, an extremely specialized non cellular basal membrane, lots of pericytes embedded in the basal membrane, and astrocytes (Fig. 1)[9, 11, 12, 13].

A multiplicity of drugs has been developed for the treatment of CNS diseases[15]. Since the BBB consists a complex obstacle for drug delivery to the CNS, each drug is designed to take advantage of a specific transport mechanism of BBB[16], which involves the following:

1. Passive diffusion: a wide range of lipophilic and low molecular weight molecules can diffuse passively across the BBB.
2. Paracellular diffusional pathways: BBB disruption due to local inflammation and circulatory factors leads to weakening of the tight junctions, which allows the entry of polar solutes between the endothelial cells of blood vessels to the brain extracellular fluid.
3. Carrier-mediated transport: small molecules such as amino acids and glucose can be transferred from blood vessels to the brain extracellular space through the endothelial cells by a carrier protein on the cell membrane.
4. Receptor-mediated transcytosis: larger and/or hydrophilic molecules such as hormones and proteins can be transported by specific receptors expressed on the luminal side of the endothelial cells.
5. Adsorptive transcytosis: this non-specific mechanism involves the endocytosis of charged substance vesicles[15, 17].

2.2 BBB and Neurodegenerative diseases

Alzheimer's disease (AD) and Parkinson's disease (PD) are progressive neurodegenerative diseases prevalent in the elderly population. Both diseases are characterized by the production of free radicals and pro-inflammatory cytokines due to neuroinflammation, and the accumulation of abnormal inclusions in the affected cells[17]. There is evidence for a strong connection between BBB dysfunction, neuroinflammation and neurodegeneration[7, 8].

Thus, BBB dysfunction has been proposed as a potential underlying pathophysiological factor in AD and PD, as it may be involved in disease progression, eliciting of peripheral immune response, and reduced drug efficacy as well (Table 1). This is

Table 1. Indications of BBB dysfunction in AD and PD (adapted from [19]).	
Disease	Indications of BBB dysfunction
Alzheimer's disease	Vascular plaques in arteries and surrounding capillaries
	Capillaries demonstrate endothelial degeneration and thickening of basement membrane
	CSF albumin concentration correlates with disease progression
Parkinson's disease	Polymorphisms in Pgp associated with increased PD risks due to exposure to pesticides
	PET scans demonstrated decrease in brain Pgp activity in PD patients
	Increased angiogenic vessels in PD patients
	LAT-1 important for delivery of L-dopa across the BBB

BBB = blood brain barrier; CSF = cerebrospinal fluid; AD = Alzheimer's disease; PD = Parkinson's disease; Pgp = P-glycoprotein; PET = positron emission tomography; LAT-1 = L-Type Amino Acid Transporter

corroborated by the increased BBB permeability, as assessed by CSF albumin concentration, observed in AD patients which correlates with disease progression[16-19].

3. Nanomedicine applications in Alzheimer's disease

Alzheimer's pathological hallmarks are: neuroinflammation leading to synaptic impairment and neuronal loss, elevated reactive oxygen species (ROS), imbalanced metal ion homeostasis (e.g., Cu, Fe, and Zn), decreased brain acetylcholine (Ach) levels, deposition of β-amyloid (Aβ) plaques and formation of intracellular neurofibrillary tangles of hyperphosphorylated tau proteins[20,21].

Treatment of Alzheimer's disease has been rather disappointing partly because of the inability of early disease detection, i.e., before behavioral symptoms and memory problems set in[22]. Therefore, recent developments in the field of nanotechnology, potentially create new opportunities for improved prevention, early detection and treatment of AD.

Nanoparticles (NPs) are increasingly recognized as promising candidates for AD diagnosis and treatment due to their nano-dimensions (below 100 nm). NPs can be used for drug delivery across the BBB, for imaging and early detection of Aβ oligomers (AβOs), inhibition of Aβ fibrilation, clearance of preformed fibrils, oxidative stress or neuroinflammation suppression, metal-chelation therapy and photothermal therapy[23-26].

Among the various NPs used in biomedical research, functionalized ultrasmall superparamagnetic iron oxide nanoparticles (USPIONs) are perceived to be very promising materials in consideration of their high biocompatibility, strong T2 effects, high sensitivity and their capacity for use as multimodal contrast agents. Currently, a handful of iron oxide nanoparticles, such as Feridex and Fereheme, have been approved for clinical application[27]. Furthermore, brain cells are particularly sensitive to magnetic nanoparticles (MNPs) in comparison with other cells of the human body. Consequently, responsiveness of MNPs to an external magnetic stimulus is an important advantage in promoting cell uptake[27]. USPIONs coupled to theranostic agents have been designed and developed.

They exhibit high fluorescence enhancement in the NIR window upon binding to aggregate β-amyloid proteins, effectively inhibit self-aggregation of Aβ and disaggregate preformed Aβ fibrils in a far superior way to phenothiazine-based small molecules[20]. They also demonstrate low cytotoxicity and may be proven to be promising drugs for the prevention of apoptosis associated with AD.

Another significant application of nanomedicine for early AD diagnosis includes a very sensitive and simple aptasensor combined with a fern leaves-like gold nanostructure. The aptasensor was fabricated for the quantitation of amyloid beta and the gold nanostructure, which was used as a transducer, was synthesized by electrodeposition, using PEG 6000 as a shape-directing agent. A specific RNA aptamer was immobilized on the surface of the fern leaves-like gold nanostructure as a recognition element. A ferro/ferricyanide redox marker was also used to detect the binding with Aβ peptide. This aptasensor can detect Aβ in a linear range from 0,002 to 1,28 ng/ml; there is a limit of detection at 0,4 pg/ml. Moreover, the easily applied aptasensor can be used for Aβ determination in CSF and serum samples and at present is probably the most efficient tool for the biomedical diagnosis of AD[28].

Furthermore, chitosan a biocompatible natural polysaccharide can be used as a nanocarrier material for nose to brain delivery of anti-Alzheimer drugs[12]. Chitosan and its biodegradation products impede the progression of various pathological processes in AD by direct or indirect actions, apart from its ability to act as a cationic surface, which gets electrostatically attached with the anionic epithelium of BBB. Also, brain targeting of drugs by using chitosan is intriguing, because it can recognize and adhere to neural tissue selectively[30]. As a result, chitosan's preferential targeting of damaged neurons, facilitates restoration of normal neural activity and physiology which is abnormal in AD.

Furthermore, a novel sensitive strategy for the determination of tau protein in AD, includes a sandwich immunoassay and amperometric detection at disposable screen- printed carbon electrodes (SPCEs) modified with gold nanoparticles-poly-amidoamine (PAMAM) dendrimer nanocomposite (3D-Au-PAMAM) covalently immobilized onto electrografted p-aminobenzoic acid (p-ABA). The immunosensor achieves a limit of detection (LOD) value of 1,7 pg/ml, which is lower than the clinical cut-off value established in plasma (5pg/ml) for AD patients and could be of particular importance for the direct determination of tau protein in undiluted human plasma and brain tissue extracts. The high analytical performance exhibited by this immunosensor and its simple operation with no need for signal amplification strategies, make this method an interesting alternative to current methods to identify AD progression[14].

Gold nanoparticles (AuNPs) have also been used in the treatment of AD with promising results. AuNPs are biocompatible, have minimum toxicity, attractive optical detection and imaging properties, easy biosynthesis and the promising ability to penetrate the blood-brain barrier by connecting several ligands or changing their sizes. Also, these nanoparticles can prevent oxidative stress, cognitive deficits and inflammatory processes in rat AD models[30]; moreover, AuNPs encapsulated with anthocyanin and peptide inhibitors were effective in inhibiting cytotoxicity and amyloid beta-protein aggregation[31].

Regarding carbon-based nanotubes (CNTs), which are important due to their unique physicochemical features, berberine-loaded MWCNTs, coated with phospholipid and polysorbate, have been developed for improved treatment of AD[32]. Also, the polysorbate and phospholipid-coated MWCNTs showed excellent recovery in memory functions between day 18 and 20[33]. Moreover, they demonstrated the potential of decreasing Aβ-induced Alzheimer's disease, which was evidenced by normalized biochemical parameters in brain tissue. A well-dispersed MWCN-hydrogel nanocomposite-based neural scaffold with structural porosity was synthesized and the results in terms

of stimulating neurite extension for nerve regeneration were promising[34].

Finally, nanosensors for AD diagnosis are of crucial importance because early detection of AD during the first phase of the illness offers better prospects for AD individuals and their families. Biosensors are devices that integrate a biochemical binding component with a signal conversion part and are already used in the evaluation of several AD biomarkers[35, 36]. The combination of biosensors with nanomaterials has been found to increase the catalytic properties and conductivity of the transducer and simultaneously facilitate the immobilization of a large number of biological recognition elements as a result of the high surface area of nanomaterials[37,38]. Graphene, AuNPs, CNTs, magnetic nanoparticles, nanopolymers, silver nanoparticles, dendrimers and quantum dots (QDs) have been used in the design of nanobiosensors for AD diagnosis[26].

5. Nanomedicine applications in Parkinson's disease

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder after Alzheimer's disease, counting more than 6.3 million people worldwide suffering from this disease[39]. It is primarily characterized by the gradual deterioration of movement due to loss of dopamine-releasing neurons in the substantia nigra pars compacta of the brain[39].

Alpha-synuclein (α-Syn) is a key protein of 140-amino acids involved in PD pathology and plays a critical role in clustering synaptic vesicles at the dopaminergic presynaptic terminals; its abnormal accumulation and aggregation in the form of Lewy bodies is associated with the dysfunctionality and degeneration of neurons in PD[40,41].

In this line of thought, graphene quantum dots (GQDs) were used to inhibit fibrillization of α-syn and at the same time to interact directly with mature fibrils, triggering them dis-aggregation[40].

Furthermore, transmission electron microscopy (TEM), among other techniques [thioflavin T (ThT) fluorescence and turbidity assays], was used to image the disaggregation of α-syn fibrils. In this in vivo study it was also observed that GQDs penetrate the BBB and prevent dopamine neuron loss induced by α-syn preformed fibrils[40].

Consequently, based on existing experimental observations showing their beneficial effects, GQDs are on the rank of candidates for the development of new therapeutic agents for PD[41].

Nanoemulsion preparations of mucoadhesive ibuprofen have been also used to enhance uptake of ibuprofen into the brain, which resulted in increased DA concentrations and improved motor coordination[42]. Another nanoemulsion of Schisantherin A, which is dishenzo cyclooctadiene lignin obtained from Schisandra Chinensis fruit, was also investigated for the treatment of PD. The pharmacokinetic profile and the bioavailability of the drug was assessed by high pressure liquid chromatography (HPLC); it was shown that the nanoemulsion drug delivery system for Schisantherin A enhances the bioavailability of the drug[42].

Besides the abnormal protein aggregation pathophysiological mechanism of PD, oxidative stress and neuroinflammation are also held responsible for PD pathology. In this context, the antioxidant curcumin has been investigated in PD treatment. Curcumin is a hydrophobic polyphenol derived from the plant curcuma longa. It has been demonstrated that nanocurcumin plays an important role in the reduction of oxidative stress and apoptosis in the brain of strains of PD flies (drosophila melanogaster)[43]. In addition, encapsulation of curcumin in alginate nanoparticles boosts neuroprotection by decreasing oxidative stress and brain cell death in a transgenic drosophila PD model[44]. Another potential therapeutic candidate is the bovine serum albumin (BSA)-based nanocurcumin, which was tested against 6-OHDA-induced cell death using SH-SY5Y cells as a cellular model of PD[45].

In theranostics, an application reported by Wen

et al. pertained to the improvement of nose-brain drug delivery using odorranalectin (OL) and the subsequent reduction in immunogenicity by using the double emulsion method[46]. Odorranalectin was conjugated to poly (ethylene glycol)-poly (lactic-co-glycolic acid) (PEG-PLGA) NPs and its bio-recognized activity on nanoparticles was verified by hemagglutination assay. This study indicated that the conjugated OL retains its hemagglutinating activity and that OL-NP synthesis has increased bioactivity[47]. Nose-to-brain delivery characteristics of OL-conjugated nanoparticles (OL-NPs) was investigated by in vivo fluorescence imaging technique using 1,1′-Dioctadecyl-3,3,3 tetramethylindotricarbocyanine iodide (DiR) as a tracer[47]. Finally, NPs were incorporated into a macromolecular drug urocortin peptide, and its efficiency in hemiparkinsonian rats was evaluated along with the intranasal administration by the rotation behavior test, tyrosine hydrolase and neurotransmitter determination tests; it was concluded that delivery of NPs to the brain increased the OL modification and enhanced the therapeutic results of urocortin-loaded NPs in PD[47].

6. Conclusions

The present brief overview of the recent advances in nanomedicine in the field of diagnosis and treatment of AD and PD shows that although nanotechnology-based diagnostic and therapeutic approaches have been promising in overcoming many long- standing challenges, there is still a long way to go. In Alzheimer's disease, the restrictions imposed by the BBB, the intracellular and extracellular localization of AD pathology and the lack of early-diagnosis biomarkers, prevent the adequate planning and strategy to be adopted; likewise, in Parkinson's disease, in vivo studies have shown that BBB dysfunction and other mechanisms are involved. Obviously, many more efficacy, safety and toxicity preclinical studies are required to confirm the usefulness of nanotechnological applications in neurodegenerative diseases.

To summarize, there are many more obstacles that have to be overcome regarding the clinical translation of experimental nanomedical innovations such as: a) find investments to support the clinical translation of promising nanomedicine formulations,

b) overcome the difficulties in performing the pre-clinical characterization and safety assessment from early stages, due to lack of protocols, c) handle the lack of knowledge in scale-up and GMP manufacturing, and at last, d) deal with the uncertainty and fragmentation in the regulatory framework[15]. □

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Learn more and access in-deep knowledge, developing your personal and professional skills and competences with the innovative (e-)learning courses from the **Supplementary Distance Education Programme (E-Learning) of the Center of Continuing Education and Lifelong Learning** . Work and study with faculty members of the University of Athens (the National and Kapodistrian University of Athens), the oldest Tertiary Education institution in Greece, as well as with renowned European experts, and enjoy the flexibility to study at your own pace, accessing anytime and anywhere learning. Over the last nineteen years, more than 80,000 people have joined the continuous professional development (CPD) and lifelong learning (LLL) courses of our *E-Learning Programme*, in a variety of academic and professional fields. Online Courses are offered through flexible and blended learning sessions, matching both the specific industry and professions' needs as well as the individuals' availability. Online Courses and Programmes offered during this academic year' s periods (of 3-4 months) fall into the following broad domains:

Courses

HEALTH, NANOTECHNOLOGY | NANOMEDICINE

Microbiome in Human Health and Diseases

HEALTH, NANOTECHNOLOGY | NANOMEDICINE

Nanomedicine: Applications in Diagnostics and Surgery

HEALTH, NANOTECHNOLOGY | NANOMEDICINE

Basic Principles of nanotechnology applications in Health Science

HEALTH, NANOTECHNOLOGY | NANOMEDICINE

Stem cells and Regenerative Medicine

HEALTH, NANOTECHNOLOGY | NANOMEDICINE

Nanotechnology and Nanomedicine

ΕΛΛΗΝΙΚΗ ΔΗΜΟΚΡΑΤΙΑ
Εθνικόν και Καποδιστριακόν Πανεπιστήμιον Αθηνών
ΙΔΡΥΘΕΝ ΤΟ 1837

ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ
Ιατρική Σχολή και Τμήμα Φαρμακευτικής

Διατμηματικό Πρόγραμμα Μεταπτυχιακών Σπουδών στη «ΝΑΝΟΪΑΤΡΙΚΗ» / "Nanomedicine"

Η Ιατρική Σχολή και το τμήμα Φαρμακευτικής του ΕΚΠΑ λειτουργούν ένα νέο Διατμηματικό Πρόγραμμα Μεταπτυχιακών Σπουδών στη **"Νανοϊατρική" / "Nanomedicine".**

Στο πρόγραμμα γίνονται δεκτοί πτυχιούχοι Ιατρικής, Φαρμακευτικής, Βιολογίας, Νοσηλευτικής, Φυσικής, Χημείας, Πολυτεχνικών Σχολών και άλλων θετικών επιστημών.

Η χρονική διάρκεια φοίτησης είναι **ένα (1) έτος**, στο οποίο περιλαμβάνεται και ο χρόνος εκπόνησης διπλωματικής εργασίας.

Το πρόγραμμα σπουδών είναι στα Αγγλικά και είναι δομημένο σε τρεις ενότητες:
UNIT 1: Introduction to Nanotechnology
UNIT 2: Applications of Nanotechnology in Medicine
UNIT 3: Nanotoxicity and Regulatory Aspects

Σκοπός του ΔΠΜΣ είναι η παροχή υψηλού επιπέδου μεταπτυχιακής εκπαίδευσης στο επιστημονικό πεδίο της Νανοϊατρικήςκαι η φοίτηση σε αυτό οδηγεί στην απονομή «Διπλώματος Μεταπτυχιακών Σπουδών» στη Νανοϊατρική (MSc in Nanomedicine) μετά την πλήρη και επιτυχή ολοκλήρωση των σπουδών.

Το ΔΠΜΣ ξεκινά το χειμερινό εξάμηνο εκάστου ακαδημαϊκού έτους.

Κατά τη διάρκεια των σπουδών, οι μεταπτυχιακοί φοιτητές υποχρεούνται σε παρακολούθηση και επιτυχή εξέταση μεταπτυχιακών μαθημάτων, ερευνητική απασχόληση, παρουσίαση και συγγραφή επιστημονικών εργασιών, καθώς και σε εκπόνηση μεταπτυχιακής διπλωματικής εργασίας. Οι φοιτητές έχουν τη δυνατότητα να παρακολουθήσουν μέρος του προγράμματος εξ' αποστάσεως σύμφωνα με τη νομοθεσία.

Πρόσκληση εκδήλωσης ενδιαφέροντος : Τετάρτη 1η Σεπτέμβρη έως 24η Σεπτέμβρη 2021

Η Ειδική Διατμηματική Επιτροπή η οποία είναι υπεύθυνη για τη λειτουργία του Προγράμματος, αποτελείται από τους:
Καθηγητή Ιατρικής κ. Ευστάθιο Ευσταθόπουλο, Πρόεδρο
Καθηγητή Φαρμακευτικής κ. Κων/νο Δεμέτζο, Αντιπρόεδρο και τα μέλη
Αναπλ. Καθηγήτρια Ιατρικής κ. Μαρία Γαζούλη
Αναπλ. Καθηγήτρια Ιατρικής κ. Όλγα Σαββίδου
Επικ. Καθηγητή Φαρμακευτικής κ. Ευάγγελο Καραλή

Περισσότερες πληροφορίες στην ιστοσελίδα του προγράμματος https://nanomed.med.uoa.gr/ και στη γραμματεία (2107462942, κ. Γεωργόπουλος Αναστάσιος)

HELLENIC REPUBLIC

National and Kapodistrian University of Athens

EST. 1837

School of Health Sciences
School of Medicine and
Department of Pharmacy

M.Sc. Program
"Nanomedicine"

Language:
English

Duration:
1 year Full Time
2 years Part Time

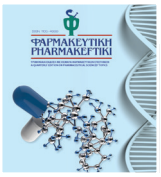
- **Applicants.** Graduates from Greek and foreign Universities with a degree in Medicine, Pharmacy, Biology, Physics, Chemistry, Engineering, and other related subjects can be accepted.
- **Distance learning.** This program provides an interactive platform that gives the possibility to students located at any distance to attend all the theoretical lectures by distance learning.
- **Cost of studies.** The very low registration fees (2.000 euros total cost) gives the opportunity to the majority of students to attend.
- **Novel subject.** There are only a few M.Sc. programs on this novel subject all over the world.
- **Benefits.** The program offers deep knowledge in advanced diagnosis, imaging and therapeutic protocols and delves into various nanomedicine and nanotechnology disciplines.
- **Research.** This program gives the opportunity to conduct high-level research work and to publish in peer-review scientific Journals.
- **Practice.** Cooperation with Pharmaceutical Industry to enhance skills and link theory to practice.

Internationally recognized professors are joined with industrial partners and medical/pharmaceutical professionals to provide a wide multidisciplinary view on this promising, modern and novel subject.

Information:

Tel: +30 210 7462942, 6947647654
Email: nanomed@med.uoa.gr
Website: https://nanomed.med.uoa.gr

Registration is open:
From Wednesday **September 1st**
until Friday **September 24th** 2021



ΕΚΔΗΛΩΣΕΙΣ - MEETINGS

Η εξάπλωση του δεύτερου κύματοςτης πανδημίας COVID-19 που δοκιμάζει τις κοινωνίες σε παγκόσμιο επίπεδο έχει στερήσει τις επιστημονικές εταιρείες από τη δυνατότητα να πραγματοποιούν επιστημονικές εκδηλώσεις και συνέδρια, με φυσική παρουσία.. Ως εκ τούτου τα συνέδρια που είχαν προγραμματιστεί μέχρι και την άνοιξη του 2021 έχουν αναβληθεί ή θα πραγματοποιηθούν on line εικονικά (virtually). Μένουμε ασφαλείς!

The spread of the second wave of COVID-19 pandemia does not allow scientific activi¬ties to take place with physical presence Thus , all scientific events scheduled till spring 2021 have been cancelled, or will be conducted virtually. while all face-to-face activities have been replaced with online meetings. Stay safe!

• AUGUST 29 - SEPTEMBER 2, 2021 - BASEL, SWITZERLAND
XXVI EFMC INTERNATIONAL SYMPOSIUM ON MEDICINAL CHEMISTRY (EFMC-ISMC 2021) EFMC SYMPOSIUM
https://www.efmc-ismc.org/

• SEPTEMBER 2 - 3, 2021, BASEL, SWITZERLAND | 8TH EFMC YOUNG MEDICINAL CHEMISTS' SYMPOSIUM
(EFMC-YMCS 2021) EFMC Symposium
https://www.efmc-ymcs.org/

• SEPTEMBER 19-23, 2021, BARCELONA, SPAIN
23RD EUROPEAN SYMPOSIUM ON QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIP (23RD EUROQSAR)
EFMC Sponsored Event
https://www.euroqsar2020.org/

• SEPTEMBER 20-23, 2021, VIRTUAL EVENT
12TH INTERNATIONAL CONFERENCE ON "INSTRUMENTAL METHODS OF ANALYSIS" (IMA-2021)
www.ima2021.gr

• SEPTEMBER 22-24, 2021 | VIRTUAL MEETING | SUMMER SCHOOL IN PHARMACEUTICAL ANALYSIS (SSPA2021)
http://www.sspaweb.com

• MAY 16-19, 2022 | VOLGOGRAD, RUSSIA
5TH RUSSIAN CONFERENCE ON MEDICINAL CHEMISTRY
Info: https://medchem21.com/index.php/en/home-english/ medchemvolga21@gmail.com

• SEPTEMBER 4-8, 2022 NICE, FRANCE
XXVII EFMC INTERNATIONAL SYMPOSIUM ON MEDICINAL CHEMISTRY
https://www.efmc-ismc.org/

• SEPTEMBER 8-9, 2022 - NICE, FRANCE
EFMC YOUNG MEDICINAL CHEMISTS' SYMPOSIUM
www.efmc-ymcs.org

• APRIL 18-20, 2022, TOKYO, JAPAN
INTERNATIONAL MEET ON PHARMACEUTICS AND DRUG DELIVERY SYSTEMS (PHARMAMEET2022)
https://www.albedomeetings.com/pharmameet/index.php