FEDERAL UNIVERSITY OF GOIÁS
PROGRAM OF PHARMACEUTICAL NANOTECHNOLOGY

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BIOLOGICAL FATE OF LIPOSOMES: ROLE OF COMPLEMENT CASCADE IN RELEASE CONTENT AND CLEARANCE IN RODENTS

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IARA MAÍRA DE OLIVEIRA VIANA

BIOLOGICAL FATE OF LIPOSOMES: ROLE OF COMPLEMENT CASCADE IN RELEASE CONTENT AND CLEARANCE IN RODENTS

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Advisor: Professor PhD Eliana Martins Lima – Federal University of Goiás (UFG, Brazil).
Co-advisor: Professor PhD Nicolas Bertrand – Laval University (ULaval, Canada).

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ATA DA DEFESA DE TESE

No dia 09 de abril de 2019, ocorreu a Defesa de Tese da aluna **Iara Maíra de Oliveira Viana** no **Programa de Pós-graduação em Nanotecnologia Farmacêutica,** em sessão presidida e registrada pela Orientadora **Profa. Dra. Eliana Martins Lima**. A Tese tem como título**: Biological fate of liposomes: role of complement cascade in release content and clearance in rodents.**
De acordo com o Regimento do Programa, o processo de defesa de tese constou de duas etapas: na primeira ocorreu a apresentação oral do estudo realizado e na segunda a sustentação do mesmo frente à arguição dos membros da Banca Examinadora. A Presidente da Banca Examinadora deu início aos trabalhos, com a abertura formal às 08:00 horas, na sala de treinamento 02 do DDRH da UFG, com a leitura da **Portaria nº. 02/2014,** de designação da Banca Examinadora, assinada pela Coordenadora Geral do **PGNANOFARMA.** A seguir, passou a palavra à doutoranda, que apresentou seu trabalho, durante 45 minutos. Após sua exposição, foi dada a palavra aos membros da Banca Examinadora, para que procedessem à arguição do doutorando, com respostas a cada um dos examinadores. Concluída esta fase, registraram-se as possíveis sugestões de alterações necessárias ao texto. De acordo com a avaliação dos membros da Banca Examinadora, à doutoranda **Iara Maíra de Oliveira Viana** foi **(X) aprovada; ( ) aprovada com alterações ou ( ) reprovada**. A aprovação será válida desde que sejam satisfeitas as condições exigidas pelos membros da Banca Examinadora, com entrega da versão final da tese, referendada pela Professora Orientadora, até o prazo máximo de **( ) 30 (trinta) ou ( ) 60 (sessenta)** dias na Secretaria Local do Programa, a contar da presente data. A doutoranda está ciente de que somente poderá usufruir de todos os direitos e prerrogativas que o Título de Doutor lhe confere quando da Homologação da Ata em Reunião da Coordenadoria Geral do PGNANOFARMA, após entrega da documentação exigida para formação do processo de concessão do Diploma de Doutor na Universidade Federal de Goiás.

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SUMMARY

^{14}C-CHOL	14 carbon-cholesterol oleate
AUC	Area under curve
ANOVA	Analysis of variance
CARPA	Complement activation-related pseudo-allergy
CHOL	Cholesterol
CVF	Cobra venom factor
DLS	Dynamic light scattering
DSPE-PEG 2000	Distearoyl-glycero-phosphoethanolamine-polyethylene glycol 2000
DPX	p-xylene-bis-pyridinium bromide
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
GVB	Gelatin veronal buffered saline without calcium and magnesium
Hb	Hemoglobin
HEPES	Hydroxy-ethyl-piperazine-ethane-sulfonic acid
HPTS	8-hydroxypyrene-1,3,6-trisulfonic acid
HSPC	Hydrogenated phosphatidylcholine
ID	Injected dose
Ke	Elimination rate
LUV	Large unilamellar vesicles
MLV	Multilamellar vesicles
MPS	Mononuclear phagocytic system
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
PdI	Polydispersity index
PEG	Poly(ethylene glycol)
RBC	Red blood cells
TCC	Terminal complement complex
v/v	Volume per volume

BIOLOGICAL FATE OF LIPOSOMES: ROLE OF COMPLEMENT CASCADE IN RELEASE CONTENT AND CLEARANCE IN RODENTS

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ABSTRACT

Introduction: Nanomedicines, including liposomes, have been studied and applied to improve clinical efficacy and safety of drugs. To tailor the pharmacokinetics of these systems, a hydrophilic polymer coating of poly(ethylene glycol) (PEG) has been used to prolong circulation time. It is believed that PEG prevents the interactions of liposomes with various clearance-enhancing proteins, therefore promoting their long circulation time. Vast literature supports that the complement cascade is responsible for the majority of the immune response against liposomes, including a rapid clearance. However, in a previous study, our group has shown, using transgenic animals unable to activate the complement cascade, that this pathway of the innate immunity could not explain the differences in early clearance observed with various non-liposomal drug delivery systems. **Objectives:** The purpose of this work is to explore the role of complement on the elimination of intravenously administered liposomes in rodents, and to study the impact of the size of liposomes in their behavior in serum. **Methodology:** Liposomes of 100 and 400 nm diameter were prepared and incubated in serum with and without EDTA in order to evaluate the effect of complement on the release of hydrophilic content. To explore the role of complement activation on the pharmacokinetics of liposomes, animals were treated intraperitoneally with cobra venom factor 24 and 12 h before the intravenous injection of radiolabeled liposomes. Radioactivity of blood samples was assessed by scintillation counting. **Results and discussion:** Complement proteins seem to trigger the release of the content from 400 nm non-PEGylated liposomes in rat serum, conversely, no effect was shown in smaller 100 nm non-PEGylated liposomes. As expected, the non-PEGylated liposomes showed faster elimination than PEGylated liposomes in rodents. At a lipid dose of around 20 $mg \cdot kg^{-1}$, the abrogation of the complement cascade did not interfere in the circulation time of either PEGylated and non-PEGylated liposomes. However, at lower doses, the complement cascade seems to play a role on the faster elimination of non-PEGylated liposomes. **Conclusion:** The activation of the complement cascade may be not enough to predict the elimination time of PEGylated and non-PEGylated liposomes.

Keywords: Complement cascade, size effect, liposomes, release content, PEG coverage, clearance, rodents.

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DESTINO BIOLÓGICO DOS LIPOSSOMAS: PAPEL DA CASCATA DO COMPLEMENTO NA LIBERAÇÃO DE CONTEÚDO E ELIMINAÇÃO EM ROEDORES

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RESUMO

Introdução: Nanomedicamentos, incluindo lipossomas, têm sido estudados e aplicados para melhorar a eficácia clínica e a segurança de fármacos. Visando alterar a farmacocinética destes sistemas, uma cobertura com polímero hidrofílico de poli(etilenoglicol) (PEG) tem sido empregado para prolongar o tempo de circulação. Acredita-se que o PEG evita as interações dos lipossomas com proteínas que aumentam a depuração, promovendo assim a sua longa circulação. Vários trabalhos sustentam que a cascata do complemento é responsável pela resposta imune contra lipossomas, incluindo a rápida eliminação. No entanto, em um estudo anterior, nosso grupo demonstrou, empregando animais transgênicos incapazes de ativar a cascata do complemento, que essa via da imunidade inata não explica as diferenças na depuração inicial observada com diferentes sistemas de liberação de fármacos. **Objetivos:** O objetivo deste trabalho é explorar o papel do complemento na eliminação de lipossomas administrados por via intravenosa em roedores, e estudar o impacto do tamanho dos lipossomas em seu comportamento no soro. **Metodologia:** Lipossomas de 100 e 400 nm de diâmetro foram preparados e incubados em soro e soro com EDTA para avaliar o efeito do complemento na liberação de conteúdo hidrofílico. Para explorar o papel da ativação do complemento na farmacocinética dos lipossomas, os animais foram tratados intraperitonealmente com fator de veneno de cobra 24 e 12 h antes da injecção intravenosa de lipossomas radiomarcados. A radioatividade das amostras de sangue foi avaliada por contagem de cintilação. **Resultados e discussão:** As proteínas do complemento parecem desencadear a liberação do conteúdo de lipossomas não-PEGuilados de 400 nm em soro de rato, por outro lado, nenhum efeito foi mostrado nos lipossomas não-PEGuilados de 100 nm. Como esperado, os lipossomas não-PEGuilados foram eliminados mais rapidamente do que os lipossomas PEGuilados em roedores. Quando cerca de 20 $mg.kg^{-1}$ de lipídeos são administrados, a cascata do complemento não interfe no tempo de circulação dos lipossomas PEGuilados e não PEGuilados. No entanto, em doses mais baixas, a cascata do complemento parece ter um papel na eliminação rápida de lipossomas não-PEGuilados. **Conclusão:** A ativação da cascata do complemento pode não ser suficiente para prever o tempo de eliminação de lipossomas PEGuilados e não-PEGuilados.

Palavras-chave: Cascata do complemento, efeito do tamanho, lipossomas, liberação de conteúdo, cobertura de PEG, eliminação, roedores.

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1 INTRODUÇÃO

Nanostructured systems used for pharmaceutical purposes are composed by particles with sizes ranging from around 1 to 100 nanometers (Initiative, 2018), up to few hundred nanometers. When compared to conventional drug products, nanostructured drug delivery systems exhibit distinct properties, some of which are very promising for the therapeutic applications (Ma *et al.*, 2009; Kim *et al.*, 2010). Enhancing drug delivery and targeting upon intravenous administration (e.g. treatment of tumors), with consequent increase of effectiveness and safety of treatment (Peer *et al.*, 2007; Bertrand *et al.*, 2014), are among the main advantages of the pharmaceutical nanotechnology approach.

Despite the great advances that nanotechnology has brought to medicine, relatively few products are commercially-available or in clinical phase (Admnistration; Fonseca *et al.*, 2014). Nanostructured systems have provided significant reduction in adverse effects, though not always associated with increased efficacy (Ernsting *et al.*, 2013; Petersen *et al.*, 2016). For instance, liposomal doxorubicin (Doxil or Caelyx®) increases in 10 to 15-fold the amount of doxorubicin in tumor(Laginha *et al.*, 2005; Ernsting *et al.*, 2013). The pharmacokinetics and pharmacodynamics of nanocarriers have a large interpersonal variability (Caron *et al.*, 2012; Anselmo e Mitragotri, 2014). Moreover, hypersensitivity reactions (Szebeni, 2018) and enhanced clearance upon subsequent doses of long-circulating nanoparticles (prepared with a hydrophilic polymer coating of poly(ethylene glycol) (PEG)) (Laverman, Boerman*, et al.*, 2001; Abu Lila *et al.*, 2013) have been reported, indicating that the *in vivo* behavior is not fully understood and predictable.

The physicochemical characteristics of the nanocarrier, such as size, shape, charge, surface modifications (such PEG coverage) and composition impact directly on their interactions with the biological environment and consequently on their biodistribution and delivery of drugs to the target tissues (Laginha *et al.*, 2005; Bertrand *et al.*, 2009; Bertrand *et al.*, 2010; Ernsting *et al.*, 2013). Many studies have shown, for example, that nanoparticles covered with hydrophilic polymers (including PEG) of the nanoparticles promote less adsorption of plasma proteins (opsonins) on the surface and less phagocytosis by macrophages, thus showing prolonged systemic circulation (Bertrand *et al.*, 2009; Jones *et al.*, 2013). However, our group had showed, using transgenic animals unable to activate the complement cascade, that the complement cascade could not explain the biological fate of

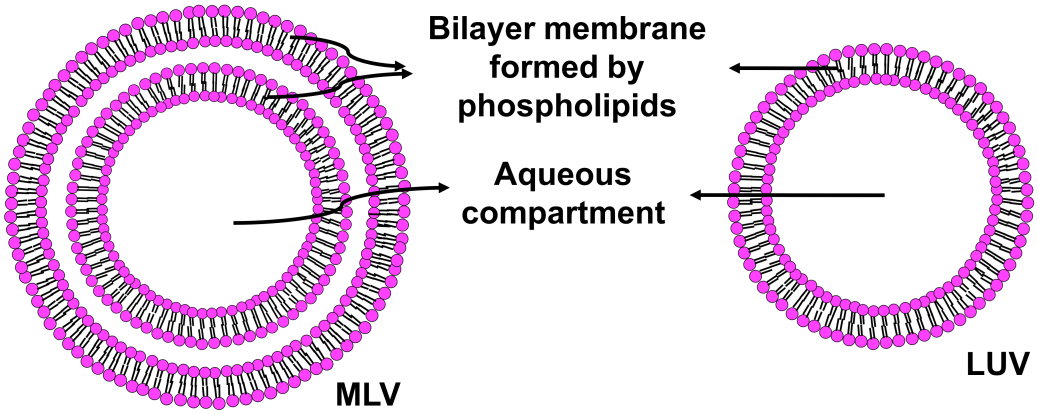
PEGylated nanoparticles (Bertrand, N. *et al.*, 2017). The circulation time might be dependent on animal model employed. For example, stealth liposomes modified with monosialoganglioside GM1 showed long circulation time in mice and fast elimination in rats (Allen e Hansen, 1991; Bertrand e Leroux, 2012).

In general, the physical and chemical properties of nanocarriers influence their biological fate. However, the effect of the size of liposomes in the release of their content remains unclear. Beyond that, the impact of the complement cascade on the pharmacokinetic profile of PEGylated and non-PEGylated liposomes has not yet been demonstrated. The understanding of the *in vitro* and *in vivo* behavior of liposomes may support the rational design of nanostructured systems for intravenous administration. Altogether, the main purpose of this work is to evaluate whether the activation of complement cascade by liposomes is relevant to the release of hydrophilic drugs and for the clearance of these nano drug carriers in rodents.

2.1 Liposomes

Nano-sized drug delivery vehicles, also known as nanomedicines, are being increasingly studied to improve the clinical efficacy and safety of drugs. Most commercially-available nanomedicines are liposomal formulations. Liposomes are vesicles made of one or multiple lipid bilayers surrounding an aqueous core (**Figure 1**). The lipid membrane and the aqueous core can load lipophilic and hydrophilic drugs respectively. The lipid membrane is formed by phospholipids, however other lipids such as cholesterol (CHOL) and lipids anchored to polymers are also widely used [16,17,18]. There are several methods for the preparation of liposomes, such as sonication (Batzri e Korn, 1973), ethanol injection method (Batzri e Korn, 1973; Jaafar-Maalej *et al.*, 2010; Shaker *et al.*, 2017), reverse-phase evaporation (Szoka e Papahadjopoulos, 1978), lipid hydration (Akbarzadeh *et al.*, 2013), freeze-thaw (Akbarzadeh *et al.*, 2013), and membrane extrusion (Hope *et al.*, 1985).

Figure 1 – Liposome vesicles of multiple lipid bilayer (multilamellar vesicles, MLV) and one bilayer (large unilamellar vesicles, LUV) [source: the author].



Multilamellar vesicles (MLV) are offen prepared by the hydration of a thin lipid film (Hope *et al.*, 1986). The extrusion of MLV through polycarbonate membranes is a method that allows good control over the diameter of vesicles. MLV extrusion through 100 nm membranes can lead to formation of large unilamellar vesicles (LUV) as well as small unilamellar vesicles (Hope *et al.*, 1985; Akbarzadeh *et al.*, 2013). LUV with size around 100 nm are often used for cancer therapy, because this size is higher than the fenestration of blood capillaries and smaller than those of the tumor vessels, which leads to particle and

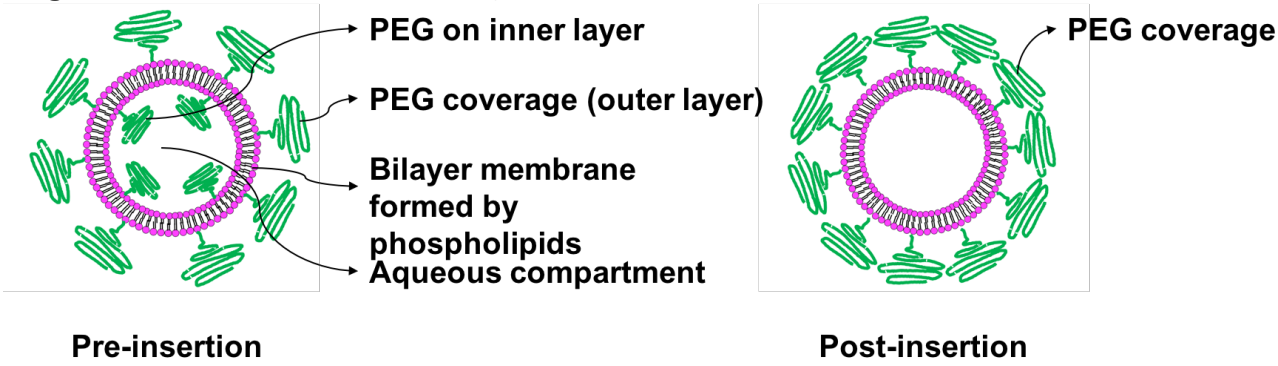
drug accumulation, a phenomenon coined as “enhanced permeability and retention” (Ernsting *et al.*, 2013). Larger liposomes (with 400 nm) may be useful for targeting lungs. Liposomes with average diameter around 50 to 200 nm can be obtained by extrusion. Liposomes prepared by extrusion with Z-average higher than 200 nm may consist in a heterogenous mixture of LUV and MLV. LUV and MLV have trapped volume between 1 and 30 µL µmol^{-1} lipid (Mayer, Bally*, et al.*, 1986). SUV has low trapping volume of 0.2-0.8 µL µmol^{-1} (Mayer, Bally*, et al.*, 1986).

2.1.1 PEGylation of liposomes

Liposomes with PEG coverage, named PEGylated, are often used due their longer circulation time following intravenous administration. The first nanomedicine released on the market (Doxil®), for example, consists in doxorubicin loaded into liposomes prepared with hydrogenated phosphatidylcholine (HSPC), CHOL and Distearoyl-glycero-phosphoethanolamine-polyethylene glycol 2000 (DSPE-PEG 2000) (Admnistration). Increasing the PEG surface coverage leads to an improved steric effect. The size of PEG chains can also enhance circulation time. Indeed, PEG 2000 and 5000 are the most widely used for intravenous administration. The higher density of PEG in the coating leads to the transition between the mushroom and brush state at around 4 mol%, resulting in a denser layer and improved properties (Garbuzenko *et al.*, 2005). Around 15-20 mol% of the PEGylated lipid DSPE-PEG 2000 can be insert in liposome membranes (Hristova *et al.*, 1995). However, the maximum membrane compressibility and highest biological stability are reached at 7 ± 2 mol% (Garbuzenko *et al.*, 2005). The improved stability is a consequence of the tight packing between acyl chains and low hydration (Garbuzenko *et al.*, 2005). Higher amounts of DSPE-PEG 2000 would induce micelle formation and bilayer solubilization (Garbuzenko *et al.*, 2005).

PEGylated liposomes can be obtained by pre- or post-insertion techniques of PEGylated lipids. The pre-insertion results in the presence of PEG on inner and outer layers of the membrane, while the post-insertion ensures that PEGylation occurs only on the exterior (Immordino *et al.*, 2006) (**Figure 2**). DSPE-PEG 2000 at 2.5 mol% might achieve high coverage of outer layer by post-insertion technique (Abe *et al.*, 2015), therefore, longer circulation time. Santos *et al.* showed that 0.5 mol% of “post-inserted” DSPE-PEG 2000 into liposomes surface is enough to prolong circulation time (Dos Santos *et al.*, 2007).

Figure 2 – Liposomes prepared by pre- and post-insertion technique [source: the author].



In general, the post-insertion of PEGylated lipids into bilayers requires temperature above the phase transition to ensure proper membrane fluidity and permeability (Abe *et al.*, 2015). Lipid membranes in the liquid-crystalline state have higher fluidity and permeability than in the gel phase (Demel *et al.*, 1968). The transition temperature of membranes varies according to lipid composition, such as phospholipid type and CHOL amount (Trauble e Sackmann, 1972). Phospholipids with saturated chains show increased intermolecular interactions and less spacing between their chains, therefore, the membrane is more compact and phase transition temperature is higher. Unsaturated phospholipids tend to have lower transition temperatures due the weaker interactions and higher space between their chains. CHOL is a component that alters the interactions between phospholipids chains, leading to lower enthalpy of transition (Demel *et al.*, 1972; Klopfenstein *et al.*, 1974). The impact of the addition of CHOL depends on the physical state of the phospholipid bilayer (Trauble e Sackmann, 1972). Vandijck *et al.* noticed that increasing CHOL content into phosphatidylcholine (PC)-phosphatidylethanolamine bilayers, the enthalpy of phase transition (and the transition temperature) decreased to the point of becoming undetectable (Vandijck *et al.*, 1976). The general effect is that above the phase transition temperature the rigidity of the bilayer increases with addition of CHOL. CHOL-rich membranes do not seem to have gel-liquid phase transition(Leonard e Dufourc, 1991), therefore, the fluidity of the membranes may not change with the increase in the energy of the system.

2.2 Liposomes for long circulation time and the immune system

The long circulation time of liposomes is desirable in cancer therapy (Gabizon, A. A. *et al.*, 1993). To achieve this long circulation time, liposomes must evade the immune system. The characteristics of liposomes, such as size and composition, may play a role in their recognition and uptake by cells of mononuclear phagocytic system (MPS) (Gabizon, Alberto A. *et al.*, 1993). The phagocytic cells delivery the liposome (with the drug entrapped)

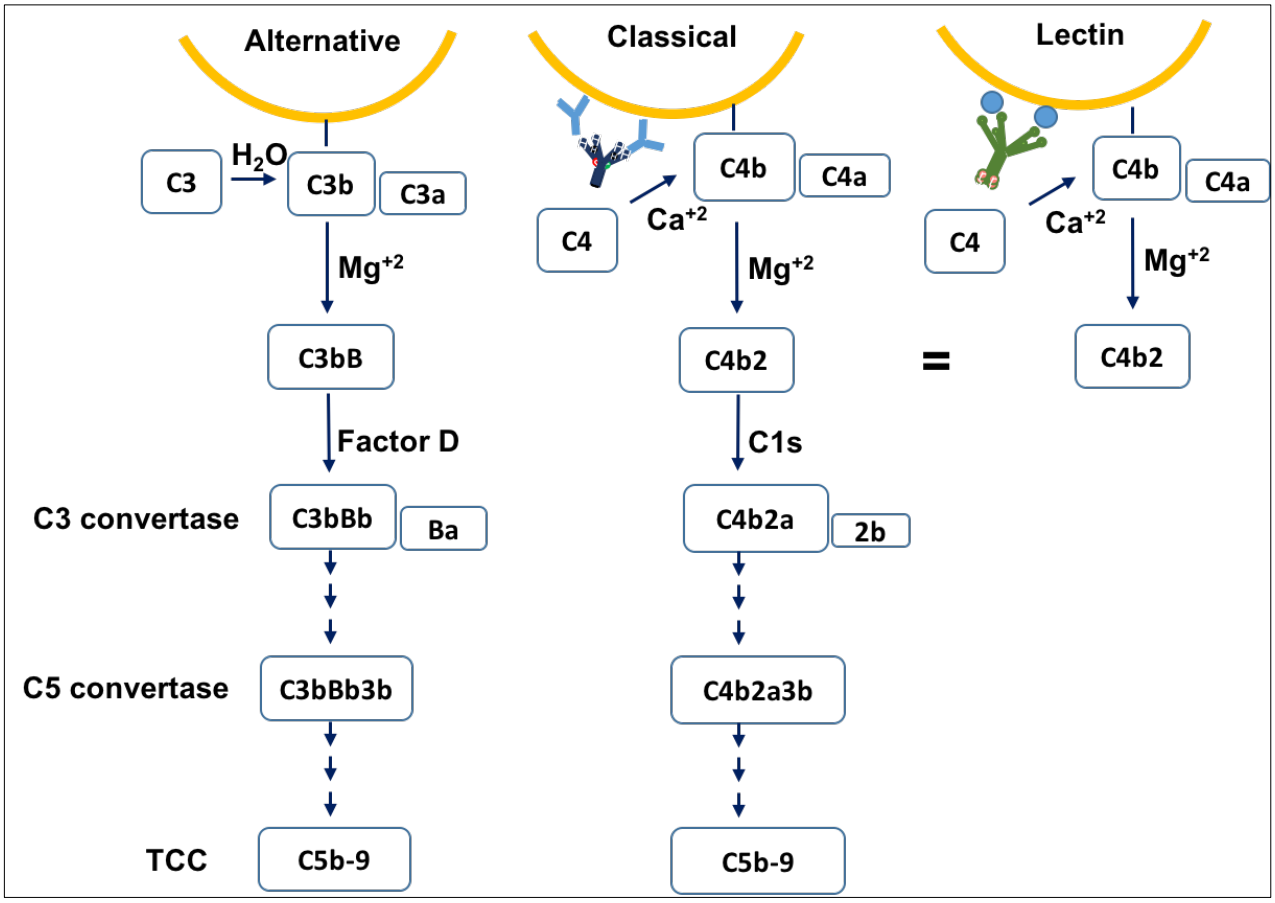
in the liver, spleen and bone marrow, thus resulting in metabolism and excretion of the drug (Gabizon, A. A. *et al.*, 1993; Gabizon, 2001). The drug elimination may also be consequence of release from liposomes into the bloodstream, allowing its faster elimination (Gabizon, A. A. *et al.*, 1993; Gabizon, 2001). As shown by Gabizon *et al.*, the clearance of doxorubicin from PEGylated liposomes (radiollabed with ^{3}H) is faster than the ^{3}H-labelled liposomes alone at 48 hours after intravenous administration in mice, suggesting that leakage of the doxorrubicin from liposomes is a mechanism of drug elimination (Gabizon, Alberto A. *et al.*, 1993).

The immune response against nanocarriers occurs similarly to invading microorganisms. Immunoglobulins and proteins of the complement cascade participate of their recognition, leading to the elimination by the cells of the MPS (Vu *et al.*, 2019). The proteins of the complement cascade are able to release cytokines, lyse and opsonize the liposomes (Szebeni *et al.*, 2003). Thereafter, phagocytic cells are recruited, and B lymphocytes start production of specific immunoglobulins. The interaction of nanocarriers with proteins of the complement cascade may also cause severe hypersensitivity reactions (Szebeni, 2018).

2.2.1 Complement cascade

Complement cascade consists in a group of around 50 proteins, among circulating and membrane proteins (Hajishengallis *et al.*, 2017). These proteins are responsible for global regulation of immunity and host defense against microorganisms (Hajishengallis *et al.*, 2017). Complement proteins can bind directly to nanoparticles or to immunoglobulins, IgG and IgM. Upon binding, complement cascade is activated by three well known pathways: alternative, classical, or lectin (Szebeni *et al.*, 2003; Ricklin *et al.*, 2010; Hajishengallis *et al.*, 2017) (**Figure 3**).

Figure 3 – Squeme highlighting the differences between the three pathways of complement cascade: alternative, classical and lectin. The single arrows symbolize one step, and three arrows symbolize more than one ligation and cleavage steps [source: the author].



Alternative pathway is initiated by spontaneous cleavage of C3 protein into C3b and C3a. C3b protein binds covalently to the antigen surface. C3b protein is responsible for opsonization of antigens, leading to phagocytosis by macrophages. C3a protein is chemotactic for neutrophils, monocytes and macrophages. After binding of C3b to surface, and in presence of Mg^{2+}, factor B binds to C3b protein. Factor B is then cleaved by factor D in Ba and Bb. Bb, major component of factor B, remains covalently bound to C3b. C3bBb protein is the C3 convertase of alternative pathway (Abbas *et al.*, 2011; Wibroe e Moghimi, 2012).

Classical pathway is triggered by IgG or IgM immunoglobulins binding to antigen surface, followed by C1q protein binding. C1q protein, when bound to Fc portion of immunoglobulins and in presence of Ca^{2+}, activates C1r associated. C1r, in turn, activates and cleaves C1s. C1s activated cleaves C4 protein in C4a and C4b. C4b binds covalently to antigen surface. Next, C2 protein binds to C4b in presence of Mg^{2+}. After C4b binding, C1s acts by cleaving C2 in C2a and C2b. The major component of C2, C2a, remains bound to C4b, forming the C3 convertase of the classical pathway, C4b2a (Abbas *et al.*, 2011;

Wibroe e Moghimi, 2012). Some authors have named the major component of C2 as C2b, therefore C3 convertase become C4b2b (Ricklin *et al.*, 2010).

Lectin pathway occurs by lectin binding to mannose residues of microbial glycoproteins and glycolipids, or by ficolines binding to N-acetylated surfaces. The enzymatic cascade is similar to classical pathway steps, once C3 convertase corresponds to C4b2a protein (Abbas *et al.,* 2011; Wibroe e Moghimi, 2012).

C3 convertase amplifies the immune response by cleaving another C3 protein and releasing more C3a and C3b. At this point of the cascade, C3b can also bind to C3 convertase, producing the protein C3bBb3b or C4b2a3b, known as C5 convertase (Abbas *et al.,* 2011).

Cleavage of C5 protein by C5 convertase leads to release of the anaphylatoxin C5a. The larger fragment, C5b protein, remains binded to C5 convertase. The enzymatic cascade ends on the formation of C5b-9, which is composed by proteins C6, C7, C8 and C9 associated to C5b (Abbas *et al.,* 2011).

Independent of the pathway, complement cascade produces cytokines, opsonins and terminal lytic complex. Cytokines C3a, C4a and C5a recruit macrophages and mast cells, which eliminate the opsonized antigens by C3b proteins. C5b-9 proteins, also known as terminal complement complex (TCC) or membrane attack complex, create a pore in the microorganism's membrane, leading to its lysis (Ricklin *et al.*, 2010). Besides these immediate actions, along with innate immune response, complement proteins stimulate B cells to produce immunoglobulins, inducing the acquired immune response (Hajishengallis *et al.*, 2017).

2.2.1.1 Complement-nanocarriers interaction and consequences

Interaction between nanocarriers and complement proteins are regulated by various dynamic interfacial forces and physicochemical properties such as size, charge, shape, hydrophobicity, hydrophilicity, chemical composition and coverage by functional groups (Milani *et al.*, 2012; Caracciolo *et al.*, 2015).

Nanocarriers with zeta potential near to neutral (± 10 mV) have been related to lower interactions with complement proteins and the MPS (Perry *et al.*, 2012; Ernsting *et al.*, 2013). In general, negatively or positively charged structures activate the complement cascade. However, this activation is not always observed (Devine *et al.*, 1994). The recently published work of Thielens *et al.* shows that anionic micelles (containing carboxylic group on the

surface) are able to activate complement cascade by alternative pathway in mice serum, while cationic micelles (with ammonium groups) do not activate complement (Thielens *et al.*, 2018). The activation of the complement cascade seems dependent on charge distribution onto structures (Sou e Tsuchida, 2008). As showed by Sou and Tsuchida, vesicles containing different acidic lipids for surface modification (conventional acidic phospholipid or carboxylic acid derivative) led to different outcomes, regardeless the charge on the surface (Sou e Tsuchida, 2008). Vesicles modified with conventional acid phospholipids led to significantly complement activation, while vesicles modified with a carboxylic acid did not induce the complement cascade (Sou e Tsuchida, 2008). The negative charge on PEGylated lipids (of the phosphate moiety) is responsible for complement activation in PEGylated vesicles (Moghimi *et al.*, 2006). The micelles produced with PEGylated phospholipid, on the other hand, did not activate complemente cascade (Moghimi *et al.*, 2006).

The composition of liposomes seems to define their interactions with complement cascade, interfering in their systemic stability and circulation time (Moghimi *et al.*, 2011). Liposomes of 800 nm in diameter containing 44 mol% of CHOL activate complement cascade preferably by alternative pathway, while 800 nm liposomes containing 22 mol% CHOL or 33 mol% CHOL activate the classical pathway (Ishida *et al.*, 2001). The resulting effect of CHOL content is an increase in the elimination of liposomes by the liver (Ishida *et al.*, 2000). Liposomes modified by PEG have a significant impact on the activation of the complement cascade. The steric effect of PEG prevents the binding of proteins and phagocytosis by MPS. Therefore, they may have longer circulation times than non-PEGylated liposomes and are known as stealth or furtive (Allen e Hansen, 1991; Allen *et al.*, 1991). Perry *et al.* studied the relationship between the PEG coverage of hydrogel nanoparticles and the phagocytosis by macrophages and pharmacokinetics profiles (Perry *et al.*, 2012). PEG 5000 was used to prepare nanoparticles with high coverage density (0.083 ± 0.006 PEG nm^{-2}) and low coverage density (0.028 ± 0.002 PEG nm^{-2}), brush and mushroom conformation respectively. *In vitro* and *in vivo* experiments indicated that increase in the surface coverage of hydrogels, decreased the adsorption of plasma proteins (opsonins) to their surface and decreased phagocytosis by the macrophages, thus resulting in prolonged systemic circulation (Perry *et al.*, 2012). In addition, the authors observed a decrease in liver accumulation and an increase in spleen accumulation as the PEG density increases, indicating that this property of the nanoparticles also interferes with the uptake by the macrophages of these organs (Perry *et al.*, 2012). As previously shown by our group, using knockout mice, the clearance of polymeric nanoparticles with low and high PEG

coverage is similar in animals with and without ability to activate complement cascade (Bertrand, N. *et al.*, 2017). Finally, PEG coverage does not have the same effect after repeated doses. PEGylated nanoparticles are cleared faster following a second dose. IgM anti-PEG immunoglobulins trigger a fast clearance of nanoparticles by classical pathway (Alberts, 1997). This effect, called the enhanced clearance effect will be discussed in the following section. The activation of complement cascade by PEGylated liposomes can also cause hypersensitivity reactions which will be discussed in section 2.2.1.1.2 (Szebeni, 2018).

2.2.1.1.1 Enhanced clearance effect

Several studies indicate that intravenous administration of a liposomal dispersion alters the pharmacokinetics of subsequent doses, accelerating the clearance of nanocarriers and increasing the accumulation in liver and spleen. This phenomenon, known as enhanced clearance effect (Laverman, Carstens*, et al.*, 2001) or accelerated blood clearance (Ishida *et al.*, 2002), has been described as consequence of activation of complement cascade. Dams *et al.* in 2000 (Dams *et al.*, 2000) were the first to observe this effect in 85 nm and 400 nm PEGylated liposomes, and 100 nm non-PEGylated liposomes upon administration in rats, rhesus monkeys and mice following 1 week intervals between doses. Wistar rats and rhesus monkeys presented changes in biodistribution of nanoparticles after the second dose, regardless of the size and surface properties of administered liposomes. However, the effect was not observed in mice. Complement activity of rat serum was investigated after subsequent doses of NaCl solution or PEGylated liposomes. The hemolytic activity remained unchanged in rats treated with NaCl solution. However, the administration of PEGylated liposomes lead to a decrease in hemolytic activity after the second dose. The authors performed serum transfusion experiments among animals, which in turn revealed that a soluble factor of 150 KDa induced the effect of enhanced clearance of the nanoparticles. Serological tests indicated that this factor does not correspond to immunoglobulins IgG and IgM (Dams *et al.*, 2000).

Enhanced clearance effect is not always observed. Goins *et al.*, for example, did not observe difference in the half-life times of repeated injections of 99M technetium-labeled PEGylated liposomes in rabbits, and only minimal effects were noticed in biodistribution. However, Goins *et al.* used intervals of 6 weeks between injections, which might explain the lack of enhanced clearance (Goins *et al.*, 1998). Oussoren and Storm, as well, did not notice changes in pharmacokinetics of subsequent doses of PEGylated liposomes upon

administration at relatively small intervals (24 and 48 h) (Oussoren e Storm, 1999). Therefore, too short (48 h) or too long (6 weeks) intervals between doses did not induce significant changes in the pharmacokinetic of the nanomedicine. Specific intervals between doses seem necessary to induce the enhanced clearance effect. As shown by Ishida *et al*, the enhanced clearance of a second dose of PEGylated liposomes may be maximal at 5 days after the first dose in male Wistar rats (Ishida *et al.*, 2003).

Besides dosing schedule, lipid dose has been related to the intensity of enhanced clearance effect (Wang *et al.*, 2007) (Laverman, Boerman*, et al.*, 2001; Ishida *et al.*, 2005). Laverman *et al.* observed that administration of first dose of 0.05, 0.5 and 5 μmol kg^{-1} of phospholipids from PEGylated liposomes induced enhanced clearance at subsequent dose of 5 μmol kg^{-1}. However, with a first dose of 5 μmol kg^{-1}, the enhanced clearance effect was attenuated at second doses greater than 15 μmol kg^{-1}, and no effect was found with doses of 50 μmol kg^{-1} (Laverman, Boerman*, et al.*, 2001; Laverman, Carstens*, et al.*, 2001). Ishida *et al.* also evaluated the effect of lipid dose on the clearance of a second dose of PEGylated liposomes. They concluded that low doses of conventional liposomes (0.001 μmol kg^{-1}) did not induce the enhanced clearance effect, while conventional liposomes at high dose (5 μmol kg^{-1}), and PEGylated liposomes at several doses (0.001 to 5 μmol kg^{-1}) may induce the enhanced clearance effect. For PEGylated liposomes, low doses (0.001, 0.01 and 0.1 μmol kg^{-1}) were more efficient than higher doses (1 and 5 μmol kg^{-1}) (Ishida *et al.*, 2005) in inducing enhanced clearance effect.

The type and constitution of nanoparticle can also affect the induction of the enhanced clearance effect. As described by Wang *et al.*, the intensity of the enhanced clearance effect is different following administration of PEGylated liposomes, PEGylated micelles, PEGylated solid lipid nanoparticles or PEGylated emulsions in beagle dogs (Wang *et al.*, 2015). Our group showed that the enhanced clearance is dependent on the architecture of nanoparticles: after administration of a first dose of PEGylated nanoparticles in mice, the enhanced clearance was not noticed in non-PEGylated liposomes, free mPEG 10,000, and PEGylated bovine serum albumin, but was noticed in PEGylated nanoparticles (high and low coverage), and PEGylated liposomes (Grenier *et al.*, 2018) (**Annex 1**). PEGylated nanoparticles are, in general, able to induce enhanced clearance effect (Laverman, Boerman*, et al.*, 2001; Ishida *et al.*, 2005; Wang *et al.*, 2007; Abu Lila *et al.*, 2013; Im *et al.*, 2016). However, according to Laverman *et al.* both non-PEGylated liposomes (fast clearance) and PEGylated liposomes (slow clearance) are able to induce enhanced clearance effect when administered 1 week before the second dose (Laverman,

Boerman*, et al.*, 2001; Laverman, Carstens*, et al.*, 2001). Laverman *et al.* have also demonstrated that formulations containing doxorubicin (Caelyx or Doxil) have their pharmacokinetics altered when administered after a first dose of empty PEGylated liposomes (without doxorubicin). Caelyx, however, did not increase clearance of a second dose of Caelyx when administered with 1-week intervals. This suggests that encapsulation of doxorubicin, due its immunosuppressive activity, avoids enhanced clearance effect (Laverman, Boerman*, et al.*, 2001; Laverman, Carstens*, et al.*, 2001). Nanoparticles coverage, such as density and size of PEG chain, has been reported as an important feature in enhanced clearance effect. PEGylated liposomes with 5 – 15 mol% PEG density appear to induce enhanced clearance effect, while non-PEGylated liposomes at same low lipid dose (0.001 μmol kg^{-1}) did not (Ishida *et al.*, 2005). The PEG size (2000 or 5000) did not impact the intensity of enhanced clearance effect (Ishida *et al.*, 2005).

Enhanced clearance effect appears to be related to the production of anti-PEG IgMs (Wang *et al.*, 2007), however it does not completely explain the phenomenon (Ishihara *et al.*, 2010). Enhanced clearance effect might also depend on immune cells (Laverman, Carstens*, et al.*, 2001; Shimizu *et al.*, 2015) and corona protein (Grenier *et al.*, 2018). According to Lila, Kiwada and Ishida, the mechanism of enhanced clearance effect starts with administration of PEGylated liposomes, and their biodistribution. Part of the PEGylated liposomes accumulate in the spleen, where they bind to the immunoglobulins present on the surfaces of B cells, sensitizing these lymphocytes. B lymphocytes, in turn, produce anti-PEG IgM immunoglobulins that remain in blood circulation for a certain time. If anti-PEG IgM immunoglobulins are present during administration of the second dose, a liposome-immunoglobulin complex will be formed with consequent activation of complement cascade by classical pathway, and opsonization of PEGylated liposomes, leading to phagocytosis (Ishihara *et al.*, 2010).

Although circulating anti-PEG IgM might be present in humans serum (Yang *et al.*, 2016), the enhanced clearance effect has not been reported in patients using doxorubicin-containing PEGylated liposomes (Gabizon *et al.*, 2008). In fact, the opposite effect has been noticed: an increase in circulation time (Gabizon *et al.*, 2008; La-Beck e Gabizon, 2017). Studies that may clarify this behavior in humans remain necessary.

2.2.1.1.2 Complement activation-related pseudoallergy

Intravenous administration of liposome formulations may trigger hypersensitivity reactions, such as type I allergy and pseudo-allergy. Type I allergy is an IgE

immunoglobulin-mediated response. The symptoms of pseudo-allergy, or idiosyncratic reaction (Waller, 2011), are similar to allergy type I, but occur without sensitization (Zhang *et al.*, 2018). The mast cells, basophil and leukocytes are responsible for allergy type I and pseudoallergy (Szebeni, 2012). However, the pseudoallergy cannot be categorized in any 4 types of hypersensibility reactions (Zhang *et al.*, 2018).

It is believed that hypersensitivity reactions such as pseudoallergies occur due to complement cascade. In 1989, Wassef *et al.* suggested involvement of complement in pseudoallergy after observation of anaphylactic reactions in pigs due intravenously administration of liposomes containing CHOL (Wassef *et al.*, 1989). Administration of liposomes caused reduction of CH50, increase of 6- keto-prostaglandin F1α and thromboxane B2 (Wassef *et al.*, 1989; Szebeni *et al.*, 1999). Ten years later, Szebeni *et al*. named this phenomenon as Complement activation-related pseudoallergy (CARPA) (Szebeni *et al.*, 1999).

The symptoms of CARPA, differently from allergic type I reactions, appear at the first exposure and disappear during re-exposure. It should be noticed, however, that there are patients in whom pseudoallergy is manifested at subsequent doses. Steroid medications and pre-medication with antihistamines are effective against pseudoallergy symptoms, but they are not effective when it comes to type I allergy (IgE-mediated). Another difference is CARPA intensity depends on infusion rate of the nanomedicine and can be resolved spontaneously. However, both reactions can lead to severe conditions (Szebeni, 2012; Szebeni e Storm, 2015).

CARPA and type I allergic reactions have symptoms in common, such as dermatitis, sweating, dyspnea, edema, erythema, sensation of impending death, fever, flushing, headache, hypertension or hypotension, hypoxemia, low back pain, metabolic acidosis, nausea, pruritus, rash, rhinitis, shock, rashes, sneezing, tingling sensations, urticaria, angioedema, asthma, bronchospasm, chest pain, hypothermia, asphyxia, confusion, conjunctivitis, cough, cyanosis and death (Szebeni, 2005).

The clinical effects of CARPA are rarely observed in patients treated with PEGylated liposomes containing doxorubicin when the initial infusion rate is lower than 0.38 mg min^{-1} (Chanan-Khan *et al.*, 2003; Szebeni, 2005; La-Beck e Gabizon, 2017). However, up to 45% of patients may have moderate to severe symptoms of CARPA when relatively fast initial infusion rate is used. Nevertheless, clinical data has shown that previous administration of corticosteroids or antihistaminics can prevent this reaction (Chanan-Khan *et al.*, 2003; Szebeni, 2005).

Because of the severity of pseudoallergy reactions, evaluating the ability of nanoparticles to trigger CARPA is crucial in pre-clinical stages of injectable products (Szebeni e Storm, 2015). Since 2013 the European Medicines Agency recommends the use of *in vitro* and *in vivo* protocols to evaluate immunostimulatory activity of intravenous liposomal products ((Ema), 2013). Enzyme-linked immunosorbent assay (ELISAs) for quantification of C3a, C5a, C4d, Bb, SC5b-9, iC3b, determination of CH50, flow cytometry of macrophages and (or) basophils can be applied as *in vitro* tests (Szebeni e Storm, 2015). Experiments in sensitive animal models ((Ema), 2013), such as pigs (Szebeni e Storm, 2015), can be performed to confirm the immunostimulatory potential by observing the symptoms of CARPA ((Ema), 2013; Szebeni e Storm, 2015). However, the evaluation of the CARPA is not a constant in preclinical studies (Petersen *et al.*, 2016). In addition, there are no standard methods that allow comparability between studies from different research groups in light of CARPA.

2.2.2 Other proteins

In addition to complement proteins, other plasma proteins may define the biological fate of nanoparticles, either by direct action or by preventing the adsorption of complement proteins (Tenzer *et al.*, 2011; Corbo *et al.*, 2016; Bertrand, N. *et al.*, 2017). This protein coating adsorbed to nanoparticles surface, also known as protein corona (Cedervall *et al.*, 2007; Caracciolo, 2015), is formed instantly upon intravenous administration (Hadjidemetriou *et al.*, 2016).

The protein corona varies qualitatively and quantitatively according to the composition of the biological fluid (Monopoli *et al.*, 2011; Caracciolo *et al.*, 2014; Hadjidemetriou *et al.*, 2015). Physicochemical characteristics of nanoparticles such as size, shape, charge, surface and composition of the nanoparticles directly affect the dynamics of protein adsorption, making the process highly specific. Therefore, it can be said that the nanoparticles have a biological identity. Palchetti *et al.* proved that positively charged PEGylated liposomes exhibit negative zeta potential after incubation with fetal bovine serum at room temperature for 90 min. As expected, proteins of opposite charge to nanoparticles adhered preferentially. Palchetti *et al.* also demonstrated the impact of the experiment design, static or dynamic (simulating the bloodstream). Corona protein was formed in less time by dynamic experiments rather than static experiments. Furthermore, the dynamic process allowed adsorption of a higher variability and amount of proteins (Palchetti *et al.*, 2016).

The adsorption of proteins to liposomes has been related to their composition, such as amount of CHOL and PEGylation. Liposomes containing CHOL have decreased protein adsorption in comparison with liposomes with only phospholipids (Semple *et al.*, 1996). The addition of CHOL to liposomes may improve their stability in serum as result of increased membrane rigidity (Mayer, Bally*, et al.*, 1986). Similarly, PEGylation has been linked to lower protein adsorption and macrophage phagocytosis (Perry *et al.*, 2012). It is believed that the longer circulation time of PEGylated liposomes is a consequence of the lower protein adsorption. However, other studies indicate that protein coverage does not interfere with elimination rate (Ke) of liposomes (Allen *et al.*, 2002). According to Semple *et al.*, the increase in binding of proteins to liposomes (> 50 g of protein per mol of lipid) led to faster clearance. Amounts of proteins less than 20 g per mole, in turn, lead to slow clearance (Semple *et al.*, 1998).

Recent studies have shown that the majority of the proteins in the hard corona are apolipoproteins, immunoglobulins and complement proteins (Hadjidemetriou *et al.*, 2016; Palchetti *et al.*, 2016). The *in vivo* composition of protein corona is dependent on time as demonstrated by Hadjidemetriou, Al-Ahmady and Kostanelos, in their work on the behavior of PEGylated liposomes in CD-1 mice (Hadjidemetriou *et al.*, 2016). The authors observed that, after 10 min, alpha-2-macroglobulin, apolipoprotein C-III and hemoglobin subunit beta-1 were preponderantly in corona protein (in descending order) (Hadjidemetriou *et al.*, 2016). After 1 h, apolipoprotein E was the main protein, followed by alpha-2-macroglobulin and apoliprotein C-III (both presented at 10min) (Hadjidemetriou *et al.*, 2016). At 3 h, hemoglobin subunit beta-1 became the major component of corona protein, followed by apolipoprotein E and apoliprotein C-III (Hadjidemetriou *et al.*, 2016). Complement proteins and immunoglobulins were also identified, but in amounts considerably lower than other plasma proteins (Hadjidemetriou *et al.*, 2016). Total amount of proteins adsorbed per lipid, however, was similar after 10 min, 1 h and 3 h (Hadjidemetriou *et al.*, 2016). Therefore, even PEGylated liposomes are not totally inert once they allow corona formation and adsorption of complement cascade proteins (Hadjidemetriou *et al.*, 2016). The functions of these proteins on liposomes behavior, however, remain unclear.

In summary, different aspects regarding the biological fate of nanocarriers have not been fully elucidated: whether the diameter (Z-average) of liposomes determine the complement activation and the release of hydrophilic content; whether the complement cascade can explain the elimination of liposomes; whether the ability to activate complement cascade of nanocarriers can be predicted by *in vitro* assays; and whether the animal model

may reflect the biological fate of liposomes in humans. Understanding the biological fate of nanoparticles may change the course of nanomedicine development for intravenous administration, such as in cancer therapy. Here, *in vitro* and *in vivo* experiments were performed in order to understand the biological fate of liposomes. 400 and 100 nm non-PEGylated liposomes, and RBC and 100 nm liposomes prepared from RBC, were used to study the impact of particle size on complement cascade activation and release of hydrophilic content.

EXPERIMENTAL SECTION

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3 CONCLUSIONS AND PERSPECTIVES

Complement cascade promoted the release of hydrophilic content from large vesicles in rat serum. These large vesicles could be used when rapid drug release is desired. However, the release from small liposomes (with size around 100 nm, PEGylated or non-PEGylated) is independent of complement cascade. Other endogeneous proteins, including the corona proteins, may promote the release of drugs *in vivo* from these small vesicles. It is also possible that the release *in vivo* rely on physico-chemical effects, such as osmotic stress or membrane desestablization.

Complement cascade is not enough to explain the clearance of PEGylated and non-PEGylated liposomes in mice and rats. The slow clearance of PEGylated liposomes is not dependent on complement proteins, either in mice and in rats. Conversely, complement proteins participated of the elimination of non-PEGylated liposomes, especially at low doses. The effect of complement cascade on clearance of non-PEGylated liposomes were more evident in rats than mice. The rat animal model may be useful for comparison between nanosystems in light of its ability to activate complement cascade. This would help the selection of the most promising nanosystems for human applications.

The complement activation by PEGylated and non-PEGylated liposomes depended on serum source: human or rodents. Rat complement proteins may be more reactive than human complement proteins, as they promoted faster release of hydrophilic content. On the other hand, mice complement proteins seem less reactive than human complement proteins. *In vitro* assays using rodent's serum cannot fully predict the behavior of liposomes *in vivo*. Nevertheless, it is an important tool to compare formulations.

Experiments with rats *in vivo* or *in vitro* may not mimic the effect in humans. It is questionable how far *in vitro* and *in vivo* experiments can predict the biological behavior of nanoparticles in humans, particularly given the complexicity of the biological responses to each kind of nanomedicine. *In vitro* and *in vivo* experiments in animals are not dispensable according to our knowledge by now, however the choice of animal model should be well thought and limitations cannot be disregarded.

4 BILIOGRAPHY

ABBAS, A. K.; LICHTMAN, A. H.; PILLAI, S. Mecanismos efetores da imunidade humoral, Imunologia Celular e molecular Chapter 12, 7th edition, 2011.

ABE, K. et al. Effects of the PEG molecular weight of a PEG-lipid and cholesterol on PEG chain flexibility on liposome surfaces. **Colloids and Surfaces a-Physicochemical and Engineering Aspects,** v. 474, p. 63-70, Jun 2015.

ABU LILA, A. S.; KIWADA, H.; ISHIDA, T. The accelerated blood clearance (ABC) phenomenon: Clinical challenge and approaches to manage. **Journal of Controlled Release,** v. 172, n. 1, p. 38-47, Nov 2013.

ADAIR, B. D.; YEAGER, M. Electron microscopy of integrins. **Integrins,** v. 426, p. 337-+, 2007.

AKBARZADEH, A. et al. Liposome: classification, preparation, and applications. **Nanoscale Research Letters,** v. 8, Feb 2013.

ALBERTS, D. S. G., D. J. Safety Aspects of PEGylated Liposomal Doxorubicin in Patients with Cancer **Drugs**. 54**:** 30-35 p. 1997.

ALLEN, C. et al. Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol). **Bioscience Reports,** v. 22, n. 2, p. 225-250, Apr 2002.

ALLEN, T. M.; HANSEN, C. Pharmacokinetics of stealth versus conventional liposomes - effect of dose. **Biochimica Et Biophysica Acta,** v. 1068, n. 2, p. 133-141, Sep 1991.

ALLEN, T. M. et al. Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives invivo. **Biochimica Et Biophysica Acta,** v. 1066, n. 1, p. 29-36, Jul 1991.

ANSELMO, A. C.; MITRAGOTRI, S. An overview of clinical and commercial impact of drug delivery systems. **Journal of Controlled Release,** v. 190, p. 15-28, Sep 2014.

BARTLETT, G. R. Phosphorus assay in column chromatography. **Journal of Biological Chemistry,** v. 234, n. 3, p. 466-468, 1959.

BATZRI, S.; KORN, E. D. Single bilayer liposomes prepared without sonication. **Biochimica Et Biophysica Acta,** v. 298, n. 4, p. 1015-1019, 1973.

BERTRAND, N. et al. Transmembrane pH-Gradient Liposomes To Treat Cardiovascular Drug Intoxication. **Acs Nano,** v. 4, n. 12, p. 7552-7558, Dec 2010.

BERTRAND, N. et al... Pharmacokinetics and biodistribution of N-isopropylacrylamide copolymers for the design of pH-sensitive liposomes. **Biomaterials,** v. 30, n. 13, p. 2598-2605, May 2009.

BERTRAND, N. et al. Mechanistic understanding of in vivo protein corona formation on polymeric nanoparticles and impact on pharmacokinetics. **Nature Communications,** v. 8, p. 8, Oct 2017.

BERTRAND, N.; LEROUX, J. C. The journey of a drug-carrier in the body: An anatomo-physiological perspective. **Journal of Controlled Release,** v. 161, n. 2, p. 152-163, Jul 2012.

BERTRAND, N.; SIMARD, P.; LEROUX, J.-C. Serum-stable, long-circulating, pH-sensitive PEGylated liposomes ,. In: D'SOUZA, G. G. (Ed.). **Liposomes: methods and protocols**. Clifton, NJ: Human Press, 2017. p.193-207.

BERTRAND, N. et al. Cancer nanotechnology: The impact of passive and active targeting in the era of modern cancer biology. **Advanced Drug Delivery Reviews,** v. 66, p. 2-25, Feb 2014.

BRULET, P.; MCCONNELL, H. M. Lateral hapten mobility and immunochemistry of model membranes. **Proceedings of the National Academy of Sciences of the United States of America,** v. 73, n. 9, p. 2977-2981, 1976.

CARACCIOLO, G. Liposome-protein corona in a physiological environment: Challenges and opportunities for targeted delivery of nanomedicines. **Nanomedicine-Nanotechnology Biology and Medicine,** v. 11, n. 3, p. 543-557, Apr 2015.

CARACCIOLO, G. et al. Stealth Effect of Biomolecular Corona on Nanoparticle Uptake by Immune Cells. **Langmuir,** v. 31, n. 39, p. 10764-10773, Oct 2015.

CARACCIOLO, G. et al. The liposome-protein corona in mice and humans and its implications for in vivo delivery. **Journal of Materials Chemistry B,** v. 2, n. 42, p. 7419-7428, 2014.

CARON, W. P. et al. Interpatient Pharmacokinetic and Pharmacodynamic Variability of Carrier-Mediated Anticancer Agents. **Clinical Pharmacology & Therapeutics,** v. 91, n. 5, p. 802-812, May 2012.

CEDERVALL, T. et al. Understanding the nanoparticle-protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. **Proceedings of the National Academy of Sciences of the United States of America,** v. 104, n. 7, p. 2050-2055, Feb 2007.

CHANAN-KHAN, A. et al. Complement activation following first exposure to PEGylated liposomal doxorubicin (Doxil): possible role in hypersensitivity reactions. **Annals of Oncology,** v. 14, n. 9, p. 1430-1437, Sep 2003.

CORBO, C. et al. Effects of the protein corona on liposome-liposome and liposome-cell interactions. **International Journal of Nanomedicine,** v. 11, p. 3049-3063, 2016.

DAMS, E. T. M. et al. Accelerated blood clearance and altered biodistribution of repeated injections of sterically stabilized liposomes. **Journal of Pharmacology and Experimental Therapeutics,** v. 292, n. 3, p. 1071-1079, Mar 2000.

DEMEL, R. A. et al. Effects of temperature and cholesterol on glucose permeability of liposomes prepared with natural and synthetic lecithins. **Biochimica Et Biophysica Acta,** v. 150, n. 4, p. 655-&, 1968.

DEMEL, R. A.; VANDEENE.LL; VANKESSE.WS. Properties of polyunsaturated lecithins in monolayers and liposomes and interactions of these lecithins with cholesterol. **Biochimica Et Biophysica Acta,** v. 266, n. 1, p. 26-+, 1972.

DEVINE, D. V. et al. Liposome-complement interactions in rat serum - implications for liposome survival studies. **Biochimica Et Biophysica Acta-Biomembranes,** v. 1191, n. 1, p. 43-51, Apr 1994.

DOS SANTOS, N. et al. Influence of poly(ethylene glycol) grafting density and polymer length on liposomes: Relating plasma circulation lifetimes to protein binding. **Biochimica Et Biophysica Acta-Biomembranes,** v. 1768, n. 6, p. 1367-1377, Jun 2007.

ERNSTING, M. J. et al. Factors controlling the pharmacokinetics, biodistribution and intratumoral penetration of nanoparticles. **Journal of Controlled Release,** v. 172, n. 3, p. 782-794, Dec 2013.

European Medicines Agency (EMA). Reflection paper on the data requirements for intravenous liposomal products developed with reference to an innovator liposomal product, 2013. Available in: < http://www.ema.europa.eu/docs/en_GB/ document_library/Scientific_guideline/2013/03/WC500140351.pdf >.

FONSECA, N. A. et al. Bridging cancer biology and the patients' needs with nanotechnology-based approaches. **Cancer Treatment Reviews,** v. 40, n. 5, p. 626-635, Jun 2014.

FOOD AND DRUG ADMNISTRATION (FDA). Drugs approvals and database: doxorubicin. Available in: < https://www.fda.gov/default.htm >.

GABIZON, A. et al. An open-label study to evaluate dose and cycle dependence of the pharmacokinetics of PEGylated liposomal doxorubicin. **Cancer Chemotherapy and Pharmacology,** v. 61, n. 4, p. 695-702, Apr 2008.

GABIZON, A. A. PEGylated liposomal doxorubicin: Metamorphosis of an old drug into a new form of chemotherapy. **Cancer Investigation,** v. 19, n. 4, p. 424-436, 2001.

GABIZON, A. A.; BARENHOLZ, Y.; BIALER, M. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid - pharmacokinetic studies in rodents and dogs. **Pharmaceutical Research,** v. 10, n. 5, p. 703-708, May 1993.

GABIZON, A. A. et al. **Preclinical Studies with doxorubicin encapsulated in polyethyleneglycol-coated liposomes**: Journal of liposome research. 3**:** 517-528 p. 1993.

GARBUZENKO, O.; BARENHOLZ, Y.; PRIEV, A. Effect of grafted PEG on liposome size and on compressibility and packing of lipid bilayer. **Chemistry and Physics of Lipids,** v. 135, n. 2, p. 117-129, Jun 2005.

GOINS, B.; PHILLIPS, W. T.; KLIPPER, R. Repeat injection studies of technetium-99m-labeled PEG-liposomes in the same animal. **Journal of Liposome Research,** v. 8, n. 2, p. 265-281, 1998.

GRENIER, P. et al. Anti-polyethylene glycol antibodies alter the protein corona deposited on nanoparticles and the physiological pathways regulating their fate in vivo. **Journal of Controlled Release,** v. 287, p. 121-131, Oct 2018.

HADJIDEMETRIOU, M.; AL-AHMADY, Z.; KOSTARELOS, K. Time-evolution of in vivo protein corona onto blood-circulating PEGylated liposomal doxorubicin (DOXIL) nanoparticles. **Nanoscale,** v. 8, n. 13, p. 6948-6957, 2016.

HADJIDEMETRIOU, M. et al. In Vivo Biomolecule Corona around Blood-Circulating, Clinically Used and Antibody-Targeted Lipid Bilayer Nanoscale Vesicles. **Acs Nano,** v. 9, n. 8, p. 8142-8156, Aug 2015.

HAJISHENGALLIS, G. et al. Novel mechanisms and functions of complement. **Nature Immunology,** v. 18, n. 12, p. 1288-1298, Dec 2017.

HAMAD, I. et al. Distinct Polymer Architecture Mediates Switching of Complement Activation Pathways at the Nanosphere-Serum Interface: Implications for Stealth Nanoparticle Engineering. **ACS Nano,** v. 4, n. 11, p. 6629-6638, Nov 2010.

HARASHIMA, H. et al. Synergistic effect between size and cholesterol content in the enhanced hepatic uptake clearance of liposomes through complement activation in rats. **Pharmaceutical Research,** v. 13, n. 11, p. 1704-1709, Nov 1996.

HOPE, M. J. et al. Generation of multilamellar and unilamellar phospholipid-vesicles. **Chemistry and Physics of Lipids,** v. 40, n. 2-4, p. 89-107, Jun-Jul 1986.

HOPE, M. J. et al. Production of large unilamellar vesicles by a rapid extrusion procedure - characterization of size distribution, trapped volume and ability to maintain a membrane-potential. **Biochimica Et Biophysica Acta,** v. 812, n. 1, p. 55-65, 1985.

HOULE, J. J.; HOFFMANN, E. M. Evidence for restriction of the ability of complement to lyse homologous erythrocytes. **Journal of Immunology,** v. 133, n. 3, p. 1444-1452, 1984.

HRISTOVA, K.; KENWORTHY, A.; MCINTOSH, T. J. Effect of bilayer composition on the phase-behavior of liposomal suspensions containing poly(ethylene glycol)-lipids. **Macromolecules,** v. 28, n. 23, p. 7693-7699, Nov 1995.

IM, H. J. et al. Accelerated Blood Clearance Phenomenon Reduces the Passive Targeting of PEGylated Nanoparticles in Peripheral Arterial Disease. **Acs Applied Materials & Interfaces,** v. 8, n. 28, p. 17955-17963, Jul 2016.

IMMORDINO, M. L.; DOSIO, F.; CATTEL, L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. **International Journal of Nanomedicine,** v. 1, n. 3, p. 297-315, 2006.

ISHIDA, T. et al. Accelerated blood clearance of PEGylated liposomes following preceding liposome injection: Effects of lipid dose and PEG surface-density and chain length of the first-dose liposomes. **Journal of Controlled Release,** v. 105, n. 3, p. 305-317, Jul 2005.

ISHIDA, T.; HARASHIMA, H.; KIWADA, H. Liposome clearance. **Bioscience Reports,** v. 22, n. 2, p. 197-224, Apr 2002.

ISHIDA, T. et al. Biodistribution of liposomes and C3 fragments associated with liposomes: evaluation of their relationship. **International Journal of Pharmaceutics,** v. 205, n. 1-2, p. 183-193, Sep 2000.

ISHIDA, T. et al. Accelerated clearance of PEGylated liposomes in rats after repeated injections. **Journal of Controlled Release,** v. 88, n. 1, p. 35-42, Feb 2003.

ISHIDA, T. et al. Effect of cholesterol content in activation of the classical versus the alternative pathway of rat complement system induced by hydrogenated egg phosphatidylcholine-based liposomes. **International Journal of Pharmaceutics,** v. 224, n. 1-2, p. 69-79, Aug 2001.

ISHIHARA, T. et al. Evasion of the Accelerated Blood Clearance Phenomenon by Coating of Nanoparticles with Various Hydrophilic Polymers. **Biomacromolecules,** v. 11, n. 10, p. 2700-2706, Oct 2010.

JAAFAR-MAALEJ, C. et al. Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. **Journal of Liposome Research,** v. 20, n. 3, p. 228-243, Sep 2010.

JONES, S. W. et al. Nanoparticle clearance is governed by Th1/Th2 immunity and strain background. **Journal of Clinical Investigation,** v. 123, n. 7, p. 3061-3073, Jul 2013.

JOYSEY, V. C. A study of the blood groups of the rabbit, with reference to the inheritance of 3 antigens, and the agglutinability of the red cells carrying them. **Journal of Experimental Biology,** v. 32, n. 2, p. 440-450, 1955.

KIM, B. Y. S.; RUTKA, J. T.; CHAN, W. C. W. Current Concepts: Nanomedicine. **New England Journal of Medicine,** v. 363, n. 25, p. 2434-2443, Dec 2010.

KIRBY, C.; GREGORIADIS, G. Plasma-induced release of solutes from small unilamellar liposomes is associated with pore formation in the bilayers. **Biochemical Journal,** v. 199, n. 1, p. 251-254, 1981.

KLAUDA, J. B. et al. Simulation-based methods for interpreting X-ray data from lipid bilayers. **Biophysical Journal,** v. 90, n. 8, p. 2796-2807, Apr 2006.

KLOPFENSTEIN, W. E. et al. Differential scanning calorimetry on mixtures of lecithin, lysolecithin and cholesterol. **Chemistry and Physics of Lipids,** v. 13, n. 3, p. 215-222, 1974.

LA-BECK, N. M.; GABIZON, A. A. Nanoparticle interactions with the immune system: clinical implications for Liposome-Based cancer chemotherapy. **Frontiers in Immunology,** v. 8, Apr 2017.

LAGINHA, K. M. et al. Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors. **Clinical Cancer Research,** v. 11, n. 19, p. 6944-6949, Oct 2005.

LAVERMAN, P. et al. In vivo applications of PEG liposomes: Unexpected observations. **Critical Reviews in Therapeutic Drug Carrier Systems,** v. 18, n. 6, p. 551-566, 2001.

LAVERMAN, P. et al. Factors affecting the accelerated blood clearance of polyethylene glycol-liposomes upon repeated injection. **Journal of Pharmacology and Experimental Therapeutics,** v. 298, n. 2, p. 607-612, Aug 2001.

LEONARD, A.; DUFOURC, E. J. Interactions of cholesterol with the membrane lipid matrix - a solid-state nmr approach. **Biochimie,** v. 73, n. 10, p. 1295-1302, Oct 1991.

MA, P. et al. Development of Idarubicin and Doxorubicin Solid Lipid Nanoparticles to Overcome Pgp-Mediated Multiple Drug Resistance in Leukemia. **Journal of Biomedical Nanotechnology,** v. 5, n. 2, p. 151-161, Apr 2009.

MAURER, N.; FENSKE, D. B.; CULLIS, P. R. Developments in liposomal drug delivery systems. **Expert Opinion on Biological Therapy,** v. 1, n. 6, p. 923-947, Nov 2001.

MAYER, L. D. et al. Techniques for encapsulating bioactive agents into liposomes. **Chemistry and Physics of Lipids,** v. 40, n. 2-4, p. 333-345, Jun-Jul 1986.

MAYER, L. D.; HOPE, M. J.; CULLIS, P. R. Vesicles of variable sizes produced by a rapid extrusion procedure. **Biochimica Et Biophysica Acta,** v. 858, n. 1, p. 161-168, Jun 1986.

MCLEAN, R. H.; WELCH, T. R. **Complement**. Boca Raton, FL: CRC Press, 1997.

MILANI, S. et al. Reversible versus Irreversible Binding of Transferrin to Polystyrene Nanoparticles: Soft and Hard Corona. **ACS Nano,** v. 6, n. 3, p. 2532-2541, Mar 2012.

MOGHIMI, S. M. et al. Material properties in complement activation. **Advanced Drug Delivery Reviews,** v. 63, n. 12, p. 1000-1007, Sep 2011.

MOGHIMI, S. M. et al. Methylation of the phosphate oxygen moiety of phospholipid-methoxy(polyethylene glycol) conjugate prevents PEGylated liposome-mediated complement activation and anaphylatoxin production. **Faseb Journal,** v. 20, n. 14, p. 2591-+, Dec 2006.

MOGHIMI, S. M.; SIMBERG, D. Complement activation turnover on surfaces of nanoparticles. **Nano Today,** v. 15, p. 8-10, Aug 2017.

MOLLNES, T. E. et al. Essential role of the C5a receptor in E-coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. **Blood,** v. 100, n. 5, p. 1869-1877, Sep 2002.

MONOPOLI, M. P. et al. Physical-Chemical Aspects of Protein Corona: Relevance to in Vitro and in Vivo Biological Impacts of Nanoparticles. **Journal of the American Chemical Society,** v. 133, n. 8, p. 2525-2534, Mar 2011.

NAMDEE, K. et al. Effect of Variation in hemorheology between human and animal blood on the binding efficacy of vascular-targeted carriers. **Scientific Reports,** v. 5, Jun 2015.

OUSSOREN, C.; STORM, G. Effect of repeated intravenous administration on the circulation kinetics of poly(ethyleneglycol)-liposomes in rats. **Journal of Liposome Research,** v. 9, n. 3, p. 349-355, 1999.

PALCHETTI, S. et al. The protein corona of circulating PEGylated liposomes. **Biochimica Et Biophysica Acta-Biomembranes,** v. 1858, n. 2, p. 189-196, Feb 2016.

PEDERSEN, M. B. et al. Curvature of Synthetic and Natural Surfaces Is an Important Target Feature in Classical Pathway Complement Activation. **Journal of Immunology,** v. 184, n. 4, p. 1931-1945, Feb 2010.

PEER, D. et al. Nanocarriers as an emerging platform for cancer therapy. **Nature Nanotechnology,** v. 2, n. 12, p. 751-760, Dec 2007.

PERRY, J. L. et al. PEGylated PRINT Nanoparticles: The Impact of PEG Density on Protein Binding, Macrophage Association, Biodistribution, and Pharmacokinetics. **Nano Letters,** v. 12, n. 10, p. 5304-5310, Oct 2012.

PETERSEN, G. H. et al. Meta-analysis of clinical and preclinical studies comparing the anticancer efficacy of liposomal versus conventional non-liposomal doxorubicin. **Journal of Controlled Release,** v. 232, p. 255-264, Jun 2016.

PLATTS-MILLS, T. A. E.; ISHIZAKA, K. **Activation of the Alternate Pathway of Human Complement by Rabbit Cells**: The Journal of Immunology 113**:** 348 - 358 p. 1974.

RATELADE, J.; VERKMAN, A. Inhibitor(s) of the classical complement pathway in mouse serum limit the utility of mice as experimental models of neuromyelitis optica. **Molecular Immunology,** v. 62, n. 1, p. 104-113, Nov 2014.

RICKLIN, D. et al. Complement: a key system for immune surveillance and homeostasis. **Nature Immunology,** v. 11, n. 9, p. 785-797, Sep 2010.

SEMPLE, S. C.; CHONN, A.; CULLIS, P. R. Influence of cholesterol on the association of plasma proteins with liposomes. **Biochemistry,** v. 35, n. 8, p. 2521-2525, Feb 1996.

SEMPLE, S. C.; CHONN, A.; CULLIS, P. R. Interactions of liposomes and lipid-based carrier systems with blood proteins: Relation to clearance behaviour in vivo. **Advanced Drug Delivery Reviews,** v. 32, n. 1-2, p. 3-17, Jun 1998.

SHAKER, S.; GARDOUH, A. R.; GHORAB, M. M. Factors affecting liposomes particle size prepared by ethanol injection method. **Research in Pharmaceutical Sciences,** v. 12, n. 5, p. 346-352, Oct 2017.

SHIMIZU, T. et al. Anti-PEG IgM and complement system are required for the association of second doses of PEGylated liposomes with splenic marginal zone B cells. **Immunobiology,** v. 220, n. 10, p. 1151-1160, Oct 2015.

SOU, K.; TSUCHIDA, E. Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: Effect of the structure of acidic groups. **Biochimica Et Biophysica Acta-Biomembranes,** v. 1778, n. 4, p. 1035-1041, Apr 2008.

SZEBENI, J. Complement activation-related pseudoallergy: A new class of drug-induced acute immune toxicity. **Toxicology,** v. 216, n. 2-3, p. 106-121, Dec 2005.

SZEBENI, J.. Hemocompatibility testing for nanomedicines and biologicals:predictive assays for complement mediated infusion reactions **European Journal of Nanomedicine**. 4**:** 33-53 p. 2012.

SZEBENI, J.. Mechanism of nanoparticle-induced hypersensitivity in pigs: complement or not complement? **Drug Discovery Today,** v. 23, n. 3, p. 487-492, Mar 2018.

SZEBENI, J. et al. The Interaction of Liposomes with the Complement System: In Vitro and In Vivo Assays. **Liposomes, Pt C,** v. 373, p. 136-154, 2003.

SZEBENI, J.. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs - A model for pseudoallergic cardiopulmonary reactions to liposomes: Role of complement and inhibition by soluble CR1 and anti-C5a antibody. **Circulation,** v. 99, n. 17, p. 2302-2309, May 1999.

SZEBENI, J.; STORM, G. Complement activation as a bioequivalence issue relevant to the development of generic liposomes and other nanoparticulate drugs. **Biochemical and Biophysical Research Communications,** v. 468, n. 3, p. 490-497, Dec 2015.

SZOKA, F.; PAPAHADJOPOULOS, D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. **Proceedings of the National Academy of Sciences of the United States of America,** v. 75, n. 9, p. 4194-4198, 1978.

TENZER, S. et al. Nanoparticle Size Is a Critical Physicochemical Determinant of the Human Blood Plasma Corona: A Comprehensive Quantitative Proteomic Analysis. **ACS Nano,** v. 5, n. 9, p. 7155-7167, Sep 2011.

THIELENS, N. M. et al. Impact of the surface charge of polydiacetylene micelles on their interaction with human innate immune protein C1q and the complement system. **International Journal of Pharmaceutics,** v. 536, n. 1, p. 434-439, Jan 2018.

TILL, G. O. et al. Activation of c5 by cobra venom factor is required in neutrophil-mediated lung injury in the rat. **American Journal of Pathology,** v. 129, n. 1, p. 44-53, Oct 1987.

TRAUBLE, H.; SACKMANN, E. Studies of crystalline-liquid crystalline phase-transition of lipid model membranes .3. Structure of a steroid-lecithin system below and above lipid-phase transition. **Journal of the American Chemical Society,** v. 94, n. 13, p. 4499-&, 1972.

VANDIJCK, P. W. M. et al. Preference of cholesterol for phosphatidylcholine in mixed phosphatidylcholine-phosphatidylethanolamine bilayers. **Biochimica Et Biophysica Acta,** v. 455, n. 2, p. 576-587, 1976.

VOGEL, C. W.; FRITZINGER, D. C. Cobra venom factor: Structure, function, and humanization for therapeutic complement depletion. **Toxicon,** v. 56, n. 7, p. 1198-1222, Dec 2010.

VOGEL, C. W. et al. Recombinant cobra venom factor. **Molecular Immunology,** v. 41, n. 2-3, p. 191-199, Jun 2004.

VU, V. P. et al. Immunoglobulin deposition on biomolecule corona determines complement opsonization efficiency of preclinical and clinical nanoparticles. **Nature Nanotechnology,** v. 14, n. 3, p. 260-+, Mar 2019.
UNITED STATES NATIONAL NANOTECHNOLOGY INITIATIVE. What is nanotechnology? 2018. Available in: < http://www.nano.gov/nanotech-101/what/definition >.

WALLER, D. G. Allergy, pseudo-allergy and non-allergy. **British Journal of Clinical Pharmacology,** v. 71, n. 5, p. 637-638, May 2011.

WANG, C. L. et al. Accelerated blood clearance phenomenon upon cross-administration of PEGylated nanocarriers in beagle dogs. **International Journal of Nanomedicine,** v. 10, p. 3533-3545, 2015.

WANG, X. Y.; ISHIDA, T.; KIWADA, H. Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. **Journal of Controlled Release,** v. 119, n. 2, p. 236-244, Jun 2007.

WASSEF, N. M. et al. Anaphylactoid reactions mediated by autoantibodies to cholesterol in miniature pigs. **Journal of Immunology,** v. 143, n. 9, p. 2990-2995, Nov 1989.

WIBROE , P. P.; MOGHIMI, S. M. Complement Sensing of Nanoparticles and Nanomedicines. Washington, DC: ACS Symposium Series; **American Chemical Society**, 2012.

YANG, Q. et al. Analysis of Pre-existing IgG and IgM Antibodies against Polyethylene Glycol (PEG) in the General Population. **Analytical Chemistry,** v. 88, n. 23, p. 11804-11812, Dec 2016.

ZHANG, B. et al. Drug-Induced Pseudoallergy: A Review of the Causes and Mechanisms. **Pharmacology,** v. 101, n. 1-2, p. 104-110, 2018.

ANNEX 1 – Paper "*Anti-polyethylene glycol antibodies alter the protein corona deposited on nanoparticles and the physiological pathways regulationg their fate in vivo*" (and supplementary information) published by Phillippe Grenier; Iara Maíra de Oliveira Viana; Eliana Martins Lima; and Nicolas Bertrand, in Journal of Controlled Release in October 2018.

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Anti-polyethylene glycol antibodies alter the protein corona deposited on nanoparticles and the physiological pathways regulating their fate *in vivo*

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ABSTRACT

Multiple studies highlight the strong prevalence of anti-poly(ethylene glycol) (anti-PEG) antibodies in the general human population. As we develop therapeutic modalities using this polymer, it is increasingly relevant to assess the importance of anti-PEG antibodies on biological performances. Here, we show that the anti-PEG Immunoglobulin M (IgM) raised in mice following the injection of polymeric nanoparticles could have significant neutralizing effects on subsequent doses of PEGylated nanosystems *in vivo*. The circulation times of PEGylated nanoparticles and liposomes were strongly reduced in animals with circulating anti-PEG IgMs, irrespective of the PEG density or the surface properties of the system. In comparison, despite that anti-PEG IgMs could bind free methoxy-terminated PEG and PEGylated bovine serum albumin, the circulation kinetics of these systems remained unaltered in the presence of antibodies. The binding of IgMs to the PEGylated surface of nanoparticles alters the nature of the proteins adsorbed in the surrounding corona, notably due to the activation of the complement cascade. These changes are responsible for the observed differences in circulation times. In comparison, the PEG-BSA is unable to activate complement, even in the presence of anti-PEG IgMs. These results inform on how anti-PEG antibodies can affect the fate of PEGylated nanomaterials and highlight how the architecture of nanoparticles impacts the deposition of the protein corona.

ANNEX 2 – Approval letter from Human Ethics Committee of UFG.

UNIVERSIDADE FEDERAL DE GOIÁS - UFG

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: NANOPARTÍCULAS DE LONGA CIRCULAÇÃO: AVALIAÇÃO DA INTERAÇÃO DO SISTEMA COMPLEMENTO COM DIFERENTES NANOPARTÍCULAS

Pesquisador: IARA MAÍRA DE OLIVEIRA VIANA

Área Temática:

Versão: 2

CAAE: 49593915.0.0000.5083

Instituição Proponente: Faculdade de Farmacia

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.355.968

Apresentação do Projeto:

O estudo pretende estabelecer as caracteristicas das nanoparticulas que permitem sua longa circulação no corpo humano, vislumbrando assim, ampliar o conhecimento sobre o comportamento do sistema imune e ativação do sistema complemento, na presença de diferentes formulações nanoestruturadas. Para isso, amostras de soro humano obtido por coleta sanguínea de 30 (trinta) voluntários com idade entre 18 e 50 anos, de ambos os sexos e em bom estado de saúde serão utilizadas.

Objetivo da Pesquisa:

O Objetivo Geral proposto constitui estudar o impacto de diferentes formulações nanoestruturadas na ativação do sistema complemento. Em especifico: - Obter diferentes formulações nanoestruturadas, tais como nanoesferas e lipossomas; - Caracterizar as formulações quanto a distribuição das particulas obtidas, tamanho médio das particulas e potencial zeta; - Avaliar o impacto do uso de diferentes formulações nanoestruturadas (com diferentes composições e métodos de obtenção) na ativação do sistema complemento por testes in vitro; - Obter uma formulação de nanoparticulas de longa circulação contendo doxorrubicina, caracterizá-la e avaliá-la quanto a sua capacidade de ativação do sistema complemento.

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Bairro: Campus Samambaia **CEP:** 74.001-970
UF: GO **Município:** GOIANIA
Telefone: (62)3521-1215 **Fax:** (62)3521-1163 **E-mail:** cep.prpi.ufg@gmail.com

Avaliação dos Riscos e Benefícios:

Como riscos, foi descrito pelo pesquisador ser baixo e de pequena gravidade, considerando que a colheita de sangue venoso (8 mL) poderá provocar leve sensação de dor local e formação de um pequeno hematoma. Para minimizar esse risco, garante-se que o procedimento será realizado por um profissional habilitado e empregando material apropriado. Ainda, na ocorrência de casos de lipotimia durante o procedimento de colheita de sangue, medidas apropriadas serão adotadas pelo profissional devidamente treinado. Os benefícios caracterizam-se como indiretos e estão descritos.

Comentários e Considerações sobre a Pesquisa:

Constitui uma investigação de relevância, apresentando literatura adequada e atualizada. A metodologia proposta está em conformidade com os objetivos propostos e os esclarecimentos solicitados, em especial referentes ao participante da pesquisa foram adequados. Consideramos assim, que as informações relativas ao participante, riscos e benefícios, instrumento de coleta de dados e garantia de sigilo estão contemplados.
O cronograma novo apresentado atende às solicitações do CEP e o orçamento proposto está adequado.
O TCLE apresentado encontra-se em linguagem clara e acessível, e as adequações solicitadas foram atendidas. Os direitos ao anonimato, de pleitear indenização e à desistência de participação foram garantidos. A documentação encontra-se completa.

Considerações sobre os Termos de apresentação obrigatória:

Foram anexados os seguintes documentos:
- Projeto de Pesquisa Completo
- Projeto Comitê Plataforma Brasil
- Folha de rosto assinada pelo pesquisador responsável e representante da instituição proponente.
- Termo de Anuência assinado também pela direção da instituição onde será desenvolvido o estudo.
- Termo de compromisso assinado pelo pesquisador responsável e outro membro da equipe.
- Currículo Lattes dos membros da equipe.
- Termo de Consentimento Livre e Esclarecido

Recomendações:

Conclusões ou Pendências e Lista de Inadequações:

Após avaliação, o pleno considera que todas as solicitações foram atendidas, estando o projeto aprovado.

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Considerações Finais a critério do CEP:

Informamos que o Comitê de Ética em Pesquisa/CEP-UFG considera o presente protocolo APROVADO, o mesmo foi considerado em acordo com os princípios éticos vigentes. Reiteramos a importância deste Parecer Consubstanciado, e lembramos que o(a) pesquisador(a) responsável deverá encaminhar ao CEP-UFG o Relatório Final baseado na conclusão do estudo e na incidência de publicações decorrentes deste, de acordo com o disposto na Resolução CNS nº. 466/12. O prazo para entrega do Relatório é de até 30 dias após o encerramento da pesquisa.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_597027.pdf	26/11/2015 08:27:35		Aceito
Outros	Questionario.pdf	26/11/2015 08:25:23	IARA MAÍRA DE OLIVEIRA VIANA	Aceito
Declaração de Instituição e Infraestrutura	TermoDeAnuenciaRomuloRocha.png	26/11/2015 08:23:21	IARA MAÍRA DE OLIVEIRA VIANA	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	26/11/2015 08:21:56	IARA MAÍRA DE OLIVEIRA VIANA	Aceito
Projeto Detalhado / Brochura Investigador	ProjetoDePesquisa.pdf	26/11/2015 08:21:27	IARA MAÍRA DE OLIVEIRA VIANA	Aceito
Folha de Rosto	FolhaDeRosto.pdf	24/09/2015 21:09:05	IARA MAÍRA DE OLIVEIRA VIANA	Aceito
Declaração de Instituição e Infraestrutura	TermoDeAnuencia.pdf	24/09/2015 13:21:42	IARA MAÍRA DE OLIVEIRA VIANA	Aceito
Declaração de Pesquisadores	TermoDeCompromisso.pdf	24/09/2015 13:17:34	IARA MAÍRA DE OLIVEIRA VIANA	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

GOIANIA, 08 de Dezembro de 2015

Assinado por:
João Batista de Souza
(Coordenador)

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ANNEX 3 – Questionnaire used as inclusion criteria.

QUESTIONÁRIO

Prezado(a) Sr.(a),

Antes de coletarmos sua amostra de sangue, solicitamos gentilmente que responda às perguntas abaixo, assinalando com um **X** em SIM para respostas afirmativas ou um **X** em NÃO para as respostas negativas. Esse questionário objetiva avaliar se o Sr.(a) pode colaborar com nossa pesquisa sem causar prejuízos a sua própria saúde. Suas respostas são sigilosas e não serão divulgadas em nenhum momento.

Qualquer dúvida, favor nos perguntar!

1. **Você está sentindo nesse momento alguma indisposição?**
   Exemplos: desconforto físico, dores de cabeça ou no corpo, tem tossido, com enjoo, resfriado ou gripado.
   ___SIM ___NÃO

2. **Está fazendo uso de algum medicamento (exceto anticoncepcional)?**
   ___SIM ___NÃO

3. **Você possui alguma doença crônico degenerativa?**
   Exemplos: Diabetes, arteriosclerose, hipertensão, doenças cardíacas, câncer, Mal de Alzheimer, reumatismo, esclerose múltipla, artrite deformante, artrose, glaucoma.
   ___SIM ___NÃO

4. **Você possui alguma doença infecciosa?**
   Exemplos: Infecção por HIV(AIDS), Hepatites A, B, C ou D, Tuberculose, Infecção por HPV, Dengue, Hanseníase, Infecção por Hbv, Infecção por Hcv, Malária, Toxoplasmose, Sífilis, Tétano.
   ___SIM ___NÃO

5. **Você possui alguma doença hematológica (no sangue)?**
   Exemplos: Agranulocitose, anemia, leucemias, baixa ferretina, doenças imunológicas, imunodeficiência primária.
   ___SIM ___NÃO

Agradecemos sua valiosa participação!

ANNEX 4 – Consent form (TCLE, *"Termo de Consentimento Livre e Esclarecido"*).

UNIVERSIDADE FEDERAL DE GOIÁS
FACULDADE DE FARMÁCIA
FARMATEC - LABORATÓRIO DE NANOTECNOLOGIA FARMACÊUTICA
E SISTEMAS DE LIBERAÇÃO DE FÁRMACOS

UFG

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO - TCLE

Prezado(a) Sr.(a),

Você está sendo convidado(a) a participar como voluntário(a), da pesquisa intitulada "Nanopartículas de longa circulação: avaliação da interação do sistema complemento com diferentes nanopartículas". Após receber os esclarecimentos e as informações a seguir, se você aceitar fazer parte do estudo, dê um visto em todas as páginas e assine ao final deste documento, que está impresso em duas vias, sendo que uma delas é sua e a outra pertence à pesquisadora responsável. Esclareço que em caso de recusa na participação você não será penalizado(a) de forma alguma. Se aceitar participar, as dúvidas *sobre a pesquisa* poderão ser esclarecidas pelo pesquisador responsável, via e-mail (iaramaira@gmail.com) e, inclusive, por telefone, através dos números (62)3209-6039 ou (62)8105-9359. Utilize o número de telefone (62)8105-9359 para efetuar chamadas a cobrar. Ao persistirem as dúvidas *sobre os seus direitos* como participante desta pesquisa, você também poderá fazer contato com o **Comitê de Ética em Pesquisa** da Universidade Federal de Goiás, pelo telefone (62)3521-1215.

1. Informações Importantes sobre a Pesquisa:

O objetivo principal da pesquisa que você está sendo convidado a participar é avaliar a resposta do sistema imune humano frente aos novos sistemas de liberação de fármacos. A melhor compreensão da resposta do sistema imune humano e sua interação com sistemas nanoestruturados poderá auxiliar no desenvolvimento de formulações mais eficazes e que são úteis no tratamento de diversas enfermidades como, por exemplo, o câncer.

Para realizar este estudo, gostaríamos de colher 8 mL do seu sangue. Na coleta de sangue pode ocorrer uma leve dor localizada associada à picada da agulha ou pequena reação local. Para reduzir esta reação local, a coleta de sangue será realizada por um profissional habilitado, empregando rotina padronizada. Serão utilizados agulhas e tubos descartáveis. Outro risco associado ao procedimento de coleta de sangue, embora raro, é a sensação momentânea de tontura. Nesse caso, o profissional adotará as medidas pertinentes.

Na divulgação dos resultados da pesquisa, os voluntários não serão identificados, garantindo sua privacidade. As amostras de sangue coletadas que não forem utilizadas nos ensaios desse projeto de pesquisa serão descartadas.

Você não terá nenhum gasto, pois a coleta do sangue será realizada no FarmaTec, de acordo com sua disponibilidade. Esclarecemos que não haverá remuneração e ressarcimento das despesas (como transporte e alimentação) decorrentes da cooperação com a pesquisa. Ademais, garantimos a liberdade de se recusar a participar ou retirar o seu consentimento, em qualquer fase da pesquisa, sem penalização alguma. Asseguramos, ainda, o direito de pleitear indenização (reparação a danos imediatos ou futuros) decorrentes de sua participação na pesquisa.

Rubricas do participante e do pesquisador: _____

1.2 Consentimento da Participação da Pessoa como Sujeito da Pesquisa:

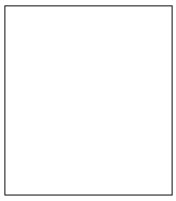
Eu,....., inscrito(a) sob o RG....., abaixo assinado, concordo em participar do estudo intitulado "Nanopartículas de longa circulação: avaliação da interação do sistema complemento com diferentes nanopartículas". Informo ter mais de 18 anos e menos de 50 anos de idade, e destaco que minha participação nesta pesquisa é de caráter voluntário. Fui, ainda, devidamente informado(a) e esclarecido(a), pela pesquisadora responsável Iara Maíra de Oliveira Viana, sobre a pesquisa, os procedimentos e métodos nela envolvidos, assim como os possíveis riscos e benefícios decorrentes de minha participação no estudo. Foi-me garantido que posso retirar meu consentimento a qualquer momento, sem que isto leve a qualquer penalidade. Além disso, foi-me assegurado o direito de pleitear indenização (reparação a danos imediatos ou futuros) decorrentes de minha participação na pesquisa. Declaro, portanto, que concordo com a minha participação no projeto de pesquisa acima descrito.

Goiânia, ... de ..... de .....

_____
Assinatura por extenso do(a) participante

_____
IARA MAÍRA DE OLIVEIRA VIANA
Pesquisadora principal

Testemunhas em caso de uso da assinatura datiloscópica



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_____

ANNEX 5 – Approval letter from Animal Ethics Committee of UFG.

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL DE GOIÁS
PRÓ-REITORIA DE PESQUISA E INOVAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS/CEUA

Goiânia, 09 de abril de 2018.

PARECER CONSUBSTANCIADO REFERENTE AO PROJETO DE PESQUISA DO PROTOCOLO N. 017/18

I - Finalidade do projeto de pesquisa: Doutorado

II - Identificação:

- **Data de apresentação a CEUA:** 06/03/2018
- **Título do projeto:** Pesquisa, desenvolvimento e inovação para a oferta de soluções tecnológicas ao setor farmacêutico. Nanopartículas de longa circulação: avaliação da interação do sistema complemento com diferentes nanopartículas.
- **Pesquisador Coordenador no SAP:** Eliana Martins Lima - Faculdade de Farmácia/UFG
- **Pesquisador Responsável/ Unidade:** Iara Maíra de Oliveira Viana – Faculdade de Farmácia/FarmaTec/UFG
- **Pesquisadores Participantes:** não há.
- **Médico Veterinário/CRMV:** Daniel Silva Goulart - CRMV/GO 4632
- **Unidade onde será realizado:** FarmaTec/Faculdade de Farmácia/UFG

III - Objetivos e justificativa do projeto: Estudar o comportamento dos sistemas nanoestruturados no meio biológico. Obter diferentes formulações nanoestruturadas PEGuiladas e não PEGuiladas. Estudar a ativação da cascata do sistema complemento pelos sistemas nanoestruturados após sua incubação in vitro com soro de roedores. Comparar os resultados obtidos em soro de roedores com soro humano. Roedores são utilizados em estudos farmacocinéticos na etapa pré-clínica como forma de avaliar novos produtos, inclusive produtos nanotecnológicos. Entretanto, esses estudos não têm sido suficientes para prever o comportamento das nanopartículas em humanos. Acredita-se que as proteínas do sistema complemento ao opsonizar a superfície das nanopartículas, as conduzam para sua eliminação. Assim a cobertura da superfície das nanoparticulas, com polímero PEG por exemplo, seria suficiente para evitar sua rápida eliminação. Entretanto, um trabalho recente de nosso grupo evidencia que o sistema complemento não é fator determinante para o clearance rápido de nanopartículas poliméricas. Com esse trabalho, objetivamos comparar o comportamento dos sistemas nanoestruturados quando incubados com soro de diferentes fontes, roedores e humana. A melhor compreensão do comportamento das nanopartículas no meio biológico auxiliará no planejamento racional de estudos in vitro e in vivo dos sistemas nanoestruturadas para administração intravenosa.

IV - Sumário do projeto:

- **Discussão sobre a possibilidade de métodos alternativos e necessidade do número de animais:** Não existem métodos alternativos que atendam ao objetivo da pesquisa de avaliar o comportamento biológico de sistemas nanoestruturadas em roedores. No entanto, a metodologia proposta emprega reduzido número de animais pois consiste em experimentos in vitro. Inicialmente o sangue de 2 ratos será coletado e empregado no desenvolvimento e validação dos ensaios in vitro. Em seguida, o sangue de 2 camundongos será coletado e testado com a metodologia pré-definida com o soro de rato. Após os testes iniciais, sangue de 4 camundongos serão coletados e os soros obtidos serão reunidos. Em paralelo será coletado o sangue de 1 rato. O soro dos roedores será incubado com sistemas

nanoestruturados PEGuilados e nãoPEGuilados (em triplicata). Os experimentos serão conduzidos empregando controles positivos e negativos. Em seguida, as amostras serão avaliadas por ensaios como Ensaio Hemolítico e ELISA. Os experimentos serão reconduzidos empregando outros sistemas nanoestruturadas após avaliação estatística do primeiro bloco de ensaios.

- **Prevê Projeto Piloto:** sim. No projeto piloto serão utilizados 2 ratos Wistar machos, com 250 gramas e idade entre 8 a 12 semanas; e 2 camundongos Balb/c machos, com 25 gramas e idade entre 4 a 8 semanas.
- **Espécie animal utilizada/ número total de animais/ Número de animais por tratamento ou grupo experimental:** No total serão utilizados 15 animais, sendo 12 camundongos Balb/c e 3 ratos Wistar.
- **Descrição do animal utilizado (Explicitar: espécie/ linhagem/ sexo (informar número por sexo)/ peso e/ou idade etc):** 12 camundongos Balb/c machos, com 25 gramas e idade entre 4 a 8 semanas; e 3 ratos Wistar machos, com 250 gramas e idade entre 8 a 12 semanas.
- **Fonte de obtenção do animal:** biotério central da UFG (Wistar) e biotério do IPTSP da UFG (Balb/c).
- **Descrição das instalações utilizadas e número de animais/área/qualidade do ambiente (ar, temperatura, umidade), alimentação/hidratação**: Os animais serão mantidos no biotério da Faculdade de Farmácia / UFG em ambiente controlado: temperatura 22±2°C, umidade 55±10% e ciclo claro/escuro de 12/12 horas. Os camundongos serão mantidos em caixas (5 por caixa) de polipropileno de dimensões (30 x 20 x 13) cm. Os ratos serão mantidos em caixas (3 por caixa) de polipropileno de dimensões (49 x 34 x 16) cm. Maravalha de madeira peneirada (para retirada do pó) e autoclavada, será utilizada como cama para os animais. As caixas para a acomodação de camundongos possuem rack com ventilação individual e serão higienizadas com hipoclorito de sódio 10%. As caixas para a acomodação de ratos serão mantidas em estantes ventiladas e serão higienizadas com hipoclorito de sódio 10%. Os animais receberão ração específica para a espécie e água ad libitum. Tubos cilíndricos de papel autoclavados serão empregados objetivando criar local de fuga para os animais. Além disso, o cuidado dos animais será realizado rotineiramente, em mesmo horário e pelo mesmo pesquisador/colaborador desta pesquisa.
- **Utilização de agente infeccioso/gravidade da infecção a ser observada e análise dos riscos aos pesquisadores/alunos:** Os riscos da pesquisa são inerentes ao uso de animais de laboratório com status sanitário convencional. Cuidados quanto à segurança dos manipuladores serão tomados utilizando equipamentos de proteção coletiva e individuais, tais como luvas de látex, máscara, jalecos, rack ventilada individualizada, sala específica para animais infectados, imunização dos pesquisadores envolvidos, e higienização correta antes, durante e depois do manuseio e experimento animal. As formulações são desenvolvidas em ambiente estéril e todos os materiais serão previamente autoclavados para evitar qualquer tipo de contaminação cruzada. A execução dos testes será realizada por equipe capacitada com experiência prática na ciência de animais de laboratório.
- **Procedimentos experimentais do projeto de pesquisa:** No mesmo dia dos ensaios in vitro, após contenção manual e higienização do local com álcool 70, os animais serão submetidos à punção cardíaca sob anestesia inalatória com isoflurano. A dose de indução de anestesia será de 5% e a de manutenção da anestesia será de 1 a 3%. Agulhas hipodérmicas 27G (em ratos) e 25G (em camundongos) serão empregadas na punção cardíaca. Logo após o procedimento de coleta de sangue (1mL/camundongo e 5 mL/rato), os animais serão eutanasiados empregando sobredose de isoflurano. A dose para eutanásia será de 5%. As carcaças dos animais serão armazenadas em freezer a -20 °C e serão encaminhados para incineração na Escola de Veterinária da Universidade Federal de Goiás.

- **Métodos utilizados para minimizar o sofrimento e aumentar o bem-estar dos animais antes, durante e após a pesquisa. Pontos Finais Humanitários:** Além do uso de anestésico geral durante as coletas, endpoints em nossos experimentos para minimizar e/ou evitar a dor e sofrimento dos animais serão empregados. Os animais serão avaliados diariamente em relação à aparência, peso, comportamento e alimentação. Caso hajam alterações, conforme as descritas abaixo, o animal será eutanasiado.

• O animal não é mais capaz de comer ou beber. • O animal apresenta sinais de dor. • O animal perdeu mais de 15% de peso corporal num relativamente curto espaço de tempo (1-2dias), ou a sua diminuição de peso em mais de 20% em relação ao seu peso no início do experimento. • O animal tem um sangramento significante. • O comportamento e locomoção do animal são totalmente anormais.

- **Grau de invasividade:** GI1
- **Material utilizado em outros projetos:** Não
- **Método de eutanásia:** punção cardíaca seguida de sobredose de isoflurano (indução com 5%, manutenção com 1-3%).
- **Destino do animal:** Os animais eutanasiados serão enviados à Faculdade de Veterinária da Universidade Federal de Goiás para a incineração.

V – Comentários do relator frente às orientações da CEUA:

- **Quanto aos documentos exigidos pela CEUA/UFG:** Todos os documentos foram apresentados adequadamente.
- **Quanto aos cuidados e manejo dos animais e riscos aos pesquisadores:** o manejo dos animais e os riscos aos pesquisadores foram adequadamente descritos.

VI - Parecer da CEUA:

De acordo com a documentação apresentada à CEUA, consideramos o projeto APROVADO, smj desta Comissão.

Informação aos pesquisadores:

Reiteramos a importância deste Parecer Consubstanciado, e lembramos que a pesquisadora responsável deverá encaminhar à CEUA-PRPI-UFG o Relatório Final baseado na conclusão do estudo e na incidência de publicações decorrentes deste, de acordo com o disposto na Lei nº. 11.794 de 08/10/2008, e Resolução Normativa nº. 01, de 09/07/2010 do Conselho Nacional de Controle de Experimentação Animal-CONCEA. O prazo para entrega do Relatório é de até 30 dias após o encerramento da pesquisa, a qual está prevista para finalizar suas ações até **28 de fevereiro de 2019**.

VII - Data da reunião: 09/04/2018.

Dra. Marina Pacheco Miguel
Coordenadora da CEUA/PRPI/UFG

Comissão de Ética no Uso de Animais/CEUA
Pró-Reitoria de Pesquisa e Inovação/PRPI-UFG, Caixa Postal: 131, Prédio da Reitoria, Piso 1, Campus Samambaia (Campus II) - CEP:74001-970, Goiânia – Goiás, Fone: (55-62) 3521-1876.
Email: ceua.ufg@gmail.com