Biocompatibility Evaluation of Engineered Amino Acid Pairing Peptides for Drug Delivery

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

To ensure the effective and safe use of nanomaterials for medical applications, the biocompatibility of the materials must be tested with particular relevance to the environment in which the material is placed. In nanoparticle-based drug delivery, it is crucial to evaluate a nanoparticle's biocompatibility to ensure minimal cytotoxicity. Of several types of nanoparticles, peptide-based nanoparticles have emerged as promising systems for targeted cancer therapy. Yet, the biocompatibility of many of these peptides and their assembled particles has not been studied. This thesis, summarizes the original contribution on the effective and safe use of the particular self/co-assembling, amino acid pairing peptides and some of their DEGylated forms (modified versions) as carriers for anticancer drug delivery application.

Therefore, the biocompatibility of the self-assembling, amino acid pairing (AAP) peptides AC8, its two DEGylated forms, as well as two related peptides, EAK16-II and EK8, is systematically investigated. The toxicity of these peptides and their complexes with pirarubicin was tested against the human adenocarcinoma lung cancer cell line, A549.The biocompatibility of the peptide-drug co-assembling complexes is assessed and the potential of these five peptides as carriers for the hydrophobic anticancer drug pirarubicin is demonstrated. For the first time experimental results on cytotoxicity, haemolytic activity, red blood cell (RBC) aggregation, complement activation and anaphylotoxin activation as an end result of complement activity for these five AAP peptides is reported. AC8, the amino end DEGylated AC8 (NP-I) and EK might be strong candidates for hydrophobic drug delivery considering their lack of toxicity and the fact that they are not recognized as a foreign molecule, inducing no considerable immune reactions. These results provide a basis for *in vivo* experiments and predict minimal in *vitro* toxicity of these peptides based delivery systems.

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List of Abbreviations

A- Alanine

AAP- Amino acid pairing

AC8H- High AC8 dose

AC8L- Low AC8 dose

APTT- Activated partial thromboplastin time

BALB mice- Bagg Albino (inbred research mouse strain)

Bb- Alternative pathway

CCK8- Cell counting kit 8

C4d- Classical pathway

DAPI- 6-diamidino-2-phenylindole

DEG- Diethylyne glycol

DMEM- Dulbecco's modified eagle's medium

D-PBS- Dulbecco`s phosphate buffered saline

E- Glutamic acid

ELISA- Enzyme-linked immunosorbent assay

FBS- Fetal Bovine Serum

Ig- Immune globulin

K- Lysine

MAC- Membrane attack complex

MBL- Mannose-binding lectinMNP- Mononuclear phagocytic cells

MPS- Mononuclear phagocyte system

MTT- (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide)

NK cell- Natural killer cell

NP- Nanoparticle

NT- Non-treated

PEG- Poly(ethylene glycol)

Peptide-pira- Peptide-pirarubicin (complex)

Pirarubicin- Tetrahydropyranyladriamycin

Pira- Pirarubicin

PF4- Human platelet factor 4

PT- Prothrombin time

R- Arginine

RBC- Red blood cell

RES- Reticulo-endothelial system

SC5b-9 - Terminal pathway

SEM- Standard error of mean

Serum control- Negative control

TT- Thrombin Time

Chapter 1

Introduction

1.1 Overview

The rapid advancement of nanotechnology has allowed the possibility of using engineered nanoparticles that interact within biological environments for treatment of diseases. As a result, nanoparticles have the potential to revolutionize a wide range of medical diagnostic and therapeutic interventions such as diagnostic aging (1-3), photothermal therapy (4), nucleic acid delivery (5-7), plantable devices, and of particular interest in this thesis, drug delivery (8). The interaction of nanoparticles interacting with cells and the extracellular environment can trigger a sequence of biological effects. These effects largely depend on the dynamic physicochemical characteristics of nanoparticles, which determine the biocompatibility and efficacy of the intended outcomes.

To ensure an effective and safe use of nanomaterials for medical applications such as drug delivery, the interaction between a material and the biological system of interest must be studied and characterized. Therefore, studies of a material's biocompatibility must be conducted with particular focus on the environment in which the biomaterial will be placed (9).

As a result, in drug delivery, it is crucial to evaluate a nanoparticle's biocompatibility to ensure safe drug release and minimize cytotoxicity. Indeed, a thorough evaluation of the factors that affect the biocompatibility of nanoparticles is central and possibly a first key step for the development of a safe delivery system for drugs. It is not surprising then that

biocompatibility evaluation of engineered nanoparticles for drug delivery applications has been expanded from being primarily investigated in a laboratory setting to being applied in the multi-billion dollar pharmaceutical industry (10). It is now well known that the inherent physical and chemical properties of nanoparticles (size, shape, surface characteristics) as well as the environment it comes into contact with, can dictate a nanoparticle's degree of biocompatibility (11-13). For instance, the route of a material's delivery into the body such as intravenous or oral intake will induce differential immune reactions (11). The immune reaction cascade can be initiated with the adsorption of opsonins to the surface of nanoparticles. Opsonins are proteins – such as immunoglobulins or complement proteins – that bind to microbes and foreign substances and in doing so, aid their clearance via phagocytosis. Opsonin adsorption, enhanced by the hydrophobicity of a particle's surface, can mark nanoparticles as foreign substances and increase their uptake by the phagocytic cells (14, 15) This uptake in turn determines the route of particle internalization (16-18) and consequently dictates the fate of nanoparticles in the body (19). This process is one of the biological barriers to nanoparticle-based controlled drug delivery (20). All of these together, highlight the importance of surface effects for nanoparticles to be used as a carrier for drug delivery. A number of studies have reported that the response of biological systems to nanoparticles is related more to its surface properties rather than its mass (21-25). For example, Nel *et al*. provided the theoretical and methodological framework that describes the biophysico-chemical interactions at the interface of nanoparticle surface and the biological environment, including contact with cells (26). As reported in most studies, nanoparticles with

no surface modification are mostly taken up by phagocytic cells, which may cause undesirable interaction between nanoparticles and the immune system, and lead to a decrease in the drug's bioavailability as well as an increase in toxicity for the host. Consequently, the question of whether nanomedicine tools could mark an end to the necessity for “smart” drug delivery systems remains until a better understanding of the concept of biocompatibility is achieved and represents a major area of interest in the field of drug delivery.

Here, some data regarding the biocompatibility and peptide- based nanoparticles in the context of drug delivery is presented. More specifically, in this research , the biocompatibility of a new class of self-assembling peptides which contains all complementary amino acid pairs in its sequence and thus the name, amino acid pairing (AAP) peptides was investigated to show their potential to be used as carriers. The selected peptides are AC8 plus its modified versions (DEGylated forms) as well as EAK16-II and EK8 *per se* and with the hydrophobic anticancer drug pirarubicin. This further allows for the testing of peptide-based nanoparticles for targeted drug delivery as well as prediction of the possible toxicological reactions to such nanomaterials (biocompatibility).

1.2 General Goal and Research Objectives

The goal of this research was to evaluate biocompatibility of amino acid pairing (AAP) peptides when they are used as a carrier for anticancer drug delivery. The hydrophobic anticancer drug Pirarubicin is the candidate to examine this purpose. The AAP peptides which will be assessed are: AC8, AC8-DEG, and DEG-AC8-DEG, EAK16-II and EK8.

The specific objectives of this thesis were to:

1- Investigate haemolytic activities of the above mentioned AAP peptides and peptide-Pirarubicin-co assemblies (complexes).

2- Study complement activation caused by AAP peptides and complexes.

3- Examine anaphylotoxin activation by these peptides.

4- Understand the toxic effects of AAP peptides and evaluate the therapeutic effect of the peptide-drug complexes *in vitro* with cancer cell line A-549, a human lung cancer cell.

5- Elucidate the potential ability of these peptides to be utilized as carriers for the delivery of the hydrophobic anticancer drug pirarubicin.

1.3 Outline of the Thesis

This thesis consists of six chapters. The scope of each chapter is listed as follows:

Chapter 1 gives an introduction to the thesis, including an overview of biocompatibility and the immune reaction cascade which can dictate the fate of nanoparticles. The significance of the study and its application in drug delivery are also addressed. The goal, objectives and the outlines of the thesis are given in this chapter.

Chapter 2 reviews the background on understanding biocompatibility of nanoparticles and relevant factors that should be considered for biocompatibility evaluation of nanoparticles. In addition, immunocompatibility- as a subcategory of biocompatibility- as well as the roles that physicochemical properties of nanoparticles in biological systems play are also reviewed. Additionally, the major obstacle to drug delivery addressed and the concept of self-assembling, ionic-complementary self-assembling and amino acid pairing (AAP) peptides is

briefly reviewed. Finally, the importance of regulatory agencies highlighted and chapter concluded.

Chapter 3 describes the *in vitro* biocompatibility and toxicity of amino acid pairing peptide AC8 and its potential application for use as a carrier for drug delivery

Chapter 4 examines how functionalization by conjugating amino acid pairing peptide AC8 (AAP8) to diethylyne glycol (DEG) in two different fashions can affect biocompatibility.

Chapter 5 investigates the nanotoxicity of self/co-assembling peptide EAK16-II and EK8 to be used as carrier for anticancer drug, Pirarubicin

Chapter 6 presents the summary of outcomes of this study, original contributions of this research and recommendations for future work.

Chapter 2[*]
Literature Review

2.1 What is biocompatibility?

Biocompatibility first drew the attention of researchers between 1940 and 1980 in the context of medical implants and their interaction, both harmful and beneficial, with the body. Only within the past two decades, has this term been formally defined under its conceptual denotation rather than practical description (9): “The ability of a material to perform with an appropriate host response in a specific situation” (27). The three dogmas which play important roles in this definition are that a material has to perform its intended functions and not merely be present in the tissue, that the induced reaction has to be proper for the intended application, and that the nature of the reaction to a particular material and its suitability may be different from one context to another (28).

In 2010, Kohane and Langer explained biocompatibility in the context of drug delivery and defined biocompatibility as “an expression of the benignity of the relation between a material and its biological environment” (10). However, some researchers have expanded that definition by denoting acceptable functionality of a biomaterial in a given biological context as important (10). As such, Williams has reviewed the biocompatibility concept for long-term implantable medical devices and tissue engineering products in detail (9). In general, a high degree of biocompatibility is achieved when a material interacts with the body without

[*] Most parts of this chapter were published as Sheva Naahidi, Mousa Jafari, Faramarz Edalat, Kevin Raymond, Ali Khademhosseini, P. Chen, Biocompatibility of engineered nanoparticle for drug delivery, Journal of Controlled Release 166 (2013) 182–194

inducing unacceptable toxic, immunogenic, thrombogenic, and carcinogenic responses (Figure 2-1).

There are a number of relevant factors that should be considered for the evaluation of biocompatibility. First, biocompatibility is highly anatomically reliant which leads to the fact that the reactions to particular materials are different from one location to another. For instance, biodegradable polymeric-based nano- and microspheres – such as those based on poly(lactic-co-glycolic acid) (PLGA) – in general make a well characterized, subjectively mild tissue reaction, whereas the same particles introduced in the loose connective tissue surrounding nerves cause fairly strong acute inflammations (10, 29-31). Therefore, another fact that one must be cognizant of is that, if a biomaterial for a particular application can cause an adverse effect in a specific tissue type, it will not necessarily provoke the same response if used for a different application or in a different tissue type.

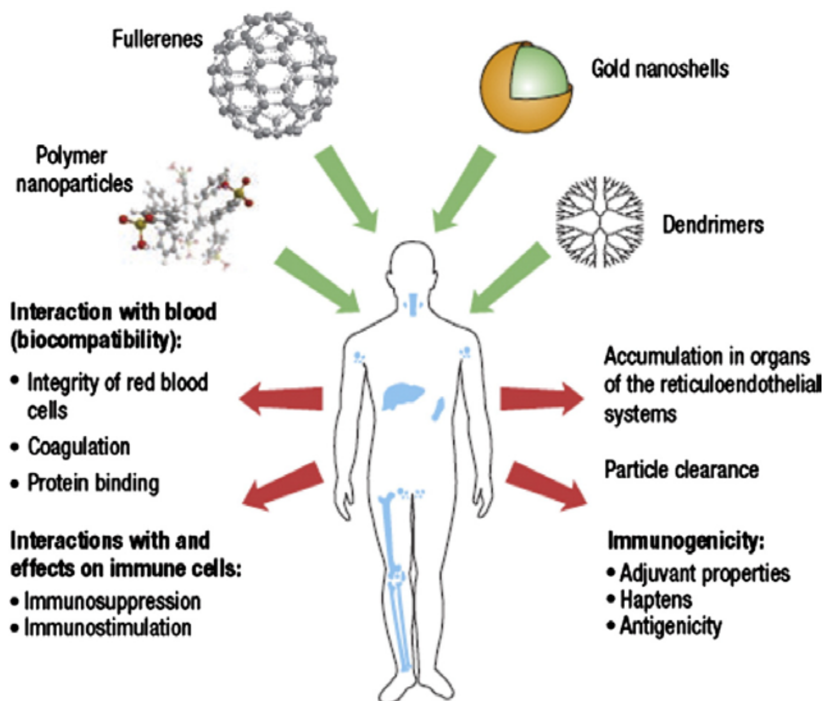

Figure 2-1 When nanoparticles interact with the body, a variety of responses may occur. These include alterations to the immune system or interaction with blood, among others. These reactions vary significantly with nanoparticle composition. For example, gold nanostructures may interact differently with the body when compared to polymeric particles. For this reason, nanoparticles have to be evaluated individually or "on a case-by-case basis" to better understand their effect on the body (32).

Second, and in an interrelated perspective, the biomaterials' intrinsic characteristics exclusively will not determine whether that particular material is biocompatible or not. For instance, PLGA nanoparticles those have a rapid clearance from the body do not usually cause peritoneal adhesion, whereas PLGA microparticles which stay longer in peritoneal cavity, do cause peritoneal adhesions (10, 33). Therefore, the exposure half-life is another important factor that deserves consideration. Third, biocompatibility is a relative matter that depends on the risk-benefit ratio, and relies on a subjective declaration since, in general, inflammation would totally vanish over time, and the neighboring tissues do not exemplify a good proof of damage. Last, but perhaps most important, the lack of adequate data regarding biological

processes in response to foreign materials, as well as the insensitive nature of the methods available for biocompatibility has limited the understanding of the biocompatibility of materials. All this together highlights the necessity of biocompatibility evaluation of biomaterials in a case-by case and tissue- and application-specific manner. Bringing all these together, I can conclude that biocompatibility of materials depends on their structure, formulation and many other factors as described above and can refer to a local or total effect on the organism. Accordingly, using biocompatible materials in an absolute sense, would be misleading (30, 31, 34-44).

2.2 Immunocompatibility

Immunocompatibility or the study of the immune response to a biomaterial, prosthesis, or medical device, as a subcategory of biocompatibility, represents a major area of interest. While factors such as the interaction with blood components, particle accumulation, and clearance in organs are indeed important, alterations to the immune system cannot be ignored. Nanoparticles have the potential to either stimulate or suppress the immune system, a property that may positively or adversely affect the function of a particle for particular applications.

2.2.1 Immunostimulation

As various compounds or materials are introduced into the body, the immune system recognizes them as foreign and elicits a multi-level immune response. When this occurs, the activity of one or more components of the immunoregulatory complexes is directly enhanced, and immunostimulatory effects such as flu-like symptoms and hypersensitivity to unrelated allergens are observed (45). Chamberlain and Mire-Sluis have described the molecular

structure, architecture of folding motifs, degradation products, formulation, package purity, and stability of pharmaceuticals as factors responsible for immunostimulation (46). Furthermore, Rihova and Kovar report that factors such as dose, route and time of administration, mechanism of action, and site of activity – all of which are extrinsic to the material – are also critical in immunostimulation (47). As part of immunostimulation, nanoparticles have displayed adjuvant properties. Adjuvants are substances that enhance the body's immune response to an antigen. In the context of cancer, pharmaceuticals are considered adjuvants when they, by stimulation of the immune system, suppress secondary tumor formation following treatment. Stieneker *et al*. showed that poly(methylmethacrylate) (PMMA) nanoparticles, when used as an adjuvant in human immunodeficiency virus (HIV) 2 whole-virus vaccine, were able to produce an antibody response in mice that was 100 times stronger than the traditional aluminum hydroxide or aqueous control vaccine (48). Caputo *et al*. showed that novel biocompatible anionic microspheres are suitable and efficient storage and delivery systems for HIV-1 Tat protein for vaccine applications that preserve protein conformation and activity (49). In particular, they have shown in vitro that anionic nano- and microspheres attach the HIV-1 Tat protein and guard it from oxidation; therefore, rising the "shelf-life" of the Tat protein vaccine (49, 50). In addition, in this group, in vivo biocompatible and novel surfactant-free polymeric core–shell nanoparticles and microparticles were developed (49-51). These particles reported to be able "to accommodate in their shell high amounts (antigen loading ability of up to 20%, w/w) of native proteins, mainly by ionic interactions, while preserving their activity" (52). However, recent progress

in the development of HIV-1 Tat-based vaccines (53) from basic science to clinical trial (54) has been reviewed elsewhere. In a similar study by Castignolles *et al*. using rabies vaccine showed that lipid-coated polysaccharide nanoparticles increased antibody response, and hence vaccine efficacy, fourfold (55). Works by Diwan *et al*. and Cui *et al*. suggest that nanoparticles exhibit adjuvant properties by enhancing antigen uptake and stimulating antigen-presenting cells (56, 57). While the mechanisms of nanoparticle-induced adjuvant properties are not fully understood, its proven effectiveness for use in vaccines has generated a great deal of interest. One of the critical components of immunostimulation is inflammation, a non-specific immune reaction whereby signaling molecules called cytokines are secreted to recruit immune cells to the location where foreign material exists. This recognition is triggered by the core composition and surface properties of the particle. Of these properties, surface charge plays a particularly important role; generally, a positively charged particle is more apt to cause inflammation than a neutral or negatively charged particle. This fact was corroborated by Tan *et al*., who showed that an anionic particle did not cause the secretion of cytokines while a cationic particle did (58). Diwan *et al*. provided further evidence of nanoparticle-induced inflammation. They showed that oligonucleotides bound to PLGA-based nanoparticles caused a larger amount of cytokine production and induced more T-cell proliferation than the naked oligonucleotide (59). Foreign material is dealt with and cleared from the body in a variety of ways. Nanoparticles can be engineered to resemble pathogens so they are dealt with in an equivalent fashion. One method of doing this is by modifying nanoparticles with Toll-like receptor (TLR) ligands, which are recognized by the immunity

system's dendritic cells. In one study, mannose was applied to modify the surface of particles, stimulating the particle's uptake by mannose receptors, a common mechanism for pathogen neutralization (60). However, there is a tremendous amount of work involved in focusing micro particle based immunostimulation against cancer cells.

2.2.2 Immunosuppression

Immunosuppression is described as the down-regulation or prevention of the activation of the immune system. Since the early 1960s, immunotoxicologists have continued to catalogue the immunosuppressive ability of drugs as well as chemicals. Immunosuppression has its drawbacks such as increasing susceptibility to infections caused by bacteria, viruses, fungi, and yeast (61), as well as the development of neoplasms (most commonly skin cancers and lymphomas) (62). While immunosuppression is undesirable in some instances, it has proven useful in the treatment of autoimmune diseases and has facilitated the acceptance of foreign tissues in organ transplant patients. As with immunostimulation, factors such as drug dose, pathway into the body, time of administration, the mechanism of action, and site of activity will affect the body's response to an immunosuppressant (47). Nanoparticles have been shown to produce immunosuppressant properties. For instance, Shaunak *et al.* (63) reported that when human macrophages and dendritic cells were exposed to the bacterial endotoxin, Generation-3.5 polyamidoamine (PAMAM) dendrimer-glucosamine conjugates – which are produced from partial modification of carboxylic acid terminated PAMAM dendrimers with glycosamine – were able to significantly inhibit cytokine- and chemokine-induced inflammation with a novel immunomodulatory and antiangiogenic properties. Interestingly,

no hematological toxicity was apparent, suggesting that the dendrimer conjugates may be able to treat and prevent the formation of scar tissue (63). In another work, PLGA nanoparticles containing collagen type II suppressed arthritis-induced inflammation in a mouse model (64). A comparable study observed similar results using PLGA nanoparticles functionalized with betamethasone in rats (65). In a similar mouse model, autoimmune diabetes was inhibited (66). Cromer *et al*. reported that amino terminated generation-5 PAMAM dendrimers modified with 2-hydrohyhexyl groups protected against fatal sepsis and in vivo and in vitro cytokine secretion caused by bacterial lipopolysaccharide (67).

In the case of allergies, the induction of immune tolerance is considered desirable. For instance, Ryan *et al*. showed that polyhydroxy C60, a type of water-soluble fullerene, was able to inhibit hypersensitivity reactions both *in vitro* and *in vivo* (68). In similar cases, it was reported that nanoparticles suppressed type I and type II allergies to common environmental and food allergens (69-73). In this scenario, however, data conflicting with the concept of desirable effects of nanomaterials have been also presented. For instance, Zogovic *et al*. investigated the influence of nanocrystalline fullerene C60 on tumor progression and reported that nanoC60, "in contrast to its potent anticancer activity in vitro, can potentiate tumor growth in vivo, possibly by causing NO-dependent suppression of anticancer immune response" (74).

2.3 PEGylation

The characteristics of a material's surface are a primary factor in the determination of the biocompatibility of that material within the body (11). This fact was recognized by

Abuchowski *et al.*, who in the 1970s, were the first to introduce the covalent bonding of poly (ethylene glycol) (PEG) to a drug or therapeutic protein in a process known today as PEGylation (75). Later on, in 1984, de Gennes described two main regimes or conformations that PEG chains can obtain which are called mushroom and brush conformation — depending on grafting density (76). If the grafting density is low, the PEG polymer is assumed to be in the mushroom regime. If the density is high the PEG polymers are assumed to be in the brush regime (77). The degree of surface coverage and distance between graft sites will depend on the molecular weight and the graft density of the PEG polymer (78); thus, requiring careful attention. Early work with PEG grafted nanoparticles pursued primarily from drug delivery (79-82). Davis and Abuchowski, as one of the first reporters on PEGylation, described covalent attachment of methoxyPEGs (mPEGs) of 1900 and 5000 Da to bovine serum albumin and to liver catalase (75, 83). It is now well known that PEGylation holds many attractive properties; for instance, it has been shown to increase a drug's half-life within the body, prolonging the activity of the drug, and thus reducing the dosing frequency (84). In addition, in drug-delivery applications, PEG grafted nanocarriers decrease MPS uptake and augments circulation time versus uncoated counterparts (11). PEG's ability to prolong the circulation lifetime of the carrier (10) has been credited principally due to its physical properties (7, 75, 84, 85) which in turn can cause the reduction or prevention of protein adsorption. To this point, Allen *et al*. addressed the question of how surface of a liposome protected with PEG molecules of different molecular weights would differ from a PEG-free liposome (78). Their work was based on a previous approach established by Torchilin and

Papisov in 1994 (86). Needham and Kim reported that PEG of a selected molecular weight and graft thickness prevents the adsorption of certain proteins to a surface (84, 85, 87, 88); yet, there is not much evidence that exist for reduction of total serum protein binding due to surface PEGylation of carrier. Ahl *et al*. has shown that PEGylation increases a colloidal carrier's stability in vivo by its steric effect which acts as a barrier for aggregation (89). Other studies have suggested that PEG endorse binding of specific proteins that mask the carrier and cause "dysopsonization" (90, 91) as well as existence of attractive interactions between poly(ethylene glycol) and proteins (87, 91, 92). Not surprisingly, PEGylation can have ability to control the physical behavior and biological performance of nanocarriers formulations and as a result substantially change their biocompatibility. Consequently, PEGylation dramatically reduces the immunogenic response to a substance's surface, including a reduction in protein adsorption, as shown in Figure 2-2 (93), as well as a reduction in platelet aggregation, neutrophil activation, hemolytic activity, and coagulation (11).

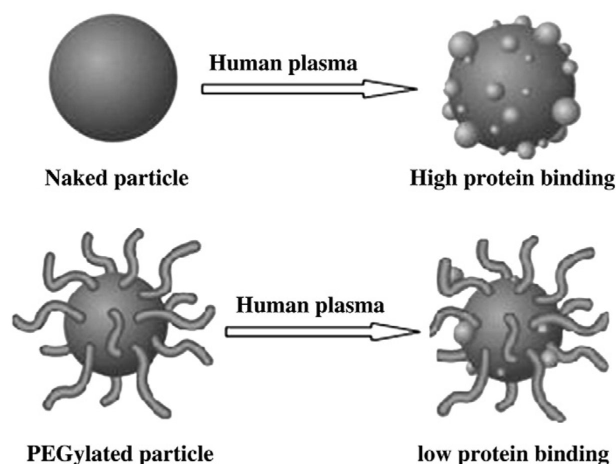

Figure 2-2 PEGylated nanoparticles are able to avoid clearance from the blood stream by repelling protein adsorption, thus prolonging nanoparticle circulation time within the body (32).

However, PEGylation also carries several disadvantages that must be considered. Recent works indicate the formation of PEG-specific antibodies, which clear the particle (along with the drug) from the body, thus reducing its effectiveness (47). In addition to this, obstacles include possible side reactions, incomplete PEGylation and the need for drug-specific tailoring (94).

2.4 Nanoparticle Interaction with Blood

The surface properties of nanoparticles can greatly affect their compatibility while in the blood stream. Interestingly, blood constituents can react immunologically to render nanoparticles and their drug complexes inactive. For instance, Gref *et al*. (79) reported that in the blood stream, macrophages rapidly clear nanoparticles that lacked surface modification to prevent the adsorption of opsonins. For this reason, preclinical examination of nanoparticle biocompatibility must include studies of hemolysis, platelet aggregation, coagulation time, complement activation, leukocyte proliferation, and uptake by macrophages (11). Therefore,

evaluation of possible toxic effects of immediate exposure of nanoparticles would be the first critical step that one would be considered. It is known that erythrocytes exist in a larger volume portion of the blood than mononuclear phagocytic (MNP) cells. Thus, nanoparticles that were injected intravenously would encounter red blood cells (RBC) before MNP cells; consequently, examination of haemolysis is an instrumental part of preclinical studies of nanoparticles (12). Many authors reported hemolytic effects of different nanoparticles in the literature — as many of the studies have been conducted with blood to see the early toxic effects of nanoparticles. As a result, a number of mechanisms for drug mediated haemolysis have been recommended, yet the true mechanism has not been clearly identified. It is now well known that surface properties (especially surface charge) play an important role for nanoparticles and can directly damage erythrocyte membranes. For instance, in the presence of certain concentrations of unprotected primary amines (positive charge), red blood cell damage was observed on the surface of poly-amidoamine, carbosilane, polypropylene imine, and poly-lysine (95-100). However, a deeper understanding and knowledge on how the particulate nature of blood would affect a nanoparticle will help for improved design of nanoparticle-based drug delivery systems. In this regard, Tan *et al*. provided the theoretical and methodological framework that help to understand how interactions between blood cells – with and without red blood cell – and NPs influence the particle motion and binding (101). They reported enhance nanoparticle dispersion as well as 50% increased nanoparticle binding upon exposure to RBC. Another study also presented erythrocyte as a vital contributor to the process of transport and primary meeting of lymphocytes to the vascular wall (102). However,

there are other studies in the literature which reported mathematical or theoretical modeling of RBC on blood flow (103, 104) which indirectly would influence nanoparticles efficacy in drug delivery system. The complement system and its activation are major characteristics of the general host response to biomaterials, including nanoparticles. Complement activation is described as the recognition, opsonization, and clearing of pathogens and foreign material by approximately 35 typically dormant proteins present within blood (either solubilized in blood or located on the surface of blood cells) (94). The complement system can be triggered by any of three different pathways: the classical pathway, alternate pathway, and lectin pathway. These pathways are activated by different criteria: the classical pathway by specific antibodies found on the surface of the intruding material, the alternate pathway by the identification of certain microbial surface structures, and the lectin pathway by mannose residues found on microbial glycoproteins and glycolipids which are identified by mannose-binding lectin (MBL), a protein found in blood plasma (105). Understanding a material's effect on the complement system is crucial to understanding the immunological response it may trigger. For this reason, reducing a surface's tendency for complement activation has been the subject of widespread interest (106).

2.5 Nanoparticles As Drug Carriers

Nanoparticles used as drug carriers are submicron-sized particles ranging 100–1000 nm. Buzea *et al*. defined nanoparticles as “particles with at least one dimension smaller than 1 micron and potentially as small as atomic and molecular length scales (~0.2 nm)” (107). Many organizations have now defined nanoparticles as the particles which should have a size

below 100 nm in at least one orthogonal direction. In fact, it is not easy to track expansions in the field of nanotechnology since the multidisciplinary nature of this field request a similar diversity of definitions in respect to each specialty or scientific discipline. In this regard, Klaessig *et al*. reported the dispute facing terminology and nomenclature efforts and listed the suggested upper boundary for the term nanoscale along with the organization, references and qualifications (108). Another example of such is the publication by the U.K. House of Lords Science and Technology Committee titled, "Nanotechnologies and Food" (109) and recommended:

…We recommend…that any regulatory definition of nanomaterials

… not include a size limit of 100 nm but instead refer to 'the nanoscale' to ensure that all materials with a dimension under 1000 nm are considered (108, 109). "The recommendation is that the term nanoscale have an upper boundary of 1,000 nm for the purpose of food regulations, rather than the ISO and ASTM International determinations that scientific usage is 100 nm." (108).

However, nanosized particles or nanoparticles used for drug delivery hold great promise for their feasibility as pharmaceutical carriers and can be prepared using a wide range of materials such as polymers, lipids, viruses, and organometalic compounds; therefore, their use in medicine is predicted to spread rapidly in the coming years (110). Studies indicate that nanoparticle-drug complexes have the ability to mitigate toxicity and side effects associated with raw pharmaceuticals such as chemotherapy drugs (11, 110), by allowing for targeted drug release and improved solubility through various methods such as encapsulation,

micellization, and protein cage architecture (111). Indeed, the potential for more precise localization and reduced toxicity of therapeutic drugs is encouraging. However, evidence suggests that nanocarriers themselves may pose a toxicological risk to patients beyond that of the taxied chemicals (110). De Jong and Bormhave summarized some of the adverse toxicological responses observed over the past decade, which include lung inflammation, platelet aggregation in blood, and impaired mitochondrial function in cells (110). As can be imagined, the toxicological effects vary with nanoparticle composition. The material composition may include metals and inorganic particles such as gold, silver, and metal oxides (112), polymer-based materials such as PLGA, and lipid-based particles such as nanoliposomes, solid lipid nanoparticles, and nanoemulsions. Each substance exhibits its own inherent physicochemical properties such as surface charge, hydrophobicity, solubility, size, shape, and aggregation tendencies which can be engineered to trigger different biological responses (8, 11). While the influence of such parameters on biocompatibility is well known in some instances, as schematically depicted in Figure 2-3 (11), investigations involving newer nanoparticle designs are still underway. As expected, the manipulation of these properties for the purpose of function and biocompatibility represents a prominent area of study in nanomedicine (8).

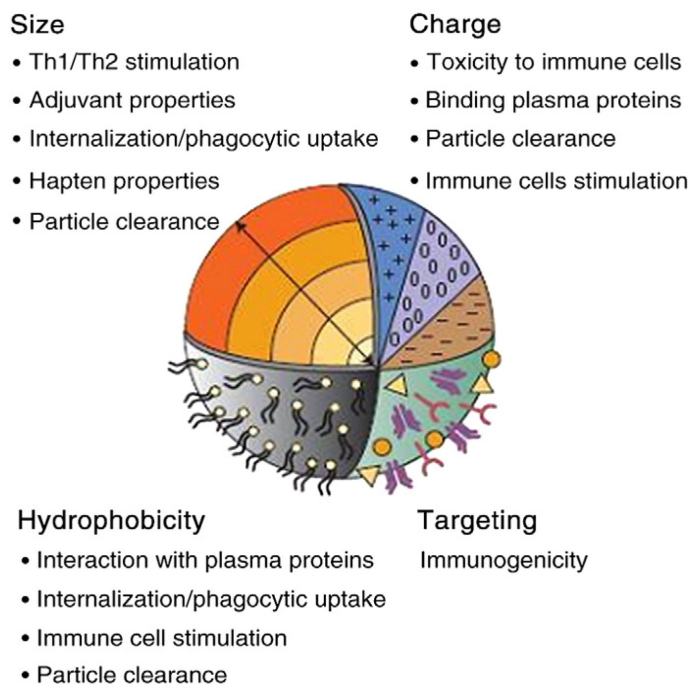

Figure 2-3 The properties of nanoparticles such as size and charge determine their effect on the body (32).

2.6 Types of Nanoparticles

Nanoparticles exist in a wide variety of sizes, shapes, and compositions (113). Nanoparticle-bound pharmaceuticals in their many forms can be found at various stages of the pharmaceutical pipeline; some have been approved for clinical use, while others are being tested and progressed through the approval process (11). In the following section, the focus will be on nanoparticles that have been widely investigated for drug delivery applications. This section also outlines a variety of approaches to nanoparticle structure and composure.

2.6.1 Carbon-based polymers

Carbon-based polymers such as fullerenes, carbon dots, nanodiamonds, and nanofoams also represent a prominent area of nanoparticle research. Of these, fullerenes are well established and consist of C60, single-walled nanotubes, and multi-walled nanotubes. Carbon nanotubes

have been proposed for use in a variety of contexts ranging from structural reinforcement of existing materials (114) to drug carriers (115). Carbon nanotubes are simple layers of graphite rolled in a tubular shape capable of exhibiting a single- or multi-walled morphology (116, 117). Their cell-penetrating and conjugative properties make them a contender for in vivo drug delivery applications (115). In addition, surface functionalization can render typically heterogeneous nanotubes water-soluble (116). With regard to biocompatibility, carbon nanotubes have been shown to activate the complement system through the classical and alternate pathways (115). Furthermore, it was found that carbon nanotubes might, in some cases, over-stimulate the complement system, resulting in inflammation and granuloma formation (11, 115, 117). Additional factors that may inhibit carbon nanotube use in humans include evidence of oxidative stress (112, 116, 117), apoptosis (116), toxicity due to metal residues from nanotube synthesis (112), lipid peroxidation, mitochondrial dysfunction, changes in cell morphology, and platelet aggregation (117). In contrast to this, some reports indicate that an inflammatory response does not occur when carbon nanotubes have been purified (118). Although the ambiguity of carbon nanotube toxicity and wide array of toxicological responses certainly warrants caution, the contradictory data on the toxic effects of carbon nanotubes also suggests a need for further research. Therefore, it is believed that modification and/or coating of carbon nanotube-based biomaterials would enhance their ability as suitable carriers for applications such as drug delivery. For instance, targeted and controlled doxorubicin delivery using modified single wall carbon nanotubes have been reported (119). In addition, Ren *et al*. summarized the data on the toxic effects of single-

walled carbon nanotube with different treatments and suggested on a standard evaluation of the effects of carbon nanotube on the cells, organs, or an entire organism (120).

Graphene is a material with a one-carbon atom thick, single layer sheet structure that occurs in nature in the form of graphite. Graphene can be used for a different range of biomedical applications due to its flexible chemical structure blend with its inherent properties. Therefore, graphene has become a potential candidate for multifunctional biomedical purposes such as biosensors (121, 122) and drug delivery (123). For instance, Hu *et al*. have reported about good antibacterial activity of graphene oxide (GO), recommending its potential for drug delivery in ophthalmology application (124). A year later, in 2011, Zhang *et al*. showed that modified GO could be used for targeted drug delivery and controlled release in the tumor therapy (125). Almost at the same time, Yang *et al*. showed that GO-based composites decreased reticuloendothelial system accumulation and remarkably enhanced tumor passive targeting effects (126).

Recently, Yan *et al*. investigated the *in vitro* and *in vivo* intraocular biocompatibility and cytotoxicity of graphene oxide (GO) (127) knowing the fact that eye is a particular organ with the presence of the blood−ocular barrier which make it to be important in targeted medical therapies such as ocular tumor-related treatments. Therefore, they investigated novel drug delivery and controlled release systems in ophthalmology and reported GO has favorable biocompatibility for retinal pigment epithelial cells with minimal adverse effects on cell viability and morphology in long-term cultures (127). Furthermore, biocompatibility and toxicity of GO on A549 cells has also been evaluated, suggesting that "GO does not enter

A549 cell and has no evident cytotoxicity”. Nevertheless, GO could cause a dose- and size-dependent oxidative stress in cell and a trivial loss of cell viability at high concentrations. These data together suggests that overall, GO has an adequate safety profile, for drug delivery application, further supported by the positive growth of cells on GO films (128).

Although there are studies that address the biocompatibility of graphene, GO and their modified versions (129-133), a great deal of research is still required in the near future prior to their application in the clinical settings. Furthermore, there is active, ongoing research in the role of carbon nanotubes in the delivery of chemotherapeutic agents while limiting systemic toxicity (134).

2.6.2 Polymeric nanoparticles

Polymeric nanoparticles include synthetic polymers, natural polymers (e.g. proteins), and pseudosynthetic polymers (such as synthetic polypeptides are broadly used for drug delivery (135). Polymer architecture, composition, backbone stability, as well as water solubility are important factors which specify the effectiveness of drug-delivery carriers (136). In this section, a selected group of polymeric nanoparticles and dendrimers that have been the most commonly used in drug delivery applications are reviewed.

Currently it is well known that polymer architecture dictates the carrier’s physicochemical properties, drug loading effectiveness, drug-release rate and biodistribution (136). Polydispersity character of polymer, defined as the heterogeneous combination of chains of altering lengths (137), makes them of particular significance for biological properties which are molecular mass dependent (138). Polymers have been found to be able to provide a

sustained release of encapsulated drugs, protect drugs from the body's enzymatic and degenerative conditions, provide targeting capabilities from a tendency for passive accumulation in tumors, and display adjuvant characteristics, meaning that it may help prevent subsequent cancer attacks. In addition, they can be used to overcome the poor aqueous solubility of certain drugs such as chemotherapeutics (139). Despite all of these benefits, polymers are frequently taken up by the immune cells and hence the immunocompatibility of these materials must be carefully considered (140).

It has been reported that polycations may not only be cytotoxic, but can also induce hemolysis and complement activation (137). It has also been observed that polyanions are less cytotoxic, but still can induce anticoagulant activity and cytokine release (137). Despite these reports, there are some reports that address the compatibility of polymers for *in vivo* applications (141). Other studies have shown that nanoparticles made from N-(2-hydroxypropyl) methacrylamide (HPMA) were able to mitigate many of the inherently toxic effects of the popular anti-cancer drug, doxorubicin (94). Furthermore, it was found that HPMA-bound doxorubicin triggered anti-cancer immunity in mice; up to 80% of cured mice were able to survive a fatal dose of cancer cells independent of further treatment (142). With regard to biocompatibility, evidence suggests that HPMA does not induce a significant response within the body, leading researchers to believe that HMPA copolymers are indeed "immunologically safe" (94).

Another type of polymeric-based particle that can be utilized as carriers for drug delivery systems is PLGA micro- or nanoparticles (30). These particles are known as clinically proven

biodegradable and biocompatible materials (35). One area in which they have been widely investigated is in the formulation of the chemotherapeutic drug, paclitaxel (Taxol®) [156]. In addition, they represent an innovative approach to adjuvant therapy in vaccination by presenting vaccine antigens (31, 34). Studies have reported that these polymeric particulate delivery systems (30) can present antigens and trigger specific humoral and/or cellular responses (34, 37), highlighting the importance of their size in the resulted outcomes (38, 40). For instance, microparticles trigger a humoral-mediated immune response whereas nanosized range PLGA particles activate cell-mediate immune responses (41, 42). Not surprisingly, it is not easy to predict the phagocytic behavior subsequent to particles' uptake. Nicolete *et al*. produced PLGA nano- and microparticles devoid of any encapsulated bioactive. They then examined these particles' uptake by macrophages as well as their effect *in vitro*, on the production of proinflammatory cytokines, TNF-α and IL-1β (30). They have reported that PLGA microparticles of size 6.5 μm, attached to the cell's surface at 2 and 4 hour incubation time and few could be seen inside the cells when compared to nanoparticles (30). Danhier *et al*. have reviewed the beneficial usage of PLGA-based nanoparticles both *in vitro* and *in vivo* as a therapeutic strategy in different diseases (43); they also reported on the characteristics of PLGA-based nanoparticle that makes them a promising candidate for targeted and untargeted drug delivery. Poly(lactic acid) (PLA) polymers have also been used in drug delivery (44, 143); however, due to their slow degradation rate, PLA polymers have not been broadly used, compared to PLGA polymers (43). PLA was used for surface modification of organic microsphere poly (hydroxyethyl methacrylate) (PHEMA). PLA modified microspheres

showed better anti-tumor effect as well as increased loading capacity in compare with unmodified one (144).

In general, one of the areas in which more work needs to be done on the development of methods for visualization of polymer-based nanoparticles. Even highly sensitive methods such as scanning- and transmission electron microscopy are limited in their capability for reliable visualization of polymer-based nanoparticles within cells, compelling the need for indirect, assay-based methods to examine nanoparticle cellular uptake by phagocytes (11).

2.6.3 Dendrimers

Dendrimers are highly branched polymers whose shape, size, branching length, density, and surface functionality can be controlled and are well defined (145). Originating from a nanosized core, polymeric branches of high specificity are grown outward, forming cavities and cages throughout the molecule (116). These channels and closed structures allow for the physical entrapment or encapsulation of pharmaceuticals (145). In addition, negatively charged drugs may associate themselves through electrostatic interactions with amine groups within the dendrite (145). Furthermore, drugs can be chemically attached to surface groups on the polymeric structure (116, 117, 145). Dendrimers are susceptive to surface group modification and can be tailored to facilitate targeting and improve biocompatibility (116, 117, 145). For instance, dendrimers with positively charged surface groups are likely to cause cell lysis. Dendrimers, like most nanoparticles used for drug delivery, aim to mitigate the inherently toxic effects of unbound drugs through targeting and subsequent accumulation in tumors; PEGylation abets or assist this process (145). On the subject of toxicity, dendrimers

cannot be classified as consistently safe or unsafe. Research suggests that dendrimers must be evaluated on a case-by-case basis to classify their particular chemistry's biocompatibility. A lack of research and clinical trial in this field deters generalization regarding safety (117). Contrarily, there are known correlations between the properties of dendrimers and their functionality and biocompatibility. For instance, size influences both solubility and cytotoxicity, and an increase in generation number leads to an increase in both of these properties. Lastly, dendrimers, possess antitumor, antiviral, and antibacterial properties, along with the capacity to enhance membrane permeability (145). These intrinsic properties have sparked interest in dendrimers for bacterial cell killing and trans-membrane transport applications (117).

Micelles act by encapsulating material within its walls. Contrarily, a micelle functions on the premise of its amphiphilic monolayer. The inner core of micelles is typically hydrophobic enabling successful encapsulation of insoluble drugs, while its hydrophilic outer core renders the encapsulated material soluble (113). Self-assembled polymeric micelles have recently attracted attention due to their special characteristics, such as high loading capacity and improved solubility of drugs, decreased systemic unfavorable effects, enhanced permeability and retention (EPR) effect which results in their accumulation at the tumor site, and lastly, their possible modification of physicochemical characteristic (33, 146-148). However, with all of the advantages, the controlled and smart release of therapeutics from traditional polymeric micelles carriers remains a challenge. Currently, nanocarriers are replaced by traditional micelle systems, since they can stably encapsulate and release therapeutics at a targeted site as

a result of external stimuli such as pH, temperature, redox, and light (149-152). However, because of their toxicity, only a small number of them have been moved to clinical studies (153) such as pH-responsive polymeric micelles (143, 154-158). Therefore, to overcome the toxicity of the carriers, polymeric segments composed of polymers with better compatibility such as poly (ethylene oxide) and biodegradable polymers like polyesters, are employed to form micelles in aqueous solutions. Lee *et al.* designed and synthesized a new class of hyperbranched double hydrophilic block copolymer of poly(ethylene oxide)-hyperbranched-polyglycerol (PEO-hb-PG) with enhanced biocompatibility, increase water solubility, and improved biodegradability after delivery of the drug (153).

2.6.4 Lipid-based nanoparticles

Lipid-based nanoparticles such as liposomes represent another category of popular drug-carrying nanostructures. Liposomes, not to be confused with micelles which are characterized by monolayers, are generally composed of one or more bilayers of an aliphatic lipid molecule arranged to form a vesicle. This vesicle formation allows for the encapsulation of drugs, vaccines, or other materials within its walls or entrapment within its layers, depending on the material to be delivered (116). A number of liposome-based formulations have gained approval for the treatment of cancer, infections, and meningitis, with prospective applications such as therapeutic vaccines currently under development (159). These include liposomal-based therapeutics containing the antifungal, amphotericin B (Abelcet®), chemotherapeutic drug doxorubicin (Myocet®), immunopotentiating reconstituted influenza virosome (Epaxal®) (160). Liposomes have been categorized as those which have been designed to

evoke an immune response to a contained antigen and those whose surface have been coated with PEG or a similar polymer to mitigate or suppress immune response. In general, liposomes with positively charged surfaces are more prone to eliciting an immune response than negatively charged or neutral particles (161). A possible downside to liposomes as pharmaceutical carriers is their selectivity with respect to functionally compatible drugs. In some cases—liposome-entrapped cisplatin, for instance—the particles are unable to release the encapsulated drug at a rate sufficient to trigger antitumor activity, despite passive accumulation at tumor sites. Nevertheless, doxorubicin-entrapped liposomes of the same composition produced effective antitumor properties, corroborating the aforementioned selectivity (116).

Solid lipid nanoparticles (SLNs) mainly consist of solid lipids, which also possess properties such as biocompatibility, biodegradability and low-toxicity. SLNs are described as colloidal particles of highly purified triglycerides, complex glyceride mixtures, or waxes stabilized by a surfactant. These are lipids whose nature allows them to remain solidified at room and body temperature (162). When regarded as a drug carrier, SLNs have undergone studies with a wide variety of pharmaceuticals ranging in structure and chemical properties (163, 164). SLNs have the advantages of both the "soft" drug carriers such as emulsions and liposomes and polymeric nanoparticles (14).

SLNs are versatile in their methods of drug incorporation; drugs can be loaded into the particle’s core or shell, between lipid layers and fatty acid chains, in the particle’s imperfections, or dispersed molecularly throughout the particle’s matrix (162). Despite this

versatility, SLNs feature a low drug loading capacity. Additionally, SLNs may undergo polymorphic transition during storage and administration; this causes gelation, size increase, and drug expulsion (162, 163). Undesired lipid-based particles such as micelles and liposomes as well as crystalline drug structures may also be formed within the complex, threatening the purity of the SLN colloid (163). Conversely, SLNs allow for a variable rate of drug release and targeting within the body, provide protection to the encapsulated drug (162), and avoid the use of harmful organic solvents, and have potential for large-scale production as a result of a streamlined production process. Another advantage lies in SLN composition. Since they are made from physiological compounds, metabolic pathways are already in place within the body (163). This anticipated biocompatibility has been corroborated through *in vitro* and *in vivo* studies of SLN toxicity. For instance, tests indicate that SLN are less toxic than polymeric nanoparticles (PLA/GA) [178]. Bolus injections in mice also showed no acute toxicity as suggested by histopathology (162, 164). Furthermore, it has been noted that SLNs are suitable for use in any parenteral application where polymeric nanoparticles are accepted (164). Recently, Qi *et al*. have provided an overview on the absorption, disposition and pharmacokinetics of SLNs (165).

In general, lipid-based nanoparticles are vulnerable to changes in temperature and osmotic pressure, among other extrinsic variables. This property, along with their inherent instability in biological media, may warrant the need for stability-enhancing alterations such as surface polymerization (145).

2.6.5 Quantum dots

The term “quantum dot” refers to a particular category of nanoparticle characterized by a crystalline structure usually composed of a semiconducting material (166). Cadmium sulfide and cadmium selenide quantum dots are among the most popular (112). Quantum dots (QDs) were discovered in the 1980s and are known to possess unique optical properties that make them ideal for imaging purpose (116, 166). In addition, quantum dots may be used for cancer detection and therapy (166), and computing applications where light is used to process signals (116).

In a drug delivery scenario, "fluorescent semiconductor nanocrystals", quantum dots, possess valuable features, such as small size, flexible surface chemistry, and valuable optical properties, making QDs not only to be a good plan for the broad characterization of nanocarrier behavior (6) but also allowing their use within almost any nanocarrier- with minimal effect on overall characteristics- and drug release at both cellular and systemic levels (167). Like all nanoparticles proposed for use in the human body, quantum dots are being tested for biocompatibility. The innate properties of the material will determine factors such as adsorption, distribution, metabolism, excretion, and toxicity, as well as the environmental conditions in which the particle is placed. Studies have shown that quantum dots themselves may induce toxic effects such as damage to plasma membranes, mitochondria and nuclei. For this reason, and for the purpose of targeting, these nanoparticles are often surface-coated; this may, however, induce additional toxicity. Furthermore, it was found that the quantum dot’s toxicity is influenced not only by its surface chemistry, but also by its core material (117). However, appropriately coated and passivated QDots do not show acute toxicity *in vivo* and in

rhesus monkey (168) regardless of the possible release of toxic chemicals such as cadmium, Cd (6) and production of reactive oxygen species (5). Modified quantum dots would permit brief nanocarrier screening without non-specific unfavorable effects (167).

P. Zrazhevskiy discussed about reducing long-term QDot toxicity (168). Such properties would have made quantum dot platform to be a potential candidate for clarifying, *in vivo* and *in vitro*, mechanisms of nanoparticle targeting, intracellular uptake, and trafficking (167). This in turn would ease assessment of the nanocarrier behavior in a range of drug delivery applications as well as contribute for design of novel nanotherapeutics, such as "NP-based antigen delivery vectors for immunotherapy" (167). As toxicity is inherent to traditional quantum dots, the search for less harmful materials is ongoing and of great interest (166).

2.6.6 Metallic nanoparticles

Metallic nanoparticles hold potential for use in both diagnostic imaging and targeted drug delivery. These nanoparticles are often delivered in solid colloidal form and aim to increase the therapeutic index of anticancer drugs through passive or active targeting while mitigating toxic effects by limiting drug exposure to healthy cells and tissues. Metal-based particles hold the potential to carry large drug doses as well as increase its circulatory half-life. Additionally, surface modification is possible due to a large surface area-to-volume ratio (6), the effects of which have been discussed in previous sections.

The use of colloidal gold in medicine can be traced back to the 1920s for the treatment of tuberculosis (112). Since then, colloidal gold nanoparticles have been widely researched as drug and gene delivery vehicles. They can be synthesized in a variety of forms (e.g. rod, dot)

(117) and are easily detectable within micromolar concentrations, warranting their use in imaging applications (112). With regard to biocompatibility, cells have been shown to intake gold nanoparticles without cytotoxic effects (112, 118). Lai *et al*. demonstrated a median lethal dose (LD50) of over 5 g/kg of body weight using a nanogold suspension with a particle diameter of 50 nm (169). Metallic nanopoarticles, including colloidal gold, continue to be actively investigated for the purpose of drug delivery and other applications. Research in this field is expected to grow over the next few decades (6).

2.7 Major Obstacles To Effective Drug Delivery

In the last several years, drug delivery research has witnessed remarkable growth due to the utilization of nanoparticles as “controlled release reservoirs” for the targeted delivery of drugs for combating many diseases (145). Evidence has shown that several different nanoparticles have been used as a carrier for drug delivery systems (145, 157, 170). The problem remains, however, that nanoparticles’ applications are still limited mainly by their unknown biocompatibility properties, which may cause their quick removal by the immune systems. Recently, the knowledge about nanoparticle interaction with components of the immune system has increased. But, still many questions such as particle immunomodulatory effects (immunostimulation and immunosuppression) need to be completely addressed. Understanding the mechanisms behind these different outcomes will allow prediction of the relationship between nanostructures and their interactions with the biological milieu. Indeed the ability of a nanoparticle to evade immune recognition represents an area of interest in the field of drug delivery. One of the promising approaches towards this goal has been through

the development of the self-assembled peptide nanomaterials due to their perceived biocompatibility and structural diversity. As a result, they have become attractive as building blocks for a variety of applications such as materials science, tissue engineering, bioengineering, and of particular interest to this research, drug delivery (27-31, 33-36, 112). The following two sections review the concept of molecular self assembly and one class of self assembling peptides, ionic complementary peptides, respectively.

2.8 Molecular Self-assembly

"There's Plenty of Room at the Bottom" is a lecture given on December 29, 1951 by physicist Richard Feynman who predicted the potential of atomic/molecular engineering and inspired the conceptual development of the nanotechnology field, which occurred decades later. Nowadays, nanotechnology is mainly based on "bottom-up" atomic/molecular development for chemical and physical construction of stable nanostructures or supramolecules. The key component of this "bottom-up" advance is molecular self-assembly. As a result, in recent years, molecular self-assembly has become a useful tool for expanding the production of new biomaterials. The self-assembly of biomolecules is a well-known phenomenon in biology, encompassing self-complementary DNA double-helix annealing and protein aggregation. Self-assembly in the most basic form consists of the "spontaneous organization of molecules under thermodynamic equilibrium conditions into structurally stable arrangements by the driving force of non-covalent interactions, including hydrogen bonds, electrostatic interaction, and van der Waals interactions" (171) .

Recently, short peptides have allowed material scientists to design functional nano/microstructures for different applications based on their self-assembly capabilities. A promising class of self-assembling peptides is the self-assembling ionic-complementary peptides (29, 30).

2.9 Ionic- Complementary Peptides

This class of peptides was discovered twenty years ago at MIT and has been used in molecular medicine and nanobiotechnology. As a result, now, this class of peptides has the potential to be used as carriers for drug delivery. Their properties, including alternating hydrophobic and hydrophilic amino acids and stable β-sheet nanofibers in aqueous solution, give them potential to encapsulate hydrophobic chemotherapeutics such as the anticancer drug pirarubicin. Three important features of ionic-complementary peptides are charge distribution, chain length as well as amino acids replacement (172).

The molecular structure of ionic-complementary peptides is characterized by an alternating arrangement of negatively and positively charged residues that must follow certain patterns. The three most simple and most studied types of charge distribution that are known facilitators of self-assembly are type I (−+ or +−), type II (−−++ or ++−−) and type IV (−−−−++++ or++++−−−−). Such ordered charge distribution promotes molecular self-assembly and results in nanostructure stabilization of ionic complementary peptides.

Therefore, ionic-complementary peptides have several important features which make them promising molecules for fabrication of materials that can form stable nano/microstructures

(74, 173). The following section reviews the ionic-complementary self-assembling peptides which will be used in this research.

2.10 EAK, EK and AC8 Peptides

The first member of the ionic-complementary peptide family is EAK16-II (EAK). This peptide is a 16 amino acid (AEAEAKAKAEAEAKAK), which has been studied since 1992 (174, 175). EAK was initially found in a region of alternating hydrophobic and hydrophilic residues in zuotin. Zuotin is a yeast protein that was initially identified for its affinity to left-handed Z-DNA. The EAK peptide consists of only three amino acids namely alanine (A), glutamine (E), and lysine (K).

EK8 peptide, as another member of ionic-complementary peptides, is an 8 amino acid peptide (EEKKEEKK), consisting of two ionic amino acids glutamine and lysine with possible enhanced water solubility and stable self-assembly. This peptide can help to investigate the effect of length of backbone, from 8 to 16 amino acids, and the presence of hydrophobic residue alanine while keeping the same charge distribution in compare with EAK.

The AC8 peptide consists of eight amino acids and contains a hydrogen bonding pair (QN), one ionic pair (EK) and four hydrophobic phenylalanine (F) side chains. Hydrophobic amino acids were incorporated to create a hydrophobic interior for encapsulation and stabilization of hydrophobic compounds such as anticancer drugs for drug delivery purposes.

However, because of limitations, when using such a self-assembling system for medical applications, a new class of ionic-complementary peptides, called amino acid pairing

peptides, has been designed (176). These limitations include but not limited to the fact that ionic complementarily has to be ensured which in turn would limit the peptide sequence design possibilities.

2.11 Amino Acid Pairing Peptide

The principle of amino acid pairing (AAP) peptides is that any self-assembling peptide can be designed through the pairing any of the three side chain interactions: hydrophobic, ionic, and hydrogen bonding among amino acid pairs. By this strategy, a large library of peptides can be made. Here, in this thesis, self-assembling peptides designed through the pairing of the side chains will be designated as “AAP peptides”. For example, in the case of AC8 (AAP8) with 8 amino acid residues, it utilizes the, three side chain interactions which would assist peptide association in addition to the backbone hydrogen bonding to form stable β-sheets. The specific side chain interactions in AAP8 are as follows: hydrophobic, electrostatic, and hydrogen bonding. The peptide contains four hydrophobic phenylalanine (F) side chains which provide strong hydrophobic interactions assisted by π stacking to stabilize the assembled nanostructures as well as to create a hydrophobic region to carry hydrophobic compounds for drug delivery applications. It also contains a hydrogen bonding pair (QN) which can increase the overall hydrophobicity and should not be significantly affected ,by solution pH and charge screen agents (i.e., salts) compared with the ionic pairing by K and E. An ionic pair (EK) which provides reasonable water solubility has also been incorporated into AAP8. The non-charged hydrogen bonding pair QN together with the ionic pair EK is expected to further enhance the stability of the assemblies. Through this design, the AAP8

peptide can self-assemble into β-sheet rich nanofibers with the potential to carry hydrophobic drugs (176).

Therefore, with respect to their advantages and potential as drug carriers, the necessity of understanding biocompatibility of these peptide based nanoparticles is undeniable, as bioincompatibility is the one drawback these systems can face. Hence, the study of this nanoparticle's interactions with blood components and immune cells, as well as its toxic effects, should be seriously considered in order to translate the use of peptide based nanoparticles from research to preclinical or clinical studies.

2.12 Regulatory Agencies

It was previously mentioned that drug delivery systems, no matter how attractive they seem, hold no weight unless they are considered adequately biocompatible. The same is true without approval from a regulatory agency such as the FDA or the European Medicines Agency (EMEA). These two qualifiers are often a function of one another. While existing guidelines awkwardly govern the use of nanomedicine, additional regulations are required to address the properties specific to nanomaterials, be it immune system or surface chemistry modification. As novel applications of nanotechnology in medicine and requests for approval continue to flow from research institutes worldwide, the need for nanotechnology-specific regulatory guidelines is made even more obvious. Regardless, standardized guidelines have yet to be established. On the whole, continued *in vitro* and *in vivo* testing is required to build a database of knowledge on the subject of nanoparticle biocompatibility. Only then, after sufficient

scientific evidence, will regulatory agencies put forth the effort of developing new guidelines (109).

2.13 Conclusions

This chapter was intended to provide an overview of recent findings of biocompatibility for several different nanoparticles. Biocompatibility is a word that is used broadly within biomaterial science, but there is still a great deal of uncertainty about its meaning as well as about the mechanisms that collectively constitute biocompatibility. Effective and biocompatible drug delivery systems based on nanoparticles as a carriers has been the dream of scientist for many years. Although we are still far from our ultimate goal of biocompatible drug delivery, progress which points to the growing importance of this research area in related to human health has been made. As biomaterials are being used in increasingly diverse and complex situations, with applications now involving tissue engineering, invasive sensors, RNA interference (siRNA) delivery and of particular interest to this thesis, drug delivery, uncertainty over the mechanisms of, and conditions for, biocompatibility is becoming a serious obstacle to the development of new techniques.

Chapter 3*

In Vitro Biocompatibility of theAC8 peptide and its potential use as a drug carrier

3.1 Introduction

Since the first application of biomaterials as conventional therapeutics, there has been tremendous interest in materials capable of sustained macromolecular delivery (177, 178). As research has progressed, modern pharmacotechnology has evolved such that the main goal now is to improve the therapeutic efficacy and controlled targeted release of drugs by using a variety of nanoparticles as carrier systems (179-185). Recent years have witnessed a expansion of the use of nanoparticles for drug delivery applications (186-189). Given their advantages and potential, the necessity of understanding biocompatibility of nanoparticles is undeniable, as bioincompatibility is the one drawback these systems can face. Hence, the study of nanoparticle interaction with blood components and immune cells, as well as its toxic effects, should be considered in order to translate the use of nanoparticles from research to preclinical or clinical studies. Haemolysis, immune system responses such as complement activation, and cytotoxicity are the main concepts considered for nanoparticle biocompatibility.

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* This chapter is based on submitted paper Sheva Naahidi, Yujie Wang , Man Zhang, Rong Wang, Mousa Jafari ,Yongfang Yuan *, Brian Dixon, P. Chen**, Evaluation of Biocompatibility of the AC8 peptide and its potential use as a drug carrier

Erythrocytes occupy a larger volume fraction of the blood than leukocytes and lysing of excessive number of red blood cells can lead to anemia; therefore, *in vitro* examination of haemolysis is an instrumental part of preclinical studies of nanoparticles (190). Additionally, recent ongoing research, for instance work by Liangfang Zhang's group, has focused on using or mimicking erythrocytes as a drug delivery system (191). A number of mechanisms for drug-mediated haemolysis have been recommended, yet the true mechanism is yet to be identified. It is now well known that surface properties (especially surface charge) play an important role for nanoparticles and can directly damage erythrocyte membranes. For instance, in the presence of certain concentrations of unprotected primary amines (positive charge), red blood cell damage was observed on the surface of poly-amidoamine, carbosilane, polypropylene imine, and poly-lysine (115, 192-196).

Another important aspect that greatly affects the delivery of drugs is their uptake by immune cells and clearance from intended target sites. It is now well known that the complement system, which plays a crucial role in this process, consists of more than 20 different plasma proteins and protein fragments as inactive zymogens in the blood (197, 198). Complement system activation results in the activation of the cell-killing membrane attack complex (MAC) (199). There are three major complement pathways that may become activated: the classical, alternative and lectin pathways. One pathway that was recently discovered but not fully understood is the ficolin innate immune recognition (200). Ficolins are oligomeric defense proteins that bind to acetylated molecules as well as neutral carbohydrates that trigger the lectin pathway (201, 202). In the classical pathway, the C1q

protein recognizes and attaches to antibody bound to the pathogen, C1r and C1s, through electrostatic and hydrophobic interactions. The lectin pathway is activated via mannan-binding lectin (MBL) binding to neutral sugar residues, while the alternative pathway is started by C3b protein binding to the hydroxyl or amino groups on a pathogen's surface (203-205). Recent studies have recognized the importance of nanoparticle surface charge in activation of the complement system. For instance, polypropylene sulfide nanoparticles, lipid nanocapsules, cyclodextrin-containing polycation-based nanoparticles and polystyrene nanospheres have shown that charged nanoparticles activate the complement system more efficiently than neutral nanoparticles (206-208).

Indeed the ability of a nanoparticle to evade immune recognition represents an area of interest in the field of drug delivery. One of the promising approaches towards this goal has been through the development of the self-assembled peptide nanomaterials due to their perceived biocompatibility and structural diversity. A new, promising class of self-assembling peptides is the self-assembling ionic-complementary peptides (209, 210). The self-assembling peptide AC8 nanoparticle is a short (eight amino acids long) peptide with the sequence Ac-FEFQFNFK-NH_2 which self-assembles into β-sheet-rich nanofibers. The schematic of the molecular structure of this peptide is shown in Figure 3-1.

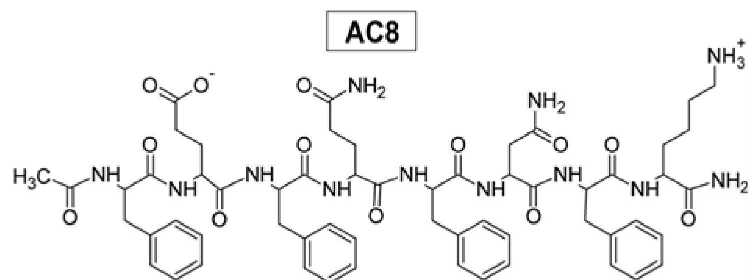

Figure 3-1 Schematic of the chemical structure of AC8. Abbreviations: AC8: Ac-FEFQFNFK-NH2.

The capability of the AC8 to stabilize the hydrophobic anticancer drug ellipticine in aqueous solution has been already investigated (211). However, the biocompatibility properties of the AC8 have not yet been explored, which is a necessary first step in systematic drug delivery application.

Here, the systematic investigation of the *in vitro* biocompatibility and immunocompatibility properties of self-assembling ionic-complementary peptide AC8 was reported. The potential ability of this peptide to be utilized as a carrier for the delivery of the hydrophobic anticancer drug pirarubicin was demonstrated. Nuclear uptake of AC8-pirarubicin complexes by A549 cells was shown by confocal microscopy. Finally, it wasdemonstrated that AC8 peptides had favorable *in vitro* biocompatibility.

3.2 Materials and Methods

3.2.1 Peptide preparation

The peptides AC8 (purity>98%) were purchased from CanPeptide Inc. (Montreal, Canada) and used without further purification. The primary structure of AC8 is FEFQFNFK and its

secondary structure is beta-strand. The N-terminus and C-terminus of the peptides were protected by acetyl and amino groups, respectively. AC8 was characterized for its self-assembling properties in aqueous solution at 0.5 mg/ml and a molecular weight of 1147.31 g/mol was confirmed by mass spectroscopy. Fresh aqueous peptide solutions was prepared by dissolving peptide powder in pure water (18.2 MV, Milli-Q A10 synthesis) at specified concentrations, followed by sonication for 10 min.

3.2.2 Cytotoxicity Assay

Non-small cell lung carcinoma cells A549 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) cell proliferation and viability assay kit (TOX1 from Sigma-Aldrich, Oakville, ON, Canada) was used to perform cytotoxicity assays. A549 cells were seeded onto 96-well plates at a density of 10^4 cells per well and treated 24 h later. 20μL of the AC8 peptide was diluted in 180μL of growth media (DMEM containing 10% FBS) and added to each well. DMEM served as the blank and untreated cells served as the control. The plates were incubated for 24 h and 48 h prior to performing the cell viability assay. Then, 100 μL of MTT substrate was added to each well and incubated for an additional 4 h at 37°C in the dark before addition of 100 μL solubilization. Absorbance was measured at a wavelength of 570 nm using a microplate reader (model 550; Bio-Red, CA, USA). Cell survival was expressed as a percentage of the absorbance value determined for control cultures using the following calculation: Viablility = (sample absorbance – blank absorbance) / (negative absorbance - blank absorbance).

3.2.3 Haemolysis Studies

Haemolysis experiments were carried out by using approximately 5mL of fresh human anticoagulated blood. The blood was centrifuge at 1500rpm for 10 minutes and washed three times by adding Dulbecco's Phosphate Buffered Saline (DPBS). After washing, red blood cells (RBCs) were isolated, suspended in DPBS to the original volume and then diluted further with DPBS to make a 5% hematocrit solution. Varying concentrations of the peptide in 800 µl of DPBS were added to the 200 µl of RBCs. DPBS and water served as positive and negative controls, respectively. After incubation for two hours all of the samples were then centrifuged after which the supernatant was removed and placed into a clean cuvette. Subsequently, the absorbance was measured at 541nm. The haemolysis in the water solution was considered to be 100% while the hemolysis in the DPBS solution was taken as 0%. The results were thus expressed as percentage hemolysis.

3.2.4 Complement system activation studies

In order to determine the amount of complement activation caused by the AC8 peptides *in vitro*, the active forms of four complement products (C4d, SC5-b9, Bb and MBL) were analyzed using enzyme-linked immunosorbent assay kits from Quidel Corp, (San Diego, CA, USA) and ALPCO Diagnostics kit (Salem, MA, USA). Peptide samples were incubated with human serum at a volume ratio of 1:5 in a shaking incubator (100 rpm) for 60 minutes at 37°C. Three time points were measured (1-3 hours); However, there was no increase at later time points. The samples were then diluted further with Sample Diluent according to the manufacturer's instructions to determine the amount of C4d, Bb, SC5b-9 or mannan-binding

protein that was formed by the complement system during the incubation. The C4d immunoassay measures the amount of active C4 fragments present in human serum which indicates the activation of the classical pathway. The Bb Plus immunoassay assesses the amount of Factor B cleavage that occurs, providing a measure of the activation of the alternative pathway. SC5b-9 measures the Terminal Complement Complex which is formed by either the classical, alternative or lectin pathways. MBL is the protein which indicats that the lectin pathway has been activated.. Zymosan (a known complement activator) was used as a positive control and untreated human serum was used as a negative control; both were assayed in the same way as all the other samples. The results were then read at 450 nm (FLUOstar OPTIMA, BMG, NC). Standard curves were made for quantification of complement activation products by using the assigned concentration of each individual standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best fit line for SC5b-9, Bb, C4d and MBL standard curves were within the manufacturer's specified range. The efficacy of the treatments was determined by comparison with baseline levels using the paired *t*-test.

3.2.5 Anaphylotoxin studies

In order to assess the amount of C3a and C5a, samples were incubated with human serum in a 1:5 ratio in a shaking incubator (100 rpm) for 60 minutes at 37°C. The samples were then tested according to manufacturer's instructions for the enzyme-linked immunosorbent assay kits from Quidel Corp. (San Diego, CA, USA). The results were then read at 450 nm (FLUOstar OPTIMA, BMG, NC). Since C3a is factor produced by the activation of the

classical, alternative or lectin complement pathways, Zymosan, from Quidel Corp. (San Diego, CA, USA), was used as the positive control and human serum was used as the negative control.

3.2.6 Statistical analysis

Statistical analysis was carried out using ANOVA or Student's t-test where applicable. *In vitro* experiments were repeated at least three times. Differences were considered significant when the p value was less than 0.05.

3.3 Results and Discussion

3.3.1 Cytotoxicity of peptide

The cellular toxicity of AC8 was determined by MTT assay as shown in Figure 3.2 The cellular viability in the high AC8 dose group (0.1 mg/mL) at both 24 and 48h time intervals were significantly lower than the non-treated cell group (0.88±0.03 and 0.62±0.03, respectively). In addition, the cellular viability of AC8 in the low dose group (0.025 mg/mL) at 48h was also significantly lower than the non-treated cell group (0.76±0.07). However, the therapeutic dose of AC8 at 24 hour did not show any impact on cellular viability. These results suggested that AC8 at therapeutic concentration was biocompatible whereas at high concentrations it significantly affected cell viability of A549 cells compared with non-treated cells. Although with increasing incubation time and dose, the anti-proliferative effect of AC8 became more significant, Figure 3-2, it is safe in low dose as a drug carrier.

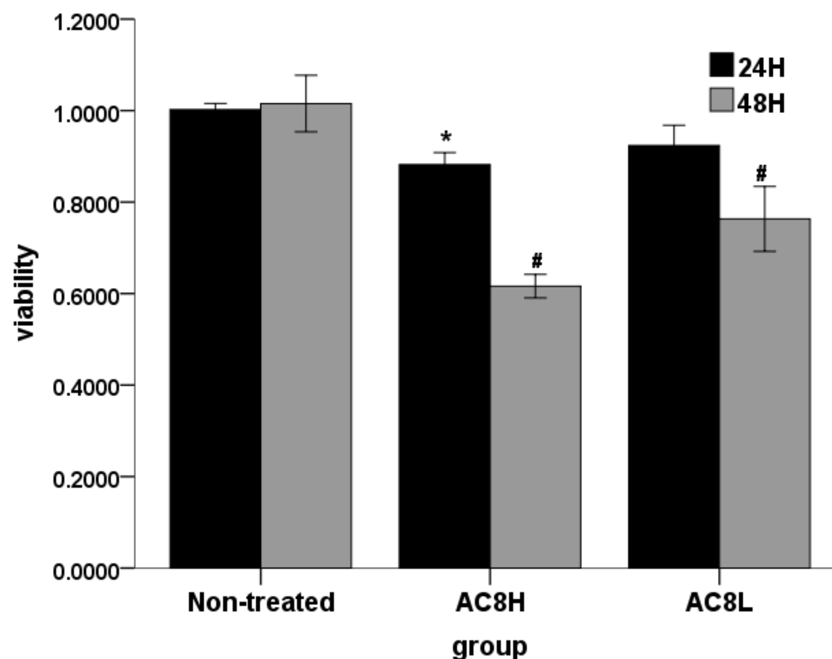

Figure 3-2 **Cytotoxicity of AC8 was measured against the lung cancer cell line A549. Three independent experiments were performed for each data point. AC8H represent group treated with high AC8dose (0.1 mg/mL) and AC8L represent group treated with low AC8dose (0.025 mg/mL). Error bars represent standard error of mean (SEM) of three independent experiments*: p<0.05 VS non-treated group in 24 hours; #: p<0.05 VS non-treated group in 48 hours.**

Although, it is preferable for carriers in a drug delivery system to have inert biological activity, it would be of interest if AC8 would contain anti-tumor activity similar to the drug for pharmacological research. In this work, it was concluded that in addition to its safety as a drug carrier, when overdose, AC8 demonstrated anti-tumor activity- at high doses.

3.3.2 Haemolysis

Hemolysis is the breakage of the RBC's membrane, causing the release of hemoglobin and other internal components into the surrounding fluid; therefore, it is a crucial factor in evaluating the biocompatibility of biomaterials. It is also a common occurrence seen in serum

samples and may compromise the laboratory's test parameters. When determining the hemolytic activity, a ratio of 5% or less haemolytic activity is considered biocompatible (212). In the present study the haemolytic potential of untreated human blood with the self-assembling peptide AC8 was investigated to see the possible hemolytic effect on RBC.

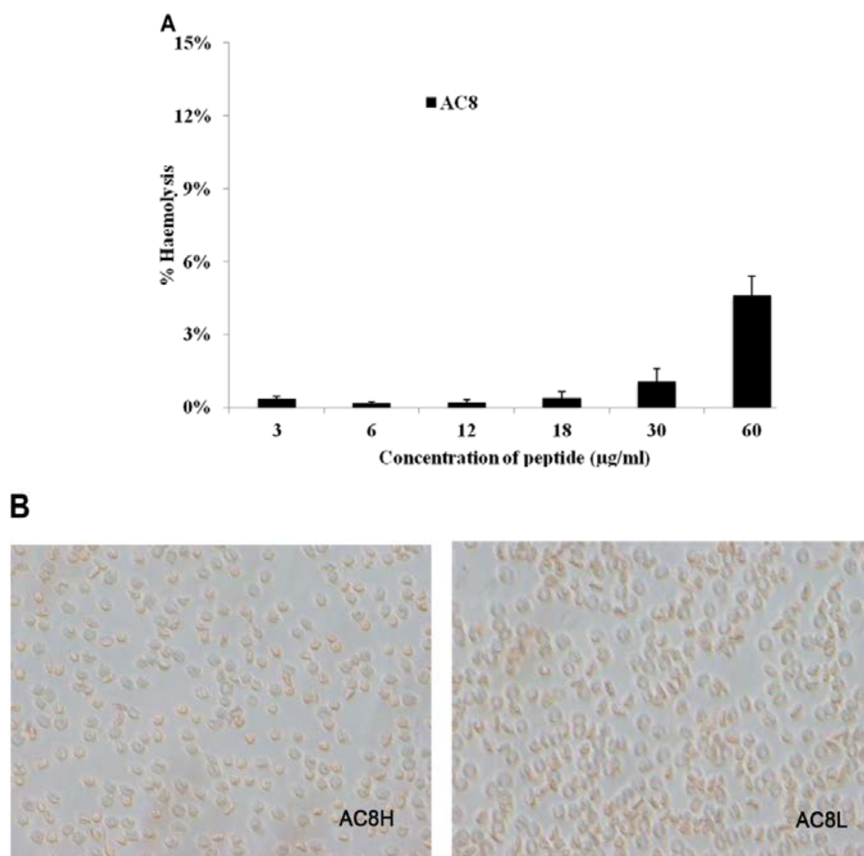

Figure 3-3 (A) Haemolytic activity of AC8. Error bars represent SEM of three independent experiments (B) Detection of RBC aggregation by light microscope (400X). AC8H represent group treated with high AC8 dose (0.1 mg/mL) and AC8L represent group treated with low AC8dose (0.025 mg/mL).

As shown in Figure 3-3A, the results of the *in vitro* hemolytic assay showed that the self-assembling peptide AC8 has no hemolytic activity within the therapeutic range of concentrations. However, at concentration as high as 0.1mg/ml, AC8 showed some haemolytic activity. Nevertheless, in terms of aggregation, there was no RBC aggregation with the AC8 peptides, neither at high nor low concentration, Figure 3-3B. Therefore, while both monomers and polymers are present in samples aggregates are not. This is important because aggregates are probably more likely to induce immune responses if they get inside the cell – HSPs will bind them and induce responses. These observations confirm that AC8 is not hemolytic and do not cause erythrocyte aggregation within the therapeutic range of concentrations.

3.3.3 Complement system activation

The complement system, as one of the key effector mechanisms of humoral and innate immunity, consists of several, generally inactive, serum and cell surface proteins which are activated only under particular conditions to generate products that mediate a variety of effector functions. The classical pathway uses a C1 plasma protein to detect IgM, IgG1 or IgG3 antibodies attached to the surface of a microbe or other structures. The alternative pathway is elicited by the spontaneous cleavage of the thioester bond in C3 ("C3-tickover") or when the internal thioester bond in the α-chain of nascent C3b go through nucleophilic attack by the direct detection of a microbial surface structure rich in nucleophilic groups (particularly hydroxyl- and amino-rich surfaces) (213, 214). The lectin pathway is activated by an MBL plasma protein which identifies terminal mannose residues on microbial

glycoproteins and glycolipids. Therefore, not surprisingly, the majority of the consequences of complement activation result from the pharmacological effect of some of the activated components. Accordingly, clarification of the potential to activate complement can be used as one decisive factor in testing the biocompatibility of a variety of nanoparticles. Different types of nanoparticles, especially polymers, have been used in medicine for targeted or controlled release of various drugs and diagnostic agents, of which many have been assessed for complement activation (215-219).

Here, the effect of AC8 peptide on the three major complement pathway is reported. After incubation with human serum, Bb and C4d concentrations were measured to assess the complement system activation via alternative and classical pathways. As shown in Figure 3-4A, complement activation via the classical pathway was not observed for AC8 peptides. Classical activation can be due to binding of C1q (main recognition unit of classical pathway) to the peptide via electrostatic or hydrophobic interactions (220).

The MBL pathway was assessed using a kit that measures the amount of functional MBL oligomer remaining in the solution. When the pathway is activated, the oligomer will bind to the surface of the invading particle rendering it dysfunctional. After incubating the samples with human serum for an hour to allow time for the oligomer to bind to the surface of the peptides and peptide complexes, the amount of remaining functional MBL oligomer was measured. Figure 3-4C shows that for the positive control, there is very little functional MBL oligomer remaining and for the negative control all the functional MBL oligomer remains. For our samples, there is activation for AC8 peptides, although it is less than the positive

control, Figure 3-4C. While there was lectin pathway activation; nevertheless, it did not reach the "threshold" level of activation required for the next step of complement activation because there was no significant Bb activation, Figure 3-4B.

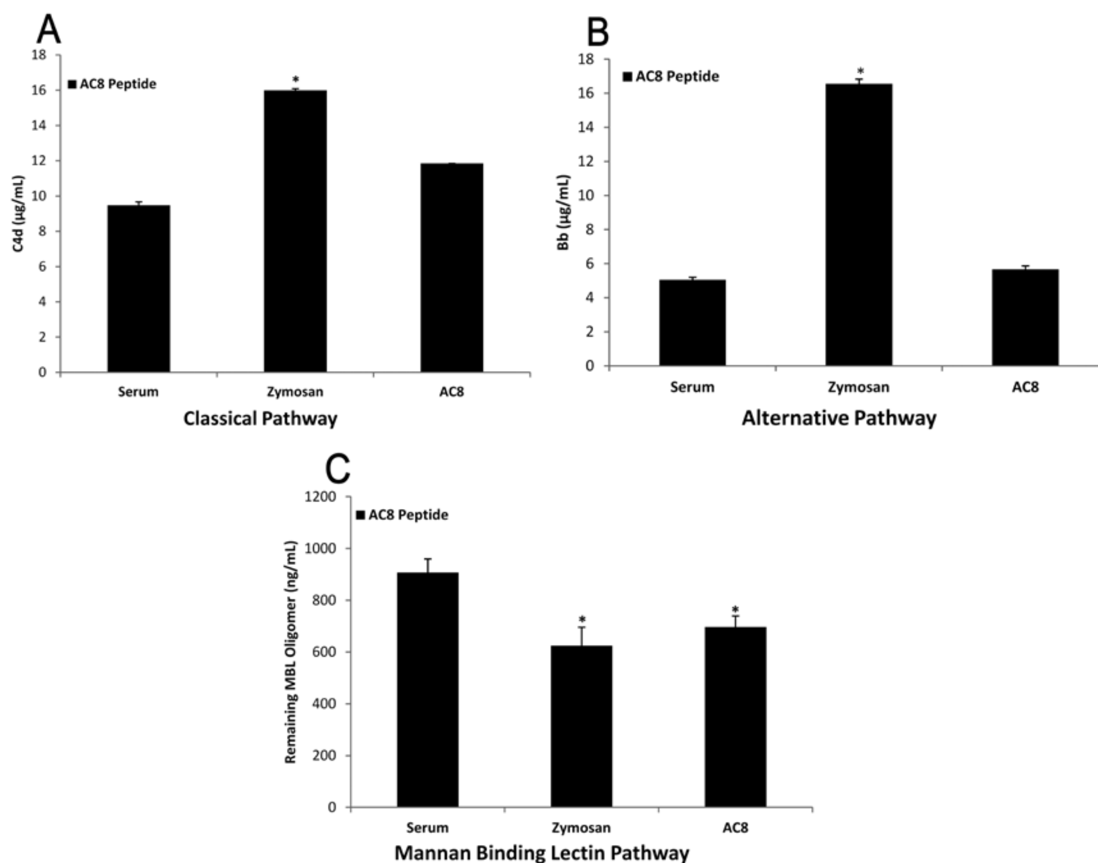

Figure 3-4 Complement activation of classical (A), alternative (B), and mannan binding lectin (C) pathways of the AC8s (60 µg/ml). Error bars represent SEM of three independent experiments. * denotes statistically significant difference compared to the negative control (human serum), as determined by two-sample t-test ($p<0.05$). Abbreviations: SEM: standard error of mean.

3.3.4 Anaphylotoxin activation

Upon either classical, lectin or alternative pathway activation, the C3 protein is split into two fragments, C3b and a potent anaphylotoxin C3a. Therefore, the presence of C3a in a test sample proves that peptide nanoparticles can activate complement by one or both pathways.

On the other hand, C3b can participate in the formation of a new enzyme, the C5 convertase, which cleaves C5 to C5b which drives the rest of the common terminal pathway, and C5a, also a very potent anaphylotoxin. One outcome of C5b production is cell killing by forming a MAC. However, a large amount of the C5b generated with *in vitro* samples is diverted to the fluid phase by reacting to S protein to form soluble SC5b-9 which can be measured as proof of terminal pathway activation. An important point is that for each mole of detected SC5b-9, an equal number of moles of C5a are generated. C5a is a small, sticky and exclusive molecule with a short half-life. In contrast, SC5b-9, as a marker for C5 cleavage, is an extremely stable soluble macromolecular complex and can be used as a marker for C5a anaphylotoxin generation. Accordingly, to further assess the *in vitro* extent of NP-induced activation of complement, SC5b-9, C3a and C5a were evaluated. SC5b-9 concentration was measured after incubation with human serum.

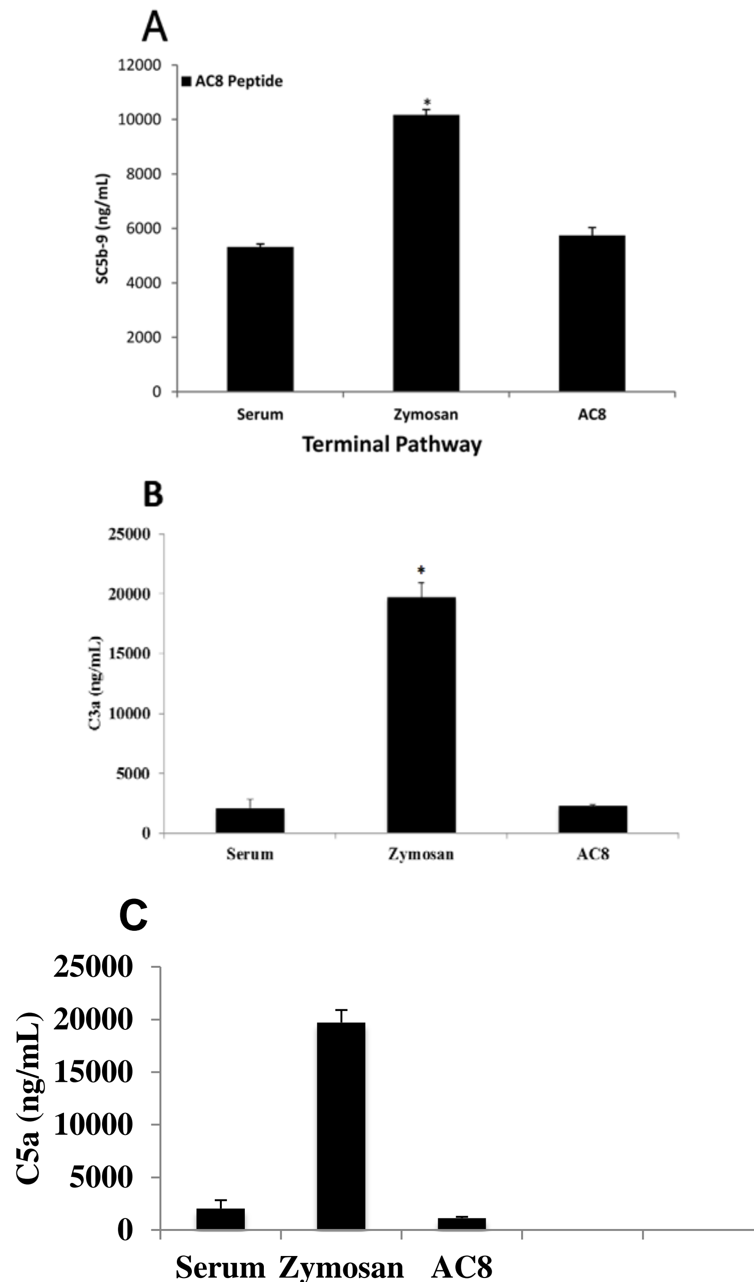

Figure 3-5 (A) Complement activation of the terminal pathway by the AC8 (60 µg/ml). Bars represent the mean concentration of SC5b-9, a biomarker of C5a through both the classical and alternative pathways. (B) Anaphylatoxin C3a assay comparing the concentration of C3a in AC8 treated samples, and positive (Zymosan) as well a negative control (Human serum). (C) Anaphylatoxin C5a assay comparing the concentration of C5a in AC8 treated samples, and positive (Zymosan) as well a negative control (Human serum). Error bars represent SEM of three independent experiments. * denotes statistical significance compared to the negative control, as determined by two-sample t-test ($p<0.05$). Abbreviations: SEM: standard error of mean.

As shown in Figure 3-5A, it was found that AC8 formulations did not activate the terminal pathway of complement system with respect to human serum (negative control) and were significantly less than Zymosan (positive control). Similar results were observed, as expected, in regards to C3a and C5a anaphylotoxin, Figure 3-5B, C. In summary, the ionic, complementary self-assembling peptide AC8 did not activate the complement system *in vitro* highlighting the potential of this peptide to be used as a drug delivery system.

3.3.5 Confocal microscopy

Confocal fluorescent microscopy confirmed the nuclear uptake of anti-cancer drug pirarubicin (tetrahydropyranyladriamycin) when complexed with the peptides as shown by its co-localization with the nucleus (Figure 3-6 A-C).

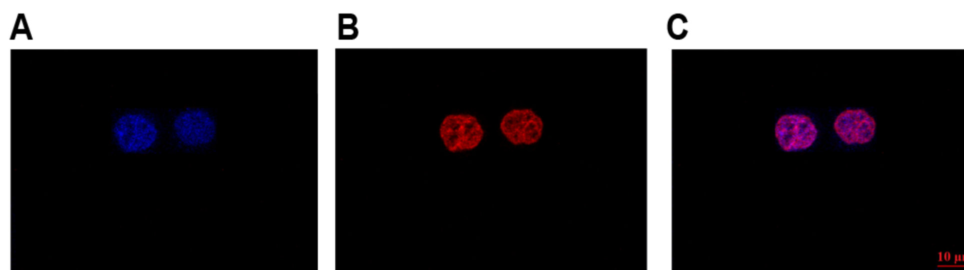

Figure 3-6 Confocal microscopy of A549 cells treated with AC8-pirarubicin complexes confirming the presence of pirarubicin inside the nucleus. Violet nucleus (C) implies the co-localization of pirarubicin (red) (B) and DAPI (blue) (A) at the same confocal layer. Abbreviations: DAPI: 6-diamidino-2-phenylindole.

Pirarubicin's hydrophobicity and fluorescence characteristics make it a good candidate to be used for our drug delivery system using AC8 as its carrier. The peptide mediated the nuclear uptake of pirarubicin, represented as violet areas (merged), as the result of localization of

pirarubicin (red) in stained nuclei (blue). It is worth mentioning that the range of concentrations used in all of conducted experiments highly exceeds the dose considered for therapeutic purposes (*i.e.* < 2.5μg/ml).

3.4 Conclusions

The *in vitro* biocompatibility of the complementary, ionic self-assembling peptide AC8 was systematically investigated. It was found that the AC8 peptide showed potential *in vitro* compatibility in terms of cytotoxicity, haemolytic activity, RBC aggregation and anaphylotoxin as an end result of complement activation. The AC8 peptide itself could significantly inhibit the proliferation of A549 cells at a high dose. Hence, AC8 could be considered as a potential anti-tumor drug in the future. It is worth mentioning that good nuclear uptake of pirarubicin was also demonstrated using AC8 as a carrier.

The *in vitro* data presented here is helpful in predicting the effects of AC8 peptide *in vivo*. Indeed, as more mechanistic studies take place in this regard, a more complete picture can be proposed for the use of AC8 in drug delivery. Based on all of *in vitro* data presented here, the AC8 peptide would be a favorable drug delivery vehicle - if treatment adheres to the recommended therapeutic concentration- for future studies.

Chapter 4[*]

Immuno- and hemocompatibility of amino acid pairing peptides and potential use for anticancer drug delivery

4.1 Introduction

One of the goals of biomaterial research is to develop novel, yet safe, therapeutics and therefore materials possessing the ability for sustained macromolecular delivery have been extensively studied (221, 222). Modern pharmacotechnology now seeks to improve the therapeutic efficacy and controlled targeted release of drugs by using a variety of nanoparticles as carrier systems (181, 182, 185, 223, 224). Given their advantages and potential in other aspects of drug delivery, the necessity of understanding biocompatibility of nanoparticles is unquestionable. . Indeed, a detailed assessment of the biocompatibility of nanoparticles is crucial for the safe development of therapeutic carriers, to identify the smallest possible defence reaction in the recipient. In order to fulfill these, experimental studies of haemolysis, complement activation, and cytotoxicity on human blood are required to determine a full picture of an *in vitro* biocompatibility study (225).

Then, all of these data together can rationalize whether *in vitro* data are related to effects would be observed *in vivo* and how to translate these data into a predicted response in human patients. As a result, these experiments are critical and essential towards our efforts to develop a novel nanomedicine approaches to effective delivery system. Indeed, as more mechanistic studies are taking place in this regard, a more complete picture can be proposed which assumes paramount importance.

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[*] **This chapter is based on a paper** Sheva Naahidi, Mousa Jafari, Megan Logan, Faramarz Edalat, Brian Dixon, P. Chen* **Immuno- and hemaocompatibility of amino acid pairing peptides and potential use for anticancer drug delivery (submitted)**

(225). For instance, some studies have shown that surface properties of nanoparticles can directly damage erythrocyte membranes and the presence of surfactants increases the amount of haemolysis; while modification with poly (ethylene glycol) (PEG) or PEGylation decreases the amount of haemolytic activity (197, 229). Another important aspect that greatly affects the application of nanoparticles as intravenous drug delivery platforms may rely on avoiding fast elimination from the systemic circulation by cells of the immune system. As soon as nanoparticles enter the bloodstream, they meet a complex environment of plasma proteins and immune cells. Nanoparticle uptake by the immune cells- can be facilitated by the adsorption of opsonins to the particle's surface - may occur both in the blood stream and in tissues through various pathways. This uptake can make a nanoparticle to be routed away from the site of its intended application and significantly decrease the number of nanoparticles available to reach the target site. Consequently, the efficacy of the drug will be efficiently dropping. As such, understanding nanoparticle immunocompatibility is an important step during the initial characterization of nanomaterials. As a first step to validate the use of any nanoparticle to be used as a carrier for drug delivery system, their interaction with a part of the human immune system, complement, has to be explored.

The complement system is part of the human innate immune system and consists of different plasma proteins and protein fragments as inactive zymogens in the blood (225). The complement system activates- by three major complement pathways- when zymogens stimulate receptors resulting in activation of the cell-killing membrane attack complex (MAC) (199). Salvador *et al.* reported that non-functionalized, high pressure carbon monoxide single-walled and double-walled carbon nanotubes activate the complement system (204), but

those whose surface is functionalized with PEG can depress or prevent such immunological responses. In contrast, other types of PEGylated nanoparticles such as clinically approved formulations of PEGylated liposomes (Doxil®) have shown that steric hindrance of PEG may not hinder complement activation and fixation (94, 236, 237). For instance, use of Doxil® in a large number of human recipients has caused immediate acute pseudo-allergic reactions and cardiopulmonary distress. These conditions are related to generation of anaphylatoxins C3a and C5a, which in turn caused release of thromboxane A2 and other inflammatory mediators from immune cells (238).

Indeed the construction of nanoparticles that are documented as self or there is a lack of immune recognition, represents a major area of interest in the field of drug delivery.

One of the promising approaches towards this goal has been through the development of self-assembled peptide nanomaterials which have gained a great amount of attention due to their possible biocompatibility and structural diversity. As a result, they have become attractive as building blocks for a variety of applications such as materials science, tissue engineering, bioengineering, and of particular interest to this thesis, drug delivery (239-248).

Recently, a new class of self-assembling peptide, the amino acid pairing peptide (AAP8) nanoparticles has been described (249). AAP8 is a short (eight amino acid) peptide with the sequence Ac-FEFQFNFK-NH_2, (NP), which self-assembles into β-sheet-rich nanofibers. In aqueous solution, the capability of the NPs to stabilize the hydrophobic anticancer drug ellipticine has been already investigated (247). Functionalization by the conjugation of a diethyl glycol (DEG) to one end of the C-terminal or both terminals (C- and N-terminals) of

the peptide AAP8—herein called as NP-I and NP-II, respectively has been reported (250), as shown in Figure 4-1.

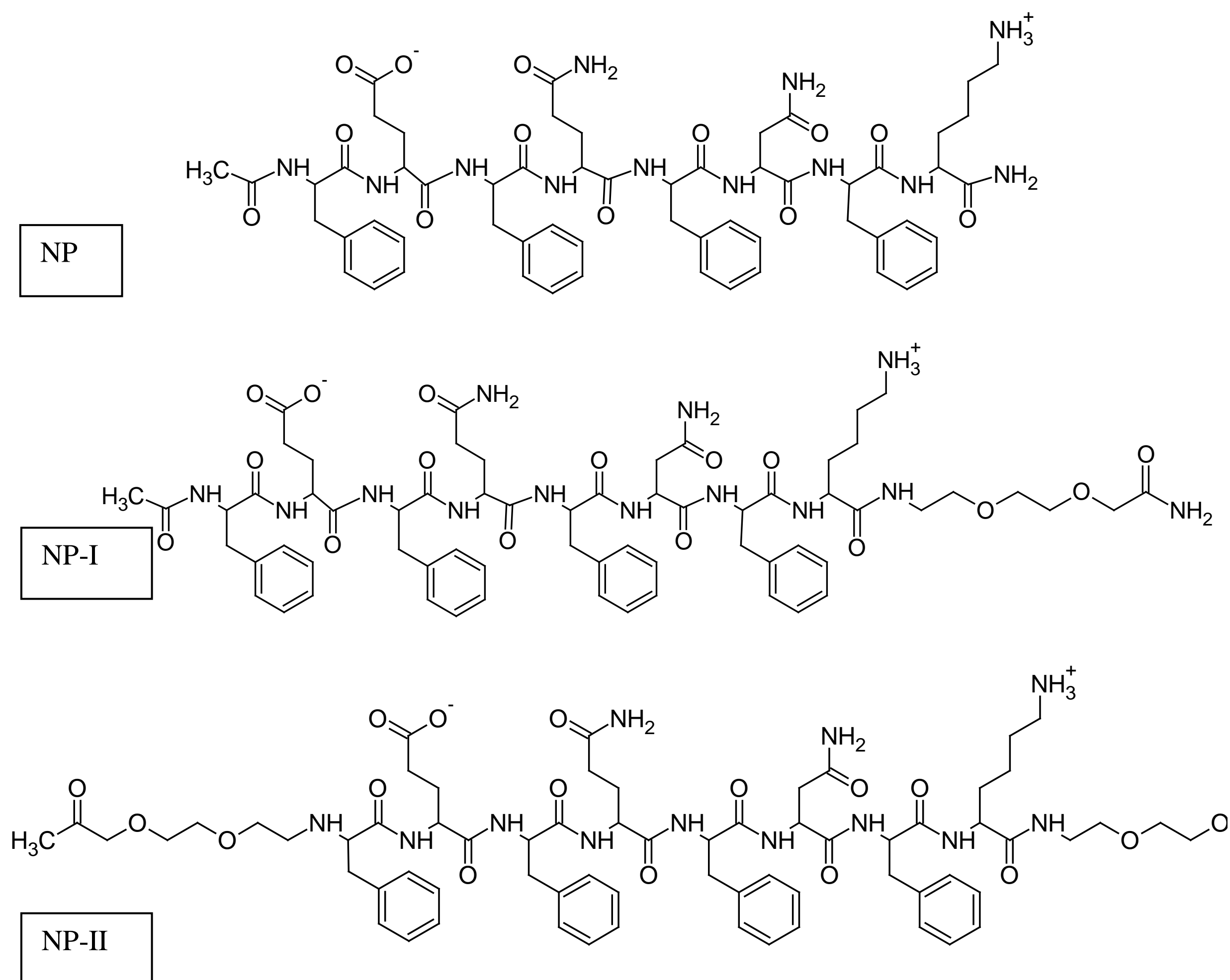


Figure 4-1 Schematic of the chemical structure of NP (Ac-FEFQFNFK-NH_2) and DEGylated forms NP-I (Ac-FEFQFNFK—DEG-NH_2) and NP-II (Ac-DEG-FEFQFNFK-DEG-NH_2).

Additionally, it has been shown that the DEG conjugate of AAP8 is associated with increased solubility, stability, and secondary structure β-sheet content of the peptide as well as reducing cellular toxicity (182). In our previous study, AAP8, was shown to be a possible carrier for anti-cancer drug delivery. Yet, the peptide had a propensity to initiate toxicity at higher concentration (0.1mg/ml). However, the biocompatibility properties of the AAP8 (NPs) in comparison with modified versions, NP-I and NP-II, have not yet been explored, which is a necessary first step in systematic drug delivery application.

Pirarubicin (tetrahydropyranyladriamycin), originally synthesized in Japan, is an anthracycline antibiotic (a derivative of adriamycin), developed to substitute for doxorubicin which was first introduced in 1979 (251-253). It is not only more effective against various tumors, but also less cardiotoxic (254-256). In addition to its anticancer benefits, pirarubicin's hydrophobicity and fluorescence characteristics make it a good candidate to be used to test in this study.

In light of the above information and literature , this study investigats the interaction of AAP8 (NP), its modified versions (NP-I and NP-II) and complexes of all three with pirarubicin with the complement system *in vitro*. The systematic investigation of the biocompatibility properties of AAP8 peptide nanoparticles: NP, NP-I and NP-II is reported The potential ability of these peptides to be utilized as carriers for the delivery of the hydrophobic anticancer drug pirarubicin is also showed.

4.2 Materials and Methods

4.2.1 Peptide-drug complex preparation

The peptide AAP8 (Ac-FEFQFNFK-NH2) and the two diethylene glycol (DEG) (–NH–CH2–CH2–O–CH2–CH2–O–CH2–CO–) conjugated versions, NP-I: Ac–FEFQFNFK–DEG–NH2 and NP-II: Ac–DEG–FEFQFNFK–DEG–NH2, were purchased from CanPeptide Inc. (Montreal, Canada). They were characterized for their self-assembling properties in aqueous solution at 0.5 mg/ml. A molecular weight of 1147.31 g/mol for AAP8, 1293 g/mol for Ac–AAP8–DEG–NH2, and 1437 g/mol for Ac–DEG–AAP8–DEG–NH2 were confirmed by mass spectroscopy. The purity of the peptides were verified as >98% by liquid chromatography–mass spectrometry. Fresh aqueous peptide solutions were prepared by dissolving peptide powder in pure water (18.2 MV, Milli-Q A10 synthesis) at specified concentrations, followed by sonication for 10 min. The anticancer agent pirarubicin (99.8% pure) was purchased from Sigma–Aldrich (Oakville, Canada) and used as received. Cell culture media, low glucose Dulbecco's modified Eagle's medium (DMEM), and phosphate-buffered saline (PBS) were purchased from HyClone (Ontario, Canada). Fetal bovine serum (FBS) and trypsin–EDTA were purchased from Invitrogen Canada Inc. (Burlington, Canada).

4.2.2 Peptide-pirarubicin complex preparation

To make the peptide-pirarubicin complexes, a pirarubicin stock solution was first prepared in pure tetrahydrofuran (THF). Aliquots of pirarubicine-THF were then transferred into 1.5 ml centrifuge tubes, and dried in a gentle stream of filtered air (0.22 mm pore size filter) to form a thin film at the bottom of tubes. The fresh peptide solutions were added into the tubes containing pirarubicin, to make peptide-pirarubicin complexes consisting of 0.3 mg/ml of

each peptide and 0.1 mg/ml pirarubicin, followed by continuous probe sonication at 6W power for 10 min. A control of pirarubicin in pure water (without peptide) at the same concentration was prepared for comparison. In order to study the toxicity of the complexes at a range of concentrations, serial dilution of the complexes in water (2, 4, and 8 times) was carried out, while keeping the peptide-pirarubicin ratio at 3:1.

4.2.3 Cytotoxicity assay

The Cell Counting Kit-8 (CCK-8; Dojindo, Japan) was used to perform cytotoxicity assays. A549 cells (ATCC, USA), a human non-small lung cancer cell line, were seeded onto 96-well plates at a density of 8×10^3 cells per well and treated 24 h later. 20µL of the peptide-drug complex was diluted in 80µl of growth media (DMEM containing 10% FBS) and added to each well. 48 h after treatment, 10µl of CCK-8 substrate was added to each well and incubated for an additional 2 h at 37°C in the dark. Absorbance was measured at a wavelength of 450nm with a reference wavelength of 620nm using a microplate reader (FLUOstar OPTIMA, BMG, NC).

4.2.4 Fluorescence, flow cytometry, and confocal microscopy

The fluorescence of pirarubicine was detected by FL2 channel (585/42 nm bandpass filter). At least 15000 events were recorded for each sample. The cellular uptake of the peptide-drug complexes by A549 cells was quantified via flow cytometry (BD Biosciences, BD FACS Vantage SE Cell Sorter, USA). Briefly, A549 cells were seeded in 24-well plates at a density of 4×10^4 cells per well, and allowed to attach for 24 h. Then the cells were washed with Phosphate Buffered Saline (PBS) and treated with peptide-drug complexes at a drug

concentration of 20µg/ml. After 20 min incubation, the cells were washed three times with PBS, detached from the culture wells by incubation with 0.25% trypsin-EDTA solution, and then fixed with 4% paraformaldehyde (PFA) in PBS prior to flow cytometry analysis. Subcellular localization of peptide-drug complexes was analyzed by confocal microscopy (Zeiss LSM 510, Canada) using a 63X objective and a slice thickness of 300nm. A549 cells were seeded in 35mm glass bottom dishes (MatTek, MA, USA) at a density of 1×10^5 cells per ml, and allowed to attach for 24 h. The cells were treated with drug complexes and controls for 20 min and washed three times with warm PBS and fixed with 4% PFA for 20 min, followed by washing with PBS and adding DAPI (Sigma-Aldrich, Oakville, Canada) to stain the cell nuclei. Pirarubicin was excited at 488 nm and detected through the 570–630 nm band path filter. DAPI was excited at 405 nm and were visualized by 420–480 nm band path filter.

4.2.5 Haemolysis studies

Haemolysis experiments were carried out by using approximately 5mL of fresh human anticoagulant treated blood. The blood is centrifuge at 1500 rpm for 10 minutes and washed three times by adding Dulbecco's Phosphate Buffered Saline (DPBS). After washing, red blood cells (RBCs) are isolated and suspended in DPBS to the original volume and then diluted further with DPBS to make a 5% hematocrit solution. Varying concentrations of the peptide (800 µl) were added to the RBCs (200 µl). DPBS and water served as positive and negative controls, respectively. After incubation for 2 hours all the samples were then centrifuged and the supernatant was removed and placed into a clean cuvette and the absorbance was measured at 541 nm. The haemolysis given by the water solution was

considered to be 100% while the hemolysis given for the DPBS solution was taken as 0%.

4.2.6 Complement system activation studies

In order to determine the amount of complement activation caused by the peptides and the peptide-drug complexes *in vitro*, four complement protease products (C4d, SC5-b9, Bb and MBL) were analyzed using enzyme-linked immunosorbent assay kits from Quidel Corp, (San Diego, CA, USA) and ALPCO Diagnostics kit (Salem, MA, USA). Peptide samples were incubated with human serum at a volume ratio of 1:5 with a final volume of 1ml in a shaking incubator (100 rpm) for 60 minutes at 37°C. The samples were then diluted further with Sample Diluents according to the manufacturer's instructions to determine the amount of C4d, Factor B, SC5b-9 or active mannan-binding protein that was formed by the complement system during the incubation. Zymosan (a known complement activator) was used as a positive control and human serum by itself was used as a negative control and both were assayed in the same way as all the other samples. Standard curves were made for quantification of complement activation products by using the assigned concentration of each individual standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best fit line for SC5b-9, Bb, C4d and MBL standard curves were within the manufacturer's specified range. The efficacy of the treatments was determined by comparison with baseline levels using paired *t*-test.

4.2.7 Anaphylotoxin studies

In order to assess the amount of C3a, samples were incubated with human serum at a 1:5 ratio in a shaking incubator (100 rpm) for 60 minutes at 37°C. The samples were then tested according to the enzyme-linked immunosorbent assay kits from Quidel Corp. (San Diego, CA, USA). The results were then read at 450nm (FLUOstar OPTIMA, BMG, NC). Since C3a is resulting factor of the activation of the classical, alternative or lectin complement pathways, Zymosan was used as the positive control and human serum was used as the negative control.

4.3 Results and Discussion

4.3.1 Cellular uptake and cytotoxicity of peptide-drug complexes

Different concentrations of pirarubicin at a fixed peptide-to-pirarubicin weight ratio of 3:1 were used to form peptide-pirarubicin complexes, and their cellular toxicity was investigated on the lung cancer cell line, A549. As shown in Figure 4-2, all the NP-pirarubicin complexes (*i.e.* NP-, NP-I- and NP-II-pirarubicin complexes and pirarubicin-water in the absence of peptide (control)) showed very acute cytotoxicity at the high pirarubicin concentration of 20 µg/ml. However at concentrations as low as 2.5 µg/ml, the viability of cells treated by NP-I- and NP-II-pirarubicin complexes is significantly lower than those treated by unmodified NP-pirarubicin or pirarubicin in water.

This might be due to two main reasons: first, the toxicity of unmodified NP is higher in comparison to the modified versions; second, the solubility of DEGylated peptides, is higher compared with naked peptides. The higher hydrophilicity in DEGylated peptides could stabilize the pirarubicin which thus remains suspended in aqueous media longer by slow release of pirarubicin from its complex, which in turn delays the drug contact with adhesive cells and thus lowers the toxcicity to non-cancer cells. It is worth to mention that the observed

results were in accordance to the observation by published paper that partly investigated the effect of DEGylation on toxicity and encapsulation of a hydrophobic drug, ellipticine

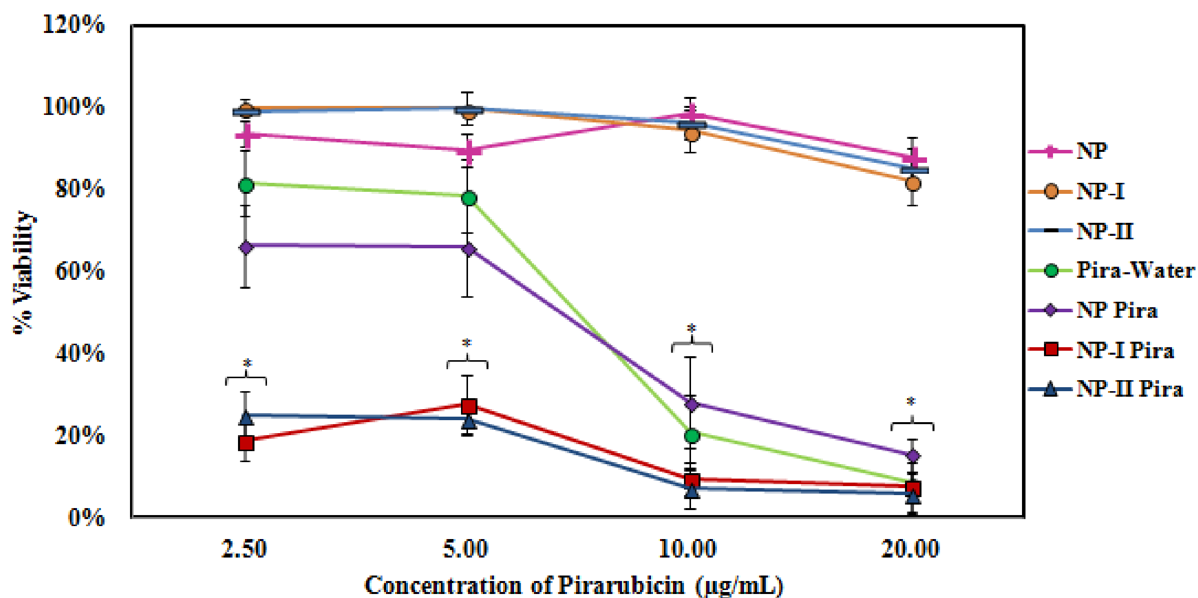


Figure 4-2 Cytotoxicity of NP, NP-I and NP-II and their complexes with the drug pirarubicin were measured against the lung cancer cell line A549. Error bars represent standard error of mean (SEM) of three independent experiments. * denotes statistically significant difference compared to the negative control, non-treated cells, as determined by two-sample t-test ($p<0.05$). Pira = pirarubicin.

Fluorescence microscopy images, Figure 4-3 A-E and flow cytometry results Figure 4-3 F also support the results of the cytotoxicity assay. As shown in Figure 4-3 F, the cellular uptake of pirarubicin at a concentration of 20µl/ml complexed with each of the three peptides is similar, as measured via flow cytometry. However, almost an 8-fold increase in pirarubicin uptake was observed when the drug is complexed with peptides compared with the drug in water. A similar conclusion can be drawn from the microscopy images. In all three cases, the

peptides mediated the nuclear uptake of pirarubicin, represented as violet areas (merged), as the result of localization of pirarubicin (red) in stained nuclei (blue). In the case of pirarubicin in water, Figure 4-3 B, the cells' nuclei were only partially covered by pirarubicin. Confocal fluorescent microscopy additionally confirmed the nuclear uptake of pirarubicin (red) when complexed with the peptides as shown by its co-localization with the nucleus (blue), creating a pink colour, Figure 4-3 G-I. Furthermore, the images demonstrated equal uptake of pirarubicin-peptide complex, irrespective of the peptide modification. It is worth mentioning that the range of concentrations used in all of conducted experiments is highly exceeding the dose used for therapeutic purposes (*i.e.* $< 2.5 \mu g/ml$).

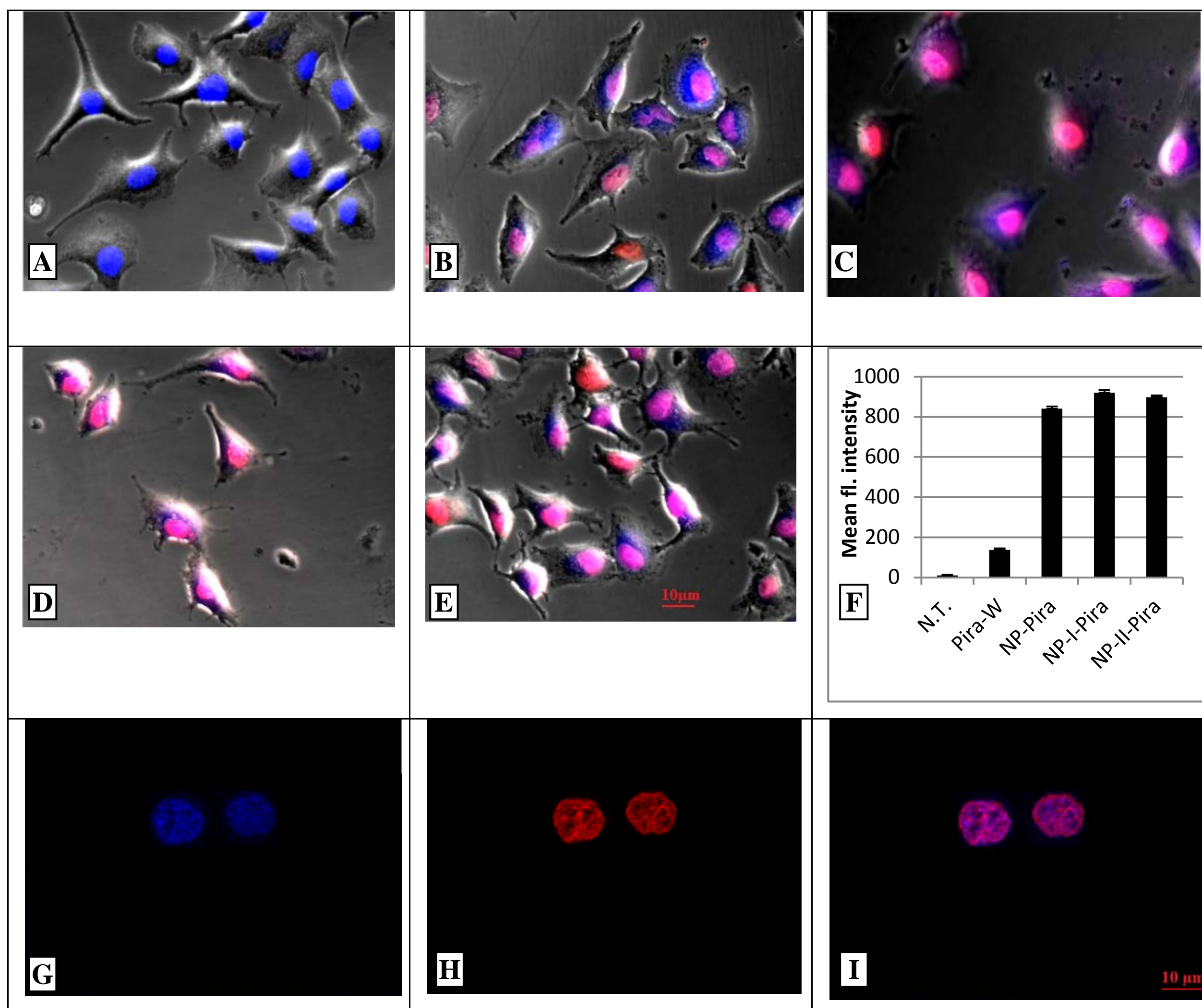


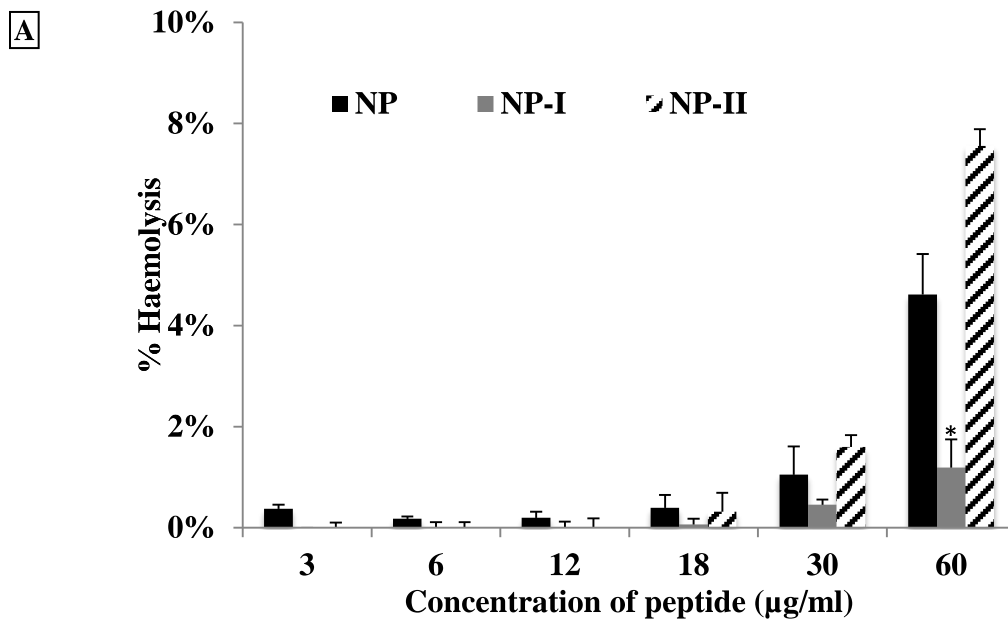
Figure 4-3 Fluorescence microscopy images (A-E) and flow cytometry results (F). Cellular uptake of pirarubicin are shown in the fluorescence microscopy images of nontreated cells (A), cells treated with pirarubicin in water (B), NP-pirarubicin complex (C), NP-I-pirarubicin complex (D), NP-II-pirarubicin complex (E). Violet areas are the result of localization of pirarubicin (red) in stained nuclei (blue). (F) Mean fluorescence intensity of uptaken pirarubicin in cells treated with different complexes and controls measured by FACS. Error bars represent standard error of mean (SEM) of three independent experiments. N.T. = non-treated cells, Pira-W= pirarubicin in water. (G-I): Confocal microscopy of A549 cells treated with NP-pirarubicin complexes. Violet nucleus (I) implies the co-localization of pirarubicin (red) (H) and DAPI (blue) (G) at the same confocal layer. Similar images were obtained for NP-I-pirarubicin and NP-II-pirarubicin complexes, confirming the presence of pirarubicin inside the nucleus.

4.4 Biocompatibility Evaluation of Self-assembling Peptides

4.4.1 Haemolysis

Haemolysis is a crucial factor in evaluating the biocompatibility of NPs. When determining the toxicity, a ratio of 5% or less haemolytic activity is considered biocompatible (257). In the present study, the haemolytic potential of the NP and its DEGylated versions with and without drug were investigated. As shown in Figure 4-4 A, NP-I did not show any haemolytic activity even at the highest measured concentration of 60µg/ml, whereas NP and NP-II showed some haemolytic activity (6.33 and 7.56%, respectively) at the same concentration. As discussed in Sadatmousavi *et al.* the critical aggregation concentration increases with increasing DEG conjugation which increases hydrophilicity. Therefore, DEGylation facilitates more organized self assembly and could prevent aggregation (43). Our results partially agree with this; however, in a biological environment, we did not observe that DEG conjugation to both ends of AAP8 (NP) provides more stable structure in terms of haemolytic activity. This could be due to the higher surface concentration of DEG in assembled NP-II polymers. Therefore, these results show that a narrow range of DEG concentration can improve the NP's haemocompatibility, and a higher concentration of DEGylation can have a drawback. This confirms the importance of careful modification of the NP and the erythrocyte's sensitivity to small changes in the NP's structure.

For drug delivery applications, the effect of the drug as well as the drug-peptide complexes should be evaluated. Figure 4B demonstrates the peptides' ability to form a non-haemolytic complex with pirarubicin. Pirarubicin itself is haemolytic at concentrations of 10 µg/mL and higher (data not shown here). Again NP-I complexed with pirarubicin shows less haemolytic activity compared to two other peptides, highlighting the importance of precise modification.



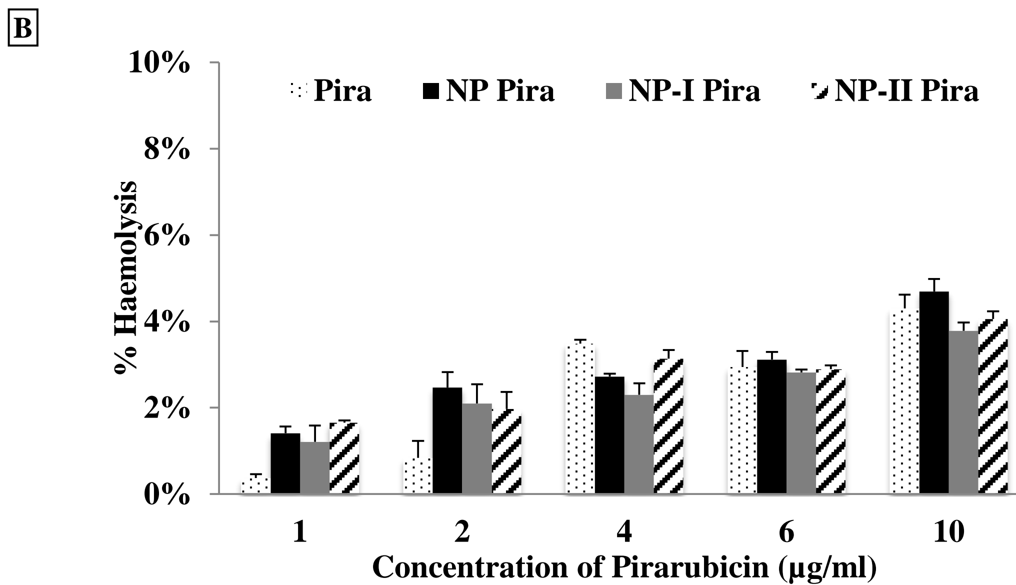


Figure 4-4 (A) Haemolytic activity of NP and its modified versions (NP-I and NP-II) alone or (B) in complex with the anticancer drug pirarubicin. Error bars represent standard error of mean (SEM) of three independent experiments. * denotes statistically significant difference compared to the non modified peptide NP, as determined by two-sample t-test ($p<0.05$).

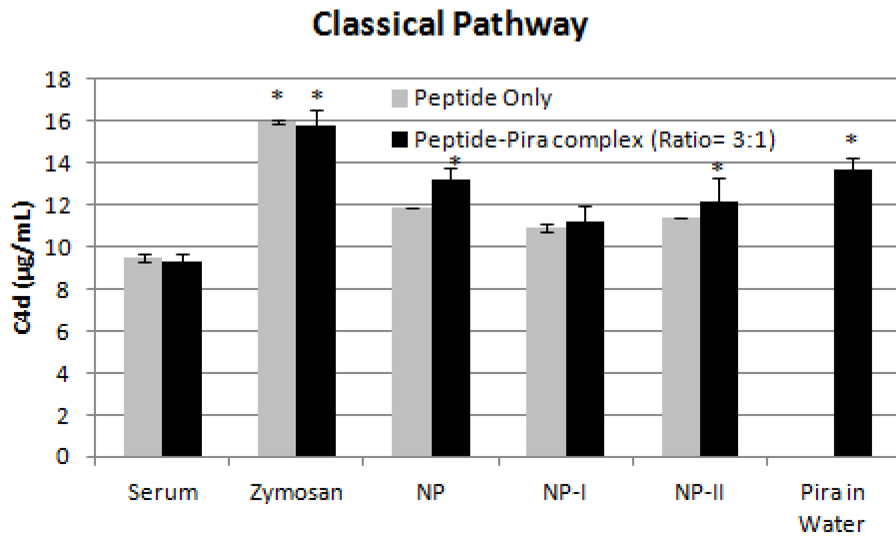
4.4.2 Complement system activation

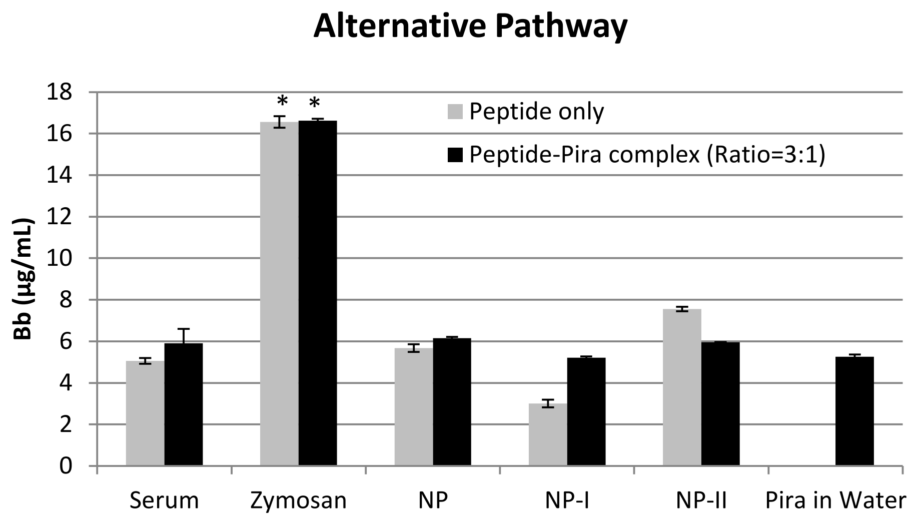
The complement system is one of the key effector mechanisms of humoral and innate immunity. It consists of several, generally inactive, serum and cell surface proteins which are activated only in the presence of pathogens to generate products that mediate a variety of effector functions. Therefore, not surprisingly, the majority of the consequences of complement activation result from the pharmacological effect of some of the activated components. Accordingly, clarification of the potential to activate complement can be used as one decisive factor in testing the biocompatibility of a variety of nanoparticles. Different types of nanoparticles especially polymers, have been used in medicine for targeted or controlled release of various drugs and diagnostic agents and thus have been assessed for complement activation (258-261). Here, the effect of NP and its modifications—with and without drug pirarubicin—on the three major complement pathways is reported .

After incubation with human serum, Bb and C4d concentrations were measured to assess the complement system activation of peptides via alternative and classical pathways, respectively. As shown in Figure 4-5 A-B, no complement activation was observed for the classical, and alternative pathway of each peptide (NP, NP-I , NP-II) and their complexes with drug. The anti-cancer drug pirarubicin had the classical activity because of its hydrophobic nature. This could be due to binding of C1q (main recognition unit of classical pathway) to the peptides surface via electrostatic or hydrophobic interactions (264). Therefore, these results at least partially agree with the published literature on complement

system activation which indicates hydrophobicity can trigger activation of the classical pathway. The MBL pathway was assessed by measuring the amount of functional MBL oligomer remaining in the solution. When the pathway is activated, MBL will bind to the surface of the invading particle making it incapable of further binding. After incubating the samples with human serum for an hour to allow time for MBL to bind to the surface of the peptides and peptide complexes, the amount of remaining functional MBL oligomer was measured. Figure 4-5 C shows that there is between 600 and 800 ng/mL of functional MBL oligomer remaining in the positive control and for the negative control all the functional MBL oligomer remains. For our samples, however, there was some lectin activation for NP *per se* with no lectin activation when complexed with drug. Complement activation via the lectin pathway was not observed for NP-I and NP-II peptides and their respective complexes, Figure 4-5 C. This could be due to the charged side of the peptide binding to the MBL (265). When the NP peptides were complexed with pirarubicin, no activation was seen for the NP, NP-I and NP-II complexes.

A





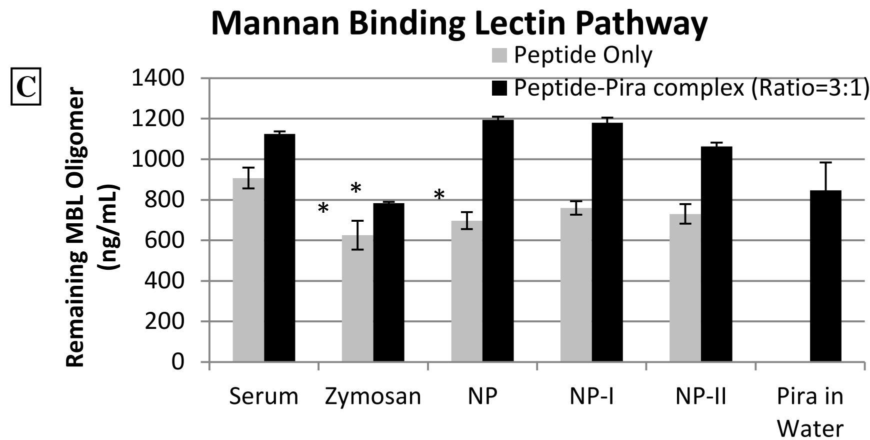


Figure 4-5 Complement activation of classical (A), alternative (B), and mannan binding lectin (C) pathways of the NPs (60 µg/ml) or in complex with pirarubicin. Error bars represent standard error of mean (SEM) of three independent experiments. * denotes statistically significant difference compared to the negative control , as determined by two-sample t-test ($p<0.05$).

These findings show that, overall, the NP-I complex is the most promising drug delivery vehicle in terms of three pathways. In addition, these results illustrate that NPs with a different combination of DEG groups produced different levels of complement activation, probably resulting from the different physico-chemical characteristics of the modified NPs. It is worth noting that the anti-cancer drug pirarubicin alone in water did not trigger the terminal, alternative, lectin and terminal attack pathways. Nevertheless, it activated the classical pathways, which is in agreement with the rest of the data.

4.4.3 Anaphylotoxin activation

After activation of any of the complement pathways, the C3 protein is eventually split into two fragments, C3b and a potent anaphylotoxin C3a. Therefore, the presence of C3a in a test sample proves that NPs can activate complement by any pathway. C3b can participate in the formation of a new enzyme, C5 convertase, which cleaves C5 to C5b, which drives the rest of the common terminal pathway, and C5a, another very potent anaphylotoxin. One outcome of C5b production is cell killing by forming a MAC. However, a large amount of the C5b generated with *in vitro* samples is diverted to the fluid phase by reacting to S protein to form soluble SC5b-9 which can be measured as proof of terminal pathway activation. An important point is that for each mole of detected SC5b-9, an equal number of moles of C5a are generated. C5a is a small, sticky and exclusive molecule with a short half-life and thus is difficult to measure directly. In contrast, SC5b-9, as a marker for C5 cleavage, is an extremely stable soluble macromolecular complex and can be used as a marker for C5a anaphylotoxin generation. Accordingly, to further assess the *in vitro* extent of NP-induced activation of complement, SC5b-9 and C3a were evaluated.

SC5b-9 concentration was measured after incubation with human serum. As shown in Figure 4-6 A, it was found that NP-II and its complex effectively activate the complement terminal attack complex. However, NP and NP-I formulations as well as their complexes with pirarubicin did not activate the terminal pathway of complement system with respect to the negative control and were significantly less than Zymosan (positive control). High activation of the terminal attack pathway by NP-II in comparison with NP-I highlights the importance of

surface density of DEG for modification of nanoparticles in order to reduce bio-incompatibility. Similar results were observed, as expected, for C3a anaphylotoxin production, Figure 4-6B.

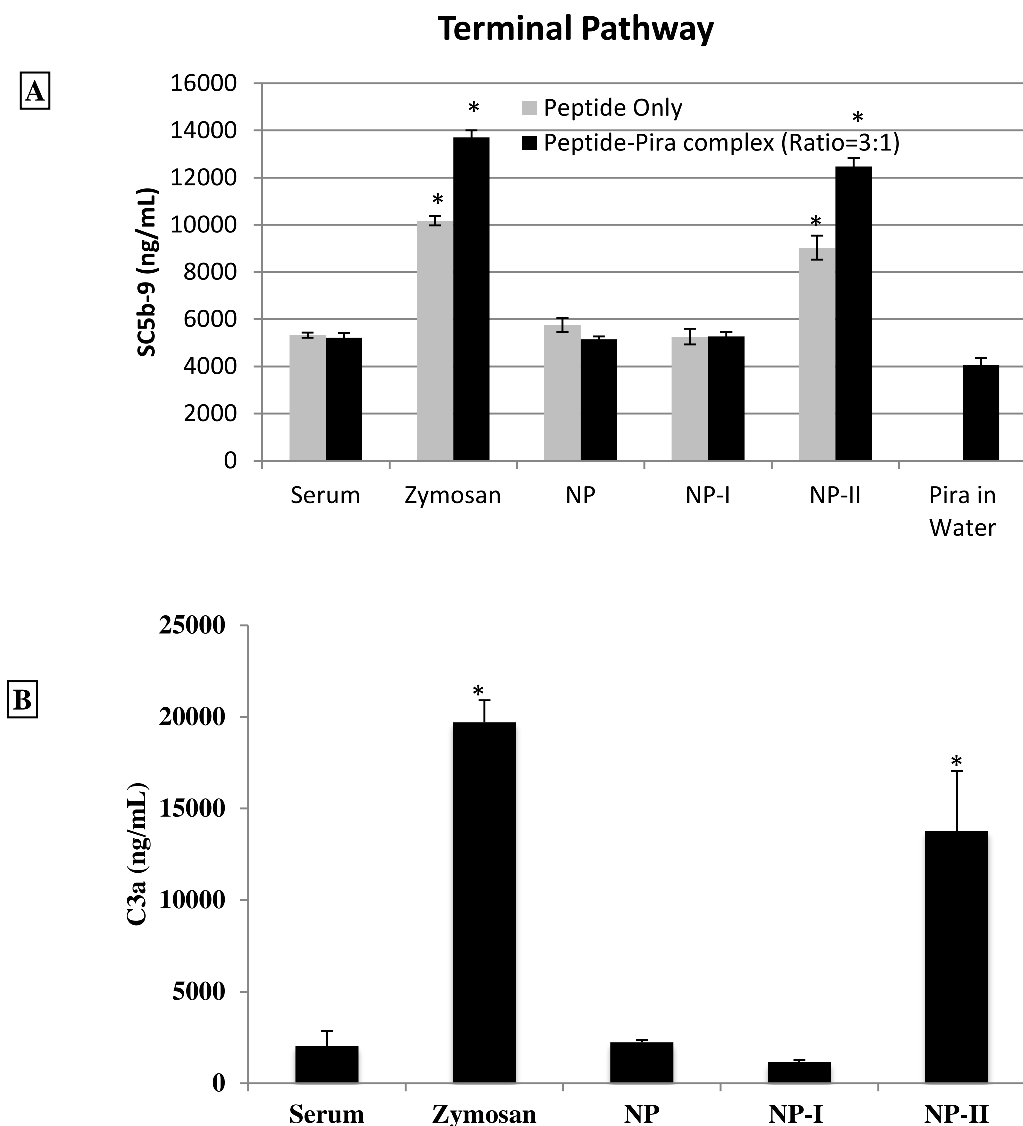


Figure 4-6 Complement (A) Complement activation of the terminal pathway by the NPs alone (60 µg/ml) or in complex with pirarubicin. Bars represent the mean concentration of SC5b-9, a biomarker of C5a through both the classical and alternative pathways. (B) Anaphylatoxin C3a assay comparing the concentration of C3a in NP, NP-I and NP-II treated samples, as well as positive (Zymosan) and negative

control (Human serum). Error bars represent standard error of mean (SEM) of three independent experiments. * denotes statistical significance compared to the negative control, as determined by two-sample t-test ($p<0.05$).

In summary, NP and NP-I performed similarly in all pathways of complement activation but the lectin pathway. NP *per se* produced the small activation of the lectin pathway with no lectin activation when complexed with drug. However, NP-I and its complex did not activate the classical, alternative, lectin and terminal pathways of complement system, most probably because of proper functionalization by DEG conjugation with more organized self assembly, and less hydrophobicity, resulting in stable fibers in the complex with drug in aqueous solution. Thus these results indicate NP-I is a potential candidate for a hydrophobic anticancer drug delivery system. This highlights the importance of proper modification in drug delivery systems.

4.5 Conclusions

The *in vitro* biocompatibility of NPs and their modified versions were systematically investigated. Their potential to be used as carriers for the anticancer drug pirarubicin was also shown. The nuclear uptake efficiency of pirarubicin was enhanced upon co-assembling with NP and its DEGylated versions (NP-I and NP-II). The DEGylated NP-pirarubicin complexes showed acute toxicity at pirarubicin concentrations as low as 2.5 µg/ml, however the toxicity of NP-pirarubicin at this concentration was 33%. It was also found that the one terminal DEGylated peptide, NP-I, , showed the best compatibility in terms of haemolytic activity, and complement activation. Furthermore, the DEGylation did not compromise the efficacy of the

nuclear uptake of its load (pirarubicin). These results highlight the importance of modification to NPs in drug delivery. Based on these data, I suggest the use of NP-I as an anticancer drug delivery vehicle for future applications. Based on these data, it is suggested that the use of NP-I as an anticancer drug delivery vehicle for future applications. It is proposed that the *in vitro* data presented here will help in predicting the effects of these NPs *in vivo*.

Chapter 5[*]
Nanotoxicity of Self-Assembling EAK16-II and EK8 Peptides and Anticancer Drug Pirarubicin

5.1 Introduction

A major goal of modern nanotoxicology in pharmaceutical applications is to enhance therapeutic safety by understanding the relationship between biophysicochemical properties of nanomaterials and the outcomes resulting from the contact of these nanosized materials with biological systems. In the past few years, cell culture systems have been the main focus of nanotoxicology studies. These data are necessary but not sufficient for assessing toxic responses. As a result, nanotoxicology studies as a sub-discipline of nanotechnology has recently undergone a dramatic expansion as the importance of biocompatibility has become obvious for the correct functioning of nano sized materials.

Currently, various drug delivery carriers have been developed for cancer chemotherapy such as liposomes, polymeric micelles, albumin-bound nanoparticles and lipoproteins, all requiring acceptable bio-compatibility and low bio-activity (266-270). Among them, self-assembling peptides- serendipitously discovered 20 years ago- have been extensively explored as one of the potential candidates that can be used as a building block for a variety of applications such as materials science (266), bioengineering, tissue engineering (271-274), cell culture and of particular interest to this thesis drug delivery (239-248, 275).

[*] This chapter is adapted from a paper draft " Sheva Naahidi, Yujie Wang, Mousa Jafari, Lu Sheng, Yongfang Yuan, P. Chen, Nanotoxicity of Self-Assembling EAK16-II and EK8 Peptides and Anticancer Drug Pirarubicin"

These peptides are capable of self-assembling spontaneously into various microstructures in aqueous solution and have triggered numerous studies aimed at synthesizing novel self-assembling peptides and characterizing the resulting microstructures (276). Other advantages of these peptide-based carriers are possible biocompatibility and low toxicity, as well as ease of preparation (277). Therefore, self-assembling peptides have obvious advantages which can play an important role in nanoparticle delivery systems. One of these promising classes of peptides is self-assembling ionic-complementary peptides (278). These ionic- complementary, self-assembling, peptides are characterized by either alternating arrangement of charged residues or hydrophobic/hydrophilic amino acid residues (279). These unique repetitive amino acid sequences, both ionic and hydrophobic, allow them to self-assemble into stable nanostructures, which may provide a protected and stable environment for the drug molecules (271-273).

Two such promising peptides are EAK16-II and EK8 which both contain all complementary amino acid pairing in the sequence and thus the name amino acid pairing (AAP) peptides. The molecular structure of both peptides are shown in Figure 5-1.

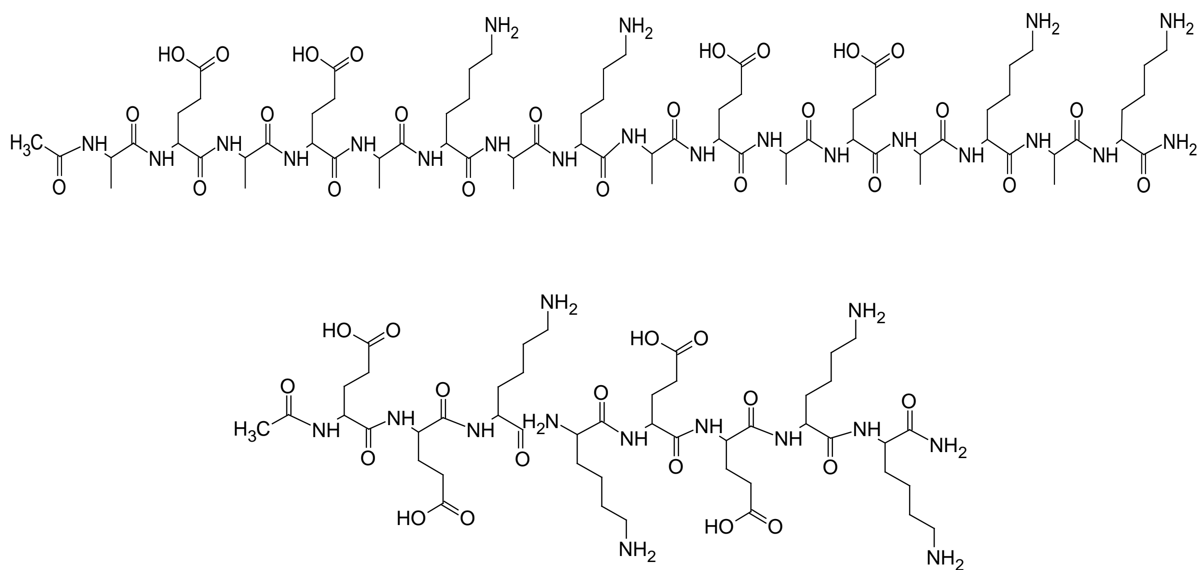


Figure 5-1 Chemical structure of ionic-complementary peptide EAK16-II (n-AEAEAKAKAEAEAKAK-c) (A) and EK8 peptide (n-EEKKEEKK-c) (B)

Pirarubicin is used as a candidate to test our drug delivery system using EAK and EK as carriers.

However, prior to their utilization as vehicles for drug delivery, the nanotoxicity of these peptides needs to be addressed by understanding their effects on biological systems. *In vivo* systems are expensive and the nanomaterial-biological entity interaction, such as peptides and cells, could trigger unique results such as biodistribution, clearance, and immune responses. Therefore, initial *in vitro* studies can precede the need for the wider range of *in vivo* studies. Additionally, *in vitro* examinations are necessary first step to rationalize whether there are effects which would be observed *in vivo* and they can be used to translate these data into a predicted response model in patients. As a result, these experiments are critical and essential towards our efforts to develop a novel, effective nano medicinal drug delivery system.

So far, there have not been any comprehensive studies on the possible nanotoxicology of EAK and EK; therefore, further study was required to understand how

to use these two peptides in systematic drug delivery. Complement is an important effector arm of both innate and acquired immunity (280). Anaphylatoxins and chemoattractants, C3a and C5a, are generated as a outcome of complement activation and can provoke anaphylaxis in systematically infected individuals (281). In addition to anaphylatoxin release, the terminal part of the complement pathway generates C5b-9 complexes (280) and these have the capability to trigger non-lytic stimulatory responses from endothelial cells (237), and modulate endothelial regulation of hemostasis and inflammatory cell recruitment.

In light of the above information and published articles, the *in vitro* biocompatibility and toxicity of both self-assembling ionic-complementary peptides EAK16-II and EK8 along with the anticancer agent Pirarubicin were evaluated. Based on reasonable and tolerable *in vitro* data between both peptides candidates, EK8 was further selected to be evaluated systematically *in vivo* for its immunocompatibility and bioavailability. The potential ability of these peptides to be utilized as carriers for the delivery of the hydrophobic anticancer drug pirarubicin is also shown. Nuclear uptake of peptide-pirarubicin complexes by A549 cells was shown and confirmed by both fluorescence and confocal microscopy respectively. Finally, EK8 peptides had favorable biocompatibility and poor bio-activity *in vivo*.

5.2 Materials and Methods

5.2.1 Sample preparation

The peptides EAK and EK (purity>98%) were purchased from CanPeptide Inc. (Montreal, Canada) and used without further purification. Both peptides were protected by acetyl and amino groups at their N-terminus and C-terminus, respectively. The anticancer agent pirarubicin (99.8% pure) was purchased from Sigma–Aldrich, (Oakville, Canada) and was used unaltered. The powdered peptides were dissolved in pure water (18 MΩ; Millipore Milli-Q system) to obtain a fresh peptide solution at a concentration of 0.5 mg/ml (source). The solution was then sonicated in a bath sonicator (Branson, model 2510) for 10 min. To make the peptide-pirarubicin complexes, a pirarubicin stock solution was first prepared in tetrahydrofuran (THF) and then transferred into a glass vial, and dried in a gentle stream of filtered air (0.22 mm pore size filter) to form a thin film at the bottom of the vial. The freshly prepared peptide solution was added into the vial containing pirarubicin, to make peptide-pirarubicin complexes consisting of 0.5 mg/ml EAK or EK8 and 0.1 mg/ml pirarubicin, followed by mechanical stirring at 900 rpm for 24 h. These complexes were then serially diluted in water 2, 4, and 8X (while keeping the peptide-pirarubicin ratio at 5:1) to prepare a range of concentrations for toxicity studies.

5.2.2 Cytotoxicity Assay

Non-small cell lung carcinoma cells (A549) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). To perform cytotoxicity assays, a Cell Counting Kit-8 (CCK-8 from Dojindo, Japan) was purchased. The human lung carcinoma cell line

A549 was seeded at a density of 8×10^3 cells per well onto 96-well plates. To each well, 20µL of the peptide-drug complex diluted in 80µl of growth media (Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum) was added, 24 h after cell seeding. After 48 h of treatment, CCK-8 substrate (10µl) was added to each well and incubated for an additional 2 h at 37°C in the dark. Using a microplate reader (FLUOstar OPTIMA, BMG, NC), absorbance measurements were taken at a wavelength of 450nm with a reference wavelength of 620nm.

5.2.3 Hemolysis studies

Approximately 5mL of anticoagulant treated, fresh human blood was used for hemolysis studies. Prior to use, the blood was centrifuged at 1500rpm for 10 minutes and washed three times with Dulbecco's PBS (DPBS). Red blood cells (RBCs) were then isolated and suspended in DPBS to the original volume and then diluted further with DPBS to make a 5% hematocrit solution. Varying concentrations of the peptide in 800µl of DPBS, water (positive control) or DPBS (negative control) were added to the 200µl of RBCs and incubated for two hours. All the samples were then centrifuged and the supernatant was removed and placed into a clean cuvette; subsequently, the absorbance was measured at 541nm. Hemolysis in the water solution was considered 100% while the hemolysis given by the DPBS solution was considered 0%. The results were expressed as percentage hemolysis.

5.2.4 Complement system activation studies

The amount of complement activation caused by each peptide and the (EAK or EK) peptide-drug complexes *in vitro* were determined by measuring the active forms of four complement products (C4d, SC5-b9, Bb and MBL) using enzyme-linked immunosorbent assay kits from

Quidel Corp, (San Diego, CA, USA) and ALPCO Diagnostics kit (Salem, MA, USA). Peptide samples were added to human serum at a volume ratio of 1:5 and incubated in a shaking incubator (100 rpm) for 60 minutes at 37°C. Three time points were measured (1-3 hours). However, there was no increase at later time points. Human serum alone and zymosan (a known complement activator) were used as negative and positive controls, respectively. The results were then read at 450nm (FLUOstar OPTIMA, BMG, NC). Standard curves were made for quantification of complement activation products by using the assigned concentration of each individual standard supplied by the manufacturer and validated. Quantification of complement activation products were made by making standard curves using the assigned concentration of each individual standard supplied by the manufacturer and validated. The slope, intercept and correlation coefficient of the derived best fit line were within the manufacturer's specified range for SC5b-9, Bb, C4d and MBL standard curves. The efficacy of the treatments was determined by comparison with baseline levels using paired *t*-test.

5.2.5 Anaphylatoxin studies

The samples were incubated with human serum in a 1:5 ratio in a shaking incubator (100 rpm) for 60 minutes at 37°C to assess C3a formation. Enzyme-linked immunosorbent assay kits from Quidel Corp. (San Diego, CA, USA) were then used to measure C3a concentrations. The results were read at 450 nm (FLUOstar OPTIMA, BMG, NC). Given that C3a and C5a can result from the activation of the classical, alternative or lectin complement pathways, human serum and zymosan were used as negative and positive controls, respectively.

5.2.6 Confocal and fluorescence microscopy

Confocal microscopy (Zeiss LSM 510, Canada) was used to analyze the subcellular localization of peptide-drug complexes, using a 63X objective and a 300nm slice thickness. A549 cells (1×10^5 cells per ml) were seeded in 35mm glass bottom dishes (MatTek, MA, USA), and allowed to attach for 24 h. After treatment with drug complexes and controls (non-treated cells and pirarubicin) for 20 min and washing three times with warm DPBS, the cells were fixed with 4% PFA for 20 min. This was followed by washing with PBS and adding DAPI (Sigma-Aldrich, Oakville, Canada) to stain the cells' nuclei. Pirarubicin and DAPI were excited at 488 nm and 405 nm, respectively, and detected through the 570–630 nm and 420-480 nm band path filters, respectively.

For fluorescence microscopy, after 20 min incubation with complexes, the cells were washed three times with PBS, and fixed with 4% PFA, followed by adding DAPI (Sigma-Aldrich, Oakville, Canada) to stain the cells' nuclei. An inverted fluorescence microscope (Zeiss AxioObserver Z1, Canada) was used to evaluate the complex uptake using a 40X objective.

5.3 Results and Discussion

5.3.1 Cytotoxicity of peptides

Cytotoxicity results in Figure 5-2 showed that the cells treated by both peptides had almost the same viability as that of non treated cells, indicating the safety of peptides even at relatively high concentrations i.e., 0.1 mg/ml. Similar results were obtained for the cells treated by Pirarubicin in water due to its very limited solubility. However, the EAK-pira and

Ek8-pira complexes showed strong toxicity at drug concentrations of 0.02 mg/ml (1xd). Diluting the complexes to half (2xd) showed better performance of EK8, compared to EAK, in based on pirarubicin delivery, leading to 55% toxicity in the treated cells (versus 38% for EAK-pira). Further dilution of the complexes (4xd and 8xd) resulted in a reduction in viability to 80% .

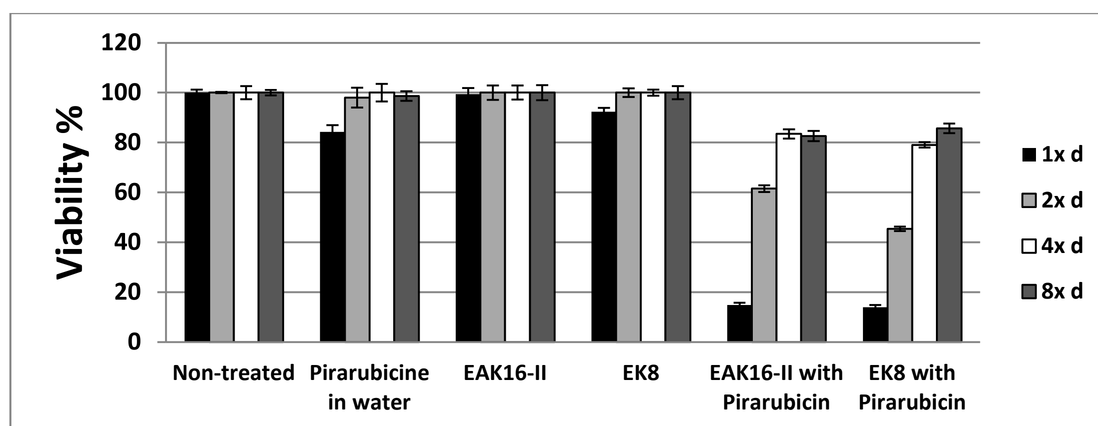


Figure 5-2 Cytotoxicity of EAK16-II and EK8 and their complexes with pirarubicin at different concentrations. Cytotoxicity of EAK16-II and EK8 per se and with pirarubicin were measured against the lung cancer cell line A549. Three independent experiments were performed for each data point. 1xd represents peptide/pirarubicin concentration of 0.1/0.02 mg/ml.

5.3.2 Haemolysis

Hemolysis is the rupture of the RBC's membrane, resulting in the release of hemoglobin and other internal components into the surrounding fluid; therefore, it is a crucial factor in evaluating the biocompatibility of biomaterials. It is also a common occurrence in serum samples and may compromise the laboratory's test parameters. When determining the hemolytic activity, a ratio of 5% or less is considered biocompatible (257). In the present study the haemolytic potential of the self-assembling/co-assembling peptides EAK and EK on

untreated human blood with was investigated. Water was used to lyse the red blood cells as positive control and D-PBS was used as the negative control. The amount of hemoglobin released was measured by the absorbance at 541 nm.

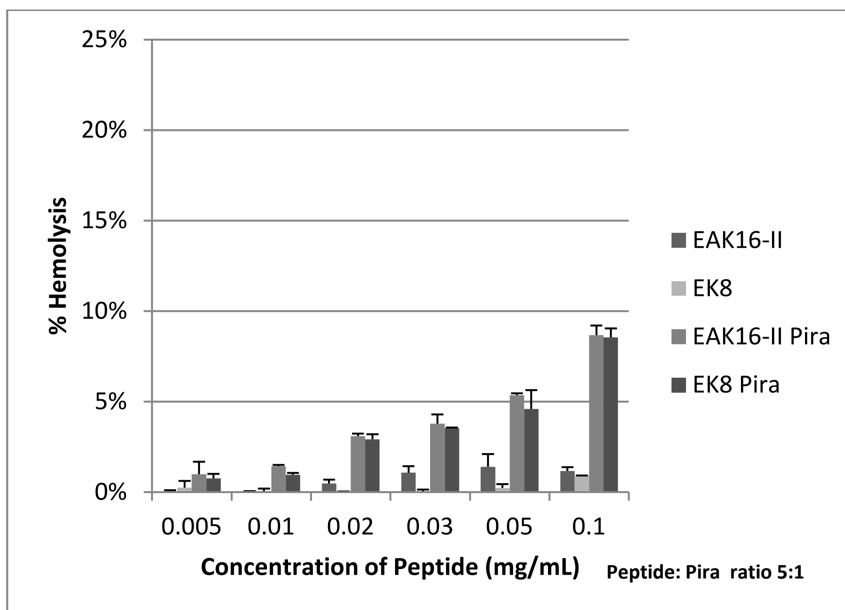


Figure 5-3 Haemolytic activity of self-assembling peptides EAK16-II and EK8 and their complexes with anticancer drug pirarubicin at different concentrations, in peptide:drug weight ratio of 5:1. Error bars represent standard error of mean of three independent experiments. Abbreviations: SEM: standard error of mean.

Although the concentration of both EAK and EK peptides dispersion was high, they did not show any observational hemolytic activities in the RBC in the experimental range Fig 5-3. Their complexes with anticancer drug Pirarubicin at concentrations as high as 0.1mg/ml which is above the therapeutic concentration (0.05mg/ml), showed mild hemolytic activity for both. However, at the therapeutic concentration, EK peptides and its complex with anti-cancer

drug Pirarubicin did not show any haemolytic activity but EAK did show small amount of activity.

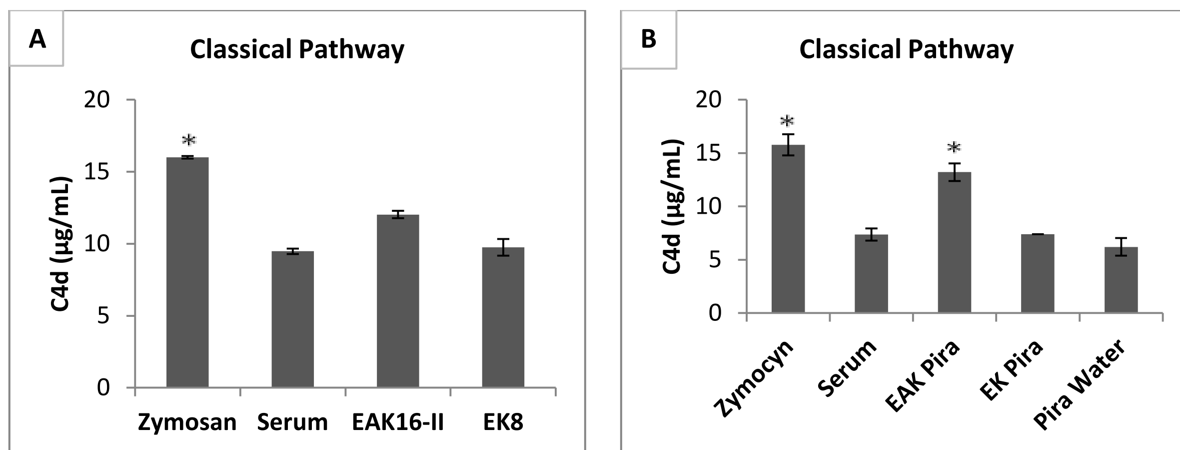
5.3.3 Complement system activation

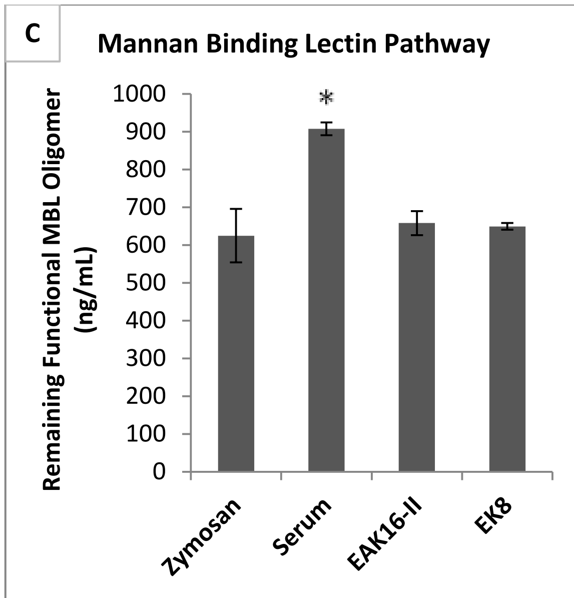
The complement system, as one of the key effector mechanisms of humoral and innate immunity, consists of several, generally inactive, serum and cell surface proteins which are activated only under particular conditions. Different types of nanoparticles, especially polymers, have been used in medicine for targeted or controlled release of various drugs and diagnostic agents, of which many have been assessed for complement activation (258-261).

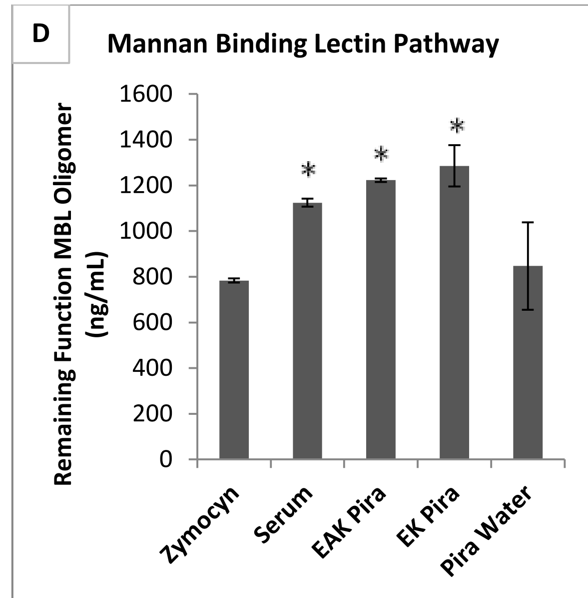
After incubation with human serum, C4d, MBL and Bb concentrations were measured to assess complement system activation via classical, lectin and alternative pathways. As shown in Fig. 5-4A, complement activation via the classical pathway was not observed for both peptides per se as well as EK complex, Fig.5-4B ; but EAK complex with pirarubicin did activate classical pathway which may be due to binding of C1q (main recognition unit of classical pathway) to the peptide via electrostatic or hydrophobic interactions (264).

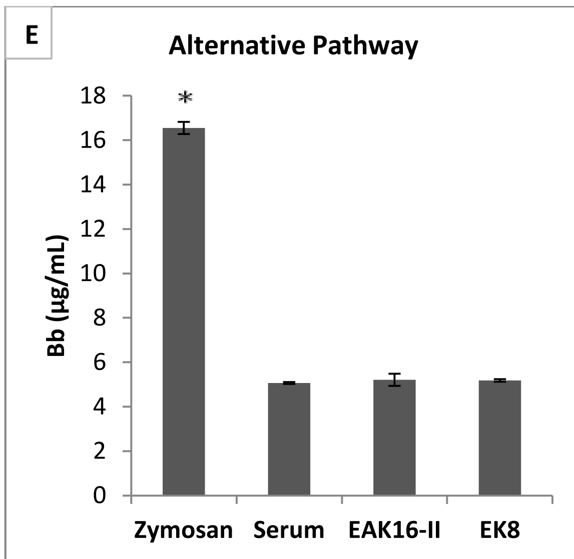
The MBL pathway was assessed using a kit that measures the amount of functional MBL oligomer remaining in the solution. When the pathway is activated, the oligomer will bind to the surface of the invading particle rendering it dysfunctional. After incubating the samples with human serum for an hour to allow time for the oligomer to bind to the surface of the peptides and peptide complexes, the amount of remaining functional MBL oligomer was measured. Figs. 5-4 C-D show that for the positive controls, there are very little functional MBL oligomer remaining and for the negative controls all the functional MBL oligomer

remained. For our samples, there is activation for both peptides EAK and EK *per se* (Fig. 5-4C). While there was lectin pathway activation; nevertheless, they did not reach the "threshold" level of activation required for the next step of complement activation because there was no Bb activation for both peptides and their complexes (Fig. 5-4E-F). It worth mentioning that even with longer incubation times the results remained the same. Finally, as shown in Fig. 5-4D, there was not any lectin activation for both peptidepira complexes. This may be due to the fact that pirarubicin can be stabilized by ionic interaction with the negatively charged residues (glutamic acid E in these cases) of the peptides. Thus, the self-assembling peptides EAK and EK could stabilize the hydrophobic drug in aqueous solution as was the case reported for EAK16-II and ellipticine (282, 283).









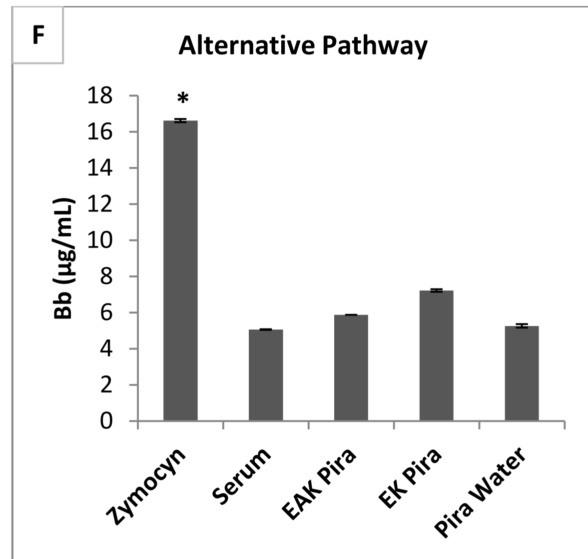


Figure 5-4 Complement activation of classical (A-B), mannan binding lectin (C-D) and alternative (E-F), pathways of the EAK16-II and EK8 (0.05 mg/ml) after 1 hour. Error bars represent SEM of three independent experiments. * denotes statistically significant difference compared to the negative control (DPBS), as determined by two-sample t-test ($p<0.05$). Abbreviations: SEM: standard error of mean.

5.3.4 Anaphylotoxin activation

As an eventual outcome of either classical, lectin or alternative pathway activation, the C3 protein is split into two fragments, C3b and a potent anaphylotoxin C3a. Therefore, the production of C3a in a test sample proves that peptide nanoparticles can activate. On the other hand, C3b can participate in the formation of a new enzyme, the C5 convertase, which cleaves C5 to C5b which drives the rest of the common terminal pathway, and C5a is also a very potent anaphylatoxin. One outcome of C5b production is cell killing by forming a MAC. However, a large amount of the C5b generated with *in vitro* samples is diverted to the fluid phase by reacting to S protein to form soluble SC5b-9 which can be measured as proof of terminal pathway activation. An important point is that for each mole of detected SC5b-9, an equal number of moles of C5a are generated. C5a is a small, sticky and exclusive molecule with a short half-life. In contrast, SC5b-9, as a marker for C5 cleavage, is an extremely stable soluble macromolecular complex and can be used as a marker for C5a anaphylotoxin generation. Accordingly, to further assess the *in vitro* extent of EAK and EK induced activation of complement, SC5b-9, C3a and C5a production were evaluated.

After incubation with human serum, the SC5b-9 concentration was measured to assess the complement system activation of EAK and EK peptides and their complexes with pirarubicin. As shown in Fig.5-5A-B, it was found that EAK and EK alone did not activate the terminal pathway of complement system similar to human serum (negative control) and were significantly less than Zymosan (positive control). Similar results were observed, as expected, in regards to C3a and C5a anaphylotoxins. However, EAK peptide complexed with anti-

cancer agent pirarubicin did activate the terminal pathway, whereas EK complex did not activate the terminal pathway Fig.5-5B. In the light of above information, further experiments examined peptides complex, peptides-pira, for their ability to activate anaphylotoxin C3a and C5a

As shown in Fig. 5-5C, initial anaphylotoxin C3a production was not observed for both peptides complexes. However, EAK complexed with pirarubicin did induce production of anaphylotoxin C5a which confirms the observations seen in the SC5b-9 testing (Fig 5-5D). In summary, the ionic, complementary self-assembling peptide EK8 did not activate the complement system *in vitro* alone or complexed with pirarubicin highlighting the potential of this peptide to be used as a drug delivery system, while EAK did induce complement activation when complexed with drug.

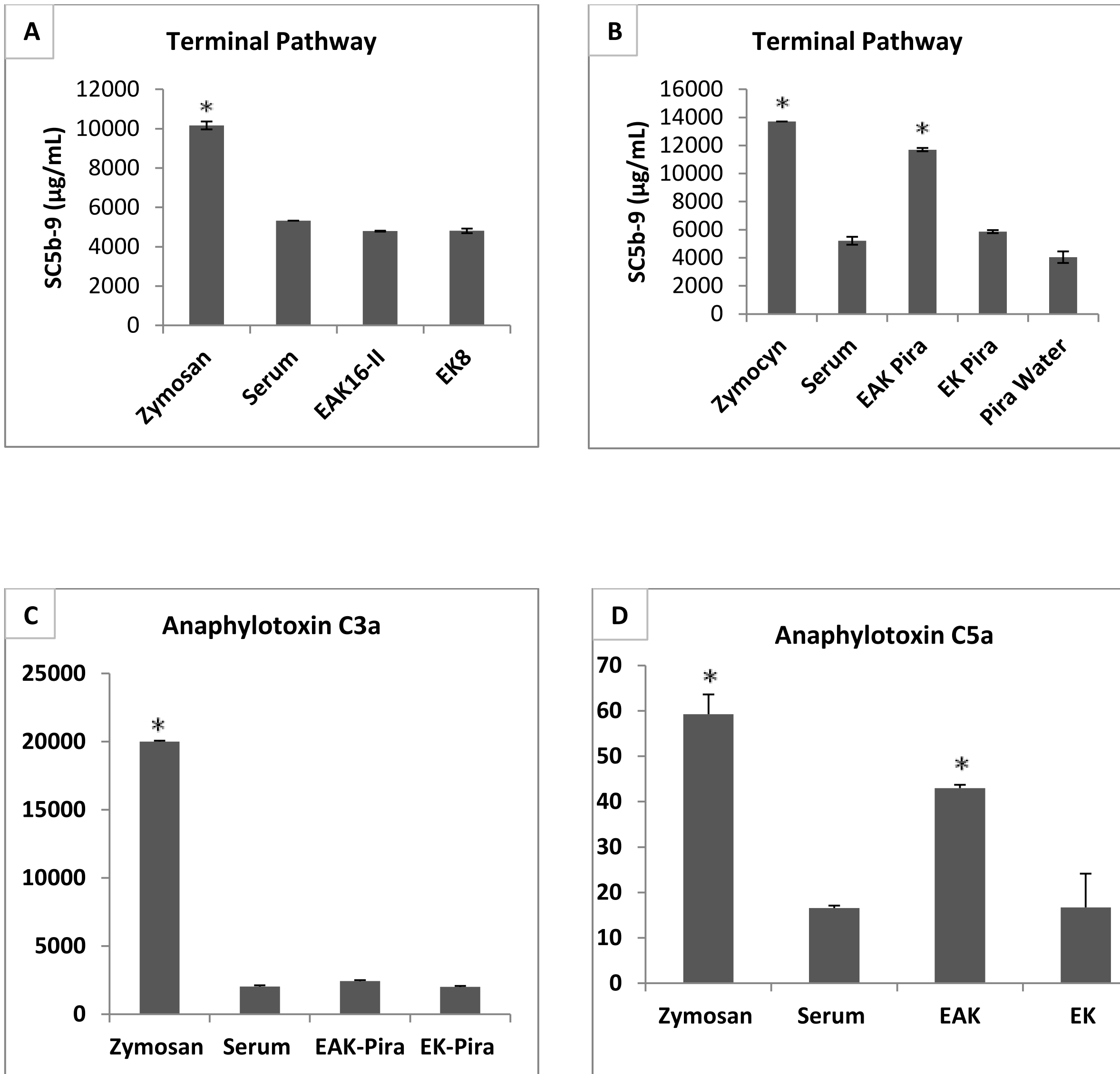


Figure 5-5 Complement activation of the terminal pathway by the EAK 16-II and EK8 peptides (A) and their complexes with pirarubicin (B) at a concentration of 0.05 mg/mlafter 1 hour. Bars represent the mean concentration of SC5b-9, a biomarker of C5a production . C-D) Anaphylatoxin assay comparing the concentration of C3a and C5a induced by both peptides-Pira treated samples, and positive (Zymosan) as well a negative control (Human serum) Error bars represent SEM of three independent experiments. * denotes statistical significance compared to the negative control, as determined by two-sample t-test ($p<0.05$). Abbreviations: SEM: standard error of mean; D-PBS: Dulbecco`s Phosphate Buffered Saline.

5.3.5 Confocal and fluorescence microscopy

Confocal fluorescent microscopy confirmed the nuclear uptake of anti-cancer drug pirarubicin (tetrahydropyranyladriamycin) when complexed with the peptides as shown by its co-localization with the nucleus (Fig. 5-6 A-C).

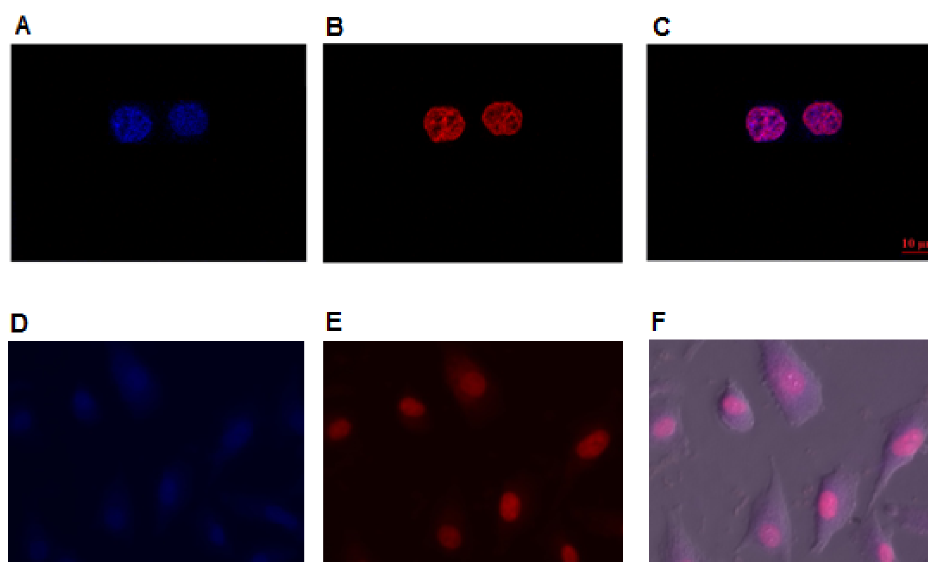


Figure 5-6 Confocal microscopy of A549 cells treated with EK-pirarubicin complexes confirming the presence of pirarubicin inside the nucleus. Violet nucleus (C) implies the co-localization of pirarubicin (red) (B) and DAPI (blue) (A) at the same confocal layer. Fluorescence microscopy images (D-F). Cellular uptake of pirarubicin are shown in the fluorescence microscopy images of nontreated cells (D), cells treated with pirarubicin in water (E), EK-pirarubicin complex (F), Violet areas are the result of localization of pirarubicin (red) in stained nuclei (blue). Abbreviations: Pira-W= pirarubicin in water. DAPI: 6-diamidino-2-phenylindole.

Pirarubicin's hydrophobicity and fluorescence characteristics make it a good candidate to be used for a drug delivery system using EAK and EK as its carrier. The peptide mediated the nuclear uptake of pirarubicin, represented as violet areas (merged), as the result of localization of pirarubicin (red) in stained nuclei (blue). The range of concentrations used in all of

conducted experiments highly exceeds the dose considered for therapeutic purposes (*i.e.* < 2.5μg/ml).

5.4 Conclusions

The EAK16-II and EK8 peptides' potential carrier for the hydrophobic anticancer drug Pirarubicin was systematically investigated for its biocompatibility and toxicity. It was found that EK and its complex with Pirarubicin caused no hemolytic, complement and anaphylotoxin activation at the concentration to be considered for a drug delivery system. EK peptide Further results also indicated its potential to deliver the drug Pirarubicin into the cell. However, EAK pirarubicin complex did activate the classical pathway in significant extent and C5a anaphylotoxin suggesting that complement activation did proceed to the later, inflammatory, stages which are confirmed by the terminal pathway. The differences in *in vitro* observations can be due to the presence of additional hydrophobic amino acid alanin or the effect of length, from 16 amino acid, EAK16-II to 8 amino acids EK.

Studies are in progress to determine the uptake pathway of the drug and increase the efficacy of this novel therapeutic system through more efficient targeting.

Chapter 6

Original Contributions and Recommendations

6.1 Original Contributions to Research

This thesis presented the biocompatibility potential of the special classes of self/co-assembling amino acid pairing peptides as carriers for hydrophobic anti-cancer drug delivery. The hematocompatibility and immunocompatibility of the designed peptides as well as their toxicity against the human adenocarcinoma lung cancer cell line, A549, were systematically investigated

This thesis investigated the following peptides: (i) AC8 (ii) Two modified (DEGylated) versions of AC8 (iii) EAK16-II and EK8. The major contributions and outcomes of each peptide are highlighted and summarized in the following parts.

Biocompatibility of the AC8 peptide as a drug carrier

Although peptide-based nanoparticles have emerged as promising drug delivery systems for targeted cancer therapy, the biocompatibility of many of these nanoparticles has not been elucidated. Therefore, in this thesis, the *in vitro* biocompatibility and toxicity of the self/co-assembling peptide AC8 were evaluated. AC8 showed minimal hemolytic activity (5%) and did not cause aggregation of red blood cells. The *in vitro* assay revealed that AC8 did not activate the complement system via the classical or alternative pathway but did activate the lectin pathway to a small extent. However, AC8 showed no C3a and C5a anaphylotoxin activation suggesting that complement activation did not proceed to the later, inflammatory,

stages. The *in vivo* immunocompatibility and bioactivity of the self/co-assembling peptide AC8 in its nanoparticle form has been also evaluated.

Immuno- and hematocompatibility of AC8-based peptides and potential use for anticancer drug delivery

Recently, amino acid pairing, peptide-based nanoparticles have been introduced as promising carriers for hydrophobic anticancer drugs in cancer therapy. The AC8 peptide, with eight amino acid in sequence, n-FEFQFNFK-c, is based on the amino acid pairing (AAP) design containing 8 amino acids and hence the designated name AAP8. In previous work in Pu Chen's lab, AAP8 nanoparticles (NPs) were modified by conjugation to diethylene glycol (DEG), a short segment of polyethylene glycol, in an amino-end-terminal or both-end-terminal fashion (DEGylated NPs). Yet, the biocompatibility of these amino acid pairing, peptide-based NPs in comparison to modified versions has not been extensively studied. Here, the *in vitro* biocompatibilities of the NPs and their modified versions were compared and the potential of these peptides as carriers for the hydrophobic anticancer drug pirarubicin was demonstrated. The biocompatibilities of the peptide-drug co-assembly complexes were also assessed. The toxicity of the NPs, DEGylated NPs, as well as their mixture with pirarubicin was tested against the human adenocarcinoma lung cancer cell line, A549. It was found that the amino-end DEGylated NP, (NP-I), had superior biocompatibility to the non-modified NPs or two-ended-terminal DEGylated NPs (NP-II). NP-I showed acceptable hemolytic activity while NP and NP-II showed marginal and acceptable hemolytic activity (5%), respectively. Nuclear uptake of NP-pirarubicin complexes indicated adequate and comparable

uptake for all three types of NPs by A549 cells. All three types of NPs did not activate the complement system via the classical and alternative pathways of complement system nor did they activate the anaphylotoxine C3a. However, NP-II and its complex effectively activate the complement terminal attack complex. The lectin pathway was not activated by NP-I and NP-II, but was to a small extent by the non-modified NPs with no lectin activation when complexed with drug. These results indicate NP-I is the most promising peptide for use as a drug delivery system, highlighting the importance of proper modification in drug delivery systems.

Nanotoxicity of self-assembling EAK16-II and EK8 peptides and anti-cancer drug pirarubicin.

Nanotoxicology testing of nanoparticles is an essential step in their safety assessment for biomedical applications. Self/co-assembling peptides, as one of the potential candidates for applications in the pharmaceutical industry, have gained considerable attention due to their structural diversity, promising diagnostic and therapeutic possibilities in cancer. In this regard, *in vitro* nanotoxicity of the self/co-assembling peptides EAK16-II (EAK) and EK8 (EK) as well as the *in vivo* immunocompatibility and bioactivity of the peptide EK8 in its nanoparticle form were evaluated to show their potential us as a carrier for the hydrophobic anticancer drug pirarubicin. Both EAK and EK self/co-assembling peptides showed no hemolytic activity at therapeutic concentrations and EK did not cause aggregation of red blood cells. The *in vitro* assay revealed that both peptides complexed with pirarubicin did not activate the complement system via the lectin or alternative pathway. EAK pirarubicin

complex did activate the classical pathway to a significant extent whereas EK peptide and its complex with pirarubicin did not activate the classical pathway. Although both EAK and EK peptides showed no C3a activation, EAK did activate C5a anaphylotoxin suggesting that complement activation did proceed to the later, inflammatory, stages which was confirmed by an assessment of terminal pathway activation. Based on the observed *in vitro* results, EK peptide was selected for further *in vivo* studies. The *in vivo* immune response assay showed that administration of EK to BALB/c mice had no effect on the weight of immune organs or body weight of mice at doses less than 0.1mg/kg. This peptide also did not have any effect on the expression of CD3+ T-cells and natural killer (NK) cells, the ratio of CD4+/CD8+ T-cells and the proliferation of B-cells. The efficiency of the each peptide-pirarubicin complex (peptide-pira) was also confirmed by showing a decrease in the viability of lung cancer cells treated with peptide-Pira. Here Ireport for the first time experimental results regarding two self-assembling peptides, EAK and EK, concluding that EK might be an ideal candidate for hydrophobic drug delivery applications given its lack of toxicity and the fact that it is not recognized as a foreign molecule, inducing no immune reaction. These results will provide a basis in the future for the clinical toxicity testing of this peptide in drug delivery.

6.2 Recommendations

The introduction of synthetic materials, used for nanoparticle fabrication among other applications, has spawned a new scientific concept within biomaterials, called biocompatibility, which delves into understanding the reciprocal relationship between materials and biological systems. Peptide based nanoparticles interacting with cells and the

extracellular environment can trigger a sequence of biological effects. These effects largely depend on their dynamic physicochemical characteristics, which in turn determine the biocompatibility and efficacy of the intended outcomes. Because of several important structural features of Ionic-complementary peptides, effects of changes on molecular design as important factors can be evaluated. These effects include amino acid replacement, alteration in charge distribution and variation in peptide chain length, from 8 to 32 amino acids in sequence. In the light of above information and based on the studies conducted in this thesis, recommended future studies include the following:

- Biocompatibility evaluation of EAR16II (EAR) as a modified form of EAK16II (replacement of lysine to arginine) and screening the effect of R (arginine) amino acid in comparison with K (lysine) on biocompatibility of this sequence.

- Replacement of R amino acid to H inEAH-16II, and screening of this replacement for its effect on biocompatibility.

- Biocompatibility evaluation of EAR8 to address the effect of length, comparing 16 amino acid EAR16-II to 8 amino acids EAR8.

    - New additional measure for biocompatibility such as thrombogenicity, to evaluate the intended peptide's propensity to stimulate platelet activation and/or aggregation, its effects on plasma coagulation time, and tendency to initiate vascular thrombosis.. Evaluation of platelet activation in response to nanoparticle introduction by measuring the release of human platelet factor 4

in human plasma (PF4). Evaluation of fibrinogen activation, one of the most plentiful proteins in human plasma which activates the coagulation cascade and plays a major role in the opsonization process of the complement system. Coagulation assays which could be conducted to measure biocompatibility are: Prothrombin Time (PT), Activated Partial Thromboplastin Time (APTT), and Thrombin Time (TT).

- 3D cell culturing and toxicity evaluation of designed peptides as opposed to 2D cell culturing. This can help to predict more accurate *in vivo* toxicity.
- After choosing proper peptide design in terms of biocompatibility, the selected peptide can be incorporated with targeting motifs. Then *in vitro* and *in vivo* biocompatibility evaluations can be applied.
- Finding a desirable anti-cancer drug which is compatible with our peptide such that in combination they trigger no toxicity or an extremely low level of toxicity with co assembly with selected targeting peptides.

In vitro and *in vivo* studies have to be performed in the final design to select and form *de novo* smart nano-delivery systems and move these smart systems from bench to clinical studies.

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