Universidade de Lisboa

Faculdade de Farmácia

Developing pharmacokinetic models for nano drug delivery systems

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Monografia orientada pelo Professor Doutor Luís Pleno de Gouveia, Categoria Professor Auxiliar

Mestrado Integrado em Ciências Farmacêuticas

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Agradecimentos

Dedico este trabalho a toda a instituição da Faculdade de Farmácia da Universidade de Lisboa. Quero agradecer todo o corpo docente desta instituição bem como os professores convidados pela sua dedicação a aquisição e transferência de conhecimentos e orientação dos descendentes no percurso académico.

Resumo em Português

A área dos nanomedicamentos é interdisciplinar e complexa com fontes de literatura terciárias, sobre a forma de manuais, emergentes desde os 2010 e, ainda assim, os processos que sustentam a farmacocinética e a farmacodinâmica de nanomedicamentos ainda não estão totalmente caracterizados.

O objetivo desta monografia é apresentar, para os indivíduos que podem ser relativamente novos na área de nanomedicamentos, as propriedades farmacocinéticas de nanopartículas, as abordagens na modelação farmacocinética, e demonstrar a aplicação destes princípios em exemplos tanto de investigação fundamental, quanto no desenvolvimento e otimização bio galénica de nanomedicamentos.

Aqui são descritas as etapas farmacocinéticas de absorção, distribuição, metabolização e eliminação referentes a nanomedicamentos, com realce nos aspetos que distinguem estes processos daquilo que é observado quando se trata de medicamentos "convencionais". É também fornecida uma discussão sobre conceitos essenciais necessários para discussão de modelação farmacocinética usados nas abordagens compartimentais, mecanísticas, e baseadas na fisiologia. Diversos assuntos tangentes como corrente interesse na área de oncologia, extrapolação interespécies em estudos pré-clínicos e aspetos regulamentares associados são também brevemente abordados.

Esta monografia foi realizada com base nas publicações disponíveis nas bases de dados de PubMed e Science Direct até ao mês de setembro do ano 2021. Este trabalho não é único e assemelha-se as revisões de Moss D. M. e Siccardi M., de Glassman P. M. e Muzakantov V. R., ou de Yuan D. et al quanto a organização bem como aos conteúdos.(1–3)

A farmacocinética que descreve os medicamentos "convencionais" baseados na distribuição de substâncias ativas começa apenas quando as etapas finais de libertação e degradação das nanopartículas já começam a ocorrer. A existência simultânea de entidades particuladas e moleculares complica a descrição, otimização, desenvolvimento e avaliação regulamentar de novas formulações de nanomedicamentos. Isto, juntamente com a falta de técnicas analíticas adequadas para a quantificação de nanopartículas em meios biológicos, torna os estudos de modelação farmacocinética de nanomedicamentos um desafio.

Palavras-chave: nanomedicamentos; nano sistemas de distribuição de fármacos; propriedades farmacocinéticas; modelação farmacocinética.

Abstract

Nanomedicines are a complex and highly interdisciplinary field with recently emerging Textbooks as tertiary literature sources since 2010s, and yet the processes that underpin the pharmacokinetics and pharmacodynamics of nano drug delivery systems are not fully characterized.

The aim of this monograph is to introduce the pharmacokinetic dispositions, pharmacokinetic modelling approaches, and to demonstrate application of these principles in examples of both basic research and NDDS development to individuals who may be relatively new to the field of nanomedicine.

In this monograph are described the pharmacokinetic steps of absorption, distribution, metabolization and elimination particular to nano drug delivery systems, primarily focusing aspects that distinguish NDDS from "conventional" drugs. A description of essential concepts necessary for discussions of PK modelling in compartmental, mechanistic, and physiology-based approaches are also provided. Various related topics including growing interest in cancer therapy, interspecies extrapolation in pre-clinical study settings, and reglementary affairs related to NDDSs are also briefly addressed.

Writing of this monograph was conducted after browsing information available in the PubMed and Science Direct databases up to September 2021. This work is not unique and resembles the reviews by Moss D. M. and Siccardi M., Glassman P. M. and Muzakantov V. R., and Yuan D. et al, in their structure, subject and contents.(1–3)

Pharmacokinetics that describes small molecule active substances, begin only when the final steps of nanoparticles fate of release and degradation had begun. Simultaneous existence of both particulate and molecular entities complicates the description, optimization, development, and regulatory assessment of new nano formulations. This together with the lack of appropriate analytical techniques for nanoparticle quantification in biologic media makes pharmacokinetic modelling studies of NDDSs challenging.

Keywords: nanomedicine, nano drug delivery systems; pharmacokinetic dispositions; pharmacokinetic modelling.

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Abbreviations

% ID/g - measure of biodistribution in percentage of dose administered per gram of tissue.
API - active pharmaceutical ingredient.
AUC – Area under the curve.
BD – biodistribution.
DE - Delivery efficiency [% of administered Dose that reaches target tissue].
DTI - drug targeting index [(AUCtargetcompartmentNP/AUCtoxicitycompartmentNP)/ [(AUCtargetcompartmentAPI/AUCtoxicitycompartmentAPI)].
ECM – extracellular matrix.
ERP – enhanced retention and permeation.
IFP – interstitial fluid pressure.
MBPK - mechanistically based pharmacokinetics.
NDDS – nano drug delivery system.
NM – nanomedicine
NP – nanoparticle.
PBPK - physiologically based pharmacokinetics.
PEG – polyethylene glycol.
PK – pharmacokinetics.
MPS – mononuclear phagocytic system
PC – protein corona.
$t_{1/2}$ – half-life.

Definitions

Delivery efficiency – fraction of administered dose that reaches the target tissue, per gram of target tissue.
Drug loading capacity – amount of drug that can be incorporated into a given formulation or nanocarrier.
Nanocarrier – a vehicle particle responsible for the biodistribution of NDDS onto which one or several API can be loaded.
Nano drug delivery system – drug formulation designed to have targeted delivery and controlled rate of drug release employing nanotechnology.
Nanomedicine – field of health sciences concerned with nanotechnology based medical applications.
Nanomaterials – material with at least one dimension comprised in the nanometer size scale.
Nanotechnology – controlled design, production, and application of nanomaterials and their properties.
Nanosizing - top-down size reduction of solid active substance from powder particles or bottom-up synthesis of nano particles comprised of active substance alone or in a mixture with excipients.
Protein corona – proteins suprastructure formed by adsorption of biologic fluid proteins to nanoparticle surfaces.

1 Introduction

Nanomaterials (NMs) are very diverse in composition, structure, characteristics, and very broad in their uses and possible applications. Particles at the nano scale are characterized by transitional physicochemical properties between those observed in molecules and those of bulk matter. These nanoparticles exhibit unique chemical, electric, magnetic, biologic, and mechanical properties that are determined by their composition and can vary greatly within the nanometer size range.

Nanomedicine is a highly multidisciplinary field at interface of physical, chemical, material, pharmacokinetic and pharmacological fields of science and engineering as well as a source of concern from toxicological and environmental standpoints. In this way nanomaterials have found application in biology and medicine research and as disease diagnosis, prevention, and treatment tools.

Nanoparticles can be used in treatment of diseases, as a carrier for active pharmaceutical ingredient (API), as bioactive materials, as implant components, and for diagnosis as in vivo contrast agents or as in vitro screening elements.(4,5) Regarding the use of nanomaterials in drug delivery, the choice of the nanocarrier is based on the physicochemical properties of the API and of the excipients to endow an ability to incorporate the therapeutic load whilst performing selective delivery and controlled release.

The application of NM in imaging and theranostic applications exploits the unique physicochemical properties of nanoparticles. Nano-systems have been developed for nuclear imaging [positron emission tomography (PET), and single photon emission computed tomography (SPECT)], magnetic resonance imaging (MRI), computed tomography (CT), ultrasound (US), optical imaging, and photoacoustic (PA) imaging. This can be archived through the use of superparamagnetic iron oxide NPs (SPIONs), quantum dots (QD), plasmonic nanoparticles, upconverting nanoparticles (UCNPs), dye-doped silicas, gas vesicles (GV), or through incorporation of radioisotopes, paramagnetic ion chelates, or fluorophores into the NPs.(4–8)

The primary objective in the development of nano drug delivery systems (NDDS) is to manipulate the pharmacokinetic (PK) characteristics of the active pharmaceutical ingredient (API) by making it follow the pharmacokinetic profile of the nanoparticle (NP), while conserving, or even enhancing its pharmacodynamic (PD) properties in the therapeutic target tissues. As such NDDS aims to provide spatial and temporal targeting of drugs in order to archive reduction in side effects and increase in effectiveness. PK optimization constitutes a crucial step in NDDS development, it consists in the selection of formula that accomplishes the desired PK behavior. PK modeling can supply greater confidence toward the efficacy and safety of the whole system even before entering the first clinical trials. This monograph will overview the main specificities of PK dispositions of NDDSs important during NM development and focus on PK modeling process.

There are numerous reviews that categorize, characterize and explore the various existing NDDSs and NMs platforms in research or products that are already approved by EMA or FDA in the respective markets, these are discussed elsewhere.(4,7,9–11)

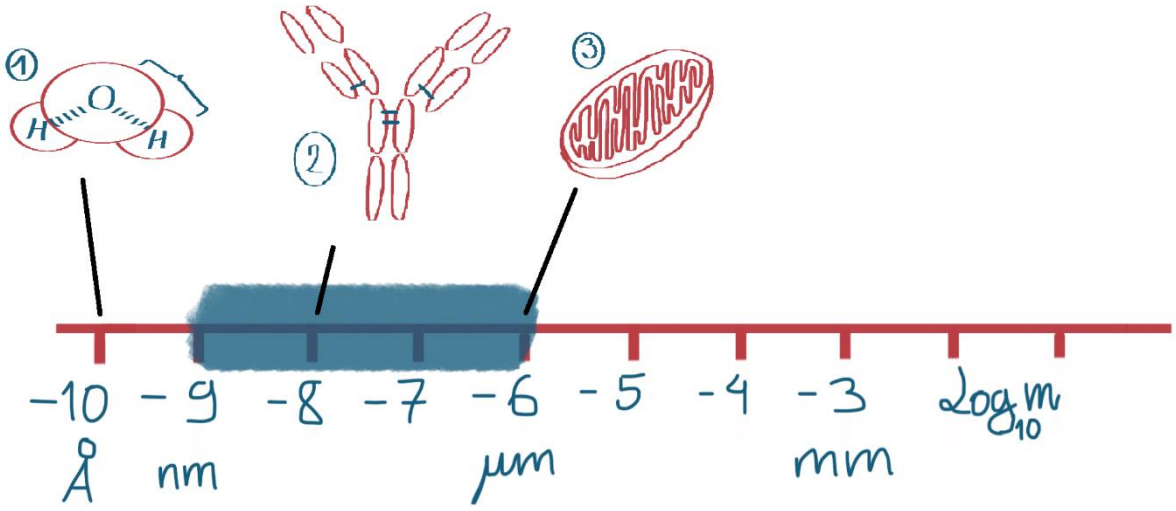

Figure 1

Pictorial representation of base ten logarithmic meter scale with 1) water molecule molecular bond at the angstrom scale; 2) an antibody averaging at 10 nm diameter; 3) mitochondrion at approximately 1 μm diameter; shaded region represents nanoparticle domain comprised of nanospheres, nanorods, nanofiber, nanosheets and any other possible geometric configurations.

2 NDDS Pharmacokinetics

In this first part is provided a general overview of the pharmacokinetic processes namely the absorption, distribution, metabolism, and elimination of pharmaceutical nanomaterials. A special emphasis is brought on how physicochemical properties of the NDDSs determine their pharmacokinetic behavior. These physicochemical properties can be regarded as critical quality attributes of the formulation thus establishing a traceable relation between in vitro characterization and in vivo performance. Most of the relationships described here where already established by fundamental research studies since 1970s on liposomes,(1) yet many new NDDS delivery systems have been developed over the years, and new studies and reviews have allowed to distill and expand beyond these observations.

Perhaps the most noticeable change in pharmacokinetics of an active substance when properly formulated as a NDDS delivery system, are the increase in area under the curve, a prolonged half-live, a decrease in apparent clearance and a reduced volume of distribution determined from measured plasma concentrations of total API. These changes can be marginal as well as several dozen fold in their magnitude for the same active substance demonstrating that bio-galenic development process of NDDS formulation is determining factor in in vivo dispositions of the selected drug.(12,13) These PK parameters alone do not reflect an actual therapeutic advantage of one nano system over another or even over a conventional drug formulation. In this regard biodistribution studies are required, and target drug delivery efficiency metric can represent a prominent quantifier of tissue exposure to the API.(14,15) Additionally if the used pharmacokinetic model is supplemented with an appropriate pharmacodynamic component, efficacy and toxicity of the formulation can be estimated from a preclinical setting.(16,17)

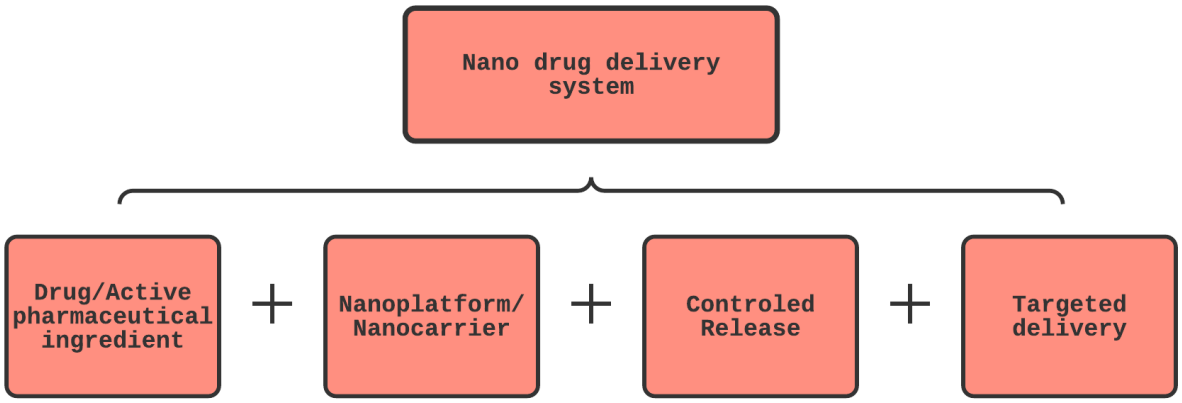

Figure 2

Concept of nano drug delivery system as a combination of physical elements, API and nanocarrier, and performance characteristics of controlled release and targeted delivery.

2.1 Absorption

Bioavailability is defined as a fraction of administered dose that reaches systemic circulation. Intravenous route does not suffer absorption and is entirely bioavailable, while other routs of administration always have a reduced effective dose. This incomplete absorption is caused by simultaneous degradation and removal of drug from the site of administration by local physiologic processes. Absorption represents the first step of drug pharmacokinetics after administration, it describes the cumulative processes that lead to drug uptake into systemic circulation and leads to a gradual increase in blood concentration until reaching a maximum, at this point absorption is no longer the presiding process and blood concentration profile is overtaken by the dominance of distribution and elimination processes that are responsible for a gradual decrease in the concentration of the drug. Administration route is the principal factor responsible for differences in bioavailability, plasma time-concentration PK profile, and consequently, performance of any formulation, NM or otherwise.

2.1.1 Oral route

Nanosizing[1] and nanoparticle design can be used to solve some of the inherent weaknesses of the API, such as, low bioavailability. Bioavailability of biopharmaceutical classification system class II (low solubility drugs), and class IV (low solubility and low permeability drugs), can be corrected by an increase in surface to volume ratio which, in turn, increases the dissolution rate and the apparent solubility of the active substance. Nanoparticles can be formulated as amorphous solid solutions or solid dispersions leading to even faster API release and dissolution rates in gastrointestinal tract (GIT).(18)

[1] Nanosizing is production of nanoparticles by top-down size reduction of solid active substance from powder particles or bottom-up synthesis of nanoparticles comprised of active substance alone or in a mixture with excipients to archive a high surface to mass ratio resulting in a higher apparent solubility of the active substance.

There are several anatomical and physiological obstacles that orally administered formulations must overcome to have appreciable bioavailability.(19) Gastric acidic environment can compromise the stability of many acid liable drugs, here lipid nano capsules and PEGylation[2] can be a great improvement for gastric stability of API as well as modify their absorption kinetics.(20) Another problem are the digestive enzymes and biliary acids compromising the integrity of most lipid based and biodegradable polymer formulations. Gastrointestinal mucus lining is a passive barrier for NPs diffusion, it is also a site of possible interactions such as adsorption that can hinder NP's approach to the enterocyte lining.(21) The transport of nanoparticles through mucosal lining is a size dependent process, because mucus possesses a matrix structure with mesh-pore spacing of 50-1800 nm, and variable thickness of 120-480 nm effectively becoming a filtering sieve.(21,22)

Choi Y.H. and Han H.K. authored a review of many nanomedicines that have been developed for increased absorption of oral formulations, and discuss how nano sizing can provide superior oral bioavailability and extended terminal half-life.(9)

Chiang P. C. et al using a surface area absorption PK model for oral or subcutaneous administered NPs, based on Noyes and Whitney dissolution equation, show that absorption efficiency increases with higher total surface area, but only in the dissolution rate limited region of administered drug. When surface area is increased further, a solubility rate limited region is observed where API absorption efficiency plateaus.(23)

Kumar S. used fibroin-casein nanoparticles to enhance oral route bioavailability of carvedilol. Sensitivity analysis simulations of their gastrointestinal absorption model suggests that increase in API release rate would increase its Cmax, AUC and bioavailability.(24)

2.1.1.1 Transcytosis

Nanosizing is a possible strategy to increase API bioavailability through increased dissolution rate and by shielding it from degradation, still for a nanoparticle to reach bloodstream it needs to cross the mucosal and enterocyte lining of GIT. For the purpose of enterocytic crossing by transcytosis a rational design of nanoparticle stability, size, and surface characteristics is necessary. (21)

For the NDDS to be bioavailable after oral administration it must remain stable until it reaches the enterocytes, maintain its integrity through the endocytosis process and in the intracellular medium, otherwise only API in its free form will be able to reach the systemic circulation. A reduction in the apparent volume of distribution and an increase in terminal half-life of API when compared with drug administered as a solution should be an indication of NP reaching the bloodstream.(25)

Transcytosis can be mediated through the different endocytic mechanisms clathrin-mediated or caveolin-mediated endocytosis or pinocytosis. Paracellular transport is a very limiting route because only 1/10 000 of the total absorptive surface comprises tight junctions and is limited to 1 nm size threshold, consequently resulting in negligible contributions for NP absorption.(9,20,22)

[2] PEGylation is the process of attachment of molecules of polyethylene glycol to the surface of a NDDS or therapeutic proteins usually by covalent bonding or incorporation as a block-copolymer.

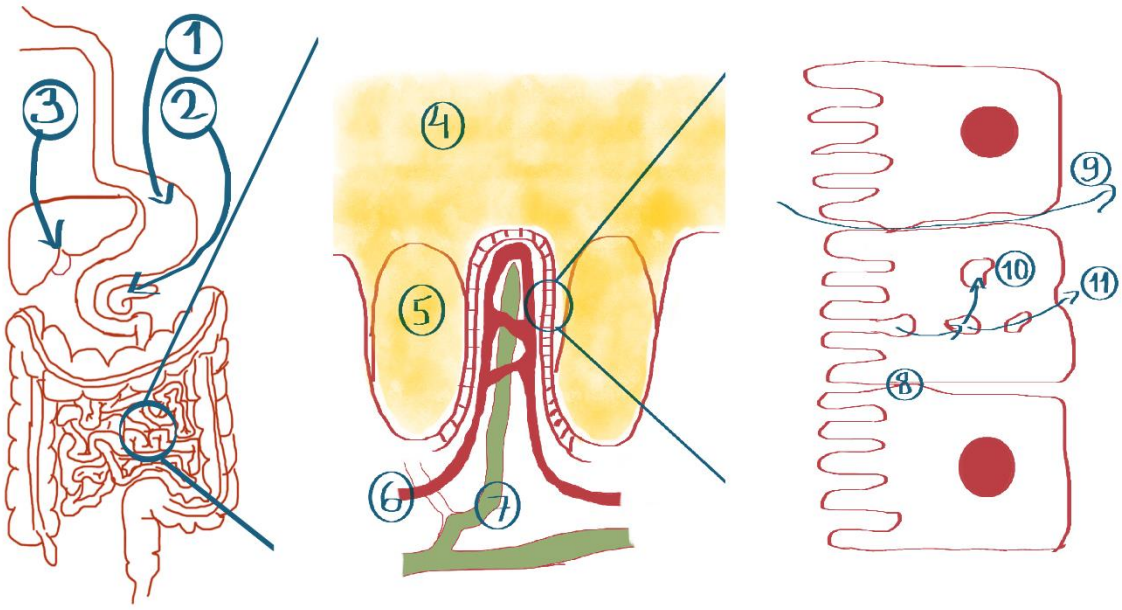

Figure 3

Pictorial representation of barriers to the design of NDDS for oral route of administration: 1) stomach; 2) pancreas; 3) liver; 4) mucus; 5) villus; 6) arteriole; 7) draining lacteal 8) enterocyte tight junction; 9) transcytosis; 10) lysosomal degradation; 11) exocytosis completing transcytosis rout.

Active targeting can be used for enhanced enterocytes transcytosis through oral route of administration. In a study by Zou D. et al, is shown that use of a surface ligand on nanoparticles improved their in vitro cellular uptake, and an increase in AUC compared to either free drug or NPs without surface conjugated ligand was observed in vivo.(26)

Employment of hydrophobic NDDS can be used to promote an alternative absorption route through lacteals and lymph drained together with chylomicrons to effectively bypass of hepatic first pass metabolization, thus increasing absorbed and bioavailable fractions of the active substance.(24,27–29)

2.1.2 Subcutaneous rout

Despite being less appealing than the oral rout, subcutaneous administration enables ambulatory treatment of chronic diseases otherwise restricted to intravenous route in a hospital setting, however there is still a high potential for immunogenicity and the bioavailability of such route is incomplete.(30)

Capillaries are restricting structures for nanoparticle diffusion, and regarding subcutaneous route of administration, only compounds with MW lower than 16 kDa, <10 nm, are freely absorbed into circulation through capillaries, while higher molecular weight entities, 10-100 nm, require lymphatic drainage to be a systemically available as a nanoparticles.(30,31)

It is also important to consider how the extent of degradation of the formulation at site of injection, first pass catabolism, and wide lateral spreading in subcutaneous tissue can affect toxicity and overall performance of the formulation.(30)

2.1.2.1 Lymphatic exposure

Due to nanoparticle size, subcutaneously administered formulations tend to drain into blind-ended lymphatic capillaries and eventually accumulate in the regional lymphatic nodes. This process increases exposure of the lymphatic system to the API and can be exploited for lymphatic targeting of drugs.(30) The effect of increased lymphatic exposure and delayed absorption of drug can be decomposed into several mechanisms: preferential lymphatic uptake, lymphatic first time pass and lymphatic retention.(32) Owing to ECM characteristics, negatively charged particles appear to have greater lymphatic exposure and uptake than neutral formulations.(30)

Regarding this observation, Ryan G. M. et al tested two formulations for their ability of lymphatic exposure to API after SC or IV administration. Results, with drug solution as the control, show that micellar formulations, 5 nm, have similar PK behavior as simple solution of the drug, while the dendrimers, 12 nm, and the liposomes, 100 nm, had several hundred times higher lymphatic recovery concentrations and a larger AUC of total API. Also the dendrimers had an overall better performance over the liposomes and the SC route was better than the IV route regarding lymphatic exposure.(33)

Likewise, several subcutaneous nanoparticle antiretroviral drugs (ARVD) have been formulated to enhance the exposure of lymph node resident mononuclear cells and provide better viral clearance. Kraft J. C. et al tested a triple ARV lipid nanosuspension in macaques following a series of similar studies confirming the superior exposure of the lymphatic system and particularly the lymphatic and plasma mononuclear cells to ARVDs.(32,34–36)

Optimal sizes for lymphatic delivery by subcutaneous route appear to be in 10-50 nm region and acceptable up to 100 nm in diameter.(28,33) Besides dendrimers, solid lipid NPs, and polymeric NPs, particularly when formulated with excipients with longer lipid chain length have shown an overall higher immune cell uptake and lymphatic system transfer.(28)

2.1.2.2 Depot systems

Many posterior segment eye diseases are treated with intraocular, mainly intravitreal injections. Sapino s. et al authored a review regarding different ophthalmic drug delivery formulations. Matrix based systems such as hydrogels and thermo responsive polymers are capable of creating a sustained release medium for drugs, including nanocarriers, greatly extending the overall API half-life.(37)

When local action is desired in the vicinity of the site of administration, larger nanoparticles with slower release rate show longer local retention and greater API exposure. This can be used to reduce the number of administrations. As an example, Amrite A. C. et al test the peri-ocular administration of 200 nm and 20 nm nanoparticles and performed simulations with different release rates. It shows that for slower release rates the reduced diffusion and clearance of larger nanoparticles increases the transscleral delivery and retinal exposure to the API.(38)

Externally controlled API release rate can be accomplished by using thermally or photo responsive polymer system. Fong W. K. et al have tested a lyotropic liquid crystalline matrix with gold nanorods as a photothermal trigger to produce a photo responsive matrix. Under laser

exposure the system had slower release rate of reverse hexagonal phase and upon laser cessation it had the faster release rate constant of continuous cubic phase.(39)

Negatively charged particles are expected to diffuse and travel faster through ECM because of repulsion from highly negatively charged glycosaminoglycans contrary to positively charged NPs that might adsorb to it.(28,30,31,40) regarding these observations larger positively charged particles can be used for retention at site of administration localized release and action.

2.2 Distribution

After reaching systemic circulation NPs, like the "conventional" drugs, can be considered bioavailable for distribution into extravascular tissue spaces, where most API exert their action. This process occurs based on the affinity and preferential extraction from blood to perfused tissues according to the physicochemical properties of the NDDS. This selective tissue accumulation can be expressed as blood/tissue partition coefficient, that is a concentration ratio representing a linear relationship in perfusion limited, highly permeable tissues. There also is the possibility of ND adsorption to the endothelium and the red blood cells as well as an accumulation inside the red blood cells, resulting in a larger nanocarrier distribution volume than just the plasma volume despite a negligible extravasation.

Regarding the vascular morphology, the continuous capillaries have tight occluding junctions sealing off all the intercellular clefts between the endothelial cells, these capillary beds are found in exocrine glands, muscle, connective, lung, and nervous tissues. Transport of most macromolecular components through continuous capillaries is restricted to transcytosis. Fenestrated capillaries present in kidneys, intestine, choroid plexus, and endocrine glands, are characterized by a sieve-like structure peppered with, on average, 80 nm diameter perforations. However basal lamina surrounding the endothelium is still continuous offering the dense, felt-like, matrix composed of laminin and type IV collagen as additional resistance to diffusion. Finally, the discontinuous capillaries or sinusoids have large, wide, irregular spaces between cells lining the vessel and their basement membranes. These sinusoids also have unusually large diameter for capillaries, of 30 to 40 μm, a very slow blood flow, and are primarily found in bone marrow, liver, and spleen.(41) However inflammation, radiation and mechanical traumas can abolish these filtering conditions and allow perfuse extravasation of large particles through any of these types of endothelial linings.(42)

After the distribution, the NDDSs can have many diverse fates: it can be cleared back into the blood flow following a wash-out concentration gradient; it can diffuse and drain into lymph capillaries; it can become entrapped in ECM and gradually release its contents; it can be endocytosed into parenchymal cells; or suffer clearance by the local macrophages.

2.2.1 Size and shape

Size is the main determining factor of NDDS distribution. Every organ's capillary beds have a pore size exclusion limit.(16) Endothelial transcytosis is also a size dependent process necessary to archive extravascular access in tight junction endothelium.(43,44) The size together with surface chemistry and opsonization rate control NDDS's rate of removal by mononuclear phagocytic system as well as any other route of elimination.(45) Size is an important factor in determining half-life of the NDDS, and an increased half-life leads to

passive increase in target and non-target accumulation and exposure to the NDDS.(46,47) This is particularly important for distribution of nanomedicines, because the tissues that are permeation limited or that have low distribution rate constants benefit most from extended plasma drug presentation. Half-life of nanoparticles tends to increase with reduction in core diameter and increase in PEG molecular weight,(13) while larger particle diameters produce higher spleen and liver accumulation and retention.(45)

According to a vascular permeability model by Kirtane A. R. et al, if the target tissue has large vascular pore diameter, most efficient particle size for target site delivery has the smallest diameter above the threshold for clearance into tissues of elimination and distribution, restricting nanoparticle removal by those organs, but still allowing target tissue distribution.(48)

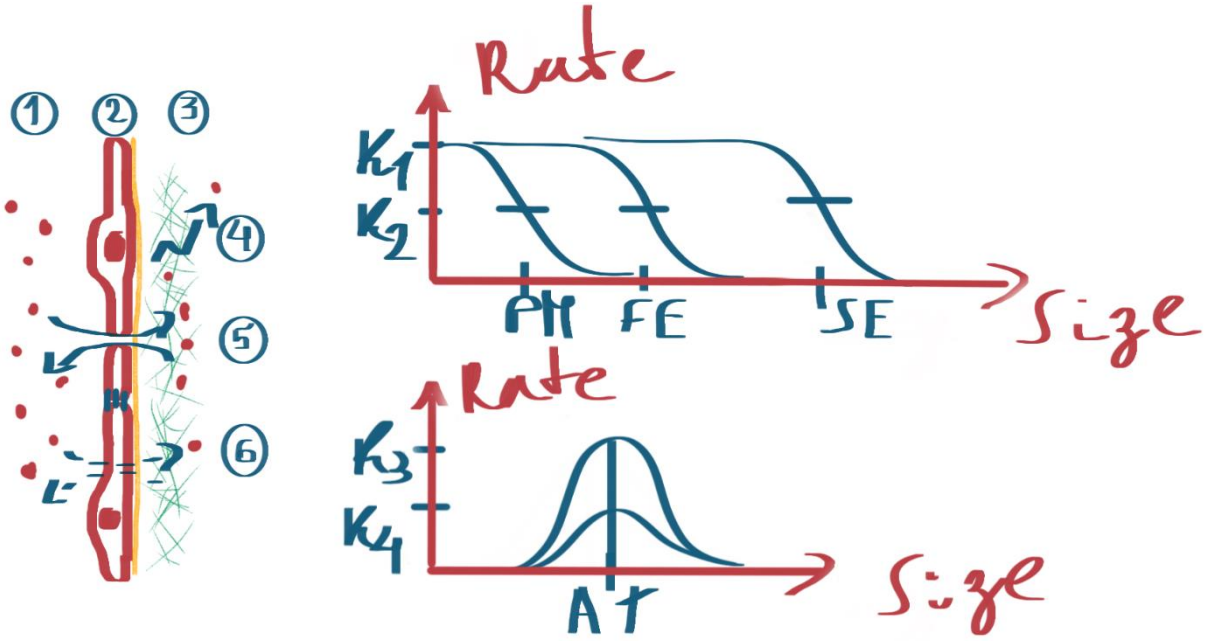

Figure 4

Left side, a pictorial representation of endothelium and NDDS extravasation processes: 1) vascular lumen; 2) endothelial cells with basement membrane; 3) extravascular space with EM; 4) diffusion; 5) extravasation and intravasation through fenestra; 6) transcytosis and simple diffusion.
Right side, relation between the process rate and size of particle: K1) perfusion limited region; K2) penetration limited region; PM) plasma membrane diffusion of small nonpolar molecules; FE) fenestrae average diameter; SE) sinusoidal gap average diameter; K3) endocytosis rate (mechanism dependent); K4) transcytosis rate; AT) optimal size for endocytosis (mechanism dependent)

Nanoparticles smaller than 10 nm have broader distribution,(49) and have a higher tumor delivery efficiency.(15) Nanoparticles of sizes 10-50 nm appear to be better suited for delivery across blood-brain-barrier due to more favored transcytosis by endothelium.(13,50) Nanoparticle with 7 to 50 nm in diameter show longer circulation time due to lower clearance by both glomerular filtration and MPS.(51) 10-100 nm size range has superior AUC, fast distribution and long half-life compared to free drug,(31) and most of the successful nanomedicines range 60-150nm in size.(11) Generally increase in size reduces distribution

volume and restricts access to organs in a stepped fashion based on capillary morphology and endothelial transcytosis but also increases clearance and retention by mononuclear phagocytic system.(45)

Shape can also have impact on distribution, PEGylated gold nanorods enter tumor cells more effectively than gold nanospheres but retain same hepatic accumulation.(52) Computational modeling by Dogra P. et al shows that the adhesion probability increases with increase in nanoparticle diameter, and gives preference to disk, rod and oblong shapes over spherical NPs. Furthermore the nanorods theoretical superiority of binding probability over nanospheres is proportional to their aspect ratio.(53) Metadata suggests that in practice tumor delivery efficiency of the spherical and rod shaped particles is superior to the particles with lamellar or any other geometry type.(15)

2.2.2 Drug Release

Dissolution or release, often represented by released fraction time curve, can be modeled by zero order kinetics for slow drug release, as first order kinetics represented by Noyes-Whitney equation, by Higuchi equation for matrix systems or by empirical models such as Weibull equation, and several others described in a review by Costa P. and Sousa Lobo MJ.(54)

The in vivo plasma half-life of total drug is proportional to the in vitro drug release half-life with a plateau at slower release rates where no further increase in plasma half-life is observed. At very slow release rates total drug closely follows NP kinetics such that total drug concentrations can serve as an approximation for NP biodistribution and clearance measurements.(55)

The release rate of NDDS has most influence on organ targeting during the distribution phase, and the faster the rate of distribution of the nanoparticle into the target tissue lesser is de impact of controlled release on NDDS delivery efficiency.(56) When a NDDS design allows for higher target tissue delivery, compared with a solution of API, delivery efficiency becomes a function of the release rate with a inversely proportional relation.(17,48) Furthermore a slow-release form ND while in blood circulation, despite important for longer half-life and increase in passive distribution in tissues, also needs to sustain an adequate rate of release of API in near the therapeutic target to archive the minimal therapeutic concentrations to be actually effective.(11) As it stands the drug release constitutes one of the most important parameter to be optimized in drug delivery.(57)

2.2.3 Surface charge and surface coating

Surface charge of NPs determines the electrostatic interactions with plasma proteins, extracellular matrix components, and cellular surface. Impact of surface charge on plasma kinetics and biodistribution of small 9-10 nm NPs has been experimentally studied by Arvizo R. R. et al.(58) They have shown that neutral, ζ=-1.1 mV, and zwitterionic, ζ=-2.0 mV, particles have larger AUC and slower elimination than the charged particles of same diameter, with positively charged particles, ζ=+24.4 mV, having the fastest clearance.(58) Positively charged formulations generally have the lowest plasma half-lives and AUCs even when compared with free drug. Together with high accumulation in lungs this can be possibly explained by fast

aggregation with anionic blood species resulting in emboli suggesting a preference for negatively charged particles in NDDS design.(59)

Charged particles have very poor absorption after intraperitoneal administration compared to their counterparts with near zero zeta potential.(58) Also Zwitterionic endow NDDS with higher brain biodistribution than their neutral and mono charged counterparts.(58)

On the contrary for the purposes of tumor delivery, meta-analysis shows a trend for positively charged NPs exhibiting grater uptake than neutral ones, which in turn perform better than negatively charged particles.(15)

2.2.4 Nanoparticles and blood flow

Advection of nanoparticles by the blood flow can be expressed as a partial contribution of convection and diffusion. Models using convection-diffusion equations can account for nanoparticle segregation towards endothelium by incorporation of a adsorption kinetics models.(53) The Brownian motion and shear induced diffusion are partial contributors to nanoparticles margination, whereas inertial lift contribution is minimal due to their small size.(60) Small enough nanoparticles like antibody conjugates could also be reasonably described by diffusion models like Krogh model, when no active extravasation mechanisms are assumed.(61,62)

Relevant parameters used in this type of modeling are Péclet number, Reyonds number, Schmidt number, and diffusion coefficient either derived by Stokes-Einstein equation or experimentally defined. Permeability of endothelium to NPs is dependent on NP diameter, vascular pore size, permeation area, while extravasation depends on oncotic pressure and concentration gradient. Several computational fluid dynamics (CFD) driven approaches have been utilized to describe NP behavior in capillary vessels in attempts to model NP PKs and to capture NP related parameters relevant for biodistribution and target delivery.(48,51,53,60,63) In a CFD model by Liu Y. et al, results show that active targeting causes uneven distribution in target tissue with deposition primarily at the vascular bed entrance.(53)

Still for these approaches to be successful it is necessary to have NPs well characterized. After administration, interactions with plasma proteins and formation of soft and hard bio-coronas, changes the effective hydrodynamic diameter of the NDDS making it a time dependent variable. As a result NDDS exists as a set of populations of particles with different eviction rates and biodistribution characteristics.(64,65)

Intra-arterial route of administration has been used for chemotherapy of primary and metastatic brain tumors, so far with moderate success.(66) However trans arterial cater infusion has the highest potential for tumor distribution and targeting with ability to archive very high tumor/blood ratios of administered nanomedicine in localized unresectable tumors.(47) The administered dose should be calculated based on tumor type, size and location.(47) The desired properties of NDDS for this route of administration are high first pass extraction of NDDS in target region and high systemic clearance to reduce off-target distribution, and for this purpose it is important to consider hydrodynamic properties to prioritize high vascular eviction and endothelial adherence.(67)

Another vascular transport based mathematical model analysis by Dorga P. et al show that tumor accumulation is directly related to vascular pore size, porosity, and inversely proportional to local capillary blood viscosity,(51) and that tumor blood flow has little impact on tumor delivery efficiency which is also supported by Thurber G. M. and Wittrup K. D. model.(61)

2.2.5 Passive targeting

In passive targeting the NDDS is designed for nonspecific eviction from the circulation based on its physicochemical properties. NDDS with passive targeting are accumulated in tissues with capillary structure that allow its extravasation. This can be complemented with active targeting strategies that are primarily based on surface functionalization with aptamer molecules, selected for specific anchoring to target site structures, usually the cell membrane proteins.

Liver and spleen are the two major sites for distribution and accumulation of NDDSs because of a combination of high extravasation rates and low intravasation rates for most NDDSs. Opposed to these, intestines, muscles, bones, and kidneys can have relatively high uptake rates for nanoparticles and contribute to distribution, but they also possess high intravasation rates as well releasing NPs back into the circulation mostly without significant accumulation.(68,69)

2.2.5.1 Cancer therapy as a focus in nanomedicines

Much of recent nanomedicine based research and development has been focused on cancer treatment.(7) Drug delivery strategies for successful solid tumor treatment can summarized by CAPIR cascade: "circulation in the blood compartments (C), accumulation in the tumor via the enhanced permeability and retention (EPR) effect (A), subsequent penetration deep into the tumor tissue (P), internalization by tumor cells (I), and finally intracellular drug release (R)" described by Sun Q. et al. They also discuss effective strategies to archive these goals.(70,71)

Several tumor and NP related characteristics pose a challenge for intratumoral diffusion from perivascular space and penetration into the less perfused areas of tumoral tissue, this can reduce effectiveness of treatment. Particle stability, cellular internalization, and intracellular drug release are important for efficient delivery of API into target cells. And the success in accomplishing these steps is a requirement for archiving a better therapeutic index. All of these PK behaviors of NDDS depend on their 3 main characteristics: size distribution, surface chemistry, and stability of the formulation.(4,71) Cheng Y.H. et al meta-analysis compares performance of different NDDS, among them, dendrimers exhibit by far the highest tumor delivery efficiency followed by iron oxide and gold nanoparticles.(15) Wei Q. Y. et al published an exhaustive review discussing recently developed NDDS in anti-cancer therapies.(10)

2.2.5.2 Enhanced permeability and retention effect

Nanomedicines approach to cancer therapy has been an extensive filed of research. The two main approaches used to enhance tumor delivery are the exploitation of passive accumulation due to highly permeable vasculature, or by active targeting of distinctive ligands usually not expressed in the normal tissues through surface functionalization of NPs. Tumor tissue often possesses structural abnormalities of vasculature such as abnormal fenestrations, structural

disorganization, irregular branching, serpentine structure, uneven distribution density, occluded or embolized blood vessels and irregular perfusion. There is also a denser extracellular matrix, impairment of lymphatic drainage, increased interstitial fluid pressure (IFP), enhanced permeability due to local vasodilating agents and increased trans-endothelial transport. These observations have led to development of anticancer nanomedicines to exploit this EPR effect.(13,47,61,72–75) In order to properly utilize this effect, transversal to solid tumors, it is nanomedicine requires a half-life long enough to provide selective accumulation.(46,47,57) Important factors impairing the effective employment of EPR effect are the variability in cancer phenotype between tumor types and tumor models, highly variable delivery, uneven intra-tumoral distribution and high interstitial fluid pressure that can significantly reduce the NM extravasation.(3,8,13,15,76) There is a big array of possibly effective strategies that have been proposed to improve the EPR effect and tumor delivery through use of drugs and nanoparticles that modulate tumor vessel penetration, reduce IFP, degrade the ECM, use of vasodilators, bubble liposomes, and microwaves. (47,72,73,76) So far studies report, on average, a relatively low tumor targeting efficiency according to several meta-analysis studies.(14,15) Good tumor penetration is a combination of high rate of extravasation of NP combined with high intratumoral diffusion rate.(46) General size range of 10–12 nm can strike a balance between good tumor permeation, penetration and retention.(77)

2.2.6 Active targeting

Active targeting molecular vectors can enhance the target cell uptake, specificity and possibly efficacy of the NDDS without altering its biodistribution.(26,38,52,74,78) Biodistribution is nonetheless expected to be increased when active targeting is directed at intravascular endothelial surface, this is a valid strategy to increase deposition on vascular beds with targeted ligands constitutive expressed on capillary endothelium eventually promoting transcytosis and extravasation.(1,46,57) In general active targeting strategies show higher tumor delivery performance when compared to formulations that only rely on passive targeting.(15) Active targeting can be used for enhanced endocytosis and transcytosis through oral route of administration. Zou D. et al have shown in vitro that gambogic acid as surface ligand improves enteric uptake of nanoparticles, and an increases of AUC in vivo compared to both free drug or NPs without conjugated ligand.(26) 1,3-β-glucan are naturally occurring in bacterial and fungal cell wall and is a ligand of TLR 2 engaging immune response and was been successfully employed by Tukulula M. et al to increase the rate of rifampicin NPs uptake by macrophages.(78) Neuropilin-1 targeting through surface conjugation of NRP-1 ligands to promote specific endothelial transcytosis.(74) However several studies also show that cellular accumulation of active targeted NDDSs can occur through pathways independent of endocytosis, phagocytosis, and pinocytosis as observed in vitro when using endocytic inhibitors.(7,26,70)

Peptides have chemically versatile structure that can mimic different endogenous ligands and for these reasons have been used as active targeting modules in NDDS. Together with nucleic acids peptides allow vast possibilities for targeting aptamer development.(27)

There have also been observed limitations to active targeting such as possible increased in blood clearance, and binding site barrier (BSB).(1,3,13,46,79) BSB phenomenon is used to describe the differential binding of antibody to antigen immediately outside of the vascular wall or in the periphery of a tumor. The differential binding is thought to be due to very high affinities of the antibody-antigen complex and nonspecific binding to normal tissue. High affinity of ligand

to the target results in low dissociation rates, local retention, and therefore, tissue penetration becomes restricted and reducing the therapeutic action only to tissues directly bathed by the capillary beds. Concluding, that the avidity of the binding should be also optimized for active targeting NDDSs.

2.2.7 Cellular uptake

Cellular uptake, particularly phagocytosis, has been incorporated into mechanistic and PBPK models in many studies, describing is as either a time dependent, a concentration dependent or even as a not saturable process. Since it is a formulation and dose dependent process, choice of equations and parameters used should be justified with support from experimental data.(20,80)

2.2.7.1 Size

Most nanoparticles, due to large size are not subject to transporter mediated nor simple passive diffusion through cellular plasma membranes. Therefore, majority of NDDS cellular uptake is mediated by endocytic processes.

Particle uptake efficiency depends on the cell type, enterocytes preferentially uptake nanoparticles of 100-200 nm in diameter while phagocytic cells uptake increases proportionally with size reaching best results at 2-3 micrometers. Regarding non-phagocytic cells, Hoshyar N et al review how size affects various in vitro properties and PKs of nanoparticles. NPs targeted for cellular uptake, have optimal diameter size of 50 nm but ranging from 30 to 100 nm by both active targeting and passive uptake.(13) Extensive review of gold nanoparticles by Dykman L. A. and Khlebtsov N. G. arrive at an optimal diameter of 30-50 nm and a review by Fröhlich E also suggest an optimal diameter of 20-50 nm for NP uptake.(52,81) Some experimental results as well as a thermodynamic model by Zhang S. et al show that maximal rate of endocytosis for nanoparticles should be around 25 nm diameter.(44) Compared with the rate of endocytosis into endothelial cells the rate of exocytosis into extravascular space can be much slower, because of a partial retention in lysosomes and a partial release back into the circulation, as shown in a blood brain barrier transcytosis model by Khan A. I. et al.(43)

2.2.7.2 Shape and Surface Charge

Cellular uptake of nanoparticles is shape and charge dependent, with greater cellular uptake of spherical and rod-shaped nanoparticles.(13) Increase in aspect ratio of NPs reduces NP uptake effectiveness.(52) Nanoparticles with hexagonal nano prism geometry have slower phagocytic rate when compared to spherical particles of same volume.(82) Cellular uptake is higher for positively charged polymer NPs coatings than NP with negatively or neutral charged surfaces possibly because of opsonization or adsorption to negatively charged cell surface glycocalyx.(52,81)

2.3 Metabolization

Since NDDS is not a chemical substance that can't be perfectly described by a molecular structural formula, neither would it be representative of these particles due to high complexity and the relevance of geometric configuration, much like biologic medicines. For the NDDS, "metabolization" can be considered any change of their initial composition and properties including gradual deterioration by dissolution of components, API release, aggregation, protein corona formation and opsonization.(3) This can be thought as a continuous process of maturation of the NP since the moment of first contact with biologic media after its administration.

2.3.1 Degradation

The temporal and spatial API release profile is determinant of its performance. The release rate of the NDDS can be conditioned by chemical composition of its environment and can be designed to release the API in response to target tissue-specific environment such as pH and enzymes.(3) Dendrimers and polymer conjugated APIs have best controlled release mechanisms for NDDS because linker bond chemistry enables high specificity towards enzymatic and nonenzymatic chemical reactions. Meanwhile the polymeric, solid-lipid, amorphous and crystalline nanoparticles have mostly time dependent release mechanisms driven by erosion, dissolution, and diffusion of the NP components on its surface that, to some extent, respond to temperature, pH, viscosity, and other medium factors.

Main route of toxicity for NDDS is the unintended accumulation of the API or NP components due to non-specific distribution. Inorganic particles like silica, iron oxide, gold and quantum dots are mostly inert and can persist in the body for a long time leading to local inflammation and foreign-body giant cell formation. For nanomedicines designed to have repeated administration, it is preferred to use biodegradable material that can be hydrolyzed to molecules removable by renal filtration or biliary excretion or further metabolized by the body as a nutrient.(3)

2.3.2 Protein corona

After administration of nanoparticles, due to their foreign surface chemistry, they spontaneously adsorb proteins from the surrounding biologic medium, this process is dynamic with qualitative and quantitative changes in protein composition over time. This surface protein coating is called protein corona and it mainly depends on the route of administration and surface properties of the NDDS. At the heart of this complex protein dynamic equilibrium lies the Vroman effect, explained as the sequential adsorption of proteins, first by proteins in greater concentration and higher medium mobility and sequentially displaced by proteins with higher affinity and slower dissociation kinetics towards a more thermodynamically favorable state. The protein corona can be subdivided into the soft and the hard shells based on presence of conformational changes in adsorbed protein structure and ease of separation from NP core by washing and centrifuging. (64,65,83–85)

This new altered surface identity changes how NPs interact with cell membrane receptors, affecting cellular adhesion, uptake, transport, and distribution previously described. Most

notably this causes enhanced particle recognition by the immune-system cells leading to macrophage activation and faster elimination from blood circulation. In case of small NPs, protein corona can significantly increase the effective hydrodynamic diameter reducing renal clearance and altering their advection in plasma. Needless to state this can have very significant effects on NP PKs and therapeutic response. Unfortunately composition of protein corona regarding protein species, the relative abundance, as well as their confirmational changes on the particle surface can vary greatly between different NPs resulting in a necessity to establish these parameters through in-silico, in-vitro, and in-vivo experiments on a case by case basis.(64,65,83)

There have been great advances in use of computational techniques such as force field, molecular dynamics, and quantitative structure-activity relation in silico simulations to rationalize nanomedicine development enabling better screening and optimization of formulations based on literature even before the NDDS reaches the lab.(5,65,83)

It has been shown that PC formed under physiologically simulated blood flow conditions is different from when performed in stationary conditions. PC formed on NPs under dynamic flow and recovered after in vivo administration has greater protein diversity and can vary based on administration rout.(63–65,85,86) Hydrophobicity is a critical factor for controlling serum protein binding and corona formation.(52,83,84) Protein adsorption interferes with surface peptide targeting reducing specificity of cellular uptake and uptake efficiency of the NDDS.(52,85) Review by Lee H. describes various relations established between NP characteristics and PC formation, (83) and a review by Jain P. et al enumerates different ways protein corona can modulate the biologic effects of the NDDS.(85)

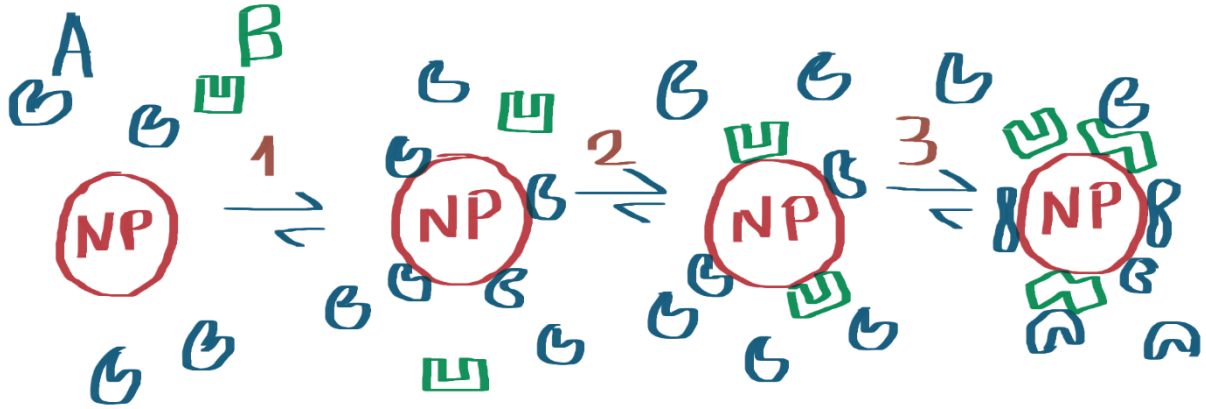

Figure 5

Protein formation process: 1) initial corona formation, adsorption of high concentration high mobility proteins; 2) corona maturation, adsorption of proteins with high affinity, reducing surface free energy 3) conformational changes of tertiary and quaternary structure of adsorbed proteins strengthening hard corona structure.

2.3.3 Prodrug activation

The phased administration of prodrug nanoparticles and catalyst nanoparticles can control the rate of release and increase the specificity of API action through a more selective accumulation. This strategy can also help maintain the required chemical stability until the prodrug reaches the target and provide sufficient reactivity to achieve therapeutically active drug concentrations

in target tissues which are a challenge for both prodrug and NDDS formulations. Miller M. A. et al used this strategy to increase the specificity of action of their NDDS. Their formulation consisted of nanoparticles with prodrug and separate nanoparticles with bio-orthogonal prodrug activator thus restricting the release of API to occur only in compartments where both entities where present at the same time, mostly due to accumulation in endosomal/lysosomal vesicles.(87)

One example of prodrug design strategy to obtain better tumor penetration while maintaining passive tumor accumulation is through a NDDS size transition mechanism.(71) Sun Q. et al developed a doxorubicin dendrimer self-assembling pegylated lipid NDDS with 30 nm diameter that, upon tumor tissue entry, would fuse with cell membranes releasing 5 nm diameter dendrimers intra- and extra-cellularly resulting in faster diffusion, higher tumor penetration, and a better performance in tumor bearing mice model.(70) Another strategy was demonstrated by Xu C. et al, they produced a nanosphere doxorubicin formula that would assemble into nanofibrils upon matrix metalloproteinase cleavage. This shape transition design was able to increase tumor retention and internalization by tumor cells, and together with pH dependent release rate of API have shown efficacy increase in two different mice tumor models.(88)

Proportion of API molecules conjugated per molecule of polymer in new molecular entity prodrugs can be linearly related to release rate, as shown by Harada M. et al, the percentage of released docetaxel decreases linearly with increase in molecules bound per PEG-poly(aspartic) block-copolymer molecule in their micellar NDDS.(89)

Masking the targeting ligands and surface charge with cleavable PEG can also be employed as a prodrug strategy to ensure better distribution and targeted cellular uptake.(71) This complex setup was shown by McNeeley N. K. and demonstrated an increase in in-vivo plasma concentration and as intended in-vitro increase in uptake when treated with unmasking solution of cystine.(13,90)

2.4 Elimination

Elimination of nanoparticles can be broadly sub-divided in two categories, in the first elimination occurs through metabolization of degraded nanoparticle's components in case of biodegradable polymers and lipidic formulations, where the metabolites end up being used as nutrients or eliminated by same route as simple molecular entities are. Second category comprises the nanoparticles that suffer excretion by removal of intact NPs or their degradation remnants, where smaller particles undergo renal filtration and urinary excretion while the larger ones undergo hepatocyte uptake and biliary excretion. Otherwise, the particles are retained indefinitely in the tissues by macrophage segregation and granulation.

2.4.1 Renal route of elimination

Renal glomeruli are composed of fenestrated endothelium with 50-100 nm pores, endothelial basement membrane with 2-8 nm mesh, that is considerably thicker than usual, 200-400 nm in length, and podocyte pedicular filtration slits with 4-11 nm in aperture, this configuration has a filtration threshold for particles of about 6 to 8 nm in diameter and restricts passage of globular proteins larger than 70 kDa in molecular weight.(16,41) As a result particles less than 5.5 nm in diameter are expected to have swift renal clearance after administration,(45) while negatively

charged particles would offer some resistance due to repulsion by the negatively charged podocyte filtering glycocalyx.

While renal clearance is undesirable in therapeutic applications it is the preferred rout of elimination for diagnostic nanoparticles design. NPs of hydrodynamic diameters smaller than 5.5 nm, are subject to swift renal filtration, greatly reducing their half-life, and reducing undesired nonspecific accumulation consequently providing a higher signal-to-background ratio attractive in imaging techniques.(6)

2.4.2 Elimination by mononuclear phagocyte system (MPS)

Particles with sizes above renal filtration threshold undergo elimination by mononuclear phagocyte system primarily in liver, spleen, kidney, and lung tissues. NPs are sequestrated in liver by Kupffer cell uptake, through sinusoidal filtration and endothelial transcytosis. After reaching liver space of Disse, NPs or their degradation products can be transported into biliary canaliculi by hepatocyte transcytosis and excreted with bile, possibly subject to enterohepatic recirculation but eventually eliminated by the fecal rout,(45) and/or degradation due to physical and chemical instability in phagosome/lysosome medium.(91) Despite liver having a large distribution volume and fast clearance, hepatobiliary elimination is slow for most nanoparticles resulting in longer retention.(3,20,45)

Phagocytic clearance of nanoparticles can be verified by reduced liver accumulation and increased plasma half-life after pre-treatment with macrophage depleting doses of clodronate liposomes.(45,91) Administration of increasing doses of NDDS causes saturation of macrophages in liver consequently increasing uptake by the spleen, lungs and other mononuclear phagocyte system associated organs.(1)

Study by Poon W. et al discusses the relationships between nanoparticle design and their elimination pathways. They show that Kupffer cells are responsible for most of the accumulation and retention of nanoparticles in liver meanwhile reducing their hepatobiliary elimination. Furthermore hepatobiliary elimination appears to be size limited by the capillary fenestration aperture to about 100 nm, while phagocytosis by Kupffer cell is more efficient for larger sized particles.(1,45)

It is important to consider the effect of cytotoxic drugs on monocyte phagocytic system when performing PK modeling. For NDDS with principal distribution and clearance mediated by phagocytosis, the degradation and release of API can cause MPS cell death with reduction in effective distribution volume and clearance rate. If this happens in therapeutic window doses of the formulation, then a robust non-linear kinetic model as well as PK monitoring should be implemented to produce dosing regimen adjustments in order to maintain efficiency of the treatment without causing more sever toxicity due to depletion of phagocytic cells.(3,55)

2.4.3 Stealth nanoparticles

Surface modification with polyethylene glycol reduces the rate of macrophage uptake and prolongs the circulation half-life of nanoparticles and loaded drugs. This non-ionic polymer coat increases the hydrodynamic diameter, modulates bio-corona formation, decreases

opsonization and reduces receptor mediated cellular uptake, consequently establishing itself as the most frequently employed surface stealth modification.(52,64,65,83,85,92)

In Shalgunov V. et al study optimizing a formulation of nanoparticles with PLGA-PEG copolymer, particles having higher surface PEG coverage had consistently lower spleen and liver distribution, and overall higher plasma concentrations and half-lives.(55) Polyethylene glycol surface coating of NPs forms an hydrophilic and steric stabilizing layer that reduces NP-protein interactions with optimal effects observed at 2-5 % (wt) and PEG MW of 5 kDa.(92) Another study that considered the balance between anti-opsonization effect and conservation of targeting ability due to selective protein corona formation arrive at preferred MW of 2kDa.(93)

Polyethylene glycol surface coating, despite being able to reduce MPS clearance of nanoparticles can be a subject to accelerated blood clearance (ABC), but this phenomenon can occur with any type of NDDS.(2) As a consequence of repeated administrations, it is possible to develop anti-PEG antibodies that greatly increase opsonization of PEG coated particles resulting in rapid clearance of administered dose rendering treatment less effective. Antibody mediated clearance of pegylated NDDS is dominated by hepatic clerance with reduction of exposure of remaining organs to the API.(94) It has been shown in rats with anti-PEG antibodies, that the plasma levels of PEGylated NPs can be restored to normal levels if pre-treated with HMW PEG (> 20 kDa) before the NDDS administration.(3,92,94,95)

Another approach for nanomedicine avoidance of immune response is through cell membrane camouflage. This biomimetic strategy has been done with nanoerythrosomes, mesenchymal stem cells, extracellular vesicles and other plasma membrane structures derived from different cell types.(8,11,96) So far this biomimicry approach is the best alternative for stealth capabilities of PEG in nanoparticle design.

2.5 Toxicity

Nanotoxicology and toxicokinetics are a cornerstone of safety in regulatory evaluations for new NDDSs.(97) Main concerns regarding nanoparticle toxicities come down to the final fate of its components, that is biodegradability and biocompatibility of the NP excipients in different tissues. Majority of inorganic material such as metals, silica and their oxides remain inert and become retained in tissues for long periods of time rising the concerns about the potential for over-accumulation and long-term toxicity by different cytotoxic, inflammatory, and oxidative stress mechanisms.

Although NPs have a huge potential for diagnosis and therapy, they also possess the capacity to cause severe toxic effects in the body and thus their formula should be carefully designed keeping their toxic properties in mind.(6) In a detailed review by Iavicoli I. et al regarding renal toxicity of nanoparticles authors show that cytotoxicity is inversely proportional to particle size, establish the importance of composition on the underling mechanism of toxicity, and how crystalline structure, solubility and dissolution rate all relate to toxic potential of the nanoparticles.(86)

It is important to have a good characterization of the NDDS and separate/purify it to a narrow size population, since different sizes and different compositions can result in an altered distribution profile.(11) For example, micelles, remnant liposomes, API crystals and other

artifacts due to compounding or bad stability can be a source of variability and reduction in expected safety as well as the overall NDDS performance.(4,20) Since even small changes in size and shape of the NPs can cause significant changes in their optical, electronic, magnetic, and biologic properties it is necessary precision synthesis to archive, and correlate with, specific functions.(4)

3. Pharmacokinetic Modelling

PK modeling is important in phases 1 and 2b of clinical trials and even in preclinical stages of drug development. Preclinical modeling is especially important for the purpose of research of PK behavior of NPs, and for rational optimization of NDDS design.

Before a NDDS centered PK study is designed it is necessary to have a sensitive and specific assay capable of measuring the parent drug, nanocarrier and all its metabolites of clinical relevance. The inputs to a PK study are the concentration-time profiles of the nano-formulation in plasma, serum, or whole blood. Nanoparticles that bind to blood cells or coagulation factors may have lower plasma concentrations than when dosed in whole blood.(31) Thus it is important to have a robust analytical procedure for NP and API quantification in various biologic media.(98) The necessity of appropriate choice of labeling and quantitative assays for ADME studies can't be overstated.

The objective of a PK study should be defined beforehand as it determines the study design. It's also important to perform a sampling design and develop a study protocol earlier on, based on number of subjects and sampling limitations in order to ensure the ability of accurate parameter estimation and hypothesis testing based on assumptions taken.

Preemptive simulation of data allows to test the selected study design, evaluate the consequences of the design factors and chosen assumptions, predict the results, and verify the ability to fulfil the pre-set study design objective. The same applies to PK model testing, simulated data can supply an objective measurement of the model performance before experimental data is available.

Perhaps the most important aspect to understand in regards to nanomedicines PK modeling interpretation is that API in its native form and in nano carrier bound state have different PD characteristics in the target tissues. API exposure quantified as AUC total, for the sum of free and carrier bound drug, is not a good predictor of NDDS efficacy or safety, and direct comparison of PK parameters with conventional formulations or other nanomedicines does not yield useful information about their performance. For example docetaxel formulated as hard copolymer nanoparticles with varying release rates, or as ester linked copolymer micelles prodrug, show much lower minimal cytotoxicity concentrations when compared to their native solution counterparts.(55,89) NDDS distinct PDs from its API solution can be attributed to the fraction of total API that is active or available to perform its pharmacologic function, but there is also the possibility that the adsorbed and endocytosed NDDS have cumulative contribution towards the therapeutic action.

Pharmacokinetic modeling resides in proper mathematical and statistical analysis of available data. There are several approaches to pharmacokinetic modeling of NDDS but no matter the approach taken it is of good practice to explicitly define and explain the objectives set,

assumption taken, and hypotheses tested. The model building process should be described as a sequence of constructed formulas, included parameters, tested models, and the used model selection criteria. The model selection is often done based on Akaike information criterion, mean absolute percentage error evaluation, coefficient of determination, and visual comparison of goodness of fit.(15,32,49,68,96,99)

Model validation consists in evaluating the ability of the model to describe a validation data set not used in the model creation and parameter determination. Usually, validation is performed with independent experimentally obtained data set, called external validation and it is the most stringent approach. There are also internal validation methods, relying on data from the initial sample through cross-validation and bootstrapping methods, these can be used when no additional data is available, by.(100)

3.1 Model types

Non-compartmental analysis is a model-independent approach that, instead of describing in vivo ADME processes, summarizes the effect of their undiscriminated contributions to the observed variables. It is more robust in estimation of apparent clearance, apparent volume of distribution and are usually employed for calculation of maintenance and loading doses.(31) This method recovers descriptive metrics like AUC, AUMC, MRT, half-life, clearance, that are different if we are measuring the NP, free drug or total drug. Use of these parameters in direct comparison of formulations becomes less informative than when conventional drugs are concerned.

Empirical models are limited to describe the observed/measured variables, they usually provide the best fit for time function of plasma concentration with the least effort, but have no direct explanatory power for the parameters employed.(5)

The classical PK models with two or three compartments can be useful to determine absorption and distribution kinetics of the NDDS.(31) Kadam R. S. et al performed classic PK modeling on various total API concentrations recovered form literature of NP formulations after intravenous or oral administration and compare them with plain API formulations. The results show there is a decrease in distribution volume, increase in AUC and reduced apparent clearance that are evident even after oral administration, probably indicating that nanoparticles reach the systemic circulation.(25)

Classical PK models provide a standard way to concisely represent preclinical and clinical results. Nevertheless, classical PK approach and plasma concentrations alone cannot supply any information about biodistribution profile nor the efficiency of cargo delivery, which is the main optimization aim of targeted NDDS designs.(5) Dynamic equilibrium between NDDS and free API makes PK modeling more challenging because this dual nature resulting in loss of conventionally attributed interpretation for several classical PK parameters over total drug concentration. Parallel measurement of both API and NP in biodistribution studies becomes required to understand underling differences in free API and carrier behavior. Carrier bound fraction has much smaller volume of distribution as it is mostly restricted to intravascular compartment and free fraction contributes only slightly to intravascular plasma concentration because of faster and more extensive distribution than nanocarrier. Still, it is in the selective distribution and accumulation of the carrier, and corresponding loaded API fraction, where NDDS advantage can be found.

The API while residing in the carrier is sheltered from its usual distribution, metabolization and elimination pathways and follows the kinetics of the NDDS. At very slow release rates total drug closely follows NP kinetics such that total drug concentrations can serve as a surrogate for determination of biodistribution and clearance of NDDS at early sampling time points.(55) On the other hand for NDDS with faster drug release or carrier degradation rates, after a certain number of corresponding half-lives PK description of total drug measurements resemble, and can be better expressed by the kinetics of free the drug.(49)

3.1.1 Mechanistically based pharmacokinetic models

Mechanistic models introduce explanatory parameters, derived mathematically and/or that can be experimentally measured such as in release rate, endocytosis rate and half-inhibitory concentration of different cell lines. These parameters can be set to describe how the nanomedicine behaves in the body and allow for hypotheses testing regarding the impacts of different formulations, special disease status or specific populations on the performance of the NDDS.

Standard approach to mechanistically based modeling starts with a description of absorption, distribution, metabolization and elimination (ADME) processes to which NDDS and API are subjected in the body and alter their measurable plasma concentration over time. Mechanistically based models are often set up with a mammillary structure by formulating encompassed processes as a set of ordinary differential equations based on conservation of mass and first order kinetics which as a linear system sums the contributions from all ADME processes. Another important extension is a catenary extension into specific sub-tissue and even subcellular compartments,(2) this addition should be able to account for targeting functionalization strategy especially when compared with identical system functionalized with a dummy moiety. Certain phenomena that do not follow linear kinetics in the clinically relevant dosing can be modeled with a Hill equation. Common examples found in literature are saturated endocytosis/phagocytosis at higher NP doses, or gradual loss of effective endocytic capacity due drug cytotoxicity in a time, or dose dependent manner.(3,55,68,80) Another example are time dependent behaviors such as gradually increasing distribution partition coefficients observed for zinc both as oxide nanoparticles or in its ionic form.(49,101) Experimentally determined API, NP and major metabolites concentrations in blood, urine, and feces, can be fed into the model to numerically or analytically recover the underling contributing model parameters. Although, in order to determine the model parameters, it is necessary to have robust analytical techniques that allow for separate determination of the released free API, and the total or NP bound API, in biologic samples. The modeling analysis can also be conducted with just the total API measurements as long as the assumptions that the API release kinetics follows the values measured in the vitro assays is held.

Mechanistic PK modeling of NDDSs is possible by partitioning every compartment into a free drug and a nanoparticle bound drug sub-compartment. Staring with data form biodistribution of simple solution of active pharmaceutical ingredient a base n-compartment model can be created. By fixing the model parameters corresponding to API in its free state a sub-model that describes dispositions and ADME processes for the NDDS can be added. This way every tissue can be represented by two compartments interlinked by the rate of release of the API from NDDS. This approach indirectly describes the evolution of the system in terms of two variables, the free drug concentration and the NDDS concentration, while only requiring the measurement of the total API concentrations from NDDS biodistribution data.(20,26,32,89,96)

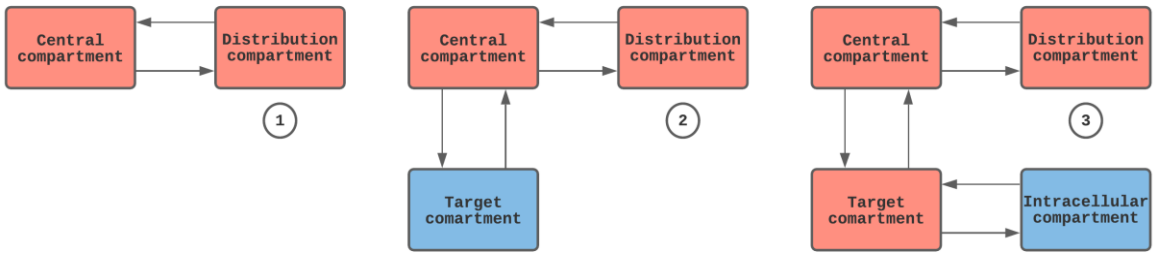

Figure 6

Schematic representation of model structure: 1) base model; 2) mamillary extension (compartment added in parallel directly connected to central compartment); 3) catenary extension (compartment added in series).

3.1.2 Physiology based pharmacokinetic modeling

Physiologically Based Pharmacokinetic models are the most extensive mechanistic model type that can accommodate a very large number of parameters and are described in a spectrum of structural complexity from reduced semi-physiologically based PK to full PBPK models.(1) PBPK models attempt to represent the body as a set of inter linked compartments based on gross organ measurements and API dosimetry in all organs and tissues of interest with most of the model parameters being based on measurable physiologic variables.(102)

The parameters usually employed are organ perfusion, macrophage density, vascular porosity, permeability, and surface areas that serve as a barrier to API extraction by the organ. Any relevant phenomena can be incorporated into the model from in vitro data of permeability, intrinsic clearance and metabolization rates of active substance, estimated from appropriate cell or enzymatic assays and usually expressed in corresponding units per gram of tissue.(2,103) These parameters for the PBPK models can be measured experimentally but, for the most common laboratory animals like mice and rats, they can also be found in data libraries and as packages for the commercially available PK software.(102)

In differential mass balance equations that describe inflow, outflow, uptake, and clearance of API, the model compartments must have justifiably attributed perfusion/blood-flow limited or permeability and/or diffusion limited distribution for each tissue.(3) The blood flow limited models assume that the leaving venous blood concentration of the drug to be at equilibrium with the tissue concentration estimated by blood/tissue partition coefficient, this is true for many small molecular active substances but rarely the case for nanoparticles. Contrary to this, in penetration and/or diffusion limited models a gradient of concentrations is established between tissue and venous blood, this results in a much slower distribution and a delayed steady state. In most frequent cases NDDS extravasation is a permeation and diffusion limited process thus increase in perfusion of the target tissue yields little to no change in NDDS accumulation, at the same time, free API being released from NDDS can wash out from the tissue reducing the local free concentration under higher blood flows.(57)

Compartments of the basic model consist of organs and tissues of interest for the model, that is they must answer one of the following questions: Is this tissue the desired therapeutic target?

Are any of the organs major sites of toxicity related to API or any of the NDDS excipient components? Are these tissues major sites for distribution and accumulation of the drug or NDDS? And are any of these organs involved in degradation and elimination routs of NDDS?

These modeled tissue compartments are often subdivided into vascular and extravascular sub-compartments to better account for distribution processes. When relevant or mechanistically justifiable the modeled tissue compartment might be further subdivided into, interstitial or extracellular, intracellular and macrophagic sub-compartments complemented with appropriate zero order or first order rate constants or nonlinear parameter terms to reflect the binding to blood and tissue components, tissue penetration and cellular uptake performance of the formulation and API. It is also necessary to consider if there is significant PK contribution of lymphatic drainage or lymphatic system involvement in the performance of the NM. At the same time, it is necessary to restrict the model only to compartments directly measured in the experiment to reduce any unjustified degrees of freedom and avoid overfitting of the model parameters.(69)

Minimal PBPK models can be employed for accurate prediction of tissue concentrations, but they are limited to the animal model used and to NDDS of same size and similar physicochemical properties.(69) While more complete models can be used to successfully describe PK behavior and biodistribution of wider range of NDDSs.(15,80)

The PBPK is a mechanistically driven modeling platform that can be used for identification of physiological factors, NP dependent factors and sources of PK variability relevant for clinical use. This makes possible to extrapolate and predict PK profiles in subpopulations with modified physiological functional states and diseases, in different routes of administration, and even cross-species by adjusting corresponding/appropriate model parameters.(5,102) Another advantage in using MBPK and PBPK is the establishment of quantitative structure–activity relationship (QSAR) in NDDS development and optimization approaches.(3,5,102)

Yuan D. et al have a review of PBPK modeling, they analyzed and compared several approaches to the model structure development and explore the diverse applications of PBPK modeling.(3)

3.2 PK-PD modeling

Main questions that a NDDS needs to answer to justify its clinical use are: Does the observed PK profile of NDDS reflects an increase in efficacy? Or does this formula reduce toxicity observed when using conventional drug? In other words, is there an actual therapeutic advantage in using NDDS over a conventional formulation? These need to be answered by clinical trials but can also be estimated by PK-PD modeling in preclinical settings.

Pharmacokinetic pharmacodynamic modeling adds another dimension by considering pharmacologic and toxicologic interaction of API with their target. This is particularly relevant in PBPK models because they are designed to predict concentrations in the site of action and at off-target tissues. By integrating both PK and PD sides into the model greater insight about NDDS performance, regarding efficacy and toxicity, can be obtained for further development.(17) This can be accomplished by estimating concentrations of bound and free API and adding a dose-time-response relationship component to the established PK model.(57) In particular PBPK can be combined with pharmacodynamic models to become a supporting

tool for toxicokinetics to assess NP associated hazards and to characterize its safety profile and pharmacological efficacy based on biodistribution data.(3,17,96)

Byun J. H. et al authored a review where they classified PD models in 6 categories depending on the mechanistic description of tumor growth and antitumor action of API. They also list the general forms adopted and exact models used in different reviewed PK-PD studies.(16)

3.3 Population based pharmacokinetics

When it is not possible to directly obtain all the necessary data because of ethical and animal welfare restrictions imposed on sampling and experimental design, it is possible, to a certain extent, resort to population based pharmacokinetic modeling. PopPK is the study of correlations in drug concentrations variability and their sources in target population. As its designed goal it can be used to identify and measure how patient demographical, pathophysiological, and therapeutical features, such as body weight, excretory and metabolic functions, and the presence of other comorbidities or therapies, can alter dose-concentration relationships.(100,104) The PopPK nonlinear mixed-effects modeling is labor intensive, however it allows for simulations using sparce sampling data form the studied population, instead of dense sampling data, to account for intraspecies variability, and has been used to circumvent some practical constraints.(33)

3.4 Model based extrapolation

In vitro-in vivo correlation is a mathematical method to establish relationships between the in vitro properties of the formula and in vivo PK parameters or therapeutic response. The extent to which in vitro-in vivo correlation can be performed is dependent on the quantity and the quality of respective in vivo and in vitro data. To establish IVIV relationships is necessary to perform a series of mathematical deconvolution techniques, that can either be model dependent or model independent. IVIVC can be used to establish relations between process rates, data sets, and model parameters for the selected NDDS pharmacokinetic model.(11,103)

The mechanistic and PB-PK frameworks allow for allometric scaling of the model through adjustment of estimated model parameters, considering obvious interspecies differences in physiology like weight, metabolic rate, and blood volumes, as well as other more subtle ones that require methodical experimental determination and thorough literature search. Models allometrically scaled this way can be used for cross-species extrapolation to perform predictions in PK profile tissue dosimetry and toxicity form one specie to another including humans.(26,99)

Due to dissimilarities in plasma composition, biodistribution and clearance kinetics the protein corona matures inconsistently across different species. These factors and the difference in basal metabolic rate of the two species can cause a significant mismatch in interspecies allometric scaling. Sahneh FD et al. simulation shows that impact of corona evolution on NP biodistribution is maximal when corona transition half-life is close to the geometric mean of NP half-lives of the two species.(105)

When simple allometric scaling is applied to the animal model parameters to extrapolate the PK profile to humans, disparities are to be expected.(57) Orally administered nanoparticles PKs were studied by Zou D. et al in rats and dogs, direct allometric scaling yielded remarkable

results for free drug in low doses based on dog model and overestimates for higher doses, but the rat model gave ten times higher estimates than dog model in the entire dose interval.(26)

Another example of IVIVE and cross-species extrapolation Lin Z. et al elaborated a PBPK model and used cross species extrapolation from mice to rats and pigs and from those to humans. Two out of five models where successful at simulating human plasma concentrations and were later used to make predictions of toxic doses based on hemolytic and cytotoxic in vitro assays.(99)

A review by Choi G. W. et al explores the methods in vitro-in vivo extrapolation of pharmacokinetic parameters and enumerate the mathematical approaches reported in literature.(103)

Data generated from both IVIV, and cross-species extrapolations can provide relevant data and better confidence in safety and doses to be used in the first clinical studies.

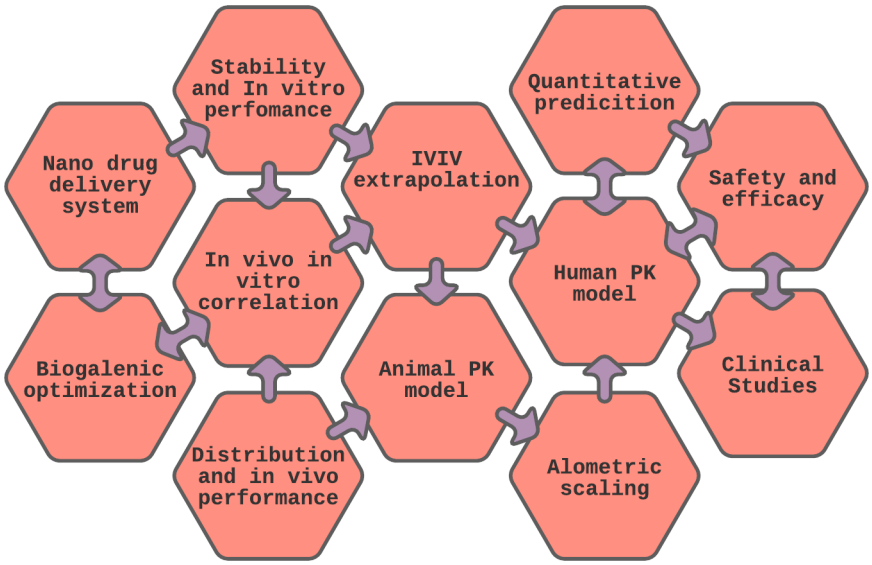

Figure 7

Abstract representation of NDDS development process from a modeling centered perspective.

3.5 Modeling Examples

Simple three-compartment models, with varying degrees of complexity, are often employed in targeted NDDS PK description because they capture the desired aspect of target exposure which these systems strive to optimize.

Non-mechanistic three-compartment models are sufficient to account for NDDS concentration in blood and in tumor. This approach has been taken in characterization of imaging nanocarriers by Sousa-Junior A. A. et al. Authors successfully determine and predict the tumor delivery efficiency, the maximum tumor concentration, and time to reach it with their erythrocyte membrane camouflaged magneto-fluorescent nanocarriers based model.(8)

In Thurber G. M. and Dane Wittrup K. D. mechanistic model for antibody conjugate drugs, they considered antibody-antigen binding in below antigen saturation concentrations with antibody clearance by uptake and by intravasation, antibody vascular transport based on Krogh model and permeability surface area per tumor volume, and low Biot number for mass transfer shown for antibodies by their previous studies. Two simplified models based on high-antigen affinity or non-binding antibodies were proposed by the authors. This approach was able to adequately fit experimental data, calculate time peak of tumor uptake and maximum concentration, as well as predict target delivery efficiency of clinical data.(61)

Another three-compartment model by Wong A. D. et al to evaluate EPR effect. They construct their model on a foundation of a bicompartmental model describing doxorubicin liposomes clinical data and adjusting tumor uptake constant. Resulting model show that when tumor uptake constant is smaller than a tenth of the elimination rate, tumor uptake contribution to the PK profile is negligible. And while extravasation and intravasation of the NDDS in tumor tissue is negligible on overall plasma kinetics, they are determinant for tumor accumulation and AUC, especially when distribution to periphery is fast and plasma half-life is low.(75)

However, these PK models describe only the disposition of the NDDS or total API, lacking release rate and simultaneous descriptions of bound and released drug for a holistic understanding of the NDDS PKs.

Chen S. et al develop a mechanistically based PK-PD model using nonlinear mixed effect modeling to investigate their NDDS with nanoparticles for controlled release and mesenchymal stem cells for tumor targeting. They accounted for API plasma PKs and biodistribution in three different states, free, NP-bound, and MSC-NP system by a parallel layer approach. It consists of PK description for each of the 3 agents interconnected by API release and NP exocytosis first order rate constants, which were determined in vitro. Addition of Michaelis-Menden equation for each of the three API species based on in-vitro IC50 studies established the PK-PD model. According to simulation results reported NP exocytosis rate from MSC is inversely proportional to tumor inhibition.(96)

Another mechanism-based PK model was used in Kraft J. C. et al study of a subcutaneous combined antiretroviral nano formulation and later by Yu J. et al for evaluation of synchronized distribution of IV administered dual anticancer drug discoid nanoparticles. This is a compound model divided in a parallel sub-model for NP disposition with a periphery compartment for pooled elimination routs with different release rates for each drug linking the NP sub-model to the free drug sub-model following their control group PKs. The lymphatic absorption follows 3 pathways with different release delays and release rates, the resulting model yielded remarkably good fit for observed data.(32,106,107)

Meta analysis by Cheng Y. H. et al 2020 explores NDDS platforms from over 3 hundred in-vivo rodent data sets and identified factors influencing tumor delivery kinetics trough evaluation of tumor delivery efficiencies in a PBPK model framework. Their parameter sensitivity analysis reveals that tumor delivery efficiency is conditioned by blood volume fraction of liver, spleen, kidneys and tumor, distribution coefficients to spleen, kidney and tumor, tumor cell uptake rate, volume fraction of tumor in the body as well as various NDDS related parameters mentioned throughout this monograph.(15)

In a Shalgunov V. et al study authored by, was used total drug of very slow-release NPs as a surrogate for NP with a PBPK model that had a good fit to experimentally obtained data. In agreement with purposed first order distribution processes, it was then used to simulate body

distribution of formulation with faster release rate with agreeable fit after introduction of a new toxicity term due to cytotoxic load that had, a time dependent detrimental effect on macrophagic cells.(55)

Benchimol M. J. et al employed a PBPK model analysis providing useful insight into relevant PK properties of NDDS. Results show that intravascular targeting is superior to extravascular targeting strategies in increasing tumor accumulation rate, anyway extravascular binding is important to explain tumor retention, while both extravasation rate and intratumoral diffusion rates have synergistic role in tumor penetration.(46)

In study by Li L. et al, they compared 5 polymeric nano-formulations to evaluate how size, composition and PEG MW affects distribution using a water quenching-NIR fluorescent dye and employment of a PBPK model. Between two models, they selected phagocytic cell PK model based on a larger coefficient of determination. Simulation showed that plasma concentration remained higher for smaller formulation, 80 nm over 200 nm, also larger size, and lower PEG molecular ratio had higher maximum uptake rates for most tissues. Sensitivity analysis shows importance of maximal uptake and release rate of phagocytic cells on respective organ concentrations, but bodyweight and the injected dose where the most dominant parameters on distribution for every organ.(68)

3.6 Regulatory considerations

Despite a very positive outlook, and growing number of NDDS types in development and in clinical trials, only a fraction of NM actually reaches the market, and even smaller number of drug products and their bioequivalents comprises the nanomedicines that are actually used in clinical practice.(11)

Requirements in assessment of nanotechnologies are a matter of balance between regulation to ensure safety of end consumer and market access to promote innovation with differing standpoints amongst authorities' policies from different regions.(108)

Currently, in the nanomedicines section of EMA site, four reflection papers are available for public consultation: on intravenous iron-based nano-colloidal products; on intravenous liposomal products; on block copolymer micelle medicinal products; and on surface coatings for NDDSs. Among the requirements to satisfy the quality, safety, and efficacy prerequisites, systemized in the common technical document by ICH, are an exhaustive physical and chemical characterization, a complete description of quality related attributes of the drug, manufacturing processes and critical process parameters, raw materials and critical material attributes, all the data on pre-clinical and clinical pharmacological studies as well as a complete description of handling and administration procedures.(109–112) Regarding non-clinical and clinical pharmacokinetic requirements set towards development of nanomedicine products only general recommendations are provided and these can depend on the type of nanoplatform in question.

4. Conclusion

The application of PK modelling and the development of NDDSs requires an overarching understanding of their unique ADME process characteristics, the ability to design an appropriate PK study, a multidisciplinary approach to nano-formulation, the analysis of large amounts of in vitro and in vivo data, and the ability to constructively integrate it into the model.

The choice of the pharmacokinetic modelling approach to take during NDDS development must be based on the objectives of the study and reflect the data provided by available analytical procedures, experimental design, and available literature. PK modeling provides the means for complete characterization of PK behavior of NDDSs, along with the ability for in vitro-in vivo and cross-species extrapolation.

Computational quantitative structure-activity relationship models can be developed on PK model foundations to explore, in silico, relationships between structural properties of NPs and their physiological behaviors based on the available data libraries. Like shown by Cheng Y.H. et al meta-analysis,(15) this approach can be systematically applied to the plentiful data from the new published NDDS biodistribution studies. Based on consulted publications PBPK modelling appears to be a highly versatile and adaptable approach as well as the one receiving most attention in the NM field over the last decade.

Pharmacokinetic modelling is the only tool that developers and industries have, to demonstrate bioequivalence, safety, and effectiveness of new drugs and NDDSs under the limitations of time, resources, and ethical constrains of pre-clinical and clinical studies, as well as being a definitive requirement by regulatory agencies for marketing applications. As such, further development of NDDS centered PK modelling is highly sought and should be further pursued.

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