Charles University
Faculty of Sciences

Study programme: Special Chemical and Biological Programmes
Branch of study: Molecular Biology and Biochemistry of Organisms

Daniela Lehutová

Therapeutic Nanoparticles and Immunotoxicity
Terapeutické Nanočástice a Imunotoxicita

Bachelor´s thesis

Supervisor: MUDr. Luca Ernesto Vannucci, Ph.D.

Prague, 2017

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, 10.5. 2017

Podpis

Abstract

Nanoparticles are submicroscopic structures made from various materials which have a huge therapeutic potential. They represent a novel tool for tissue targeting and drug delivery. Many attempts have been made to develop therapeutics more compatible with the body and to enhance the efficacy of modern drugs. Unlike the commonly used drug delivery methods, encapsulated drug nowadays display a large advantage in a matter of reducing the side effects of traditional medicaments.

In the present thesis, advantages of nanoparticles utilisation are compared with their potential hazards, especially with their influence on the immune system. As toxicity differs according to the nanoparticles chemicophysical characteristics and the tissue in which NP may accumulate, convenient strategy has to be chosen in order to prevent any harmful effects. For this reason, precautions have to be considered and nanoparticles correctly modified. Furthermore, induction of particular response and its consequence has to be appropriately monitored. High attention has to be given to nanoparticle preparation to avoid contamination and danger for operators.

Key words

Nanoparticles, drug delivery, immunity, toxicity, theranostics

Abstrakt

Nanočastice sú submikroskopické štruktúry vyhotovené z rôznych materiálov s obrovským terapeutickým potenciálom. Rovnako predstavujú nový nástroj pre lepšie cielenie a podávanie liečiv do jednotlivých tkanív. Bolo vynaložených mnoho pokusov pre vyvinutie terapeutík, ktoré budú lepšie kompatibilné v organizme a zároveň by zlepšili účinnosť moderných liekov. Na rozdiel od bežne používaných spôsobov pre podávanie liečiv, enkapsulovaná forma predstavuje obrovskú výhodu pri znižovaní vedľajších účinkov oproti bežne používaných liekoch.

V tejto práci sú porovnávané výhody použitia nanočastíc spolu s ich možným rizikom, najmä na ich vplyv na imunitný systém. Vzhľadom k tomu, že toxicita sa líši v závislosti na chemicko-fyzikálnych vlastnostiach a tkanivách, v ktorých sa akumulovali, musí byť zvolená vhodná stratégia, aby sa predišlo možných nežiaducim účinkom. Z tohto dôvodu je potrebné zvážiť preventívne opatrenia a správne upraviť dané nanočástice predtým, než budú použité v organizme, rovnako ako spôsobená odpoveď organizmu a jej dôsledok musia byť vhodne monitorované. Zvýšená pozornosť musí byť venovaná príprave nanočastíc, aby sa predišlo prípadnej kontaminácii a nebezpečenstvu pre laboratórny personál.

Kľúčové slová

nanočástice, podávanie liečiv, imunita, toxicita, teranostika

Contents

List of Acronyms

APC	antigen presenting cell
DNA	deoxyribonucleic acid
DOX	doxorubicin
EPR	(effect) enhanced permeability and retention
HFt	human ferritin protein
MRI	magnetic resonance imaging
MWCNT	multi wall carbon nanotubes
NP	nanoparticles
PEG	polyethylene glycol
REC	reticuloendothelial cells
ROS	reactive oxygen species
SWCNTs	Single-wall carbon nanotubes
TLR	toll-like receptors

1. Introduction

Nanotechnology is currently gaining much more interest and novel applications in biomedical sciences. This fact is a result of demand for more selective therapies, improvement of drug efficacy, reduction of side effects and toxicity of traditional drugs as well as more accurate diagnostic imaging. For this reason, pharmaceutical industry was improving the use of nanotechnology in the last decades and many formulations are already under clinical approval. However, lack of still appropriate information about nanoparticle interaction with the immune system and the potential risk of toxicity continues to pose questions about the limits of their application.

Cancer is a second leading cause of death worldwide. It is estimated that there are 3,7 million cases and 1,9 million deaths each year (WHO & Cancer 2017). In order to reduce these numbers, development of new tools for early diagnosis and to ameliorate targeting of pathological tissues by even encapsulated drugs represent promising option. Nanotechnology can assist the development of these strategies and, for example, it can overwhelm classical drug delivery methods and develop into more practicable therapeutics. In this thesis, I intend to briefly overview nanoparticle drug delivery method, its possible advantages and disadvantages, and particularly its relationship with immune system and risk of induced toxicity.

2. Generalities on nanoconstructs

Nanoparticles (NP) are defined as tiny material of organic or inorganic origin with a size ranging up to 100 nm and in simple or complex structured form. Some NP can be created with a hollow to permit loading with therapeutic or diagnostic molecules. The possibility to encapsulate macromolecular drugs gives them unique properties which are attractive for therapeutic use in intracellular delivery systems (Medina et al., 2007; Mohanraj et Chen, 2006). Currently, its relationship and interaction with biological system represent an important issue and thus characteristics of particles are a key factor to determine the interaction with elements of the immune system (Gioacchino et al., 2015). Additionally, particle proportion affects the distribution, accumulation, cellular uptake and can influence even metabolism (Smith et al., 2014). However, stability and behavior of NP need to be tested for physiological conditions in vivo, in various animal models. This is important also for defining their characteristics (Dobrovolskaia et McNeil, 2007). NP can be used as carriers to modify drug delivery and drug pharmacokinetics or, per se, as immunologically active materials with adjuvant properties (Dobrovolskaia et al., 2016). In conclusion, this field requires far more investigation as this interaction is not well understood.

2.1. Physicochemical properties

Features of optimal NP are biocompatibility, simple production, possibility of easy functionalization of their surface, good degradation, high stability, ability to penetrate directly to tumour site without damaging healthy tissues, controlled drug release under certain stimuli and undetectability by the immune cells with phagocytic activity. Moreover, by adding fluorescent dye or magnetic materials we can provide better tracking and broaden NP possible utilisation also as a diagnostic agent (Vannucci *et al.*, 2014; Mohanraj *et* Chen, 2006).

These properties are influenced by several factors and NP surface and size play the key role. For example, the amount of reactive groups on the nanoparticle surface (Gioacchino *et al.*, 2015). The possibility to functionalize NP with various molecules, for instance, polymers that can increase the capability to link more molecules or expose more reactive groups on the surface; allow providing specific targeting capacity. Decoration with special molecules, for example, PEGylation permits also to reduce the possible immunogenicity of NP, to prevent uptake by the reticuloendothelial cells (REC) and by this way to increase permanence in the circulation and bioavailability of NP (Vannucci *et al.*, 2015). The electromagnetic charge can significantly influence both particle toxicity (more precisely described in chapter 5.2.) and the grade of its capability to adsorb plasma proteins when in circulation. For example, it was shown that polymer-based NP can adsorb proteins inversely proportional to the

charge. Moreover, the shape of NP surface highly contributes to the initiation of phagocytosis (Luck *et* al., 1998).

Size of NP is crucial not only in term of immunotoxicity and their *in vivo* performance, but also their distribution and accumulation. Depending the dimension, NP may present different characteristic in drug loading and release (Mohanraj *et* Chen, 2006). Generally, nanocarriers should to be small enough to easily penetrate through the endothelium, but in the same time, big enough not to be quickly filtered out of bloodstream (Vannucci *et al.*, 2014). Regarding the uptake by REC, it is higher when NP are bigger even in relation to their lower mobility in comparison to smaller particles (Mohanraj *et* Chen, 2006). For instance, small NP reach lymph nodes easier and and have faster distribution within residents dendritic cells, macrophages and B cells. Bigger NP more easily interact with tissue resident immune cells (antigen presenting cells (APC) like macrophages) while small NP quickly following the lymphatic drainage and the migration inside DC can reach lymph nodes and help antigen presentation and immune activation. In fact, it was found that small NP were more related to Th1 and CD8+ T cells responses while bigger NP were responsible for Th2 responses (Fifis *et al*., 2004; Mottram *et al.*, 2007; Manolova *et al.*, 2008). Study of influence of physicochemical properties to immune system can lead to better understanding of avoidance of hypersensitivity reaction (Dobrovolskaia *et* McNeil, 2007). Despite the dimension, the surface is sufficiently large for allowing coating with molecules and the volume small enough to permit faster diffusion of loaded material (Redhead *et al.*, 2001).

2.1.1. Administration

Generally, NP can be locally (for example, intratumorally) or systemically administered (for example, intravenously). The intratumoral approach might be a possibility to enhance concentration of the carried drug reducing its possible side effects compared to same load systemically administered. Various routes of administration are under study like oral, nasal, intramuscular, intraocular or intracardiac route (Dobrovolskaia *et* McNeil, 2007; Xie *et al.*, 2011; Nelson *et al.*, 2014). General blood stream can be reached by NP after inhalation for translocation throughout alveolar epithelia into the capillary system. Other source of penetration can be through the skin. According to the dimension and the charge, NP can even pass through the blood brain barrier (Gioacchino *et al.*, 2015).

2.2.Overview of materials

Nanoparticles, according to their components, are organized into four groups: synthetic, organic, inorganic particles (fig. 1) or composites (Mishra *et al.*, 2013) and each category has particular advantages or disadvantages related to the immune system.

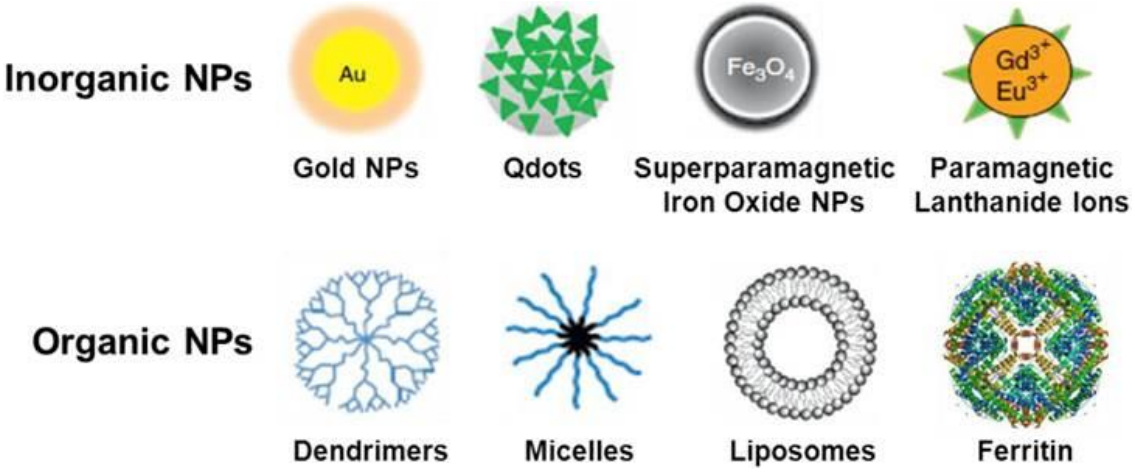

Fig.1: Examples of NP (Xing *et al.*, 2014).

Inorganic NP are widely used for therapeutic applications due to their unique properties, for example, being well-tolerated in the biological systems. These types of nanocarriers involve metallic NP (such as gold, silver, platinum), ceramic NP (for example silica, alumina or titanium), quantum dots (fluorescent semiconductor particles) and carbon particles including single wall or double wall nanotubes and fullerenes. Main disadvantages of this NP are lower biodegradability and lower cargo packing inside the particle (Mishra *et al.,* 2013; Medina *et al.*, 2007). However, there are many desirable utilisations of inorganic NP and this issue is more discussed later.

Synthetic particles are created from polymers and we can further distinguish micelles, polymersomes and dendrimers (Mishra *et al.,* 2013). Advantages are for example low immunogenicity and enhanced capability of penetration in the tissues (permeability) but most importantly, these particles are biocompatible and easily biodegradable (Lee, 2006).

In order to enhance particles` characteristics such as solubility, lower their toxicity or better functionalization, hybrid NP - composites seem to be a possibly efficient solution. This particles consist of more materials and combinations as silica with inorganic component, silica with polymer or polymer with inorganic element (Janczak *et* Aspinwall, 2012).

Last category of NP includes particles made out of organic materials, particularly based on lipid, virus capsids, polysaccharide or protein. Lipid based NP (liposomes) form single and double bilayer

membrane and encase both hydrophobic (in the lipid material or lamella) and hydrophilic material (in the aqueous space). According to their lipidic composition and integration of polyethylene glycol (PEG), liposomes with enhanced longevity and ability to avoid immune system interactions can be produced (Moghimi *et* Szebeni, 2003; Mishra *et al*., 2013). Organic NP are also of viral origin by the use of viral capsids. They can be of various dimensions, accounting from several hundreds to thousands protein molecules with ability to self-assemble. The result is a carrier with a hollow that can be filled with a cargo. These NP are used for their characteristics to penetrate inside the cell and have the advantage of being easily biodegradable. Viral NP are used especially for transfection procedures. The disadvantage is their possible immunogenicity that can limit the repetition for their use. Induced immunogenicity is possible to avoid by using bacteriophages and plant viruses (Vannucci *et al.*, 2014). Polysaccharides NP (for example chitosan, pectin) with their many side chains provide huge variety of options for linking molecules. Generally, these nanoconstructs are stable, highly biocompatible because of their hydrophilic properties as well as protein-based particles and do not lead to higher risk of toxicity (Liu *et al.*, 2008).

Protein-based NP consist of protein subunits which aggregates into a particle structure. Utilized proteins vary depending on desired scaffold to be produced for the therapeutic application. These NP, in the term of rising immunotoxicity in comparison with inorganic NP, are preferably used for various reasons. Their immunogenicity can be variable according to the type of chosen proteins, origin and volume of NP. In order to reduce the immunogenicity, protections of NP by PEGylation, as already described above, are used. The bio-inspired particles can be suitable for controlled drug release since they provide unique properties such as great drug loading, wide spectrum of administration and functionalization versatility with consequent possibility of an efficient specific targeting activity. What is more, production of these NP can be easy and cheap due to their production as recombinant protein, high stability at various temperatures and pH (Tarhini *et al.*, 2017; Mohanraj *et* Chen, 2006; Vannucci *et al.*, 2014). A good example is ferritin-based NP which are given a closer look in our laboratory.

Based on human ferritin protein (HFt), use of these nanoconstructs is profitable due to its biological properties - they are physiological proteins, stable and soluble. Ferritin protein consists of 24 subunits formed into spherical structure with a cavity. Furthermore, it is possible to functionalize them chemically or genetically. Biological function of HFt is the heavy metal uptake which prevents releasing metal ions into blood during blood circulation. By this way NP can be charged by ferromagnetic proteins that can be used for theranostics.

HFt NP have outer diameter of 12 nm and cavity of 8 nm making them relevant to penetrate through the various barriers and is relatively limiting the filtration by kidneys as already told in the previous chapter. Furthermore, their high stability related to the temperature (80 - 100°C) and pH (3 - 10) makes these NP suitable for easy chemical manipulation (Vannucci *et al.*, 2014).

HFt NP are suitable for various types of applications, especially for theranostic interventions. For example, these NP are perspective for the treatment of cutaneous melanoma when functionalized for specific targeting. Functionalized NP were demonstrated to accumulate inside primary melanoma and its spontaneous metastasis. This accumulation is the highest two days after systemic administration. When targeting (decorated with alpha-melanocyte-stimulating hormone) and simple HFt NP are compared, the first show wider and more prolonged accumulation in the tumor sites including metastasis. Moreover, when NP coupled with ferromagnetic load they resulted to be efficient for magnetic resonance imaging (MRI). Ferromagnetic load of NP can be used also for producing heat in electromagnetic field induced hyperthermia like theranostic application as also later described (Vannucci *et al.*, 2014; Vannucci *et al.*, 2015).

3. Mechanisms of tissue targeting by nanoconstructs

There are two means to distinguish selective delivery into desired site (for example tumors) namely active and passive targeting.

Passive diffusion inside the tumor microenvironment is happening for larger molecules / structures (the best size is 100 nm) by the so-called mechanism of enhanced permeability and retention, the EPR effect, which is favorably used in current cancer therapy. Due to this effect, NP can accumulate in the interstitial spaces because of the porosity of the tumor vessels and the differences of pressure between two compartments helping NP translocation from the blood flow into the interstitial spaces. The use of this effect can be limited by the anatomical characteristics of the cancers, more specifically the extension of their vascularisation as well as the presence of fibrotic processes or necrotic areas (Rossin *et al.*, 2005; Danhier *et al.*, 2010).

Active targeting is based on receptor-ligand interaction. Either ligand or receptors are structures that are linked to the NP surface to enable binding on the counterpart on the expressing cell. Molecules are chosen in the repertoire not naturally expressed on the normally cell but overexpressed by the targeted tumor. By this way it is obtained high specificity of targeting and a better uptake elicited by the receptor-ligand interaction (Friedman *et al.*, 2014; Danhier *et al.*, 2010).

On the surface of the NP targeting ligands are attached; which are either peptidic ligands, monoclonal antibodies or just their fragments which can be further conjugated with therapeutic molecules. Various immunoconjugates are being developed, but not many of them met with clinical approval yet. (Vannucci *et al.*, 2014; Danhier *et al.*, 2010).

Possible use of the targeting properties can be inhibiting the neoangiogenesis in tumors. Targeting the endothelium can limit the neoangiogenesis deprivating the tumor of its support (Folkman, 1971).

Other delivery systems with multiple targeting capability are produced. These multifunctional NP when combined with chemotherapy or radiotherapy are also a perspective strategy for cancer treatment (Danhier *et al.*, 2010).

Unprotected NP can be opsonized soon after administration becoming detectable for REC and later on processed by phagocytes (Redhead *et al.*, 2001). This clearance from the bloodstream is another factor which significantly limits NP application (fig. 2) if they are not protected by PEGylation.

When NP are unable to accumulate in targeted site they can diffuse within organs. This might lead to inflammatory reactions becoming chronic if NP are not easily biodegradable, with subsequent possible toxicity. The ability to subtract NP from the circulation, for example by liver Kupffer cells, should be used as a new approach for the treatment of REC related diseases like leishmaniasis or candidiasis (Gustafson *et al.*, 2015; Redhead *et al.*, 2001).

The possibility to reduce phagocytic activity of macrophages during NP treatment can enhance the bioavailability of NP and enhance NP circulation. One of the most used systems to obtain this effect is, as more times cited, the addition of hydrophilic polymers such as PEG with the effect to increase NP circulation and to prevent protein adsorption (Gustafson *et al.*, 2015). Another possibility is to mask particle surface with self identification proteins, for example, cofactors in order to inhibit complement pathway (as Factor H in HIV which attenuate alternative pathway) (Moghimi *et al.*, 2011) or anti-phagocytic CD47 signal which prevents internalisation by macrophages (Weiskopf *et al.*, 2014).

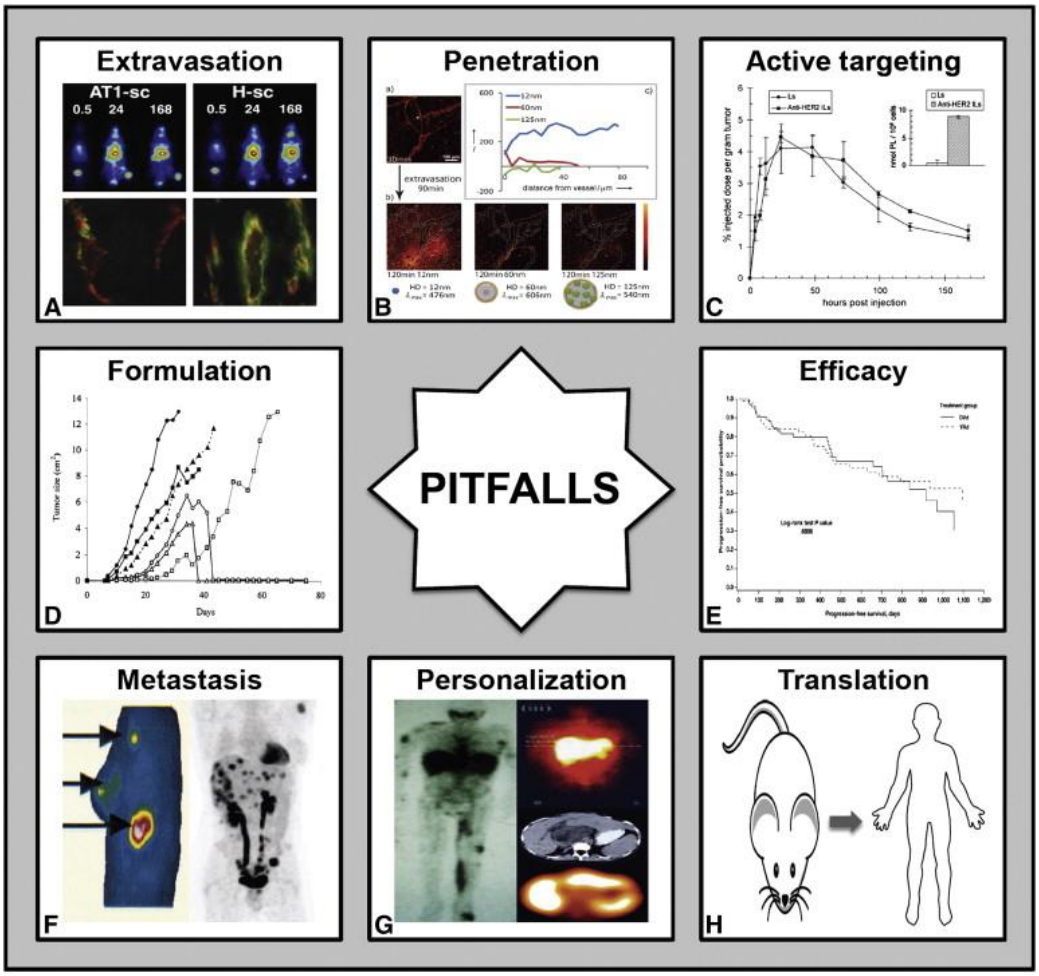

Fig. 2: Problems connected to drug targeting to tumors. A: Variability of the EPR effect. B: Efficacy of penetration of NP depending on their size. C: Comparison of efficacy of liposomes with or without targeting. D: Adequate formulation - different potency in encapsulated and free doxorubicin. E: Results in animal model and human patients may vary. Efficient NP in mouse model did not give advantage in treatment of multiple myeloma patients, as shown. F: Primary versus metastatic tumors - effective distribution in primary versus metastatic tumors. G: Patient on the picture is suffering from Kaposi sarcoma and the image shows that NP were accumulated in the healthy tissues. In this case, it could have been predicted that the treatment was not going to be successful. H: Better models for testing should be considered (Lammers *et al.,* 2012).

4. NP and their bio-medical utilization

Variety of fields in which NP can be applicable is extremely huge and NP are mostly used as a therapeutic or diagnostic agent. In this chapter we will better focus on the immune system.

Modulating immune response in order to reach desired therapeutic effect is a long-time proven approach. Generally, NP has been applied in vaccine delivery, as an adjuvant to existing therapeutics

or as a structural mimicry (Dobrovolskaia *et al.*, 2016; Diwan *et al.*, 2003). For instance, gold NPs increase immune response and further antibody and cytokine production. In most cases, enhanced reaction was observed when bigger NP were used. Another mechanism of modulation which contributes to the efficacy of vaccines is activation of inflammasome. Inflammasome is activated after destruction of lysosome provided by NP escape through its bilayer. Hence, lysosomal proteases are released into cytosol and NP toxicity is increased. However, not every lysosomal escape causes inflammasome activation. There is a possibility that as different shapes of NP are recognised by APC, different cytokines are produced (Niikura *et al.*, 2013; Smith *et al.*, 2014).

Moreover, NP are advantageously used as a complement to existing drugs where their participation can considerably prevent side effects, lower immunotoxicity and upgrade pharmacokinetics (Dobrovolskaia *et al.*, 2016). Various studies show, that this utilisation as adjuvants leads to enhanced production of antibodies making the immunisation more efficient. Similarly, required doses for adequate response were significantly lower comparing to Freund`s adjuvants (Dykman *et al.*, 2004 as cited in Dobrovolskaia *et* McNeil, 2007).

Likewise, NP can be specially prepared to mimic some pathogens in order to induce relevant immune response. More efficient activation of dendritic cells and priming T cells with greater proliferation was induced when NP were modified with toll-like receptors (TLR), which are responsible for pathogen detection in the body, together with MUC1 lipopeptide (Diwan *et al.*, 2003).

4.1. Cancer immunotherapy

Another potential use of NP stands for cancer immunotherapy. Used therapeutics significantly differ in their properties and action. A good example with potential clinical application is iron oxide NP when used together with alternating magnetic fields to cause hyperthermia in tumours. Phenomenon following hyperthermia application can induce dendritic and CD8+ T cells resulting in prevention of secondary tumour development (Toraya-Brown *et al.*, 2014). Typically, immunotherapy can be either passive or active, depending on the type of therapeutics that are applied, for example antibodies or vaccinations. Particularly, most of the immunotherapeutics function in an active way which boosts the immune system to enhance its responses against malignant cells (Galluzzi *et al.*, 2014).

4.2. Theranosis

The final aim of theranosis, by joining diagnosis and therapy into one procedure, is to create more personalised medicine and to increase the capability of treating disease in each specific person. Therefore the theranostic approach therapeutics facilitated by NP represents an attractive option. These nanoplatforms provide both imaging and therapeutic action and many materials which are already used

for diagnosis can be improved in order to reach therapeutic effect. Materials utilised are, for example, iron oxide NP, gold, carbon nanotubes, silica-based NP or quantum dots. Regarding each type of materials must be every time considered their potential toxicity and their cost. (Xie *et al.*, 2010).

For example, MRI is a useful procedure in which magnetic contrast agents allows better distinguishment between normal and malignant tissue. The most used contrast agent is made of paramagnetic materials, gadolinium and its salts. In fact, gadolinium is applied in a form of chelated chemical complex followed by functionalization due to high toxicity in its free form (Hong *et al.*, 2016). Similarly, iron oxide NP show similar capability in MRI imaging and can be eventually used also for hyperthermic purposes. Use of these NP provides early diagnosis when other contrast agents do not succeed in visualisation of metastasis and degenerative diseases (Gobbo *et al.*, 2015).

4.3. Major advantages

One of the most immediate advantages of NP for therapeutic use is the possibility to load and locally deliver drugs. New products are under study and some are already commercialised (liposomal doxorubicin (DOX) (Rivera, 2003)). In order to demonstrate the benefits of enclosed drugs, a brief comparison of diverse ways of doxorubicin administration is furnished.

DOX is one of the most widely used chemotherapeutic drugs nowadays. However, as one of the most effective anticancer drugs, DOX can be cytotoxic also for healthy cells. Its toxicity affects cardiac muscle, redox cycle in the cells, causes DNA damage and induces multidrug resistance (Mao *et al.*, 2016; Heart *et al.*, 2016).

As an antibiotic, it is isolated from *Streptomyces peucetius var. caesius*. For cancer treatment its hydrochloride form is used which is a derivative of danorubicin (Doxorubicin, PubChem Compound Database).

Currently, DOX is being administrated intravenously. Its target is rapidly dividing cells like cancer cells, but also its action can affect blood cells, hair follicles or stomach and bowel mucosa cells. It interrupts cell division and induces damage of DNA or RNA by intercalating between the base pairs. Furthermore, DOX inhibits topoisomerase II, prevents DNA ligation after double strand DNA breakage and induce formation of free oxygen radicals. When the treatment is finished, normal cells grow back regularly (Doxorubicin. Lexi-Drugs®, 2015; Doxorubicin, PubChem Compound Database).

The final therapeutic effect can be influenced by enhancing the temperature (hypethermia). For example, for DOX, increased toxicity was observed at 42 °C compared to 37 °C *in vitro* utilizing PEG

and poly-L-lactide copolymers (Na *et al.*, 2006). Therefore this drug might be suitable also for theranostic applications.

From preliminary experiments performed in the Laboratory of Immunotherapy, Institute of Microbiology, Czech Academy of Science, we can see the effect on the tumor growth by DOX after intracardiac administration (fig. 3). DOX loaded in NP reduced the tumor growth in comparison to its free form at an equivalent molar dose (Vannucci, unpublished data).

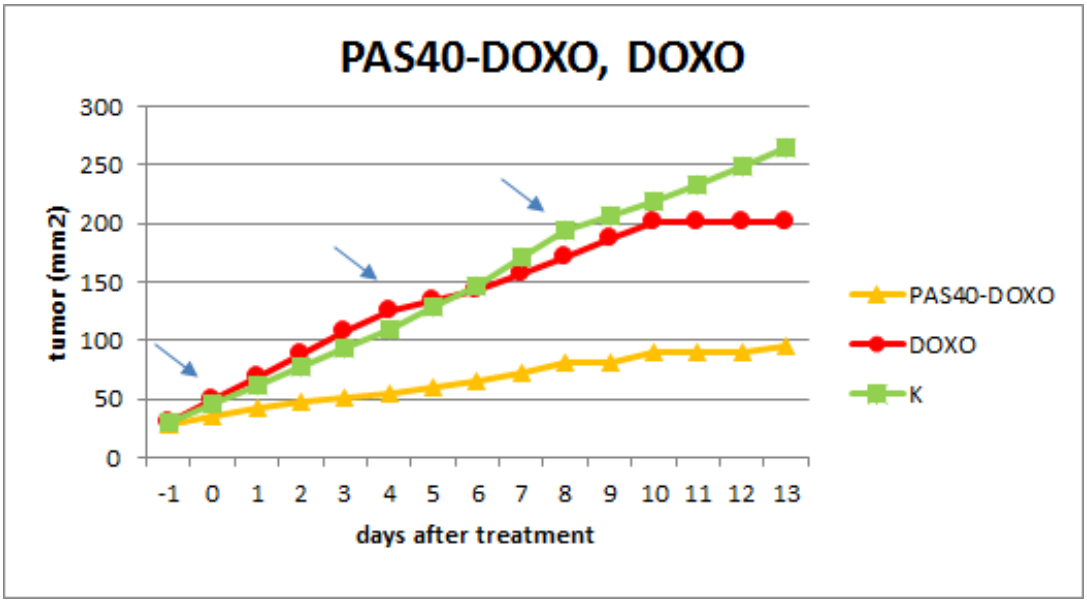

Fig. 3: Efficacy of free DOX and enclosed DOX (PAS40-DOXO) treatment after intracardiac administration. CT26 syngeneic colorectal carcinoma subcutaneously developed in BALB/c mice. The treatment was applied in day 0, 4 and 8 (Vannucci, unpublished data).

These initial results are promising; however, they must be not only better confirmed in the animal model, but also in human, since possible disparities between the animal and the clinical realities may occur.

5. NP and interactions with immune system

The immune system is using the innate immunity cells with phagocytic activity to defend the organism from undesired threats. The phagocytes (granulocytes, macrophages and dendritic cells) internalise the threatening elements (for example parasites, dead cells, particles and other foreign materials) trying to digest and to eliminate them. Macrophages, in particular, are heterogeneous cells which are able to adapt their function to environmental conditions to exert phagocytosis and to mediate inflammation (Murray *et* Wynn, 2011). The phagocytes including REC (for example Kupffer cells) are the main sector of immunity to be involved and to react after NP administration (Gustafson *et al.*, 2015).

There are various factors how NP interact with the biological tissues and specifically, immune system. More precise mapping of this interaction is required as NP can cause permanent changes in the tissues at nanolevel. As mentioned in the previous chapter, catalytic activity, composition, electronic structure or hydrophobicity have significant influence. Generally, toxicity differs according to the nanomaterial and tissue type (Gustafson *et al.*, 2015), but the dimension and surface area as above discussed (chapter 2.1.) turn out to be the key factors in the interactions with the immune system (Medina *et al.*, 2007; Gioacchino *et al.*, 2015).

NP can be engineered to interact or, on contrary, to prevent the interaction with the immune system. In the first case this effect is produced on purpose and the consequence is to obtain stimulation - upregulation or suppression. In the second case the goal is to avoid quick elimination of NP from the circulation not to impede the efficacy the aimed action. As before discussed, these approaches can be used for inducing adjuvant effect in vaccination or for carrying drugs in a specific part of the organism. Overload of NP can result in undesirable accumulation inside the organs, including immune organs, leading to possible chronic inflammation (Fig. 4) (Zolnik *et al.*, 2010; Dobrovolskaia *et* McNeil, 2007; Gioacchino *et al.*, 2015).

NP immune modulating activity together with their possibility of mimicry can potentially induce autoimmune reactions. This can be obtained with the support of mast cells and basophils (Smith *et al.*, 2014). In other studies was suggested differential responsiveness to different kind of immune cells to the same type of NP with necessity to clarify this point with relation to general potential immunotoxicity of NP (Zamboni *et al.*, 2011).

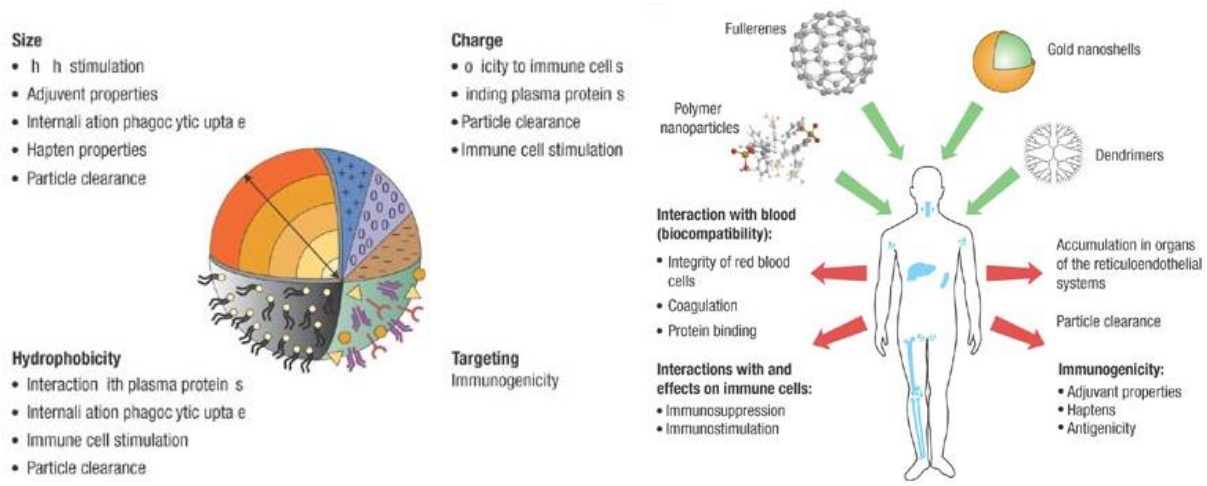

Fig. 4: NP properties determine their interaction with immune system (Dobrovolskaia *et* McNeil, 2007).

5.1. Modulation of immune response

5.1.1. Immunostimulation

Upregulation of the immune system is following the detection of extraneous element in the body with consequent stimulatory reaction of the immune system (Dobrovolskaia *et* McNeil, 2007).

According to the type of stimulation, for example, bacteria or NP exposing bacterial toxins derivatives the complement cascade can be involved. According to the type of NP and their composition the distribution or accumulation in the organs of the immune system can modify their activity with effect that be alternatively enhancing or reducing the anticancer responses when NP are applied as anticancer drug carrier. NP with low solubility or elevated antigenicity can elicit allergic responses as a consequence of inflammatory reactions. For this reason it is sometimes suggested that treatments with therapeutic or diagnostic NP might be accompanied by the use of antiallergic medicament as a prevention of unwanted cell responses (Zolnik *et al.*, 2010; Dobrovolskaia *et al.*, 2016)

5.1.2. Immunosuppression

Immunosuppression, mostly understood as not desirable effect as it can lower the organism`s possibility to fight against cancer or other diseases, has advantageous effects in terms of autoimmune disorders and transplantations, for example, suppression of T cells function and development (Zolnik *et al.*, 2010). Similarly, mast cells and basophils were inhibited by water-soluble fullerene derivative which led to reduce type 1 allergens hypersensitivity (Ryan *et al.*, 2007). These preliminary experiences are perspective for the development of new approaches and new class of treatment for allergic and autoimmune diseases.

5.2. Risk of immunotoxicity

Crucial factors to identify NP immunotoxicity are physicochemical properties and NP mechanism of action (Fig. 5) (Dobrovolskaia *et al.*, 2016). As more times mentioned, the immunotoxic potential need to be evaluated according to immunogenicity, adjuvant properties, capacity to induce inflammatory reaction and to stimulate phagocytosis. In fact, studies suggest that NP are not more toxic than regular drugs (Dobrovolskaia *et* McNeil, 2007).

Experimental effects	***Pathophysiological outcomes***
*ROS generation**	Protein, DNA and membrane injury,* oxidative stress†
*Oxidative stress**	Phase II enzyme induction, inflammation,† mitochondrial perturbation*
*Mitochondrial perturbation**	Inner membrane damage,* permeability transition pore opening,* energy failure,* apoptosis,* apo-necrosis, cytotoxicity
*Inflammation**	Tissue infiltration with inflammatory cells,† fibrosis,† granulomas,† atherogenesis,† acute phase protein expression (e.g., C-reactive protein)
*Uptake by reticulo-endothelial system**	Asymptomatic sequestration and storage in liver,* spleen, lymph nodes,† possible organ enlargement and dysfunction
*Protein denaturation, degradation**	Loss of enzyme activity,* auto-antigenicity
*Nuclear uptake**	DNA damage, nucleoprotein clumping,* autoantigens
*Uptake in neuronal tissue**	Brain and peripheral nervous system injury
Perturbation of phagocytic function, "particle overload," mediator release**	Chronic inflammation,† fibrosis,† granulomas,† interference in clearance of infectious agents†
*Endothelial dysfunction, effects on blood clotting**	Atherogenesis,* thrombosis,* stroke, myocardial infarction
Generation of neoantigens, breakdown in immune tolerance	Autoimmunity, adjuvant effects
Altered cell cycle regulation	Proliferation, cell cycle arrest, senescence
DNA damage	Mutagenesis, metaplasia, carcinogenesis

Fig. 5: Assumptive (*) and demonstrated (†) experimental effects and pathophysiological outcomes after introduction of NP (Petrarca *et al.*, 2006).

A factor which can considerably influence NP toxicity, and that is not predictable in *in vivo* use, is the bonding of proteins on NP surface. Apart from the proteins that functionalize the NP and obviously modify its original size, charge, capability of circulation and to be uptaken by phagocytes, proteins that from the tissues or plasma can stick and remain linked to the NP may deeply modify its properties. A "clean" and PEGylated NP has the possibility to remain longer in the blood stream otherwise its time of circulation can be affected by the additional elements that may join the NP surface (Dobrovolskaia *et al.*, 2016).

NP functionalization with various types of molecules can reduce the possibility of some adverse effect already cited (Albini *et al.*, 2010). Aside PEGylation, another technique, PASylation that is an addition of Pro, Ala and Ser amino acids, enhance halftime of the nanocarrier without increasing toxicity against tissues (Vannucci *et al.*, 2014).

Toxic danger may come also from NP with cations bond on their surfaces as cationic NP were found to be more able to elicit inflammatory response than anionic and neutral particles. Additionally, therapeutic nucleic acids interact with their nanocarrier electrostatically. These interactions among NP and cells cause cells` rupture and other harmful processes such as hemolysis. Moreover, therapeutic nucleic acids lead to inflammatory reaction, secretion of cytokines or type I interferons, complement activation or prolongation of plasma coagulation time (Greish *et al.,* 2012; Dobrovolskaia *et* McNeil, 2007).

5.2.1. Biocompatibility

NP biocompatibility is a factor reducing their possible toxicity. Biodegradable NP able to dissolve faster in the body are preferred to non-biodegradable NP which can represent a risk for the organism. (Dobrovolskaia *et al.*, 2016). Nevertheless, carbon nanotubes were found to be specifically degraded by activated neutrophils through myeloperoxidase reactive intermediates and peroxynitrite driven oxidation, found also in activated macrophages (Kagan *et al.*, 2010).

Many studies have been conducted regarding higher toxicity of non-biodegradable inorganic NP. The chemical constitution can be a critical factor. For instance, cobalt NP which bond to DNA helix might cause genotoxicity, influence cell cycle and cause aneuploidy alongside its downregulation. Extensive toxicity - induction of proinflammatory cytokines such as TNF-α and INF-γ was observed when palladium or nickel NP were applied. Silica NP, on contrary, induced lung inflammation or pulmonary chronic disease (Gioacchino *et al.*, 2015). Blood coagulation was affected by gadolinium or polyvinyl chloride resin particles administration with pro-thrombotic effects that were prevented by PEGylation of the NP (Balakrishnan *et al.*, 2005). Elevation of oxidative stress and increase of reactive oxygen species production was observed in human monocytes after zinc oxide NP administration with activation of pro-inflammatory cytokines (IL-1β, TNF-α) production as well as induction of NF-κB and MAPK signalling pathways. The small size of this NP contributed to the effect. Additionally, zinc oxide NP were shown to induce cytotoxicity and genotoxicity (Gioacchino *et al.*, 2015; Senapati *et al.*, 2015).

Engineered carbon NP influence and activate classical and alternative complement pathway rarely causing harmful effects. Carbon NP can be divided in single and double walled particles and their impact on the pathway significantly differs. However, both activate classical pathway, but only double

walled were noted to activate alternative pathway (Salvador-Morales *et al.*, 2006). Another study shows that carbon nanotubes via myeloid-derived suppressor cells induced TGF-β which assists in activating immunosuppressive reaction that can be involved in enhancing tumor development (Shvedova *et al.*, 2013). However, single-wall carbon nanotubes (SWCNTs) are shown to inhibit the angiogenesis, which represents a bright option for cancer therapy (Albini *et al.*, 2010).

Carbon can potentially cause inflammatory reaction which can be reduced by its oxidation. Performed tests do not demonstrate any major toxicity except from findings related to cellular metabolism or membrane permeability. The fact that SWCNTs were taken into lysosomal compartment and later normalized shows, that the nanotubes were either degraded by the endothelial tissue, which is considering their stability an important point as a possibility by cell to destroy highly stable structures, or transported throughout the cell. (Albini *et al.*, 2010).

Additional toxicity that can hamper further testing and development of the causative NP accounting also thrombogenicity, inflammation, pyrogenicity as a result of contamination, anaphylaxis and harmful impact on erythrocytes (Dobrovolskaia *et al.*, 2016).

5.2.2. Toxicity induced by contamination

Purity and sterility of NP are another important issue in order not to trigger inflammatory response and related immunotoxicity. Contamination by bacterial endotoxins (for NP produced by transfection in *E. coli*) products and linkers which might have an unsterile phase of work during synthesis and purification NP can be source of inflammatory response and pyrogenicity. Silicon oil, carbon, fluoropolymers, glass, cellulose, hydrophobic metal and metal oxides, rubber, plastics and polystyrene are partly responsible for protein aggregation leading to immunogenicity (Dobrovolskaia *et* McNeil, 2007; Crist *et al.*, 2013; Dobrovolskaia *et al.*, 2016).

5.2.3. Risk for operators

Currently, multi wall carbon nanotubes (MWCNT) are most used among NP. However, their potential toxicity does not only refer to patients, but can present a risk to operators, too, principally, by aerosol inhalation. Another risk is their biopersistence and induction of inflammation as well as neurodegenerative diseases as a result of protein and MWCNT aggregation. Amyloid deposition might cause damage to the organs, mainly to brain**.** Same toxic effect was observed after utilisation of different routes of administration. Generally, amyloidogenesis is caused by oxidative stress and inflammation. Furthermore, amyloid fibril formation was associated with recruitment of macrophages (Albini *et al.*, 2015).

After administration of MWCNT into mice, at first they accumulated in lungs and brain - which represent an important fact as general problem is NP ability to pass through blood brain barrier - and later on were able to reach liver and kidney via bloodstream. It is suggested that MWCNT entered brain through olfactory nerve. Taken together, MWCNT caused inflammation, respiratory, neurodegenerative and digestive problems. Induced toxicity might be reduced by enzymatic degradation (Albini *et al.*, 2015).

6. Evaluation of immunotoxicity

Assessment of immunotoxicity can meet with obstacles and as various testing strategies are practised, the results even for the same particles may differ. Generally, *in vitro* testing does not represent an ideal method as NP may interact with reagents or detection assays and cause undesirable reactions. Cytokines, endotoxins or other contamination should be used to evaluate the presence of inflammatory response but these systems have to be advanced in order to provide comparable culture conditions, exposure times and cell types, accurate and multiple sampling of tissues and reduction of all possible factors that can modify absorbance, luminescence and fluorescence in the analytical systems (Dobrovolskaia *et al.*, 2009; Smith *et al.*, 2014).

Regarding *in vivo* testing, standard tests must be evaluated to the relation to the physicochemical properties of the used NP. For instance, lymph node proliferation assay is applicable to NP when they are in a size <100 nm and after subcutaneous administration. Another possible method is to monitor uptake by macrophages. Tests based on this approach together with histopathology show relevant alterations in immune organs. The trend is to use *in vitro* tests as they are faster and relatively inexpensive but they cannot work as a suitable replacement. However, combination of both tests seems to be the most appropriate option (Manolova *et al.*, 2008; Dobrovolskaia *et al.*, 2009).

7. Perspectives and conclusions

Recently, utilisation of nanotechnology is greatly escalating. However, nanocarriers introduce different types of hazards compared to the standard drugs and it is crucial to understand better the concepts of drug delivery in order to prevent wrong interpretation. In fact, there are just novel findings that NP have harmful effects for patients, operators or can influence the therapeutic effect of the enclosed drug. More investigation has to be done to pharmacokinetics and to rising risks as it is not possible to prevent the interactions between NP and immune system. Taken together, NP are shown to be promising invention but more studies on their toxicity are required in order to achieve the most favorable results.

8. Literature

"Cancer." *World Health Organization*. World Health Organization, Feb. 2017, <http://www.who.int/mediacentre/factsheets/fs297/en/>

Albini A, Pagani A, Pulze L, *et al*. Environmental impact of multi-wall carbon nanotubes in a novel model of exposure: systemic distribution, macrophage accumulation, and amyloid deposition. *International Journal of Nanomedicine*. 2015;10:6133-6145

Albini, Adriana, Valentina Mussi, Alessandro Parodi, *et al*. Interactions of single-wall carbon nanotubes with endothelial cells. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2010, 6(2), 277-288

Balakrishnan, B., Kumar, D.S., Yoshida, Y. & Jayakrishnan, A. Chemical modification of poly(vinyl chloride) resin using poly(ethylene glycol) to improve blood compatibility. *Biomaterials* 26,3495–3502 (2005)

Christie M. Sayes, Kenneth L. Reed, David B. Warheit; Assessing Toxicity of Fine and Nanoparticles: Comparing *In Vitro* Measurements to *In Vivo* Pulmonary Toxicity Profiles. *Toxicological Sciences* 2007; 97 (1): 163-180

Crist, Rachael M., Jennifer Hall Grossman, Anil K. Patri, Stephan T. STERN, Marina A. Dobrovolskaia, Pavan P. Adiseshaiah, Jeffrey D. Clogston, Scott E. Mcneil. Common pitfalls in nanotechnology: lessons learned from NCI's Nanotechnology Characterization Laboratory. *Integrative Biology*. 2013, 5(1), 66-73

Danhier, Fabienne, Olivier Feron, Véronique Préat. To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery. *Journal of Controlled Release*. 2010, 148(2), 135-146

Diwan, Manish, Praveen Elamanchili, Helena Lane, Anita Gainer, John Samuel. Biodegradable Nanoparticle Mediated Antigen Delivery to Human Cord Blood Derived Dendritic Cells for Induction of Primary T Cell Responses. *Journal of Drug Targeting*. 2008, 11(8-10), 495-507

Dobrovolskaia, M. and McNeil, S. (2007). Immunological properties of engineered nanomaterials. *Nature Nanotechnology*, 2(8), pp.469-478

Dobrovolskaia, M., Germolec, D. Weaver, J. (2009). Evaluation of nanoparticle immunotoxicity. *Nature Nanotechnology*, 4(7), pp.411-414

Dobrovolskaia, Marina A., Michael Shurin, Anna A. Shvedova. Current understanding of interactions between nanoparticles and the immune system. *Toxicology and Applied Pharmacology*. 2016, 299, 78-89

Doxorubicin. Lexicomp Online®. Lexi-Drugs®. Hudson, Ohio: Lexi-Comp, Inc.; 2015

Dykman, L.A. *et al*. Immunogenic properties of colloidal gold. *Izvestiya Rossiiskoi Akademii Nauk – Seriya Biologicheskaya*.1,86–91 (2004)*

Fifis Theodora, Gamvrellis Anita, Crimeen-Irwin Blessing, A. Pietersz Geoffrey, Jie Li, Mottram Patricia L., McKenzie Ian, Plebanski Magdalena. *The Journal of Immunology*. 2004, 173 (5) 3148-3154

Folkman, Judah. Transplacental Carcinogenesis by Stilbestrol. *New England Journal of Medicine*. 1971, 285(7), 404-405

Friedman, Adam, Sarah Claypool, Rihe Liu. The Smart Targeting of Nanoparticles. *Current Pharmaceutical Design*. 2013, 19(35), 6315-6329

Galluzzi L, Vacchelli E, Pedro J-MB-S, et al. Classification of current anticancer immunotherapies. *Oncotarget*. 2014;5(24)

Gobbo OL, Sjaastad K, Radomski MW, Volkov Y, Prina-Mello A. Magnetic Nanoparticles in Cancer Theranostics. *Theranostics* 2015; 5(11):1249-1263

Greish, Khaled, Giridhar Thiagarajan, Heather Herd, *et al*. Size and surface charge significantly influence the toxicity of silica and dendritic nanoparticles. *Nanotoxicology*. 2011**,** 6(7), 713-723

Gustafson HH, Holt-Casper D, Grainger DW, Ghandehari H. Nanoparticle Uptake: The Phagocyte Problem. *Nano today*. 2015;10(4):487-510

Heart Emma A., Karandrea Shpetim, Liang Xiaomei *et al*. Mechanisms of Doxorubicin Toxicity in Pancreatic β-Cells, *Toxicological science*. 152(2), 2016, 395–405

Hong, Seong-Pyo, Seong Hee Kang, Do Kyung Kim, Bo Sun Kang. Paramagnetic G d 2 O 3 Nanoparticle-Based Targeting Theranostic Agent for C6 Rat Glioma Cell. *Journal of Nanomaterials*. 2016, 2016, 1-10

Janczak, Colleen M., Craig A. Aspinwall. Composite nanoparticles: the best of two worlds. *Analytical and Bioanalytical Chemistry*. 2012, 402(1), 83-89

Kagan, Valerian E., Nagarjun V. Konduru, Weihong Feng, *et al*. Carbon nanotubes degraded by neutrophil myeloperoxidase induce less pulmonary inflammation. *Nature Nanotechnology*. 2010-4-4, 5(5), 354-359

Lammers, Twan, Fabian Kiessling, Wim E. Hennink, Gert Storm. Drug targeting to tumors: Principles, pitfalls and (pre-) clinical progress. *Journal of Controlled Release*. 2012, 161(2), 175-187

Lee, L. James. Polymer Nanoengineering for Biomedical Applications. *Annals of Biomedical Engineering*. 2006-3-22, 34(1), 75-88

Liu, Zonghua, Yanpeng Jiao, Yifei Wang, Changren Zhou, Ziyong Zhang. Polysaccharides-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*. 2008, 60(15), 1650-1662

Luck, M. *et al*. Analysis of plasma protein adsorption on polymeric nanoparticles with different surface characteristics. *Journal of Biomedical Materials Research*.39, 478–485 (1998)

Manolova, V., Flace, A., Bauer, M., Schwarz, K., Saudan, P. and Bachmann, Martin F. (2008), Nanoparticles target distinct dendritic cell populations according to their size. Eur. *The Journal of Immunology*. 38: 1404–1413

Mao, Xiaoman, Jingxing Si, Qian Huang, *et al*. Self-Assembling Doxorubicin Prodrug Forming Nanoparticles and Effectively Reversing Drug Resistance In Vitro and In Vivo. *Advanced Healthcare Materials*. 2016, 5(19), 2517-2527

Mario Di Gioacchino, Francesco Lazzarin: Immunotoxicity of nanoparticles, *International journal of immunopathology and pharmacology* 2011 24(1 Suppl):65S-71S

Medina C, Santos-Martinez MJ, Radomski A, Corrigan OI, Radomski MW. Nanoparticles: pharmacological and toxicological significance. *British Journal of Pharmacology*. 2007;150(5):552-558

Mishra D, Hubenak JR, Mathur AB. Nanoparticle systems as tools to improve drug delivery and therapeutic efficacy. *Journal of Biomedical Material Research* Part A 2013:101A:3646–3660

Moghimi, S. Moein, Alina J. Andersen, Davoud Ahmadvand, Peter P. Wibroe, Thomas L. Andresen, A. Christy Hunter. Material properties in complement activation. *Advanced Drug Delivery Reviews*. 2011, 63(12), 1000-1007

Moghimi, S.M. Chemical camouflage of nanospheres with a poorly reactive surface: towards development of stealth and target-specific nanocarriers. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2002, 1590(1-3), 131-139

Moghimi, S.M., J. Szebeni. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Progress in Lipid Research*. 2003, 42(6), 463-478

Mottram Patricia L., Leong David, Crimeen-Irwin Blessing, Gloster Simone, D. Xiang Sue D, Meanger Jayesh, Ghildyal Reena, Vardaxis Nicholas, Plebanski Magdalena. *Molecular Pharmaceutics*, 2007, 4 (1), 73–84

Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. *Nature reviews Immunology*. 2011;11(11):723-737

Na, Kun, Kwang Hee Lee, Don Haeng Lee, You Han Bae. Biodegradable thermo-sensitive nanoparticles from poly(l-lactic acid)/poly(ethylene glycol) alternating multi-block copolymer for potential anti-cancer drug carrier. *European Journal of Pharmaceutical Sciences*. 2006, 27(2-3), 115-122

National Center for Biotechnology Information. PubChem Compound Database; CID=31703

Nelson Delia, Fisher Scott, Robinson Bruce, The "Trojan Horse" Approach to Tumor Immunotherapy: Targeting the Tumor Microenvironment, *Journal of Immunology Research*. 2014, 1-14

Niikura, Kenichi, Tatsuya Matsunaga, Tadaki Suzuki, Shintaro Kobayashi, Hiroki Yamaguchi, Yasuko Orba, Akira Kawaguchi, Hideki Hasegawa, Kiichi Kajino, Takafumi Ninomiya, Kuniharu Ijiro, and Hirofumi Sawa. Gold Nanoparticles as a Vaccine Platform: Influence of Size and Shape on Immunological Responsesin Vitroandin Vivo. *ACS Nano* 7, no. 5 (2013): 3926-938

Petrarca, C., Perrone, A., Verna, N., Verginelli, F., Ponti, J., Sabbioni, E., Di Giampaolo, L., Dadorante, V., Schiavone, C., Boscolo, P., Mariani Constantini, R., Di Gioacchino, M. Cobalt nano-particles modulate cytokine in vitro release by human mononuclear cells mimicking autoimmune disease. *International Journal of Immunopathology and Pharmacology*. 2006, 19:11-4

Redhead, H.M., S.S. Davis, L. Illum. Drug delivery in poly(lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: in vitro characterisation and in vivo evaluation. *Journal of Controlled Release*. 2001, 70(3), 353-363

Rivera, E. Liposomal Anthracyclines in Metastatic Breast Cancer: Clinical Update. *The Oncologist*. 2003, 8(90002), 3-9

Rossin R, Pan D, Qi K, Turner JL, Sun X, Wooley KL, Welch MJ. 64Cu-labeled folate-conjugated shell cross-linked nanoparticles for tumor imaging and radiotherapy: synthesis, radiolabeling, and biologic evaluation. *The Journal of Nuclear Medicine*. 2005 Jul;46(7):1210-8

Ryan John J., Bateman Henry R., Stover Alex, Gomez Greg, Norton Sarah K., Zhao Wei, Schwartz Lawrence B., Lenk Robert, L. Kepley Christopher. Fullerene Nanomaterials Inhibit the Allergic Response. *The Journal of Immunology* 2007, 179 (1) 665-672

Salvadormorales, C, E Flahaut, E Sim, J Sloan, M Hgreen, R Sim. Complement activation and protein adsorption by carbon nanotubes. *Molecular Immunology*. 2006, 43(3), 193-201

Senapati, Violet Aileen, Ashutosh Kumar, Govind Sharan Gupta, Alok Kumar Pandey, Alok Dhawan. ZnO nanoparticles induced inflammatory response and genotoxicity in human blood cells: A mechanistic approach. *Food and Chemical Toxicology*. 2015, 85, 61-70

Shvedova, Anna A., Alexey V. Tkach, Elena R. Kisin, *et al*. Carbon Nanotubes Enhance Metastatic Growth of Lung Carcinoma via Up-Regulation of Myeloid-Derived Suppressor Cells. *Small*. 2013, 9(9-10), 1691-1695

Smith, Matthew J. *et al*. "From Immunotoxicity to Nanotherapy: The Effects of Nanomaterials on the Immune System." *Toxicological Sciences* 138.2 (2014): 249–255

Tarhini, Mohamad, Hélène Greige-Gerges, Abdelhamid Elaissari. Protein-based nanoparticles: From preparation to encapsulation of active molecules. *International Journal of Pharmaceutics*. 2017, 522(1-2), 172-197

Toraya-Brown, Seiko, Mee Rie Sheen, Peisheng Zhang, *et al*. Local hyperthermia treatment of tumors induces CD8+ T cell-mediated resistance against distal and secondary tumors. *Nanomedicine: Nanotechnology, Biology and Medicine*. 2014, 10(6), 1273-1285

Van Zijverden, Maaike, Berit Granum. Adjuvant activity of particulate pollutants in different mouse models. *Toxicology*. 2000, 152(1-3), 69-77

Vannucci, Luca, Elisabetta Falvo, Cristina Maria Failla, *et al*. *In Vivo* Targeting of Cutaneous Melanoma Using an Melanoma Stimulating Hormone-Engineered Human Protein Cage with Fluorophore and Magnetic Resonance Imaging Tracers. *Journal of Biomedical Nanotechnology*. 2015, 11(1), 81-92

Vannucci, Luca, Elisabetta Falvo, Pierpaolo Ceci. *Multifunctional Protein-Based Nanoparticles for Cancer Theranosis*. p. 231

VJ Mohanraj, Y Chen. Nanoparticles – A Review. *Tropical Journal of Pharmaceutical Research*. June 2006; 5 (1): 561-573

Weiskopf K, Ring AM, Ho CCM, et al. Engineered SIRPα variants as immunotherapeutic adjuvants to anti-cancer antibodies. *Science (New York, NY)*. 2013;341(6141)

Xie J, Lee S, Chen X. Nanoparticle-based theranostic agents. *Advanced drug delivery reviews*. 2010; 62(11):1064-1079

Xie Y, Bagby TR, Cohen M, Forrest ML. Drug delivery to the lymphatic system: importance in future cancer diagnosis and therapies. *Expert opinion on drug delivery*. 2009;6(8):785-792

Xing Y, Zhao J, Conti PS, Chen K. Radiolabeled Nanoparticles for Multimodality Tumor Imaging. *Theranostics* 2014; 4(3):290-306

Zamboni, William C., Lauren J. Maruca, Sandra Strychor, *et al*. Bidirectional pharmacodynamic interaction between pegylated liposomal CKD-602 (S-CKD602) and monocytes in patients with refractory solid tumors. *Journal of Liposome Research*. 2010, 21(2), 158-165

Zolnik, Banu S., África González-Fernández, Nakissa Sadrieh, Marina A. Dobrovolskaia. Minireview: Nanoparticles and the Immune System. *Endocrinology*. 2010, 151(2), 458-465

*secondary citation