

SELF-ASSEMBLED PEPTIDE NANOSTRUCTURE DELIVERY SYSTEMS FOR
OLIGONUCLEOTIDE THERAPY

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By

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June, 2014

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ABSTRACT

SELF-ASSEMBLED PEPTIDE NANOSTRUCTURE DELIVERY SYSTEMS FOR OLIGONUCLEOTIDE THERAPY

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M.S. in Materials Science and Nanotechnology

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Oligonucleotides are potent therapeutic agents in the treatment of cancer, metabolic, cardiovascular and various hereditary diseases. Despite their great potential, oligonucleotide-based drugs have failed in clinical and pre-clinical studies due to their low cell penetration capacities, short plasma half-lives and rapid clearances. As such, development of delivery systems for oligonucleotide drugs is necessary to protect oligonucleotide based-drugs from renal and reticulo-endothelial system (RES) clearance and as well as to facilitate their delivery within target sites. In this thesis, a peptide nanostructure delivery system was developed to improve these deficiencies and to create an effective carrier for oligonucleotide therapy. Cell penetration capacity and silencing efficiency of a model oligonucleotide drug, Bcl-2 antisense oligonucleotide, was shown to be increased following encapsulation within cell penetrating peptides. In addition, the importance of the geometry of the delivery system in cellular internalization was investigated. The geometry of the nanostructure was shown to be critical in cellular internalization, where nanofibers were observed to be internalized to a greater extent compared to nanospheres. Their cellular uptake mechanisms were also studied and internalization of nanofibers was found to depend on an endocytic pathway whereas nanospheres were internalized via a non-endocytic pathway.

Keywords: Peptide amphiphiles, oligonucleotide delivery, self-assembly, geometry of the delivery system, nanofibers, nanospheres, cellular internalization mechanism, cell penetrating peptides, transfection.

ÖZET

KENDİLİĞİNDEN BİR ARAYA GELEN PEPTİT NANOYAPI TAŞIMA SİSTEMİ İLE OLİGONÜKLEOTİT TEDAVİSİ

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Oligonükleotitler, kanser, metabolik, kardiyovasküler ve birçok kalıtsal hastalığın tedavisi için potansiyel terapötik ajanlardır. Ancak yüksek potansiyele sahip olmalarına rağmen, bu ilaçların çoğu düşük hücre penetrasyon kapasitesi, kısa plazma yarı-ömrü, hızla vücuttan atılımı nedenleriyle klinik ve klinik öncesi araştırmalarda başarısız olmuştur. Dolayısıyla oligonükleotit ilaçlarının idrar ve RES (retikülo-endotel system) yoluyla atılmasının engellenmesi ve hücre penetrasyon kabiliyetinin artırılması için uygun ilaç taşıma sistemlerinin geliştirilmesi gereklidir. Bu çalışmada, oligonükleotitler için peptit nanoyapı taşıma sistemi geliştirilerek, bu etkiler engellenmiş ve oligonükleotitler için etkili bir taşıma sistemi yaratılmıştır. Hücre içine girebilen peptitlerin içine yerleştirilen model oligonükleotit ilacının (Bcl-2 antisens oligonükleotitin) hücre penetrasyon kapasitesinin ve susturma etkisinin artırıldığı gözlemlenmiştir. Ayrıca, taşıma sisteminin geometrisinin, ilacın hücre içine girmesindeki önemi araştırılmıştır. Nanoşekillerin geometrilerinin hücre içine girişi için önemli olduğu bulunurken, nanofiberlerin nanokürelere göre daha çok hücre içine alındığı gözlemlenmiştir. Hücre içine alım mekanizmaları incelendiğinde, nanofiberlerin endositoz yolağı ile hücre içine girişini gerçekleştirdiği, nanokürelerin endositoz olmayan mekanizmalarla hücre içine girdiği bulunmuştur.

Anahtar kelimeler: Peptit amfifil, oligonükleotit iletimi, kendiliğinden oluşma, taşıma sisteminin geometrisi, nanofiberler, nanoküreler, hücre içine girme mekanizması, hücre içine girebilen peptitler, transfeksiyon.

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CHAPTER 1. INTRODUCTION

1.1. Therapeutic oligonucleotides

Therapeutic oligonucleotides are short nucleic acids classified under three different subclasses; antisense oligonucleotides (AON), small interfering RNAs (siRNA), aptamers and CpG oligonucleotides. In general, oligonucleotides are single stranded or double stranded ribonucleic acids (RNA) or deoxyribonucleic acids (DNA), and may have different mechanisms of action¹. AONs and siRNA drugs act by modulating the expression level of a target gene and are described in the following section. Aptamers and CpG oligonucleotides are also regarded as oligonucleotide therapeutics but their function does not involve direct regulation of gene expression. Aptamers are short DNA or RNA molecules that are designed to bind specific target molecules. Unlike AON and siRNA, target molecule of an aptamer is not necessarily a nucleic acid, instead any biomolecule can be targeted using the SELEX (systemic evolution of ligands by exponential enrichment) strategy². CpG oligonucleotides are short DNA molecules containing CpG motifs which induce specific inflammatory responses in cells. Based on this mechanism, CpG oligonucleotides are designed for the treatment of various diseases by boosting the immune system^{3,4}.

1.1.1. AONs and siRNAs

AONs and siRNAs can be designed to bind a specific messenger RNA (mRNA) and decrease the expression of the corresponding gene. Thus, they are promising therapeutics for treatment of several diseases including cancer, AIDS, cardiovascular diseases and metabolic diseases. In addition, antisense oligonucleotides can be designed to correct mis-splicing and used for the treatment of genetic disorders⁵.

Differences between AONs and siRNAs are present in their respective structures and mechanisms of action. Structure-wise, AONs are single stranded DNA molecules that are designed to complement with target gene. The length of an AON can be longer than that of an

siRNA but is less than 40 bases. siRNAs, on the other hand, are double stranded RNA molecules that mimic the endogenous microRNA (miRNA) mediated RNA interference mechanism⁶.

1.1.2. Mechanisms of action of AONs and siRNAs

Expression of a gene starts with the synthesis of messenger RNA (mRNA) molecules from genomic DNA. Synthesized mRNA is processed and transported to the cytosol where it can be used as a template for protein synthesis. Generally, AONs are designed to decrease the expression of their targets by undergoing Watson-Crick base pairing with their corresponding mRNAs. The general mechanism of gene expression and the inhibition thereof by AONs is illustrated in Figure 1.1.

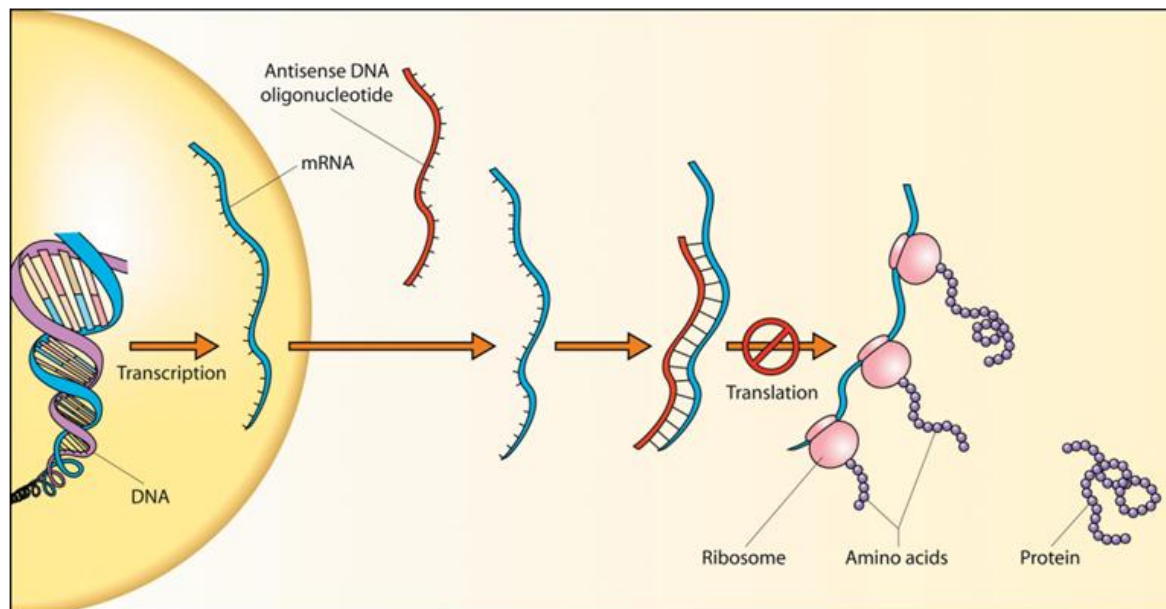


Figure 1.1. Antisense mechanism. Reproduced from Robinson R.⁷

siRNAs, on the other hand, are designed to mimic the endogenous RNA interference mechanism. In nature, microRNAs (miRNA) are double stranded RNA molecules that can bind to target mRNAs generally in the 3' UTR (untranslated region). After dsRNA (the miRNA progenitor) is cleaved by the protein Dicer, one strand of miRNA can bind to mRNA to initiate the interference pathway. Following binding, this strand recruits proteins to form what is called RISC (RNA-Induced Silencing Complex). The protein AGO2, a component of RISC, then cleaves the target mRNA, thus effecting the modulation of gene expression by mRNA degradation⁸. The same

RNA interference mechanism is valid for synthetic siRNA. Fig 1.2. illustrates the silencing mechanism of siRNA.

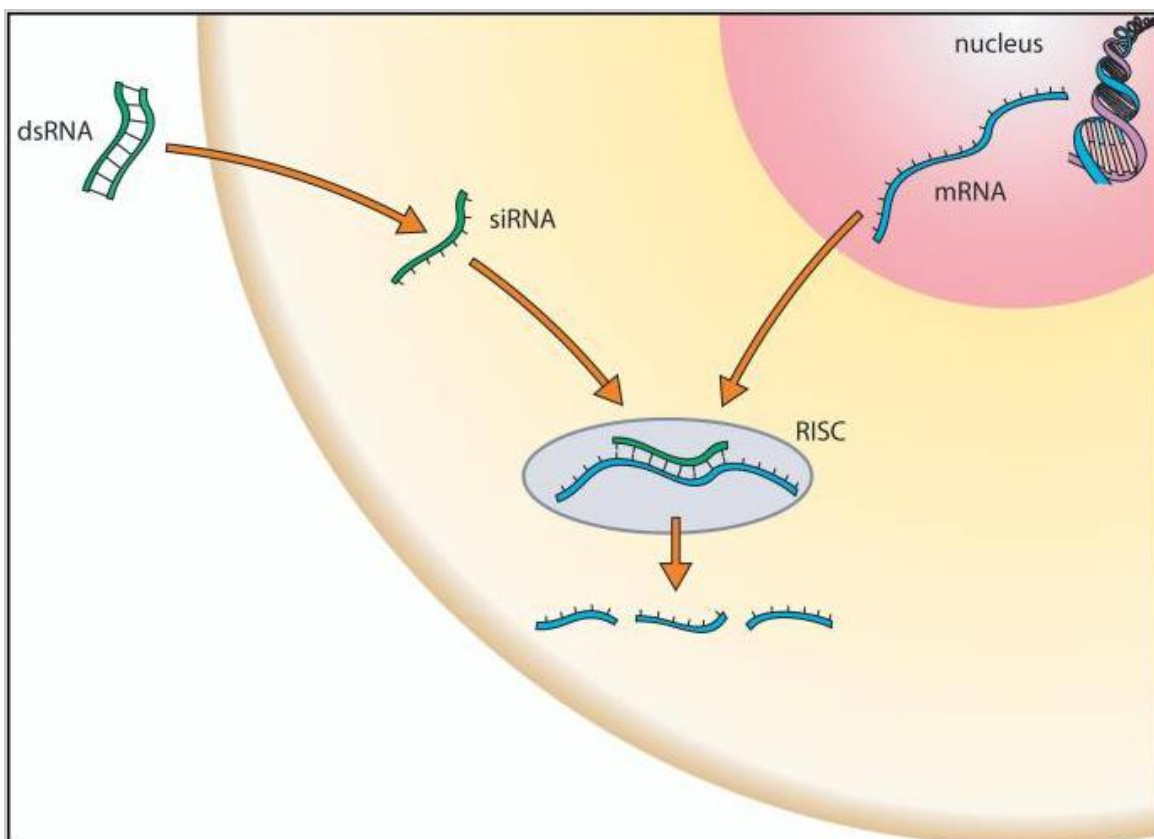


Figure 1.2. siRNA-based RNA interference mechanism. Reproduced from Robinson R. ⁷

Unlike siRNA silencing, AONs are fully complementary to target mRNA and bind to it by Watson-Crick base-pairing. Following binding, several mechanisms are responsible for disabling the target mRNA molecule. In the RNase H dependent pathway, the target mRNA is degraded by the enzyme RNase H, thus gene expression is decreased. In addition to RNase H dependent gene silencing, AONs can promote the dsRNase or RISC dependent degradation of mRNA. AONs can also be designed to inhibit polyadenylation, or translation to silence their target. AONs, designed to bind mRNA in exon-intron boundaries, can also alter the intrinsic RNA splicing patterns, and are used in the treatment of congenital splicing disorders. As such, genetic diseases such as muscular dystrophy can be treated with splice-correction antisenses⁹. (Figure 1.3.)

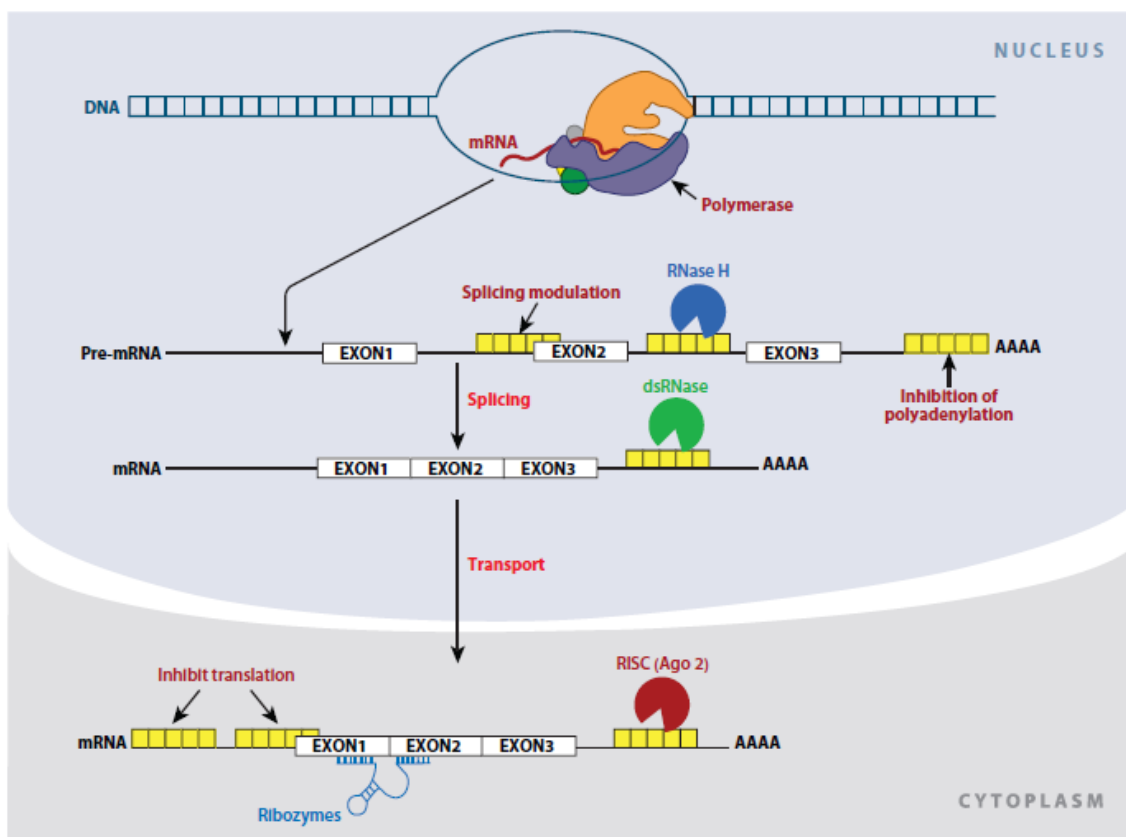


Figure 1.3. Different antisense oligonucleotide silencing mechanisms. Reproduced from Bennett *et al*⁶.

Steric blocking is another antisense-mediated mechanism and does not require RNase H or RISC dependent pathways for silencing. Nonsense-mediated decay was proposed as a mechanism for steric blocking AONs¹⁰. Steric blocking AONS might be advantageous due to their increased chemical stability, as chemical modifications such as 2' MOE are not restricted to the 5' and 3' ends and can be utilized to confer greater stability to these molecules..

In terms of their specificity, siRNAs are more prone to binding non-target molecules compared to AONs. While they show their effects via base-pairing, siRNAs are not required to be fully complementary with target mRNAs, and may tolerate mismatches and gaps except in a critical “seed region” of 5-6 bases¹¹. Therefore, siRNAs can bind to different target mRNA and may have off-target effects¹².

1.1.3. *In vivo* barriers for oligonucleotide delivery

In order to show its activity, an oligonucleotide drug has to overcome several *in vivo* barriers following its administration. When administered intravenously, the oligonucleotide might be degraded to some extent by enzymes in the blood plasma and tissues. These enzymes are primarily nucleases which cleave the phosphodiester bond of the oligonucleotide¹³. In order to combat these effects, the stability of the drug can be enhanced with chemical modifications that are discussed in the next section.

Due to their small size, oligonucleotides are readily excreted by renal filtration and their half lives in the plasma are generally short. In addition to enzymatic and excretory barrier, a third obstacle that drug has to overcome is the reticulo-endothelial system (RES). Due to the recognition of oligonucleotides as foreign molecules by phagocytic cells, RES clearance may lead to oligonucleotide accumulation in liver and spleen¹⁴. Yet another impediment to effective oligonucleotide function is the physical barrier formed by endothelial cells. The drug or the delivery system needs to exit from the blood vessel in order to function. After reaching the target tissue, the drug must also be internalized into cells and escape from endolysosomal complex inside the cell. Only after overcoming these barriers, will the oligonucleotide be functional and able to decrease the gene expression in the cytosol. There is another barrier for a specific type of AON, which is splice-correction antisense. As this AON functions in the nucleus, it needs to pass nuclear membrane as well¹⁵.

1.1.4. Chemical Modifications

To improve the stability of the drug and decrease its cleavage by nucleases, chemical modifications are frequently introduced into the structures of the nucleic acid drugs. These modifications serve to endow oligonucleotide drugs with improved stability, longer half life, better tissue distributions and better pharmacokinetic and pharmacodynamic properties. Two common strategies are used in nucleic acid modification: Backbone modifications (such as phosphorothioate modification) and sugar modifications (such as 2'-O-methyl or 2'-O-methoxyethyl)¹⁶. (Figure 1.4.)

Backbone modifications are generally introduced throughout the oligonucleotide, whereas sugar modifications are limited to the 5' and 3' ends of the nucleic acid, because sugar modifications may decrease the affinity of the drug to the target transcript.

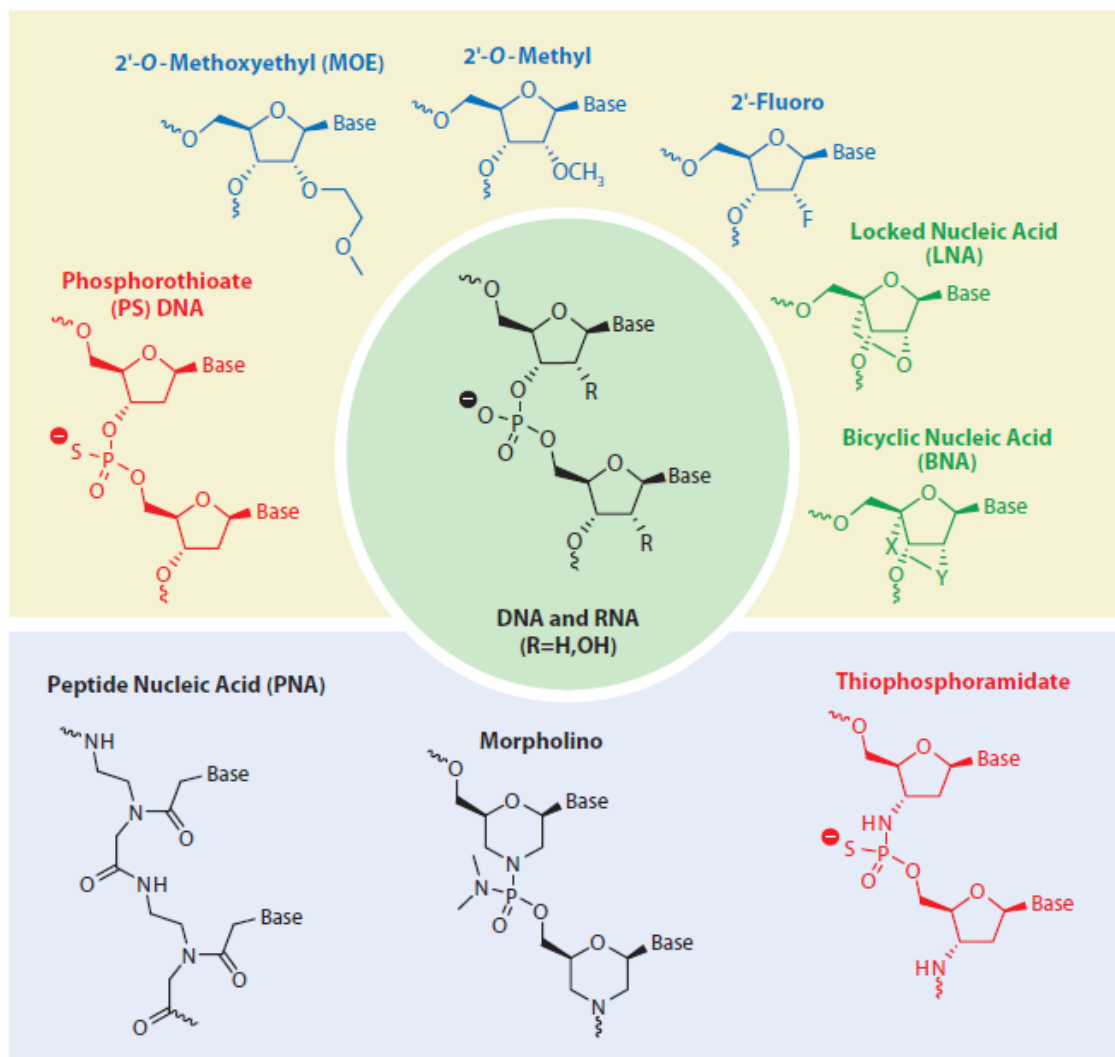


Figure 1.4. Chemical modifications in antisense oligonucleotides. Reproduced from Bennett *et al*.⁶

1.2. Drug Delivery Strategies

An efficient delivery strategy is indispensable in oligonucleotide therapy due to the limitations of naked oligonucleotide delivery and the undesired side effects often displayed by the free drug. These limitations include the low stability of the drug, poor pharmacodynamic and pharmacokinetic properties, and low cellular internalization¹⁴. As biological macromolecules, oligonucleotides are prone to nuclease attacks in biological fluids. Although the stability of the drug is improved with chemical modifications, the half life can still be below the desired level.

Many clinical trials have failed due to low pharmacokinetic and pharmacodynamic properties of these naked oligonucleotides. Due to the small size of oligonucleotide drugs, they can be eliminated by renal filtration or RES clearance. In many *in vivo* studies and clinical trials, oligonucleotides were observed to accumulate in liver and spleen¹⁷. In addition to these problems, tissue distributions of oligonucleotide drugs are often poor, and the cellular internalization of free nucleic acids is challenging due to their high negative charges. Even in *in vitro* studies, greater concentrations of the oligonucleotide drug are needed for naked oligonucleotide delivery to obtain same efficiency as carrier delivered oligonucleotide. Higher concentrations, however, may create more non-specific binding, rendering them disadvantageous¹⁸. Up to now, only two AONs were able to obtain Food and Drug Administration (FDA) approval while a vast majority of the clinical trials were terminated due to the ineffectiveness of the tested drugs. Biodistribution of the drug and low plasma half life appeared to be major hurdle in clinical studies, and low pharmacokinetic and pharmacodynamic properties were frequently observed in intravenous (IV) administration because of renal and RES clearance. Only two FDA approved drugs were successful in clinical trials according to their administration route and efficient localization to the target tissue. From these two, Fomivirsen was locally administered to the patients; therefore, the biodistribution of the drug was not a major concern as it is for IV administration. The target molecule of Mipoversen, the other approved molecule, was a liver protein, thus, liver accumulation of the IV-injected drug turned out to be an advantage for the effectiveness of drug. Apart from these two drugs, the IV injected AONs could be re-developed with a suitable targeted drug delivery strategy to overcome aforementioned obstacles.

1.2.1. Advantages of encapsulated delivery systems

The above-mentioned limitations in free nucleic acid delivery can be avoided with the use of a biocompatible and bioactive delivery system. There are several advantages of drug delivery systems, and these advantages are particularly effective when used to remedy the deficits of free oligonucleotide delivery systems. Carrier-based drug delivery approaches are capable of increasing drug efficiency by several means. The encapsulated drug is less prone to nucleases, so delivery system protects the oligonucleotide from degradation. Plasma half-lives are also extended due to greater protection from nucleases, less RES clearance and less renal excretion. As cellular internalization is enhanced under carried-enhanced delivery systems, lower drug

concentrations are required to be administrated in these approaches, which prevent non-specific interactions and unforeseen side effects. With a targeted delivery system, oligonucleotides can be delivered to a specific tissue or cell type, local concentrations of the drug in the target tissue can be increased and therefore side-effects are largely eliminated. Intracellular localization can also be adjusted by altering the design of the carrier material. Finally, the slow release of the drug can be achieved with an appropriate delivery system ¹⁹.

1.2.2. Properties of the delivery system

The choice of carrier material is an important issue, as the carrier is the major determinant of the composite drug's biocompatibility and biodegradability. The material used must be inert, but more importantly, it must be biodegradable.

The charge of the system is another property that is important for oligonucleotide complexation. Cationic lipids and polymers have been proven to complex with DNA more efficiently. Their positive charges can better facilitate electrostatic interactions between the material and the highly-negatively charged DNA molecules. Further, cationic materials can enhance cell surface binding and are shown to be efficient in transfection.

Bioactivity can be provided to the system by appending biologically important molecules on the surface. These molecules can be introduced for targeting strategies or for cellular internalization purposes. Stimuli responsive materials are also used for targeting or endosomal escape-related purposes.

1.2.3. Biomaterials as delivery systems

Different biomaterials such as lipids, polymers, peptides and inorganic nanoparticles are used as a non-viral nucleic acid carrier. More primitive delivery system designs were based on cationic lipids and polymers²⁰. These molecules can bind to negatively charged oligonucleotides via electrostatic interactions. Also positive charge of the molecule can increase cell surface binding and cellular internalization. Due to non-specific binding of cationic liposomes and polymers, neutral or negatively charged materials were also developed. In oligonucleotide delivery, there

has been an effort to balance off the transfection efficiency of the material and cytotoxicity with different delivery systems which are described below.

1.2.3.1. Lipids

Lipids can form monolayers, bilayers or multilayers in water. The formation of these molecules depends on the chemical structure of the lipid. Liposomes, various type of micelles and lipid nanoparticles have previously been used for drug delivery purposes^{21,22,23}. In addition, nucleic acid delivery using lipid based formulations has been the subject of extensive research²⁴⁻²⁶. As a lipid based delivery system, cationic lipids have been widely used for oligonucleotide and gene delivery. Cationic molecules can form complexes with nucleic acids via electrostatic interactions. In addition, interaction of cationic complexes with the cell membrane is enhanced compared to naked nucleic acids. The use of lipids for DNA delivery was first accomplished in 1987 for DNA delivery with a N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) based cationic lipid²⁷. This was followed by several other lipid formulations such as 2,3-dioleoyloxy-N-[2-spermine carboxamide] ethyl-N,N-dimethyl-1-propanammonium trifluoroacetate (DOSPA); 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); dioctadecyl dimethylammonium bromide (DODAB); N-[1-(2,3-dimyristyloxy) propyl]-N,N-dimethyl-N-(2-hydroxyethyl) ammonium bromide (DMRIE) and dioctadecyl amidoglycyl spermine (DOGS)²⁴⁻²⁶. Although cationic lipids can provide high transfection efficiencies, their positive charge may increase their binding to serum proteins and lead to aggregation^{28,29}.

1.2.3.2. Polymers

Cationic polymers have also been extensively studied for gene delivery. Polymers have been an area of interest due to their ease of synthesis, low costs and ability to form stable complexes with DNA (polyplexes). Cationic polymers such as poly-L-lysine (PLL), polyethyleneimine (PEI) and polyamidoamine dendrimers (e.g. PAMAM) have been efficient vectors for DNA delivery. PEI is a pH responsive polymer; it is protonated in the acidic organelles and hence facilitates the endosomal escape of its cargo²⁰. However, the cytotoxicity of cationic polymers such as PEI is proportional to their transfection efficiency^{30,31}. Besides their toxicity, polymers possess other

disadvantages as delivery vehicles, such as concerns about quality control and strong batch-to-batch variances in their molecular weight³².

1.2.3.3. Nanoparticles

Gold nanoparticles^{33,34}, iron oxide nanoparticles³⁵ and silica nanoparticles³⁶ are the main inorganic nanoparticles investigated for DNA delivery applications. These nanoparticles have been functionalized with different biocompatible materials; usually with polymers like chitosan, poly-L-lysine (PLL), poly-L-lactic acid (PLLA) and polylactic-co-glycolic acid (PLGA)^{37,36}. Different strategies were used to allow the covalent binding of oligonucleotide to the particles; for example, oligonucleotides were reported to be conjugated to gold nanoparticles using dithiolphosphoramidite (DTPA)³⁴.

1.2.3.4. Peptides

Peptide based materials have been widely used in DNA delivery³⁸ due to their biocompatibility and bioactivity. Among bioactive peptides, cell penetrating peptides (CPPs) in particular have received much attention as potential delivery vectors³⁹. CPPs, also known as protein transduction domains (PTD), facilitate transport of the cargo through the cell membrane. There are different classes of CPPs and one classification is made depending on the chemical structure of the peptide: amphipathic, hydrophobic and cationic. Cationic CPPs constitute 83% of all CPPs, amphipathic peptides 44% and hydrophobic peptides only constitute 15% of CPPs⁴⁰.

Another classification system is based on the design of the CPP: protein derived, synthetic or chimeric⁴¹. Penetratins and TAT derived peptides are well known examples of protein-derived CPPs. Penetratins are derived from the homeodomains of homeoproteins, which are transcriptional factors that show strong DNA-binding capacity. Tat is a transcription activating factor involved in the replication of HIV virus. It contains three domains; an acidic domain for transactivation, a DNA binding region and a basic domain for nuclear transport which functions as a PTD⁴².

Peptide sequences derived from signal sequences (membrane translocation sequences) of proteins are another major source of protein derived CPPs. Nuclear localization sequence (NLS)

is a short peptide signal recognized by the nuclear pore complex. NLS is composed of arginine proline and lysine rich motifs. There are a variety of NLS derived sequences such as PKKKRKV (derived from simian virus 40), VQRKRQKLMP (derived from NF- κ B), GRKRKKRT (derived from Oct-6) and SKKKKTKV (derived from TFIIE-beta)⁴⁰.

Transportan, as an example of a chimeric peptide, is designed from galanin and mastoparan with an amino acid substitution. There are also completely synthetic peptides such as KLALKLALKALKAAALKLA, which is a CPP designed to model alpha-helical amphipathic peptide⁴³. In addition, CPPs may be designed to bind specific molecules on the cell surface, or to bind nucleic acids, and the peptide sequences of such peptide vectors are derived from protein domains that are known to be responsible for these types of binding. Some cationic peptides, for example, were designed to bind to negatively charged proteoglycans (heparin proteoglycans) on the cell surface. Their sequence therefore contained positively charged amino acids; arginine and lysine.

The most well known example of synthetic peptides is oligoarginines which are designed as cationic model peptides. Oligoarginines are known to be adsorbed to the negatively charged cell surface and after accumulation on the membrane, can penetrate through it. The accumulation of oligoarginines on phospholipid membranes was studied with molecular dynamics by Vazdar *et al.* Their model illustrated that oligoarginines aggregate more on the phospholipid membrane compared to oligolysines which are also cationic peptides⁴⁴. Oligoarginines can form complexes with nucleic acids via electrostatic interaction. It is also proposed that the stable complex formation between arginine and the phosphate backbone of nucleic acids is favored through hydrogen bonding⁴⁵. The entry mechanism of PHEA-Arg8 was suggested to be adsorptive endocytosis by Yang *et al.*, which outlined a potential cell entry method involving the adsorption of a cell-penetrating peptide on the cell membrane followed by internalization⁴⁶.

There are also proline rich amphipathic CPPs in nature and proline, alongside arginine and lysine, is considered to be one of the most important amino acid residues for membrane penetration⁴⁰. Proline is a rigid hydrophobic amino acid that readily assembles into polyproline II secondary structure which is a left-handed helix with 3.0 amino acids per turn. This type of secondary structure (PPII) is specific to prolines and it is different from well known right handed alpha helix secondary structure. The membrane penetration capacity of proline-rich sequences is

also enhanced by their association with recognition sites in protein-protein interactions⁴⁷. Several proline-rich CPPs have been investigated; bactenecin 7 (Bac-7) and sweet arrow peptide (SAP) are well known examples of this group. Bac-7 sequences from different bacterial species were compared in the literature, and the general structure of this protein was shown to be composed of a (PR) region, one or more (PX) repeats, and a basic site. The proline rich sequence has been reported to be important not only for largely determining the protein secondary structure, but also for providing hydrophobic domains that are important for membrane penetration⁴⁷. The SAP sequence (VRLPPPVRLLLLPPPVRLPPP) is also composed of proline- rich regions and features several P₃ repeats. While proline provides hydrophobic domain and creates a distinct secondary structure in large assemblies, single prolines are also important for providing a hinge to the structure. For instance, the single proline residue in buforin II was shown to be important for protein folding and the determination of its final structure; when the proline residue was removed, peptide lost its cell penetrating ability⁴⁸.

CPPs that are derived from natural proteins might be associated with toxicity depending on the concentration and membrane permeability^{49,50}. Thus, optimization of the delivery system with a non-toxic but efficient CPP is of great importance in the field of oligonucleotide delivery.

1.2.4. Cellular internalization strategies of nanocarriers

After reaching the target tissue, the carrier should be internalized into cells to display its effects. While there are many different pathways for internalization, nanocarriers are typically taken up by cells via endocytosis. Clathrin mediated endocytosis, caveole mediated endocytosis and micropinocytosis are well known types of endocytosis⁵¹. (Figure 1.5.) Phagocytosis and direct transduction are other pathways that might be involved in the internalization of the drug-encapsulated molecules.

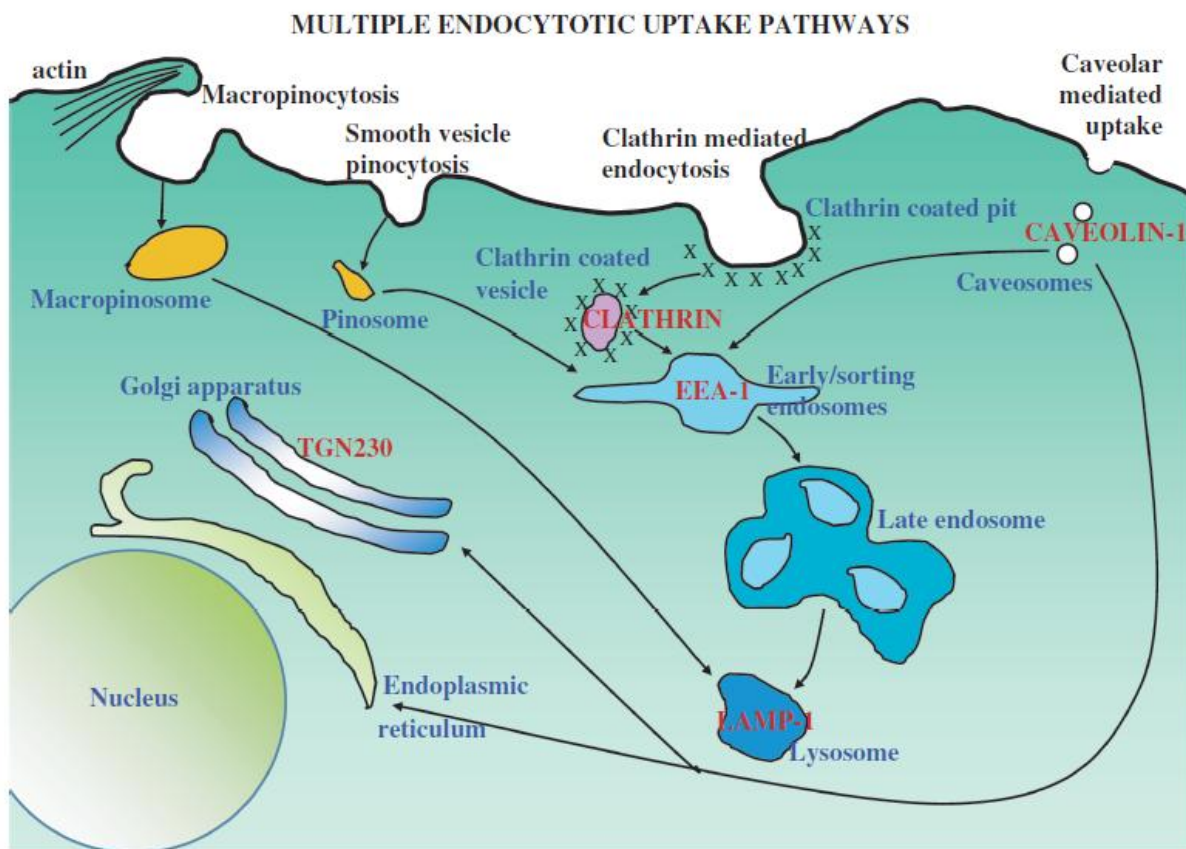


Figure 1.5. Mechanisms in oligonucleotide uptake. Reproduced with permission from Juliano *et al.*⁵²

Unlike other types of nanocarriers, cell penetrating peptides can enter cells via penetration or pore formation on the membrane. It was initially believed that CPPs can form pores on the membrane or directly translocate through membrane, and that endocytosis was not a common mechanism. However, it was later demonstrated that CPPs can be internalized by various endocytosis pathways as well, including clathrin-mediated endocytosis, caveolae-mediated endocytosis and micropinocytosis⁵³.

The corpus of knowledge regarding the internalization of CPPs has been contradictory and not very well-established. The entry mechanism may differ depending on the effect of peptide concentration, serum presence, cell type and the cargo carried by the peptide vector. Regarding the effect of peptide concentration, it was previously claimed that endocytosis might be the main mechanism at high concentrations, whereas direct translocation is the dominant mechanism at

lower concentrations⁵⁴. On the other hand, it was also shown that fluorescently labeled arginine-rich peptides can enter cells directly at high peptide concentrations^{53,55-57}.

The internalization was also shown to depend on the type of cargo. For TAT peptide, direct translocation was claimed to be the preferred mechanism only for small molecules. Moreover, CPP-mediated uptake of large molecules such as proteins depends on the endocytic ability of the cell type⁵⁸. Richard *et al.* studied the mechanism of unconjugated TAT peptide internalization and they proposed that the uptake is mediated by clathrin dependent endocytosis and that it involves heparan sulfate receptors⁵⁹.

In addition to cell penetration, CPPs can facilitate the endosomal escape of the cargo due to their membrane penetration ability. Several modifications have been made to CPPs to enhance the endosomal escape of their cargo⁶⁰. Recently one of these modifications, stearic acid was shown to be effective for oligonucleotide delivery⁶¹.

Multivalency is another strategy to induce endosomal escape in CPP-mediated delivery systems. Multivalent CPPs that are presented on the surface of liposomes or dendrimers have been reported to decrease endosomal entrapment⁶².

1.2.5. Targeted Delivery Strategies

The targeted delivery approach has been a major focus of nucleic acid delivery investigations due to the poor biodistribution and rapid elimination of oligonucleotides. Specific targeting of an oligonucleotide drug to its target tissue would serve to decrease drug over-accumulation in the liver and kidney, side effects associated with non-specific binding, and high dose-related toxicity⁶³.

A variety of strategies have been devised to direct oligonucleotides to a specific organ or tissue. One such strategy is the conjugation of a ligand to the oligonucleotide. Small molecules, peptides and aptamers are suitable ligands for this purpose. Another method is the decoration of the delivery system (liposomes, polymers or nanoparticles) with a ligand or bulky proteins⁶⁴. This strategy is based on the non-covalent interaction between the carrier and oligonucleotide, which is loaded to the delivery system instead of chemical coupling to the targeting molecule.

1.2.5.1. Covalent binding strategies

In this approach, the targeting ligand must be a highly selective molecule that recognizes a specific receptor or protein. The conjugation of cholesterol to the nucleic acid is one example of this method. siRNA-cholesterol conjugates have been shown to target low-density lipoproteins (LDL) and high-density lipoproteins (HDL), and are, therefore, efficient in silencing apolipoprotein B (apoB) in the liver of mice⁶⁵. There are other ligands that have been used to target tumor tissue, benefiting from the fact that specific cell surface markers are overexpressed in cancer cells. One of these ligands is folic acid, which binds to folate receptors that are overexpressed in many cancer cells⁶⁶. In the breast cancer model Her-2, AONs were delivered with a liposome functionalized with a folate ligand. Under *in vitro* conditions, this system was shown to be more effective than Lipofectin transfection and the stability of the system was shown to be increased in circulation⁶⁷.

The best-known strategy of covalent binding is the peptide modification of oligonucleotides, also known as peptide nucleic acids (PNA)⁶⁸. Targeting peptides can be used in this modification⁶⁹. For example, the muscle targeting peptide ASSLNIA, identified by phage display,⁷⁰ has been used for delivery of a splice correcting morpholino oligonucleotide for the treatment of muscular dystrophy⁷¹. Upon the systemic administration of the peptide-oligonucleotide conjugate, dystrophin expression was reported to be restored in muscular dystrophy model (mdx) mice.

Compared to traditional delivery systems, the bioelimination of PNA complexes are expected to be more rapid, owing to their small sizes. Therefore, including a targeting moiety on the surface of a conventional delivery system might be a better strategy for combining the advantages of the delivery vesicle and the targeting molecule. The major concern about the conjugation of the ligand to the oligonucleotide is that the conjugation may decrease the efficiency of the oligonucleotide by decreasing its binding to the complementary strand. Non-covalent interactions between peptide and oligonucleotide is more advantageous in terms of the ease under which the carrier complex can be generated and the nuclease protection that the carrier system affords to its oligonucleotide cargo.

1.2.5.2. Non-covalent binding strategies

Antibody targeting (immunotargeting) is a specific targeting mechanism for oligonucleotides⁶⁴. A delivery system can be functionalized with an antibody to selectively bind to a specific tissue or cell type that possesses a specific protein on its cell membrane. In breast cancer, for instance, the Her-2 receptor is frequently overexpressed, and carrier molecules can be functionalized with the Her-2 antibody to selectively deliver oligonucleotides to the tumor site⁷². Although immunotargeting is very specific, it has a significant drawback in that it is difficult to attach a bulky antibody on the surface of the delivery system. Instead of proteins, the targeting peptides or cell surface binding epitopes of the proteins can be used. For example, the RGD peptide, which targets integrin alpha (V) beta (3) that is overexpressed in certain tumors, has been used for functionalization of carriers and the delivery of the cargo to the tumor tissue⁷³.

Targeting ligands are also used for the functionalization of the carrier. For cancer targeting, glucose has been widely used as a targeting ligand to deliver cargo selectively to the tumor tissue^{74,75}. Increased glucose uptake is one of the hallmarks of cancer⁷⁶ and occurs as a result of increased metabolic rates, also known as the Warburg effect. The Warburg hypothesis describes enhanced glucose metabolism in cancer cells, in which glucose uptake is increased due to overexpression of glucose transporters. The metabolic switch from oxidative phosphorylation to glycolysis occurs due to the high energy demands of dividing cancer cells⁷⁷. Based on this property, glucose analogues are used in clinics for monitoring tumor progression⁷⁸. Similarly, glucose-conjugated molecules are more inclined to localize in tumor tissues⁷⁴.

1.2.6. Peptide amphiphiles (PA)

Bioinspiration, the mimicry of natural designs for material engineering, has allowed the design of molecules that can self-assemble into well-defined macromolecular structures. Peptide amphiphiles, which consist of a hydrophobic alkyl tail and a hydrophilic peptide sequence, form micelles in the aqueous media due to electrostatic interactions that force their hydrophobic sections away from the water⁷⁹. β -sheet forming amino acids facilitate the formation of elongated micelles and, by extension, nanofibers⁸⁰. Proline rich peptide amphiphiles, on the other hand, can form spherical nanostructures instead of nanofibers⁸¹. The shape of a self-assembled peptide nanostructure can therefore be modulated by changing the amino acid compositions. Self-assembling peptide amphiphiles have been shown to be effective carriers in drug delivery^{82,83}. In

addition, controlled release of AON from self-assembled hydrogel was demonstrated previously⁸⁴.

The present study details the first investigation of the effect of self-assembled PA nanocarrier morphology on the cellular delivery of AONs. In addition to forming two distinct nanostructure geometries, the delivery system was decorated with targeting peptides and cell penetrating peptides to enhance AON delivery. The effect of different bioactive epitopes on the delivery of oligonucleotides was investigated, and the internalization of the vehicle, the intracellular localization, the mechanism of internalization and the effect of the drug in gene silencing were evaluated. Overall, our conclusions from these investigations have allowed the design and development of a novel biocompatible bioactive oligonucleotide delivery system using peptide amphiphiles.

CHAPTER 2. MATERIALS AND METHODS

2.1. Materials

9-Fluorenylmethoxycarbonyl (Fmoc) and tert-butoxycarbonyl (Boc) protected amino acids except glyco amino acid, [4-[α -(2',4'-dimethoxyphenyl) Fmoc-aminomethyl]phenoxy]acetamidonorleucyl-MBHA resin (Rink amide MBHA resin), and 2-(1H-Benzotriazol-1-yl)-1,1,3,3 tetramethyluronium hexafluorophosphate (HBTU) were purchased from NovaBiochem. Fmoc-Ser[β -Glc(OAc)₄]-OH was purchased from AAPPTec. Lauric acid and N,N-Diisopropylethylamine (DIEA) were purchased from Merck. Other chemicals were purchased from Alfa Aesar or Sigma-Aldrich and used as provided. All water used was deionized water with a resistance of 18 M Ω .cm (Millipore Milli-Q). Cell culture media, fetal bovine serum, penicillin-streptomycin and trypsin were purchased from Gibco, Life Technologies. Lipofectamine and Alamar Blue were purchased from Invitrogen, uptake inhibitors were purchased from Sigma. Bcl-2 antisense oligonucleotide (G-3129, Genasense) and Fluorescein-6-amide (FAM) labeled antisense oligonucleotide having the sequence: 5'-tct ccc agc gtg cgc cat-3' were donated by Genta Inc. G4126, the mismatch (MM) control with a sequence 5'-tct ccc agc atg tgc cat-3', was used in expression studies.

2.2. Synthesis and purification of peptide amphiphiles

2.2.1. Synthesis of peptide amphiphiles

Peptide amphiphiles were synthesized with standard Fmoc solid phase peptide synthesis method. As a solid phase Rink Amide MBHA (0.59 mmol g⁻¹ loading) resin was used. The resin was swelled in DCM (Dichloromethane) and the solvent was subsequently exchanged to DMF (N,N-dimethylformamide), in which all remaining reactions were carried out. Between each step, the solid phase was washed three times with DMF, three times with DCM and three times with DMF to remove unreacted chemicals. The first step of synthesis comprised the cleavage of Fmoc groups, and was done by addition of 20% piperidine in DMF and treating for a period of 20 minutes. Then, Fmoc protected amino acids were dissolved in 8 ml DMF and amino acids were

activated with O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU), and N-ethyl-diisopropylamine (DIEA). Fmoc protected amino acid, HBTU and DIEA were added in an equivalency of 2:1.95:3 respectively. Dissolved and activated amino acid was coupled to resin or previous amino acid for 2 hours. Third, to determine the completeness of coupling, Kaiser test was performed after each coupling. Forth, to block the unreacted amine groups, 10% acetic anhydride was used for 30 minutes. After repeating these four steps for coupling of each amino acid, peptide chain grew on the resin and alkyl tail (lauric acid) was coupled with same method. To collect the peptide amphiphile from solid phase, it was cleaved from resin for 2 hours with trifluoroacetic acid (TFA): triisopropylsilane (TIS): water at the ratio of 95:2.5:2.5. The solvent (DCM) and TFA were evaporated with rotary evaporator. Ice cold diethylether was added on the remaining viscous solution and left overnight at -20 °C. After centrifugation at 8000 rpm for 15 minutes, peptide amphiphile precipitated, diethylether was decanted and remaining was evaporated in chemical hood. Peptide amphiphile was dissolved in ddH₂O, after leaving -80 °C four hours, it was freeze dried for three days.

2.2.2. Purification of peptide amphiphiles

Peptide amphiphiles were purified on an Agilent 1200 HPLC having Agilent Zorbax 300SB-C8 (21.2 x 150 mm) column with water (0.1% TFA)/acetonitrile(0.1% TFA) gradient. The purified peptides were characterized by reverse phase HPLC on an Agilent 6530 accurate-Mass Q-TOF LC/MS equipped with an Agilent 1200 HPLC. A phenomenex Luna 3 μ C8 100A (50 x 3.00 mm) column and water (0.1% formic acid)/acetonitrile (0.1% formic acid) gradient were used as stationary phase and mobile phase, respectively. After HPLC purification and lyophilization, remaining TFA was exchanged with chloride ions by addition of 1 mL, 1 mM HCl to 5 mg peptide. Then peptide was lyophilized again and stored at -20 °C.

2.3. Characterization of PAs and PA/AON complexes

2.3.1. Circular Dichroism

PA samples were prepared at a final concentration of 200 μ M and for the PA/AON combinations samples were prepared to have 30:1 PA/AON molar ratio keeping the PA concentration same. Quartz cuvette with 1 mm pathlength was used for measurements. Circular Dichroism analysis

was performed with J-815 Jasco spectrophotometer in the far UV region. Parameters were selected as following: Integration time of 4 s, bandwidth of 1 nm, and data pitch of 0.1 nm. The average of three readings between 190 and 300 nm were collected. Ellipticity was converted to molar ellipticity with the unit degree cm² mol⁻¹ using following formula.

$$[\theta] = \frac{100 \times \theta}{(C \times l)}$$

[θ]: Molar ellipticity, θ: Ellipticity in degrees, C: Concentration in M, l: length in cm

2.3.2. Zeta Potential and DLS

A Malvern Zetasizer nano-ZS ZEN 3600 (Malvern Instruments, USA) instrument was used for zeta potential and Dynamic Light Scattering (DLS) experiments with detector angle of 173°. Glass cuvette was used for DLS measurements and zeta potential disposable cuvette was used for zeta potential measurements. Samples were prepared to have AON concentration of 1 μM. Peptide concentration was calculated accordingly to have a 3:1, 10:1, 30:1, 100:1 and 300:1 PA/AON concentration. Only PA sample was prepared to have a 100 μM PA concentration. At least three measurements were done with automatic subreadings.

2.3.3. Morphological characterization of peptide amphiphiles and peptide amphiphile oligonucleotide complexes

TEM samples were prepared on a 200-mesh copper grid. Dilute solutions of PA to have 0.05% (w/v) and PA/AON complexes to have 0.05% (w/v) and 50 ng/μL AON concentrations were prepared. Sample was dropped onto the grid after 5 min, sample is removed and grid is stained with 2% uranyl acetate for 3 min. After washing, samples were air dried and images were obtained with a FEI Tecnai G2 F30 TEM.

2.4. Maintenance of cells and transfection with PA and PA/AON complexes

Cells were maintained in 75 cm² and 162 cm² flasks with standard medium, containing Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), and 1%

penicillin–streptomycin and passaged at a cell confluency between 80 and 90% using trypsin–EDTA. In transfection experiments PA/AON complexes were prepared in ddH₂O to have molar ratio of 30:1 and these complexes were administered in serum free medium (1% penicillin–streptomycin containing DMEM).

2.5. Cell viability assay

To investigate the effect of PA solutions on cellular viability, MCF-7 cells were seeded at a cell density of 1×10^4 cells per well in 96 well-plate with standard medium (DMEM, 10% FBS ,1% PS). After 24 h medium was exchanged with serum free medium and PA solutions were administered to have final concentration of 200 μ M, 100 μ M, 50 μ M and 25 μ M. After 24 h or 48 h of incubation, medium was discarded and Alamar Blue assay was performed. Cells were incubated with 10% Alamar Blue in serum free media for 4 h. Then, fluorescence at 560/590 nm excitation/emission was measured with a microplate reader (Molecular Devices Spectramax M5). Fluorescence showing the viability of cells was normalized to non-treated control.

2.6. Cellular internalization of PA/AON complexes observed with confocal microscopy

13 mm glass coverslips were placed in 24-well plates and 4×10^4 MCF7 cells per well were seeded in standard medium. After 24 h medium was discarded and PA/AON complexes were administered in serum free medium at molar ratio as indicated in the graphs. AON final concentration was 1 μ M and PA concentrations were calculated accordingly. After 4 h or 24 h, cells were washed with PBS three times, fixed with 4% PFA, stained with phalloidin and TO-PRO-3 for counter-staining. Samples were visualized with Laser Scanning Confocal Microscope (LSM 510, Zeiss).

2.7. Cellular internalization of PA/AON complexes quantified with flow cytometry

For flow cytometry, 8×10^4 MCF-7 cells per well in 24 well plates or 2×10^4 MCF-7 cells in 96 well plates were seeded. After 24 h of cell seeding, medium was discarded and serum free medium was added. Prepared PA/AON complexes to have final concentrations of 30 μ M PA and 1 μ M AON were administered. Cells were collected with trypsinization, dissolved in PBS, centrifuged again, washed once again with PBS, and finally dissolved in PBS. Cells were

analyzed with Guava easycyte flow cytometer (Milipore). Cells were gated by SSC (side scatter channel) and FSC (forward scatter channel) using non-treated control. Fluorescence intensity of FAM-AON was measured with Green channel and the voltage of green channel was adjusted using non-treated cells.

2.8. Mechanisms of cellular internalization

Small molecule chemical inhibitors were used to inhibit specific pathways and study the internalization mechanism of PA/AON complexes. Inhibitor concentrations were adjusted as the maximum dose that did not induce cell death.

2×10^4 MCF-7 cells in 96 well plates were seeded. After 24 h of cell seeding, medium was discarded and serum free medium was added. Inhibitor solutions were administered to have a final concentration of 100 μ M amiloride, 20 μ M dynasore, 0.2 μ g/mL chlorpromazine, 10 μ g/mL nystatin. In glucose transporter inhibition studies, 1000 μ M phloridzin dihydrate, 50 μ M cytochalasin B final concentrations were used. After 1 h of inhibitor administration, prepared PA/AON complexes to have final concentrations of 30 μ M PA and 1 μ M AON were administered. Cells were collected with trypsinization dissolved in PBS and centrifuged again. Cells were washed once again with PBS, centrifuged, and finally dissolved in PBS. Internalized AON in cells were analyzed with Guava easycyte flow cytometer (Milipore). Cells were gated by SSC (side scatter channel) and FSC (forward scatter channel) using non-treated control. Fluorescence intensity of FAM-AON was measured with Green channel.

2.9. Gene Expression studies

Expression levels of targeted gene (Bcl-2) in AON-PA transfected cells were investigated. 4×10^5 MCF-7 cells per well in 6 well-plate were seeded with standard medium. After 24 h medium was discarded and PA/AON complexes were administered in serum free medium to have a final concentration of 2 μ M AON and 60 μ M PA. Complexes were incubated for 24 h except R₈-Pro-PA, which was administered at 20 μ M concentration and incubated for 4 h due to its toxicity. Lipofectamine control, only AON and mismatch (MM) were also administered to have a final concentration of 2 μ M oligonucleotide. Lipofectamine used according to the manufacturer's instructions. After incubation, cells were washed with PBS and RNA was extracted with TRIzol

reagent (Invitrogen). Expression levels of Bcl-2 gene were investigated with Biorad C1000 thermal cycler using one step qRT-PCR kit (Invitrogen). Expression levels were normalized to GAPDH gene.

2.10. Monitoring internalization of PA/AON complexes with confocal microscopy

2×10^5 MCF-7 cells per well in 6 well-plate were seeded with standard medium. Plates with glass coverslip-bottoms that were designed for live cell imaging were used. After 24 h of cell seeding, medium was discarded, serum free medium was added and LysoTracker Red DND-99 (Life-Technologies) was added to have a final concentration of 50 nM. After 30 min, PA/AON complexes were administered in serum free medium to have a final concentration of 1 μ M AON and 30 μ M PA. Using Laser Scanning Confocal Microscope (LSM 510, Zeiss) acquisition was done with 5 min intervals. Cells are incubated at 37 °C, 4% CO₂ in a humidity chamber attached to the microscope.

CHAPTER 3. THE EFFECT OF SHAPE OF PEPTIDE NANOSTRUCTURES IN CELLULAR INTERNALIZATION AND TARGETED DELIVERY SYSTEM FOR OLIGONUCLEOTIDE THERAPY

3.1. Background

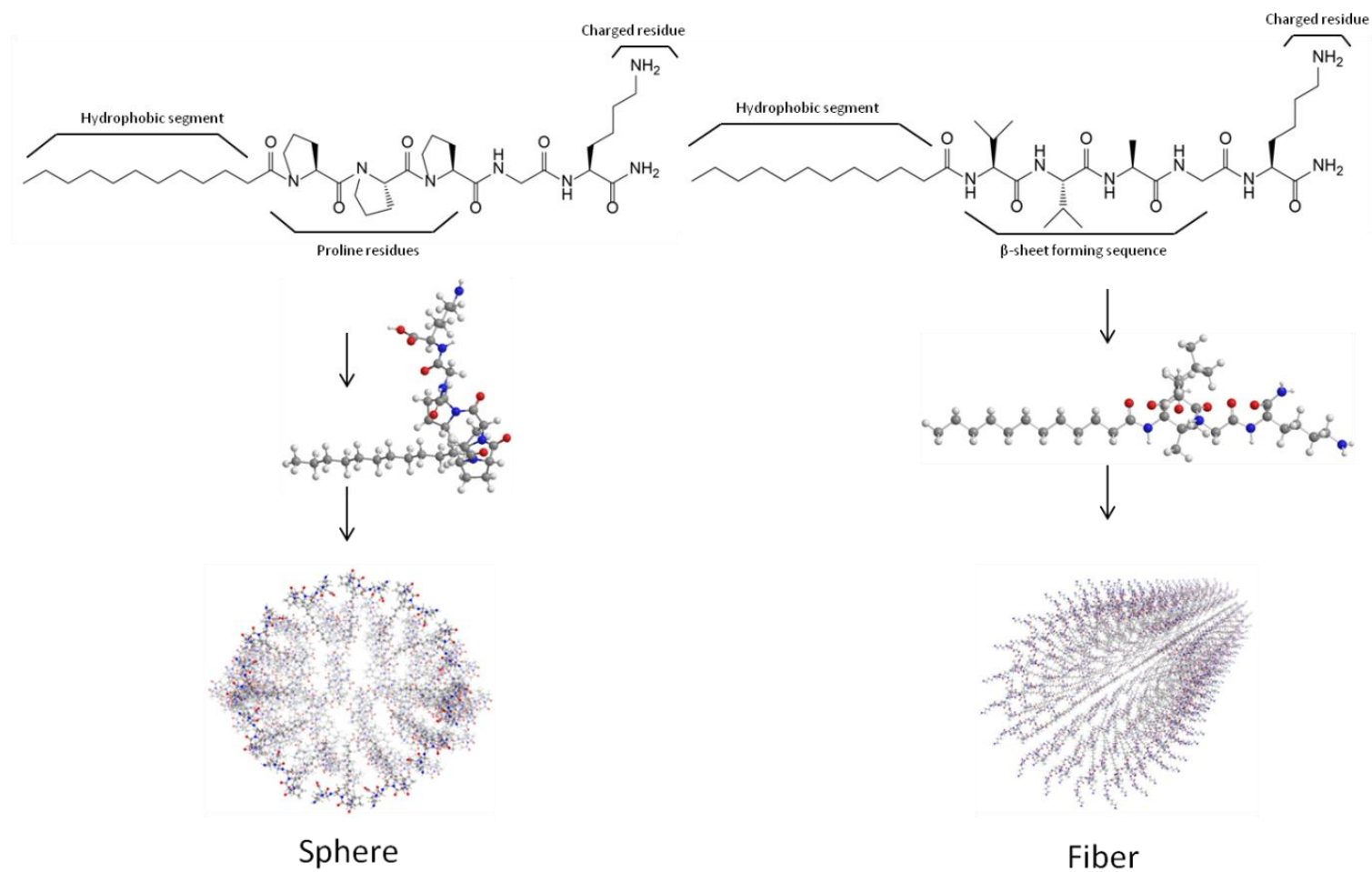
Development of an appropriate delivery system is important to overcome limitations in free oligonucleotide delivery. Therefore, we developed a self-assembled peptide amphiphile delivery system for oligonucleotides. The shape of the delivery system is a key determinant in the cellular internalization of drug molecules,^{85,86} and the effect of geometry in cellular internalization was not previously studied with peptide based delivery systems. Modulating the cellular internalization pathway according to the shape of the delivery system is important to develop advanced delivery materials. Therefore, we designed peptide amphiphiles that can self-assemble to two distinct nanostructures via minor alterations in their amino acid sequence. As targeted delivery is crucial to eliminate side effects and to increase the effectiveness of the drug, the peptide amphiphile delivery system was functionalized with a glucose targeting moiety to allow specific targeting to tumor sites.

3.2. Design and synthesis of peptide amphiphiles

Peptide amphiphiles were designed to form spherical or fibrous nanostructures. Two different nanostructures were formed depending on peptide sequence; K-PA and Glc-K-PA formed nanofibers due to the presence of VVA (β -sheet forming sequence) whereas P-PA and Glc-P-PA formed nanospheres due to the PPP (proline rich sequence) motif. Scheme 1 details the self-assembly of these peptide amphiphiles. PAs were designed to contain two distinct sections that possess different functions. PAs contain a hydrophobic tail formed by an alkyl tail (C_{12}), and a peptide sequence to drive the self-assembly. In addition, a positively charged amino acid (lysine) is added to the sequence to allow the complexation of PAs with AONs through electrostatic

interactions. Hydrophobic forces and electrostatic interactions drive the self-assembly of these structures, burying the hydrophobic alkyl tail towards the interior and forcing bioactive peptide part towards the exterior of the resulting network.

Figure 3.1. illustrates the PAs that were used in this part of the study. Glucose unit was used as a targeting moiety. Glc-K-PA and Glc-P-PA were designed to contain a glyco-serine amino acid which would be presented on the surface after self-assembly.



Scheme 3.1. Schematic representation of peptide amphiphiles that can self-assemble to form nanospheres or nanofibers.

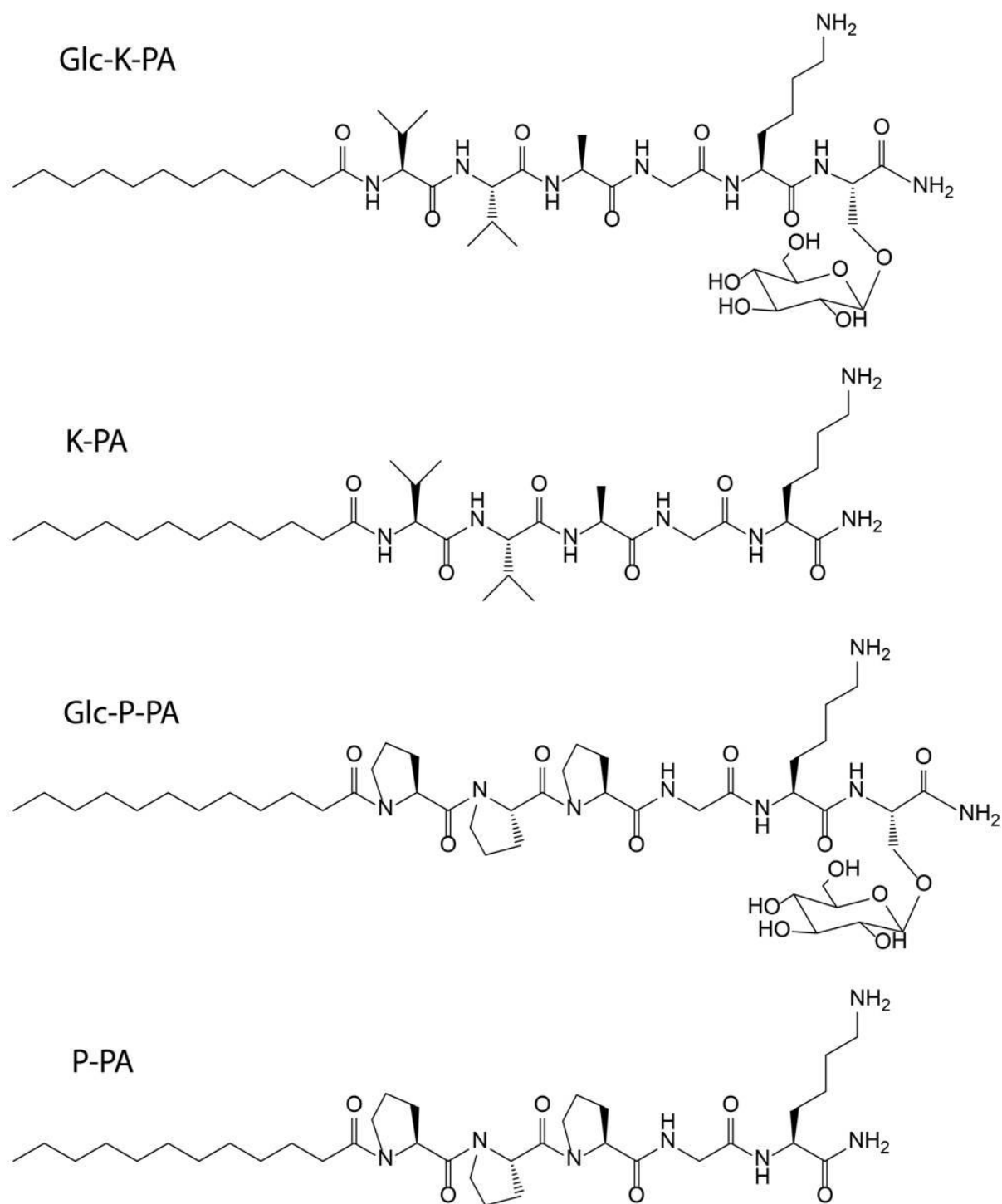


Figure 3.1. PAs used in this study. Glc-K-PA (C_{12} -VVAGKS(Glc)-Am), K-PA (C_{12} -VVAGK-Am), Glc-P-PA (C_{12} -P₃GKS(Glc)-Am), P-PA (C_{12} -P₃GK-Am)

After synthesis and purification of PAs, chemical characterization was performed by LC-MS as demonstrated between Figure 3.2. and Figure 3.9.

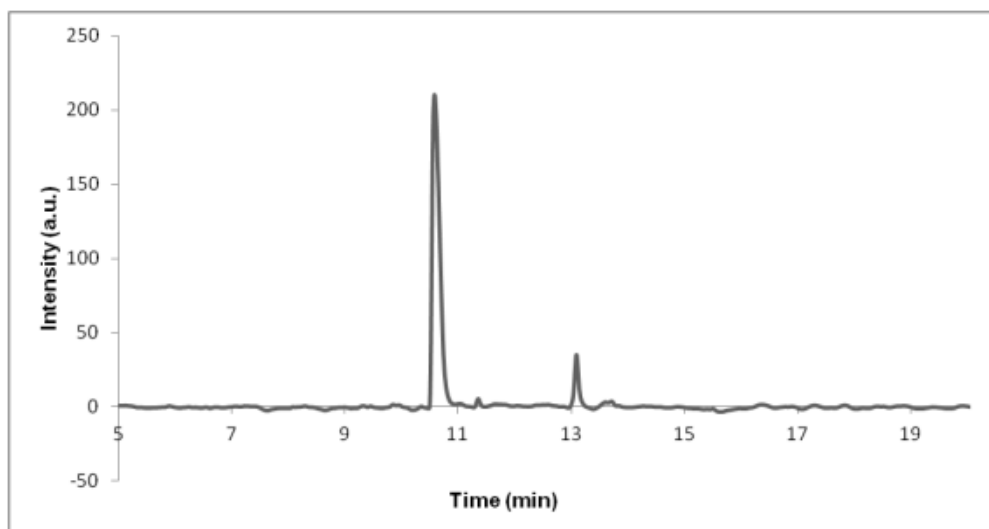


Figure 3.2. Liquid chromatogram of C₁₂-VVAGK-Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 89%.

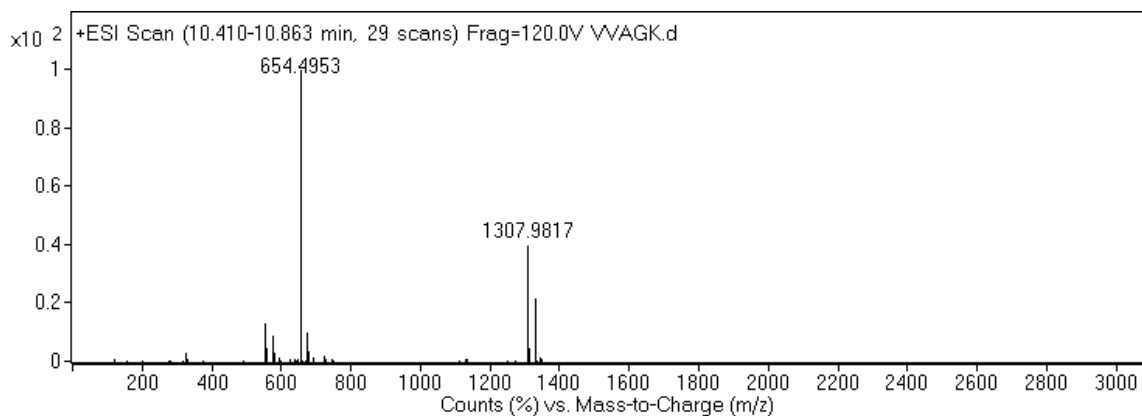


Figure 3.3. Mass spectrum of C₁₂-VVAGK-Am. Mass data [M+H]⁺ (calculated): 654.48, [M+H]⁺ (observed): 654.49, [2M+H]⁺ (calculated): 1307.96, [2M+H]⁺ (observed): 1307.98, [2M+Na]⁺ (calculated): 1329.96, [2M+Na]⁺ (observed): 1329.98.

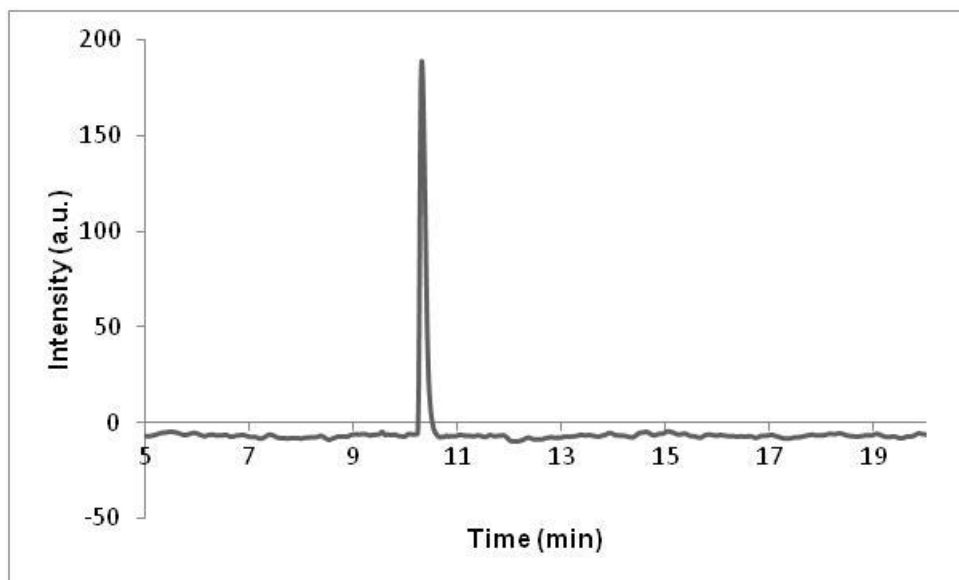


Figure 3.4. Liquid chromatogram of C_{12} -VVAGKS(Glc)-Am after subtraction of water, the change of response units with respect to time at 220 nm.

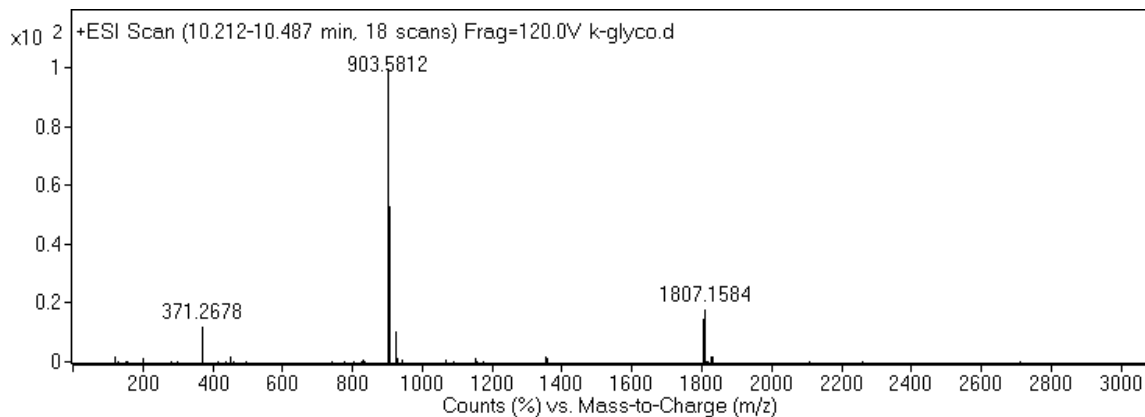


Figure 3.5. Mass spectrum of C_{12} -VVAGKS(Glc)-Am. Mass data $[M+H]^+$ (calculated): 903.57, $[M+H]^+$ (observed): 903.58, $2[M+H]^+$ (calculated): 1807.14, $2[M+H]^+$ (observed): 1807.16.

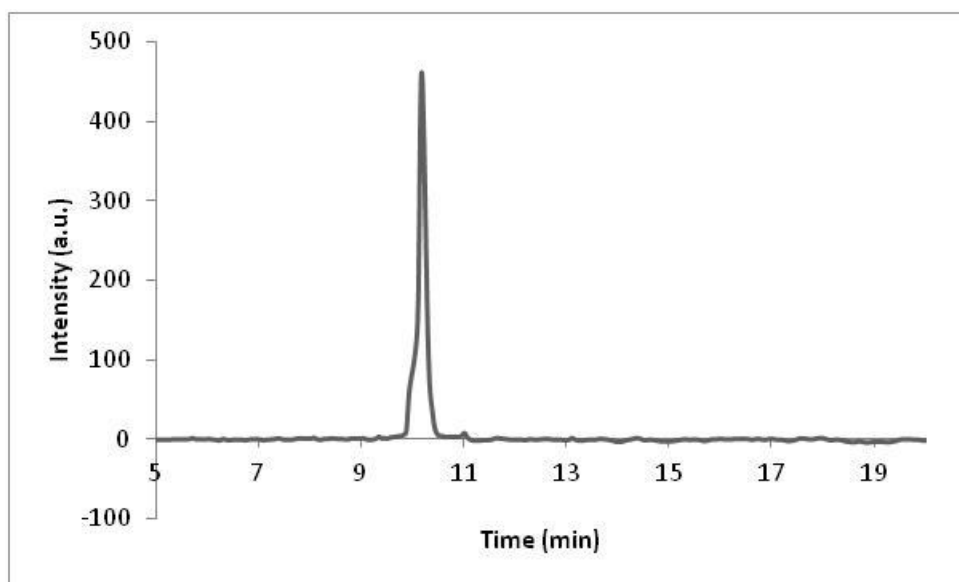


Figure 3.6. Liquid chromatogram of C_{12} -P₃GK-Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 98%.

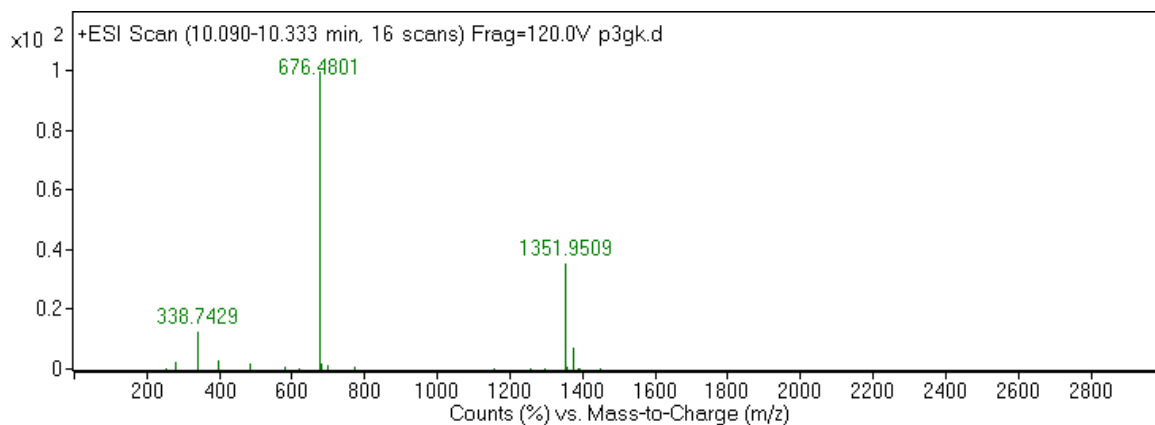


Figure 3.7. Mass spectrum of C_{12} -P₃GK-Am. Mass data $[M+H]^+$ (calculated): 676.47, $[M+H]^+$ (observed): 676.48, $[2M+H]^+$ (calculated): 1351.94, $[2M+H]^+$ (observed): 1351.95, $[M+2H]^{+}/2$ (calculated): 338.73, $[M+2H]^{+}/2$ (observed): 338.74.

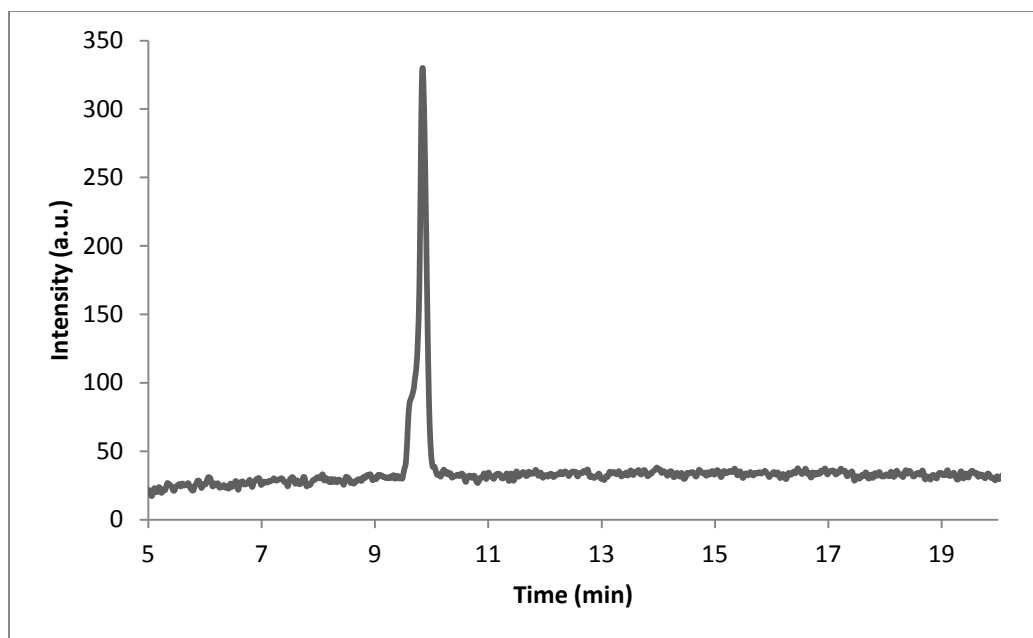


Figure 3.8. Liquid chromatogram of C_{12} -P₃GKS(Glc)-Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 99%.

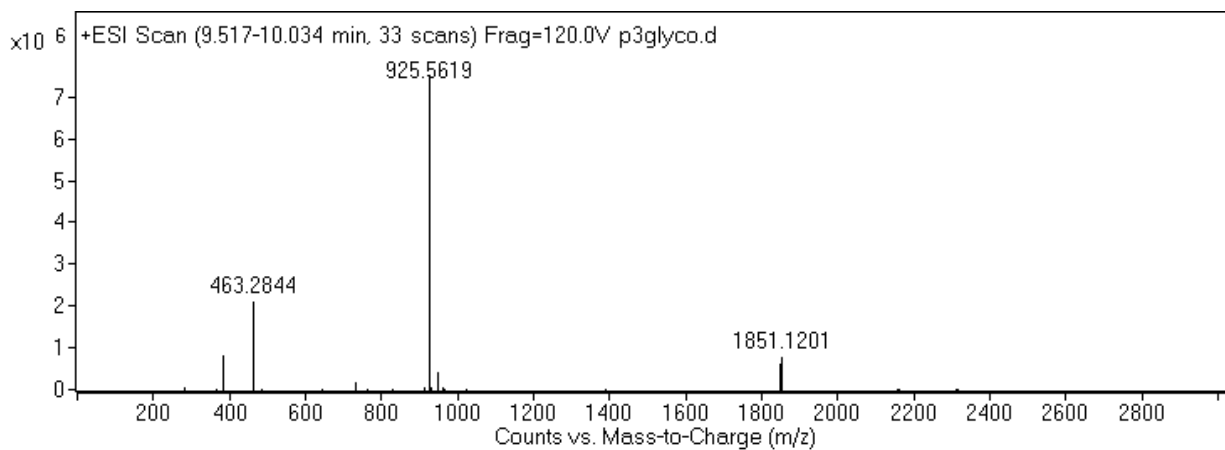


Figure 3.9. Mass spectrum of C_{12} -P₃GKS(Glc)-Am. Mass data $[M+H]^+$ (calculated): 925.55, $[M+H]^+$ (observed): 925.56, $2[M+H]^+$ (calculated): 1851.10, $2[M+H]^+$ (observed): 1851.12 , $[M+2H]^+/2$ (calculated): 463.27, $[M+2H]^+/2$ (observed): 463.28.

3.3. Characterization of PA/AON complexes

PA and PA/AON complexes were characterized following PA synthesis and assembly. Secondary structure characterization was performed by circular dichroism (Figure 3.10). CD spectra demonstrated a negative peak at 197 nm for K-PA and Glc-P-PA, which suggests random coil formations in PAs without AON. Upon complexation with the oligonucleotide, K-PA/AON and Glc-K-PA/AON both formed β -sheet structure, with a positive peak around 193 nm and a negative peak around 217. Therefore, oligonucleotide addition appears to have induced the self-assembly of K-PA and Glc-K-PA, both of which form nanofibers upon assembly. Unlike fiber forming PAs, proline rich amino acids form a different secondary structure known as the polyproline helix (PPII). This secondary structure gives a negative peak around 204 nm⁸⁷. Glc-P-PA and P-PA gave a negative peak at 203 nm both in the presence and absence of AON, which showed that the predominant secondary structure in nanosphere forming PAs is the PPII structure.

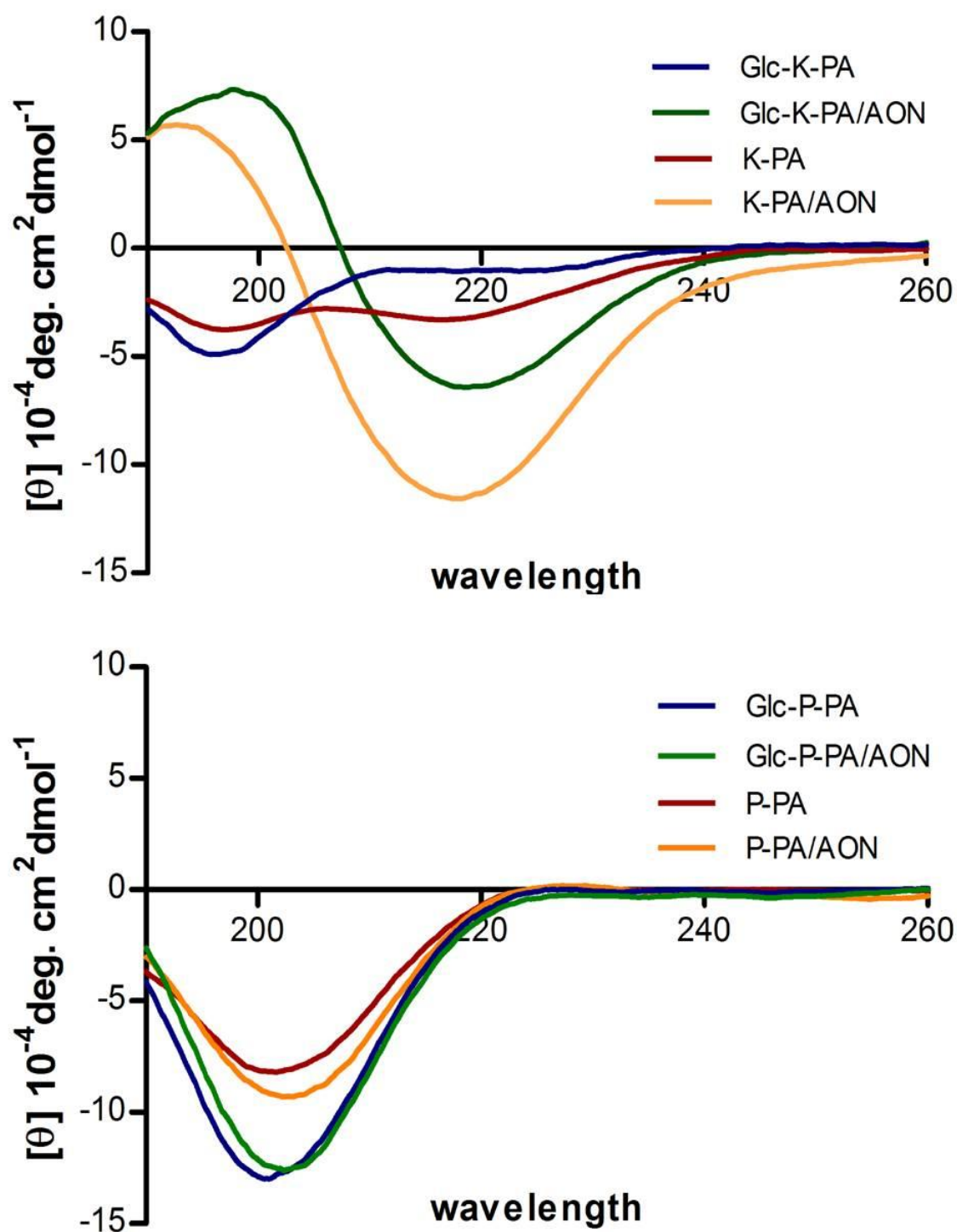


Figure 3.10. CD spectra of PA/AON complexes.

Zeta potentials of PAs and PA/AON complexes at different molar ratios were also investigated. As illustrated by Table 3.1., K-PA and Glc-PA were positively charged, while P-PA and Glc-P-PA were almost neutral. When AON was added to the system, negativity was increased, as would be expected, due to the negative charge of oligonucleotide. All PA/AON complexes were negative at 30:1 molar ratio, which was the ratio used *in vitro* experiments, except K-PA/AON, which was neutral.

Table 3.1. Charges of PAs and PA/AON complexes at different molar ratios. At a 30:1 molar ratio, PA/AON complexes are all slightly negatively charged, except for K-PA/AON.

	Only PA	PA/AON (30:1)	PA/AON (100:1)	PA/AON (300:1)
K-PA	29.0 ± 2.7 mV	1.4 ± 0.3 mV	42.6 ± 0.7 mV	43.3 ± 1.3 mV
Glc-K-PA	24.9 ± 3.0 mV	(-)22.2 ± 1.8 mV	(-)6.3 ± 0.4 mV	17.8 ± 0.7 mV
P-PA	2.7 ± 1.1 mV	(-)15.5 ± 1.7 mV	(-)26.9 ± 4.6 mV	(-)14.7 ± 1.0 mV
Glc-P-PA	0.6±3.9 mV	-16.3±1.7 mV	-23.2±6.6 mV	-27.5±2.2 mV

The morphology of nanofibers and nanospheres formed by PA/AON complexes were investigated by TEM imaging. (Figure 3.11.) K-PA/AON and Glc-PA/AON formed nanofibers with a fiber diameter of 10 nm. P-PA/AON and Glc-P-PA/AON, on the other hand, formed nanospheres around 70 nm. The presence of AON on these nanospheres was demonstrated by the electron mapping of phosphorus. (Figure 3.12)

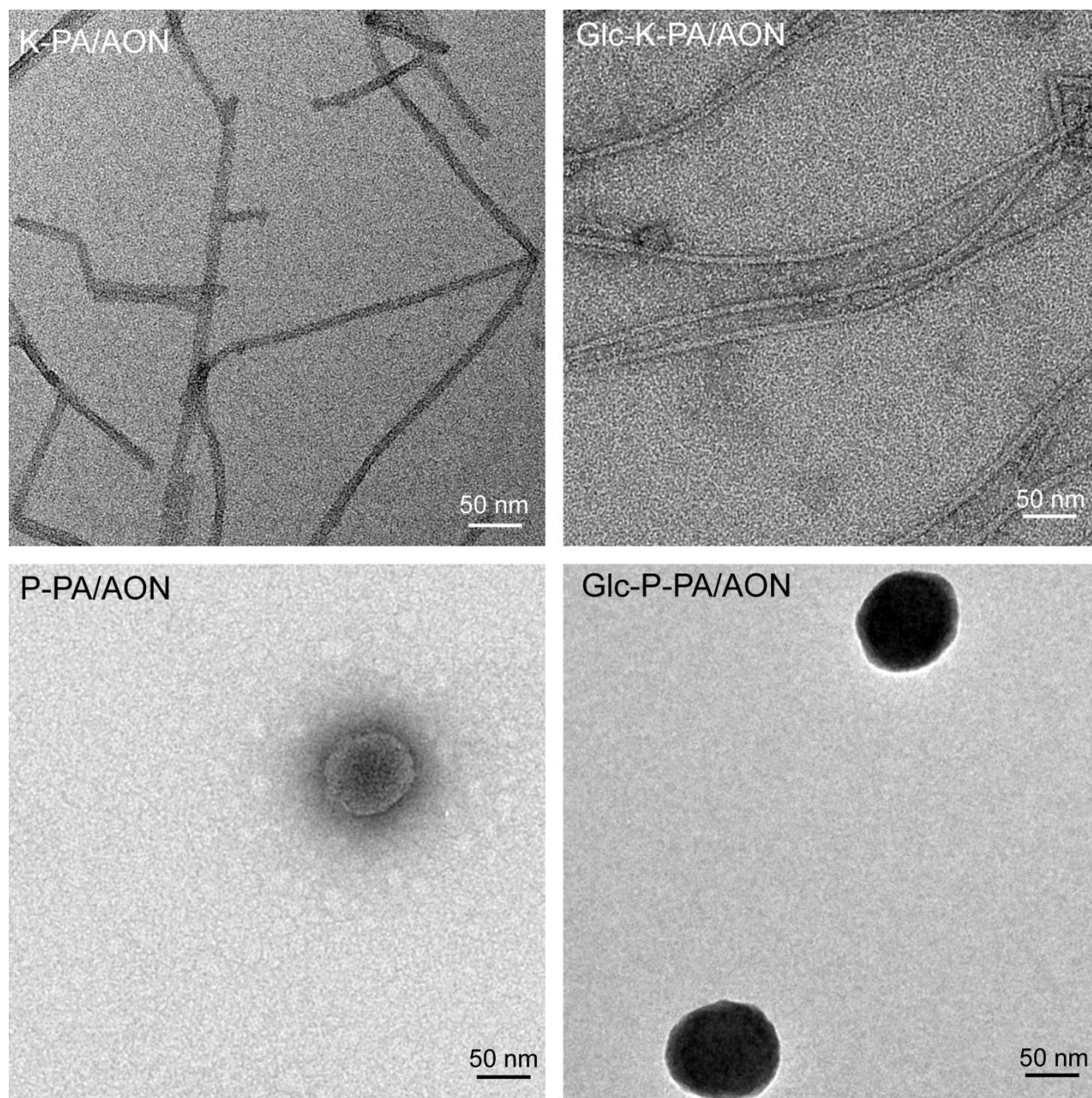


Figure 3.11. TEM of PA/AON complexes.

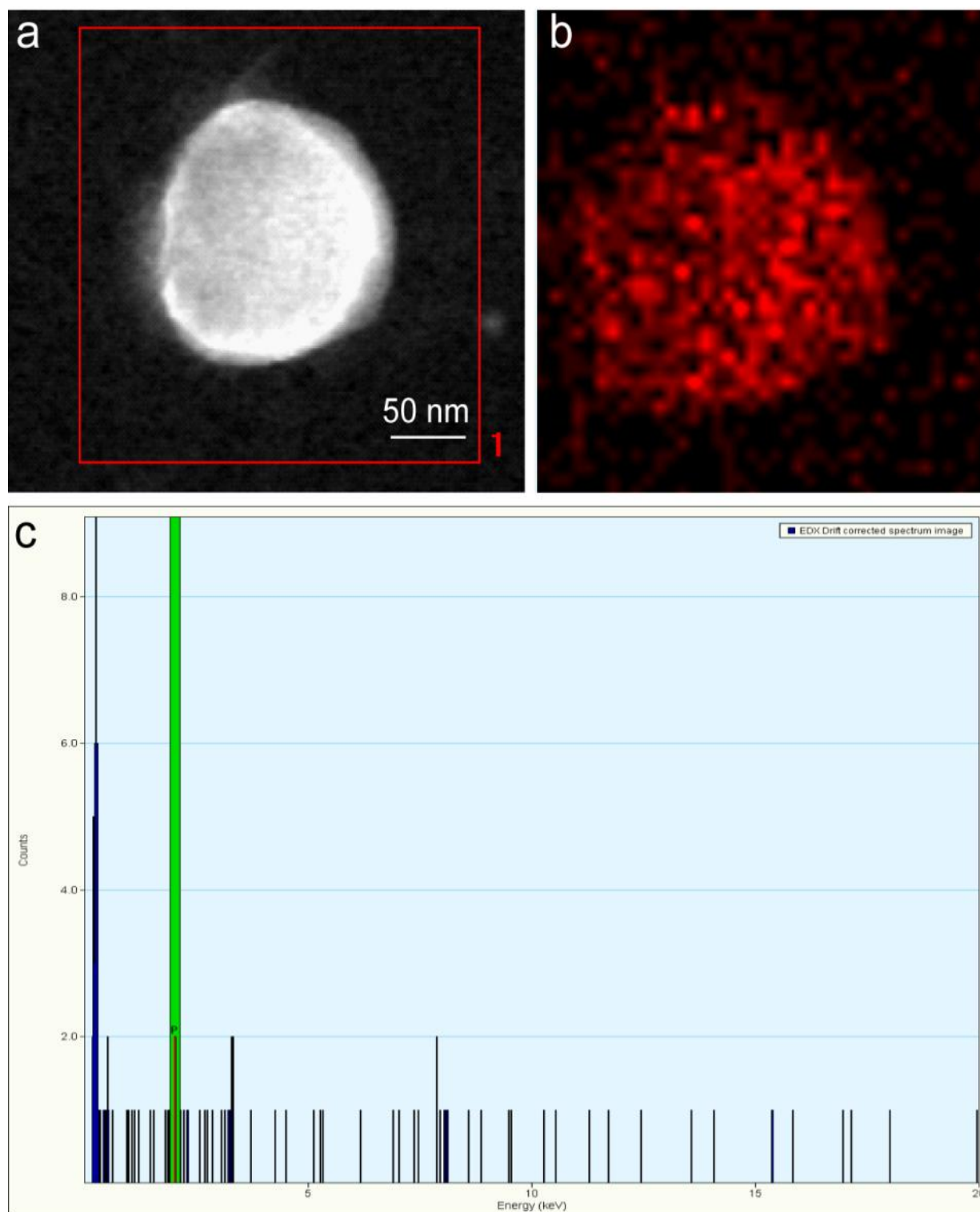


Figure 3.12. a. TEM image of Glc-P-PA/AON nanosphere. b. Electron mapping of the corresponding nanosphere for phosphorus. c. EDX spectra of the corresponding area.

The hydrodynamic size of the nanosphere forming PAs and PA/AON complexes was investigated with DLS. (Figure 3.13.) P-PA and Glc-PA with and without AON formed nanospheres with a diameter around 70 nm.

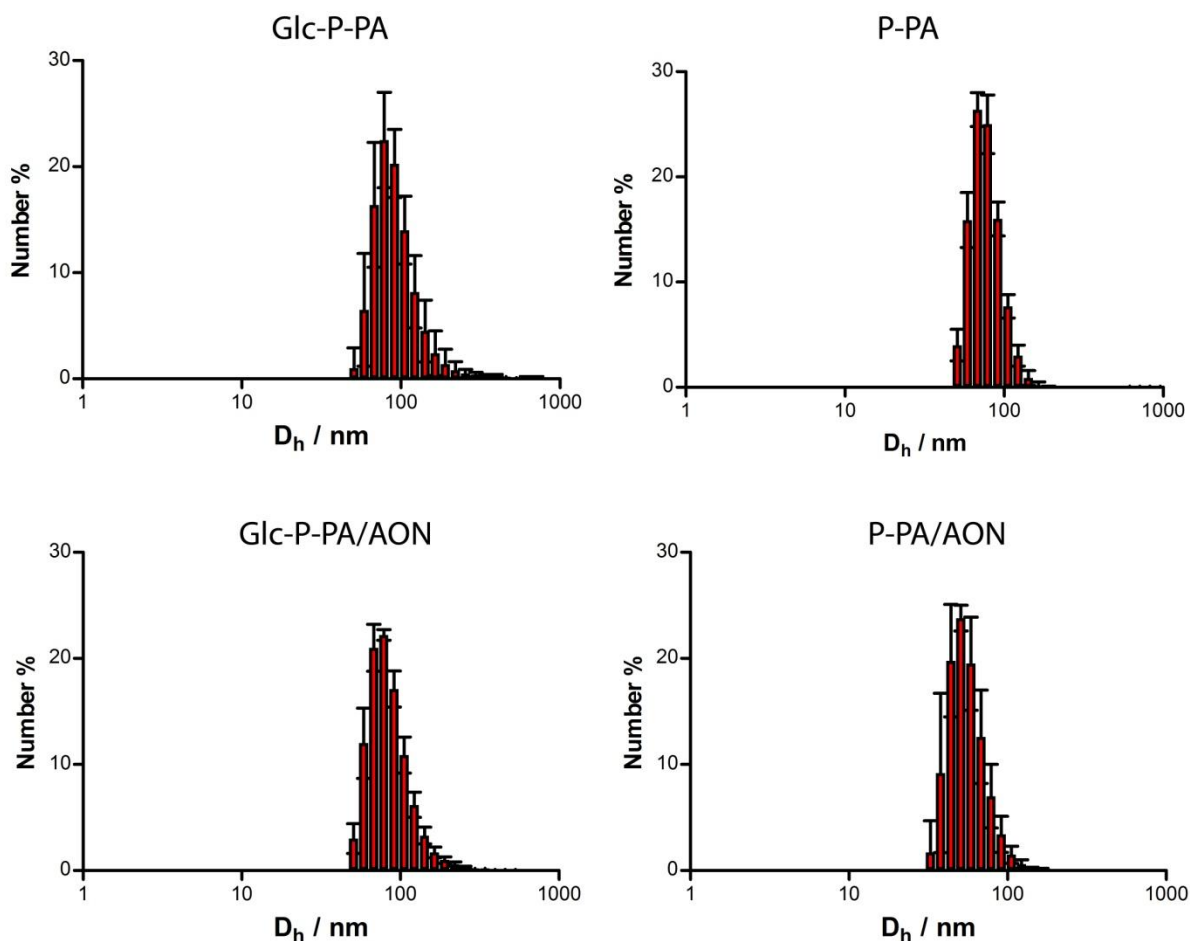


Figure 3.13. Hydrodynamic size distribution of PA and PA/AON nanospheres measured with DLS.

3.4. Cytotoxicity of PAs

MCF-7 viability treated with PAs was investigated to investigate the cytotoxicity of the material. At *in vitro* experimental concentrations (between 30 -60 μ M), PAs did not induce a significant cell death. As the concentration of PAs was increased, there was a decrease in the viability of cells.

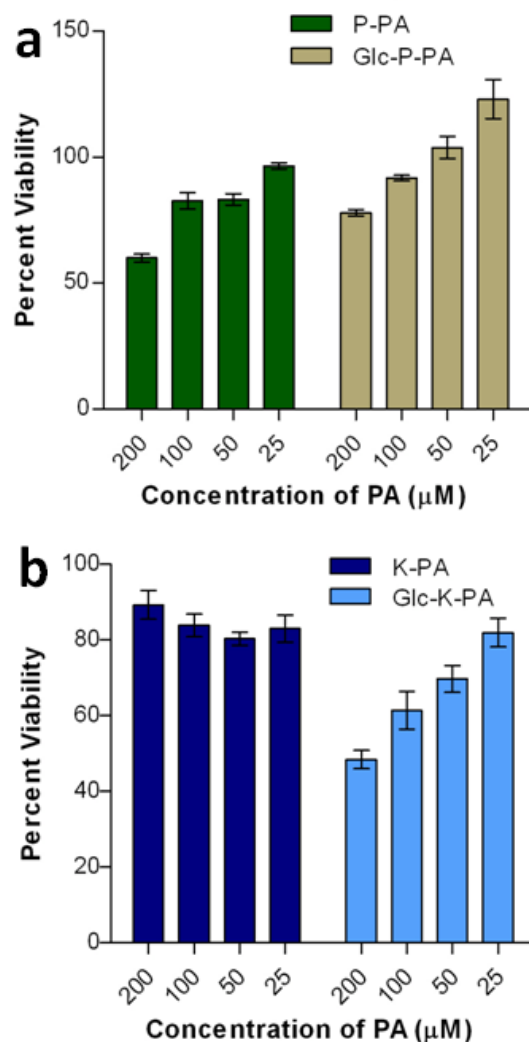


Figure 3.14. Viability of MCF-7 cells treated with peptide amphiphiles in serum free media. **a** 24 h MCF-7 viability treated with P-PA and Glc-P-PA. **b** 48 h MCF-7 viability treated with K-PA and Glc-K-PA. (Error bars show SEM, $n=4$)

3.5. Cellular internalization of PA/AON complexes

Cellular internalization of the PA/AON complexes was observed by confocal microscopy. (Figure 3. 15.) At 4 h, cellular internalization was lower compared to the extent of internalization at 24 h. Fibers were shown to be bound to cell membrane to a greater extent at 4 h and internalization in fibrous networks might be slower compared to micelles. At 24 h, the internalization of Glc-K-PA/AON was drastically high compared to other PA/AON complexes.

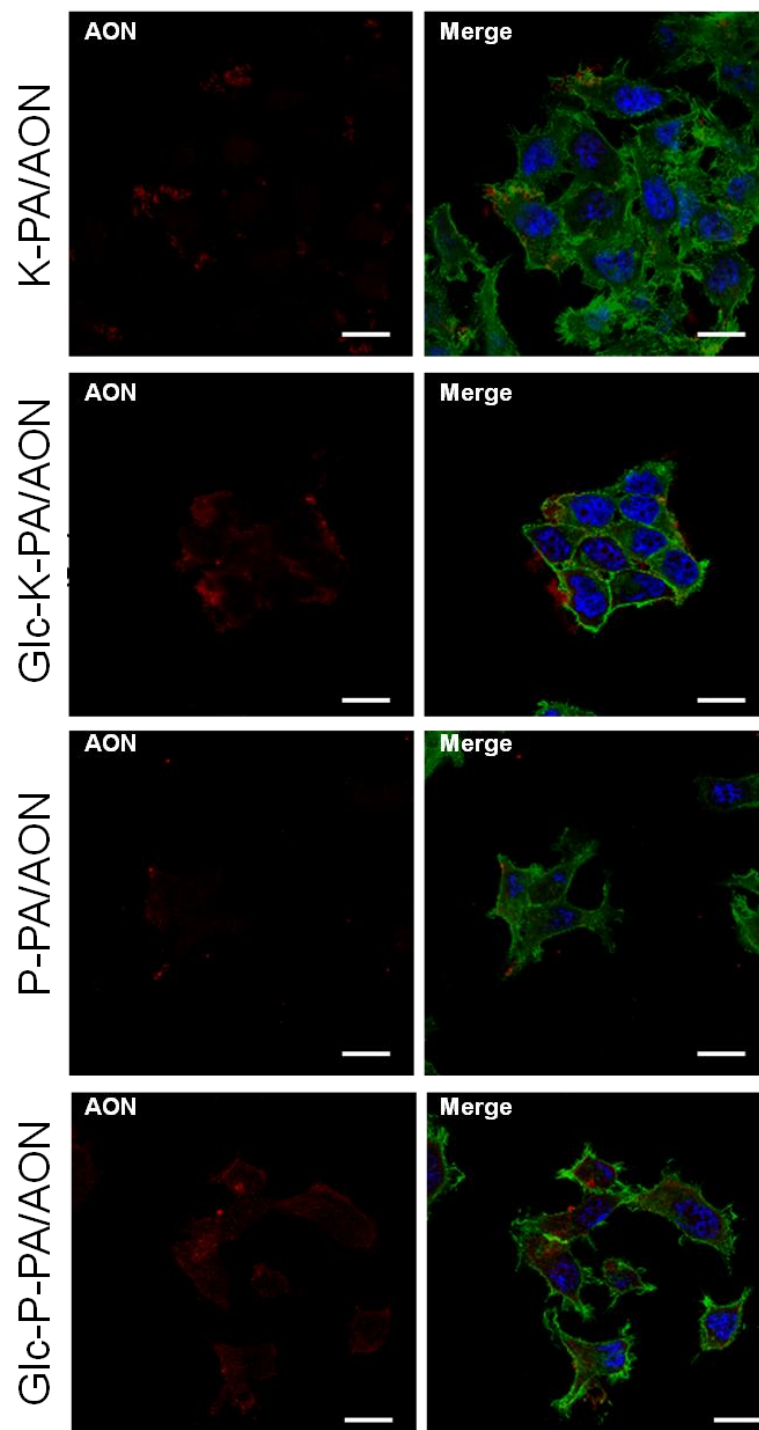


Figure 3.15. 4 h cellular internalization of PA/AON complexes in MCF-7 cells. FAM-AON (red), phalloidin (green), TO-PRO-3 (blue). Scale bar 20 μ m.

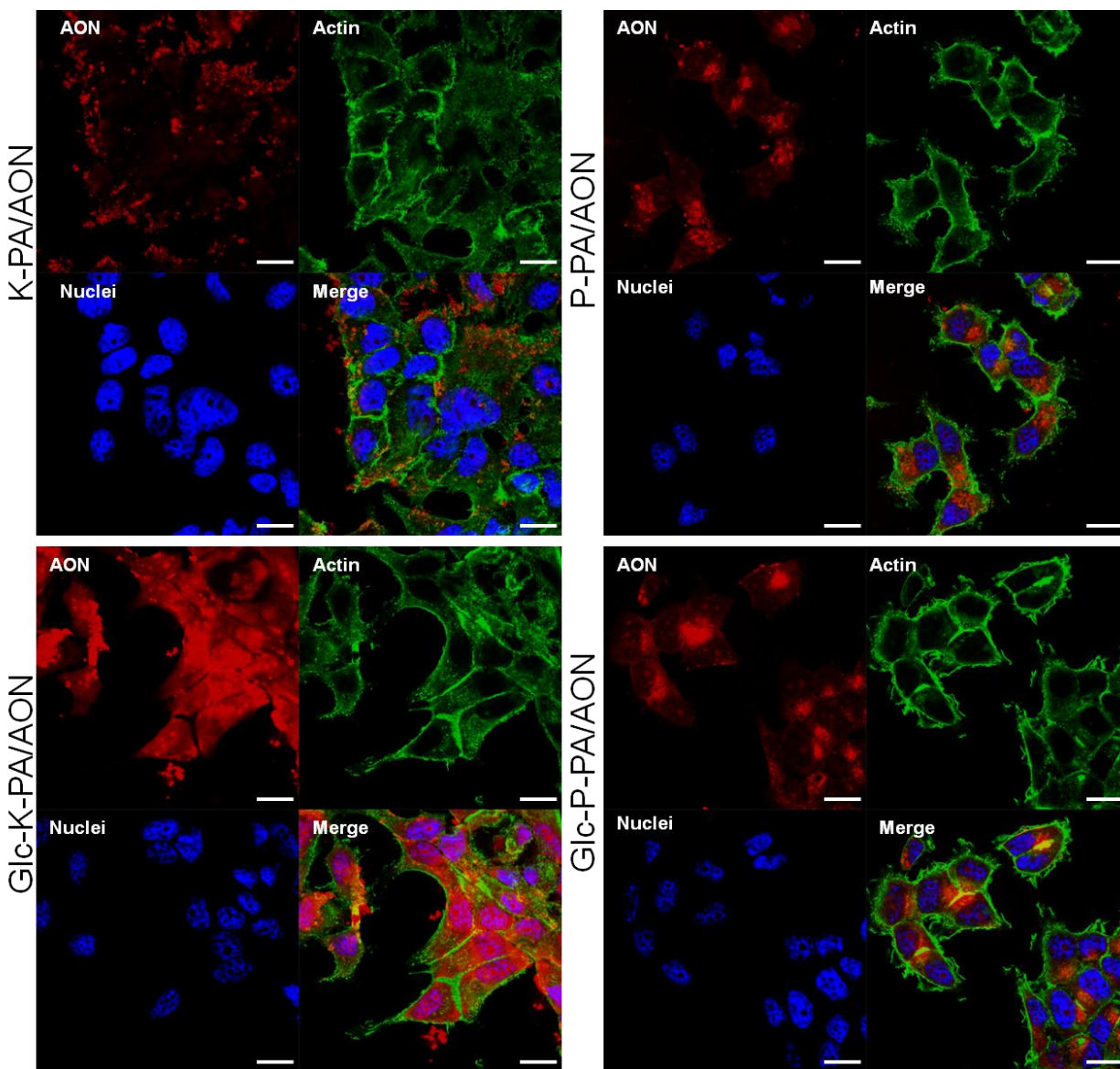


Figure 3.16. 24 h cellular internalization of PA/AON complexes in MCF-7 cells. FAM-AON (red), phalloidin (green), TO-PRO-3 (blue). Scale bar 20 μ m.

After confocal imaging of cellular internalization, the uptake of PA/AON complexes by MCF-7 cells was quantified by flow cytometry. (Figure 3.17.) Flow cytometry results were then used to quantify the amount of FAM-AON (fluorescein-6-amide-AON) inside the cells. Parallel to our confocal microscopy results, the cellular internalization of nanofiber-forming PAs were greater

compared to that of nanosphere-forming PAs. In addition, Glc-P-PA/AON again had the greatest internalization efficiency in MCF-7 cells.

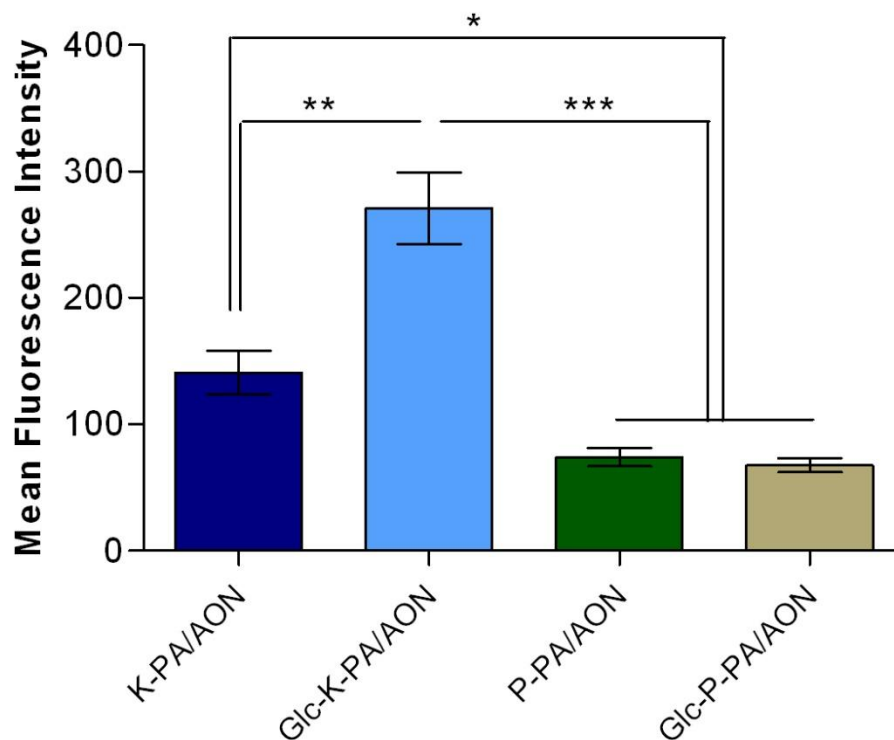


Figure 3.17. Cellular internalization of PA/AON complexes after 24 h quantified with flow cytometry. (Error bars show SEM, two independent experiments were repeated with $n=3$ in each experiment. Student's *t*-test shows statistical significance with * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.6. Cellular internalization mechanisms

The internalization mechanism of a delivery system is crucial for determining its efficiency. As such, it is vital to understand how a particular delivery system is internalized, and to determine whether the morphology of the carrier molecule alters the pathways involved in its internalization. As the possible mechanisms of internalization are direct penetration and endocytosis (*e.g.* clathrin-mediated endocytosis, caveolae-mediated endocytosis and micropinocytosis), several endocytosis inhibitors were used in this study to determine the extent of endocytosis experienced by our delivery systems. Four drugs; amiloride, dynasore,

chlorpromazine and nystatin were used to block specific endostatic pathways. Amiloride inhibits macropinocytosis, dynasore inhibits dynamin-dependent endocytosis, chlorpromazine inhibits clathrin-mediated endocytosis and nystatin inhibits caveolae-mediated endocytosis⁵¹.

Nanofiber and nanospherical PA/AON complexes displayed different internalization patterns (Fig 3. 18.) The uptake of nanosphere-delivered oligonucleotides was not substantially decreased by endocytosis inhibitors, suggesting that these complexes can directly translocate through the cellular membrane. However, there was a slight decrease in the internalization of P-PA/AON under amiloride and dynasore inhibition. As such, macropinocytosis and dynamin mediated endocytosis play a role in the internalization of sphere forming PA/AON complexes, yet their contribution is relatively minor and the main route of internalization appears to be direct translocation.

Fiber-forming PA/AON complexes, on the other hand, can enter cells via endocytosis, as demonstrated by the inhibition of their internalization in the presence of amiloride, dynasore and chlorpromazine. Clathrin- and dynamin-mediated endocytosis in particular were the main internalization pathways of fiber PA/AON complexes. As nystatin did not inhibit the internalization, caveolin-dependent uptake was not involved in the uptake of PA/AON complexes.

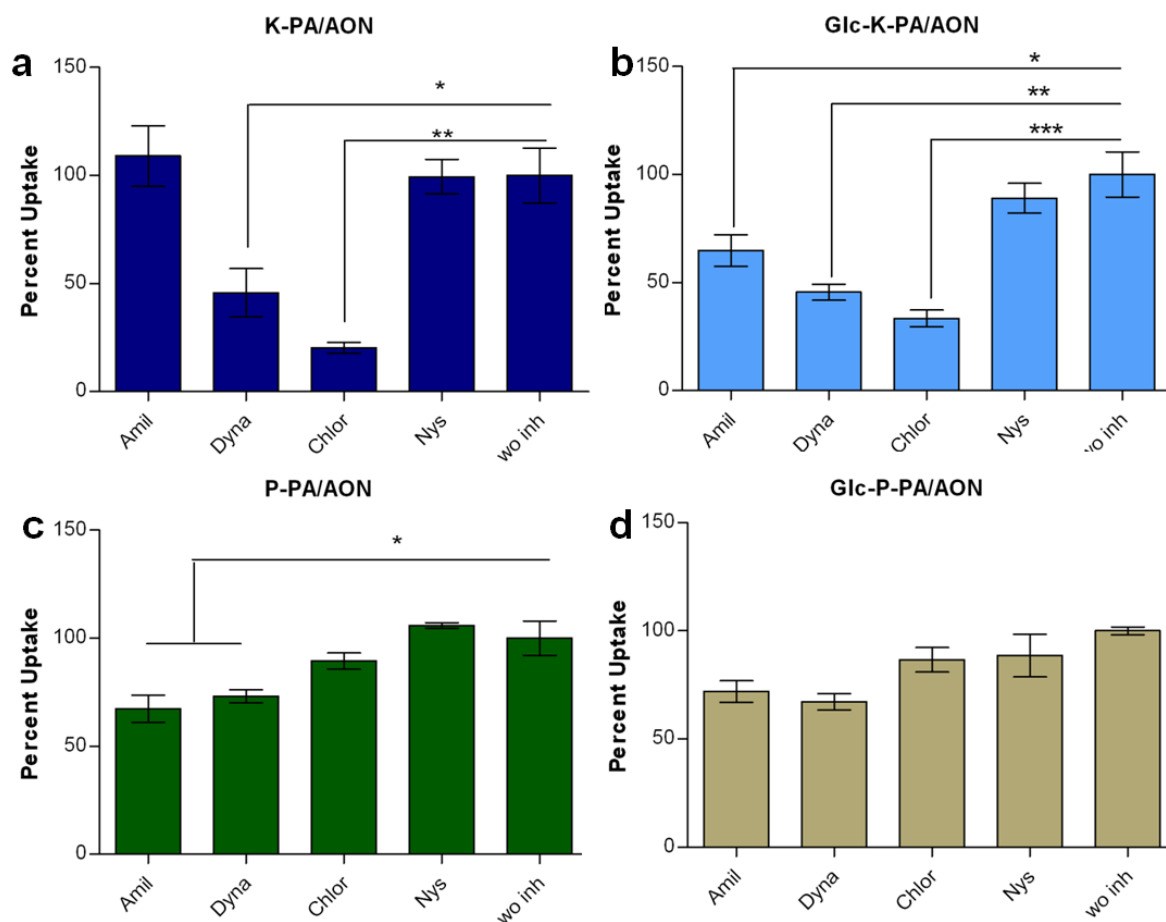


Figure 3.18. Uptake mechanism of PA/AON complexes. Percent uptake in MCF-7 cells after 24 h normalized to without inhibitor group (wo inh). Amiloride (Amil), dynasore (Dyna), chlorpromazine (Chlor) and nystatin (Nys) were administered to cells to inhibit endocytosis pathways. (Error bars show SEM, two independent experiments were repeated with n=3 in each experiment. One way ANOVA shows statistical significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

3.7. Cellular internalization mechanisms of glyco-PA/AON complexes

The involvement of glucose transporters in the internalization of glyco-PA/AON complexes was investigated by using GLUT (glucose transporter) and SGLT (sodium dependent glucose transporter) inhibitors. Phloridzin dihydrate specifically inhibits SGLTs⁸⁸ whereas cytochalasin

B inhibits GLUT transporters⁸⁹. In MCF-7 cells as a glucose transporter, GLUT1, GLUT2, and GLUT5⁹⁰, GLUT12⁹¹ and SGLT1⁹² expression was previously shown.

Figure 3.19. shows that internalization of Glc-K-PA/AON was reduced under phloridzin inhibition, whereas the internalization of K-PA/AON was not affected by this inhibition. This result demonstrates that the internalization of Glc-K-PA/AON is SGLT transporter-dependent and the binding of glucose to SGLTs increases the internalization of PA/AON complexes. As cytochalasin B is the inhibitor of actin filaments⁸⁹, this result also suggests that the internalization of fiber-forming PAs are actin dependent, whereas the internalization of sphere forming PAs are not inhibited with cytochalasin B and therefore independent of actin filaments. This observation is in line with our finding that fiber-forming PAs utilize endocytic pathways, as the inhibition of actin would block the movement of the endocytic vesicles that fiber-forming PAs rely on. Spherical PAs, in contrast, are not affected by cytoskeletal inhibition due to their ability to directly translocate through the cell membrane.

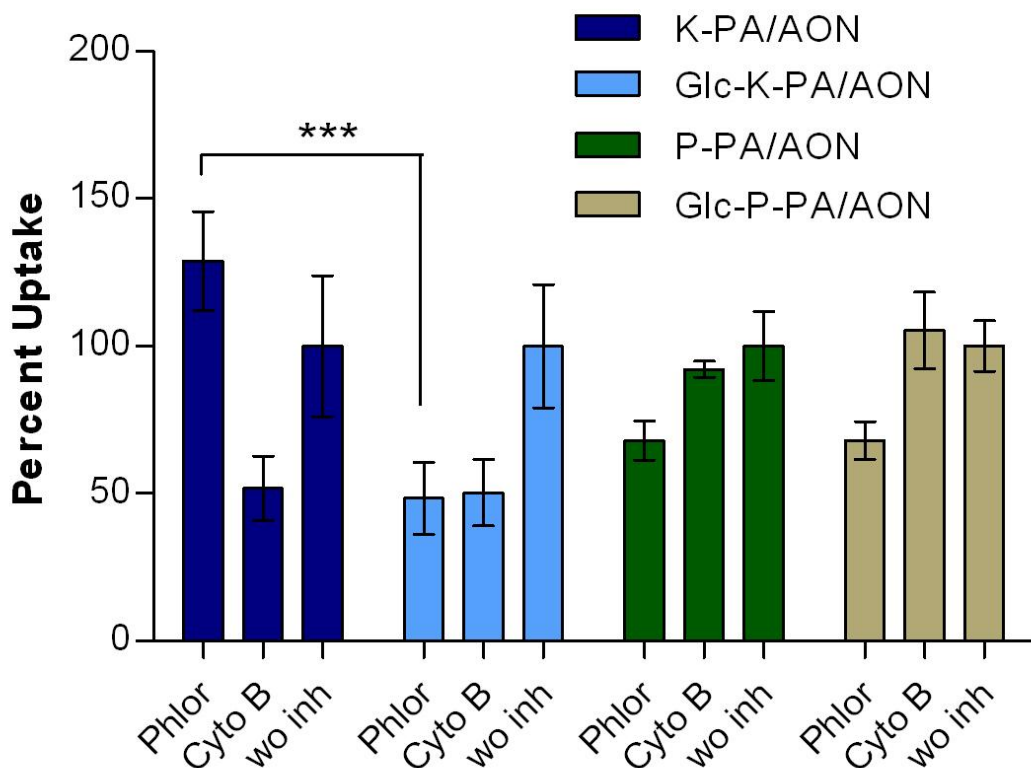


Figure 3.19. Glucose transporter-mediated uptake mechanisms of Glc-PA/AON complexes. Percent uptake in MCF-7 cells after 24 h normalized to without inhibitor group (wo inh). Phloridzin dihydrate (Phlor) and cytochalasin B (Cyto B) were administered to cells to inhibit glucose transporters. (Error bars show SEM with n=3. Two way ANOVA shows statistical significance with * $p<0.05$, ** $p<0.01$, *** $p<0.001$)

3.8. Expression of Bcl-2 in MCF-7 cells incubated with PA/AON complexes

The target gene expression of antisense oligonucleotide in cells treated with PA/AON complexes was investigated to evaluate the therapeutic efficiency of our PA-based delivery system. However, only a slight decrease in expression was observed in the K-PA/AON treated group. Although glyco-PAs delivered more AON inside the cells, they were unable to decrease the target gene expression. There was even an increase in Bcl-2 expression for Glc-P-PA/AON treated group. This unexpected result might stem from the relation of glucose internalization with Bcl-2 expression levels. *In vitro* glucose levels were found to be correlated with Bcl-2 gene expression, and glucose deprivation is known to induce cell death in cancer treatment⁹³. As such, glucose binding might initiate a signaling pathway, may lead to a resistance to apoptosis and increase the expression of Bcl-2.

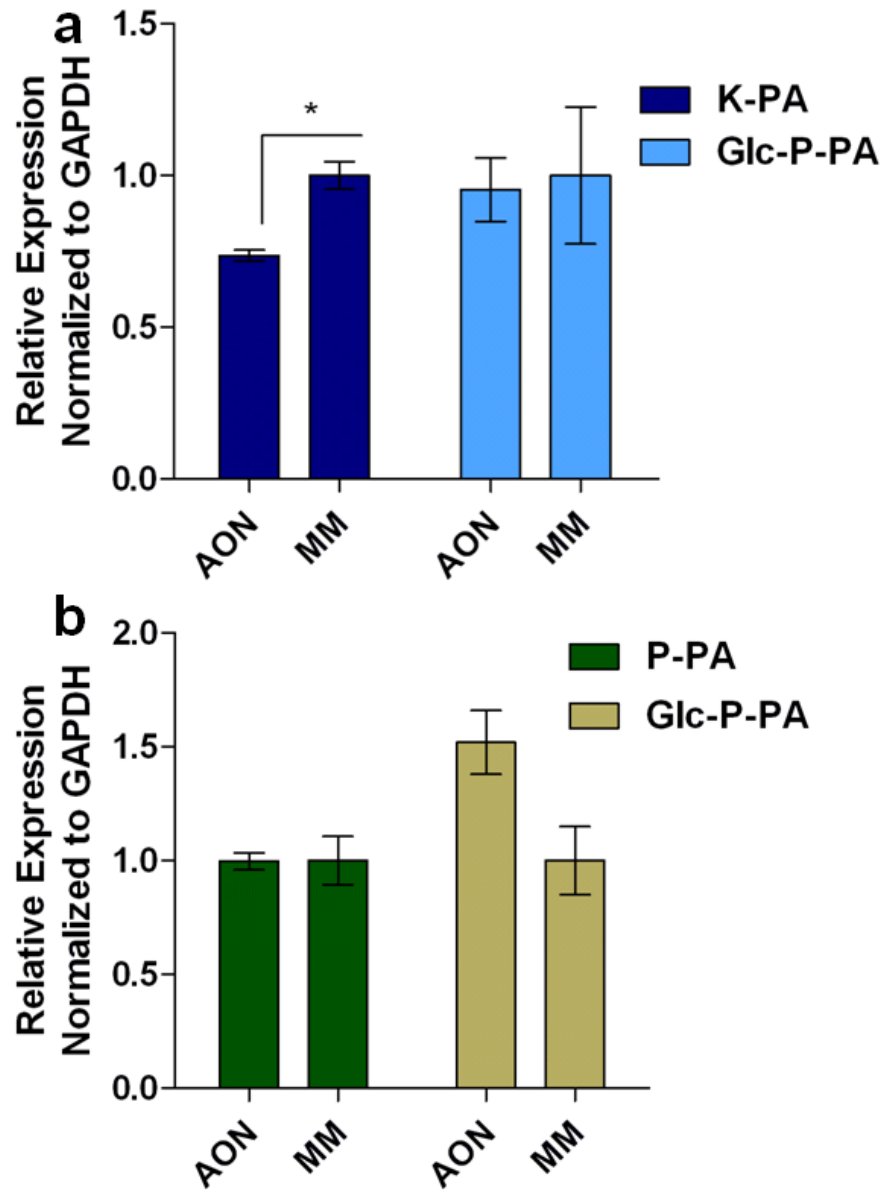


Figure 3.20. Bcl-2 expression of MCF-7 cells transfected with PA/AON or PA/MM complexes for 48h. Gene expression is normalized to GAPDH. (Error bars show SEM, two independent experiments were repeated with n=3 in each experiment. Student's t-test shows statistical significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

CHAPTER 4. OLIGONUCLEOTIDE DELIVERY WITH BIOACTIVE PEPTIDE AMPHIPHILES

4.1. Background

Oligonucleotides have limited cell penetration capacity and therefore high doses of naked AON is required for silencing of the target gene⁹⁴. A delivery system that can increase the cellular penetration is desirable for AON delivery, since it would increase the efficiency of the drug and even at low concentrations of oligonucleotide, silencing can be achieved. Moreover, low dose of AON would result in fewer side effects and less off-target effects.

Our aim in this study was to increase the cellular penetration of AON with a bioactive self-assembled peptide amphiphile delivery system. For this purpose, PA delivery system was decorated with cell penetrating peptides and cell surface binding peptide.

4.2. Design and synthesis of peptide amphiphiles

For oligonucleotide delivery, self-assembling peptide amphiphiles were designed to form a nanosphere structure decorated with bioactive peptides to increase cellular internalization. Peptide amphiphiles were designed to contain an alkyl tail, a proline rich sequence and a bioactive unit. (Figure 4.1.) The proline residues, known as β -sheet and α -helix breakers, enable the final self-assembled structure to be spherical⁸¹. The hydrophobic and electrostatic interactions drive the self-assembly of peptide amphiphiles and as a result of the self-assembly, hydrophobic alkyl tail is buried inside and bioactive peptides are presented on the surface. In order to increase the cellular internalization of PA/AON complexes, PAs were designed to contain cell penetrating peptides (R4 and R8) or a cell surface binding peptide (KRSR). R4 and R8 are polyarginine CPPs that have been shown to be effective in delivery of therapeutics inside cells⁹⁵⁻⁹⁸. KRSR is a cell surface binding peptide designed based on the common sequence BBXB which was found to be consensus in heparin binding peptides and proteins⁹⁹. This

sequence was previously suggested as a common receptor binding peptide for drug delivery applications¹⁰⁰.



Figure 4.1. Chemical structures of PAs that are used in this study: C₁₂-P₄GK (K-Pro-PA), C₁₂-P₄GKRSR (KR SR-Pro-PA), C₁₂-P₄R₄ (R₄-Pro-PA) and C₁₂-P₄K₂R₈ (R₈-Pro-PA).

Designed molecules were synthesized and purified with HPLC. Chemical characterization was done by LC-MS, whose results are demonstrated between Figure 4.2. and Figure 4.9.

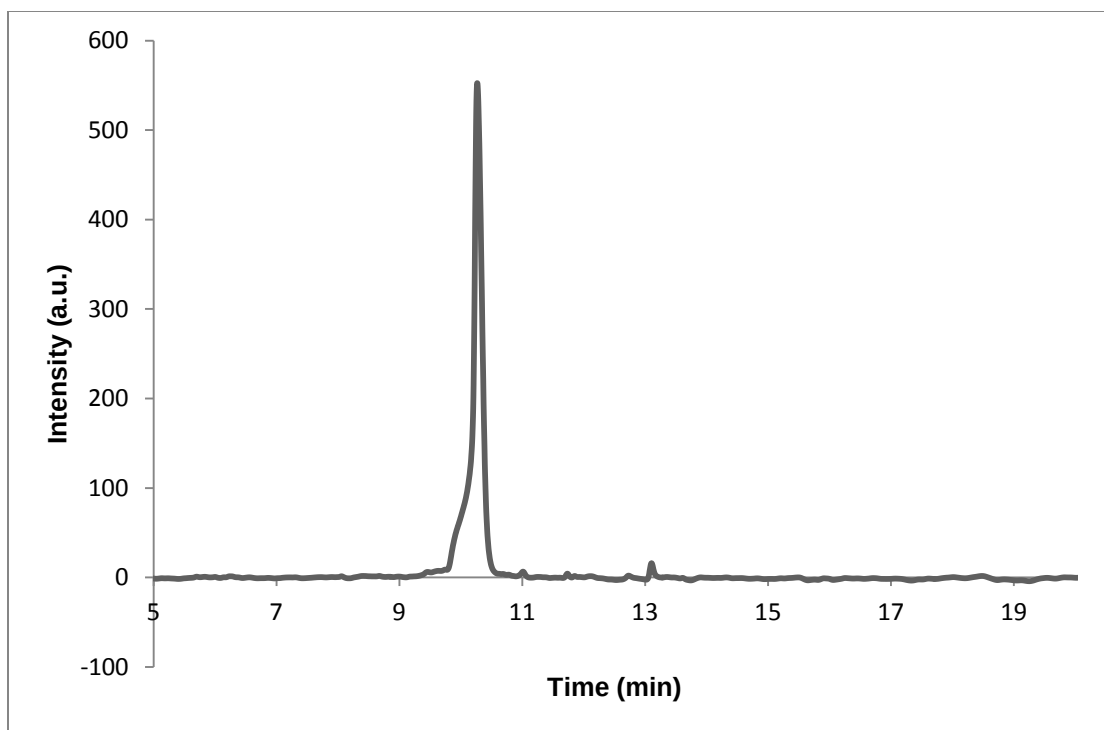


Figure 4.2. Liquid chromatogram of C₁₂-P₄GK-Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 98%.

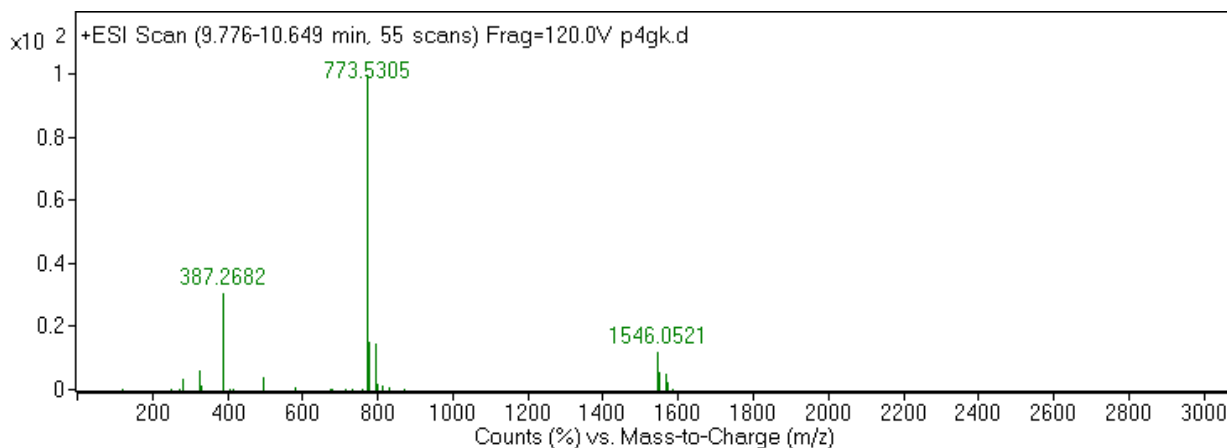


Figure 4.3. Mass spectrum of C₁₂-P₄GK-Am. Mass result [M+H]⁺ (calculated): 773.52, [M+H]⁺ (observed): 773.53, [2M+H]⁺ (calculated): 1546.04, [M+H]⁺ (observed): 1546.05, [M+2H]⁺/2 (calculated): 387.26, [M+2H]⁺/2 (observed): 387.27, [M+Na]⁺ (calculated): 795.52, [M+Na]⁺ (observed): 795.53, [2M+H]⁺

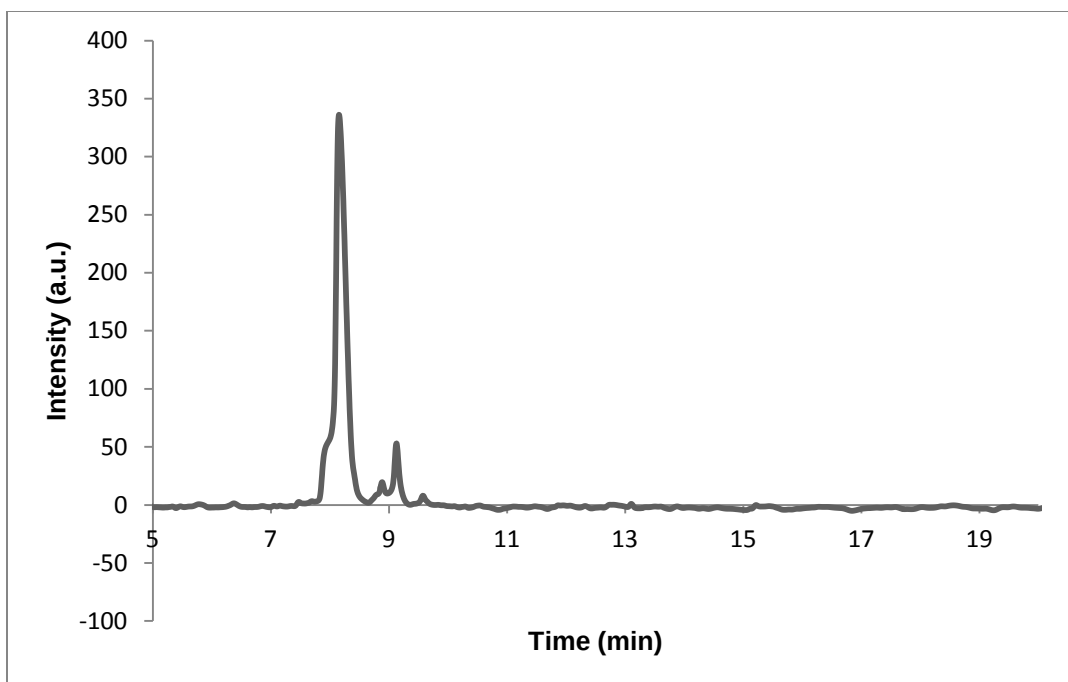


Figure 4.4. Liquid chromatogram of C₁₂-P₄GKR SR-Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 90%.

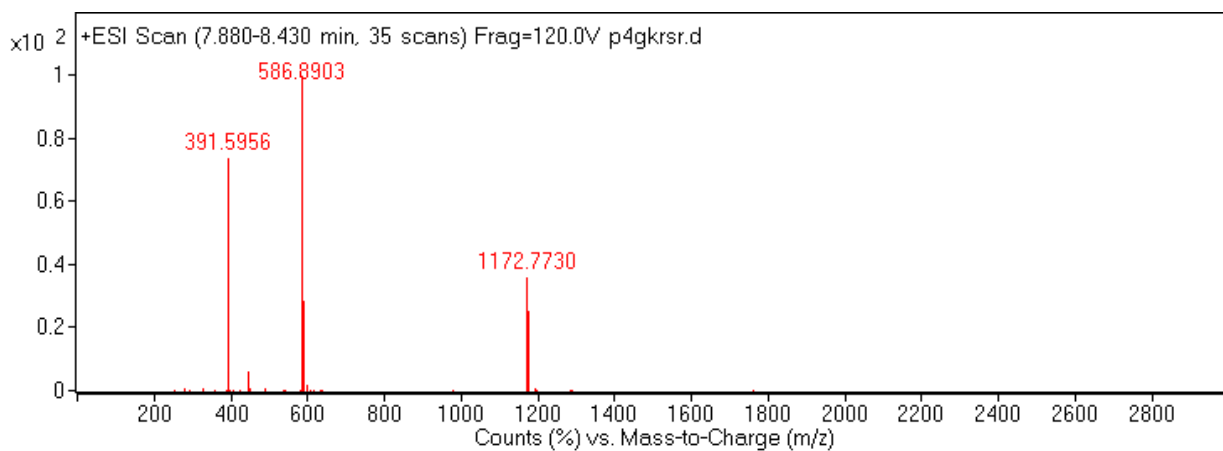


Figure 4.5. Mass spectrum of C₁₂-P₄GKR SR-Am. Mass result [M+H]⁺ (calculated): 1172.76, [M+H]⁺ (observed): 1172.77, [M+2H]⁺/2 (calculated): 586.88, [M+2H]⁺/2 (observed): 586.89, [M+3H]⁺/3 calculated): 391.59, [M+3H]⁺/3 observed): 391.59.

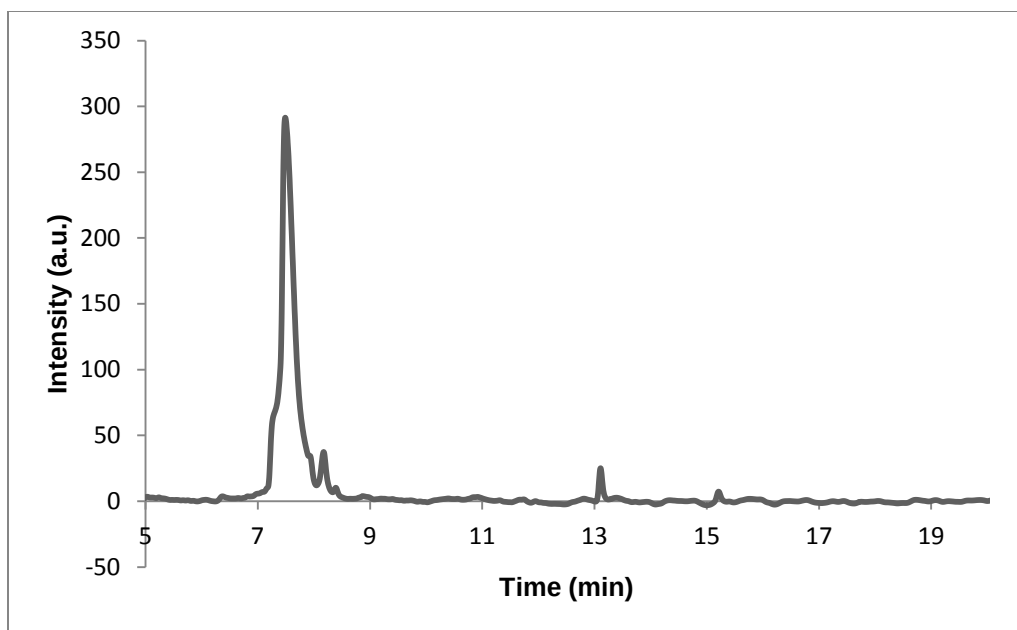


Figure 4.6. Liquid chromatogram of C_{12} - P_4R_4 -Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 91%.

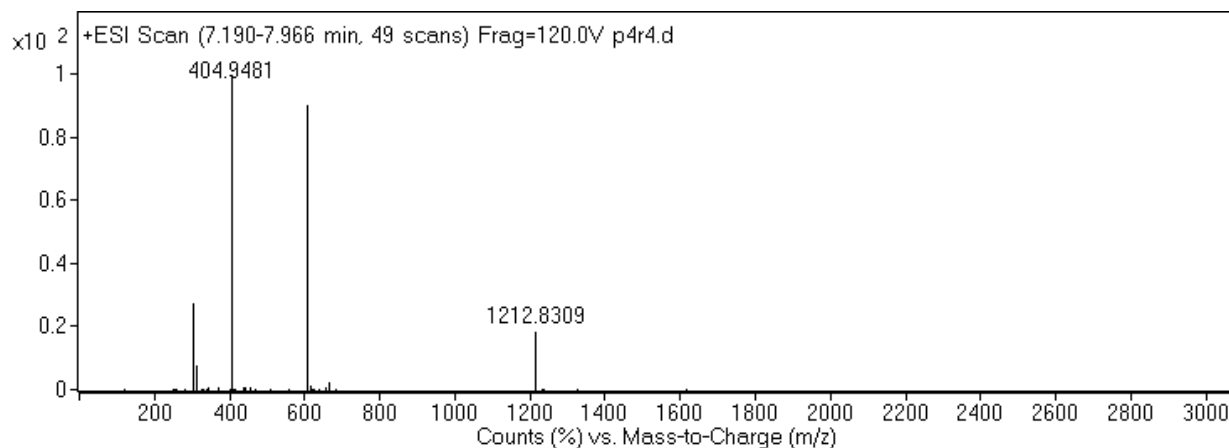


Figure 4.7. Mass spectrum of C_{12} - P_4R_4 -Am. Mass result $[M+H]^+$ (calculated): 1212.81, $[M+H]^+$ (observed): 1212.83, $[M+3H]^+/3$ (calculated): 404.94, $[M+3H]^+/3$ (observed): 404.95, $[M+4H]^+/4$ (calculated): 303.95, $[M+4H]^+/4$ (observed): 303.96

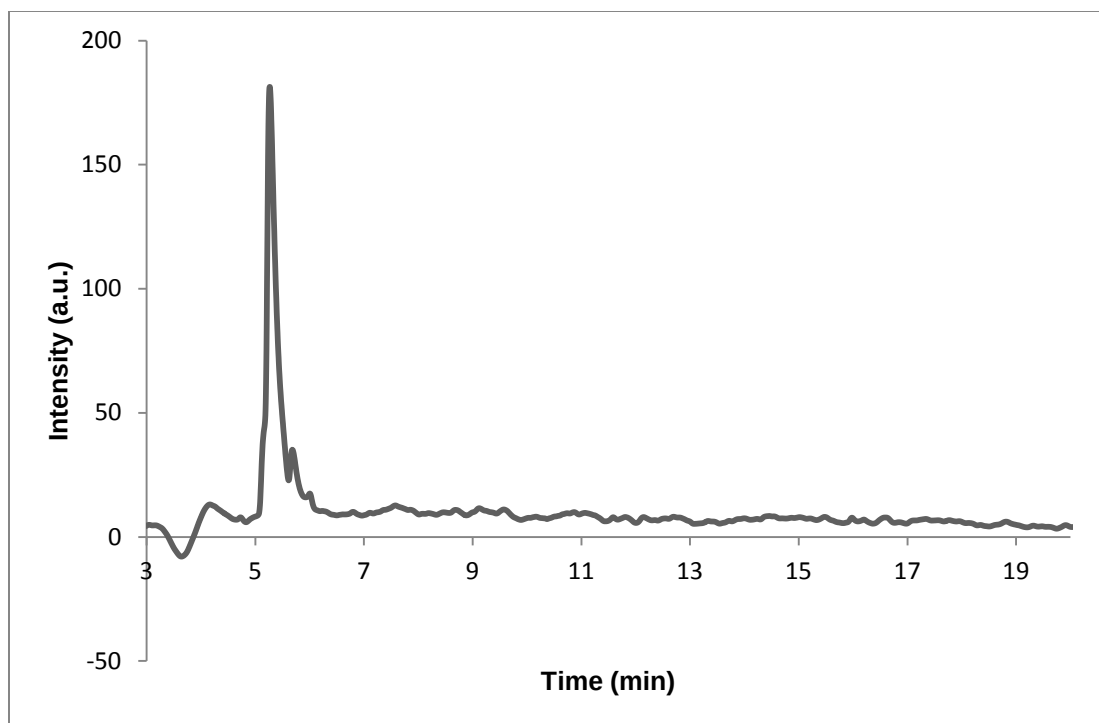


Figure 4.8. Liquid chromatogram of C_{12} - $P_4K_2R_8$ -Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 94%.

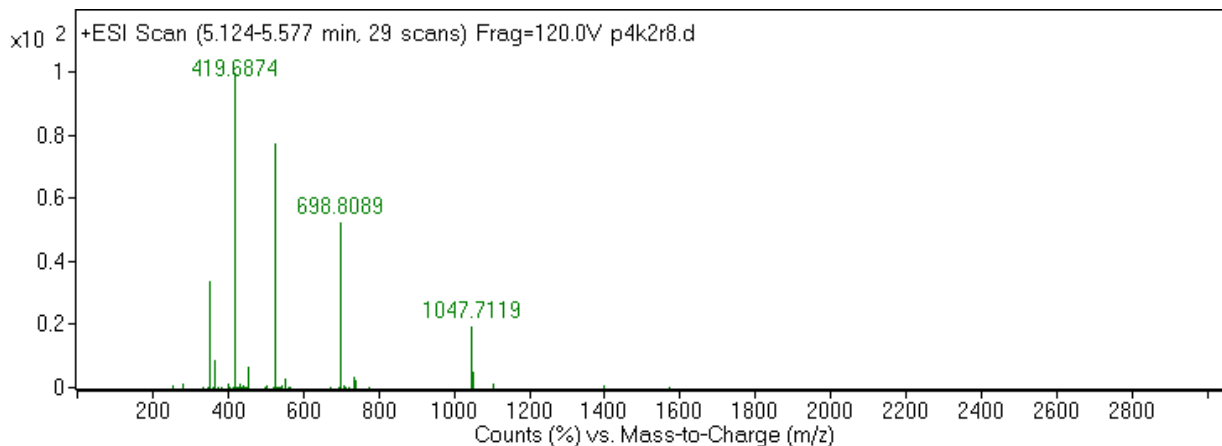


Figure 4.9. Mass spectrum of C_{12} - $P_4K_2R_8$ -Am. Mass result $[M+3H]^+/2$ (calculated): 1047.70, $[M+3H]^+/2$ (observed): 1047.71, $[M+4H]^+/3$ (calculated): 698.80, $[M+4H]^+/3$ (observed): 698.81, $[M+5H]^+/4$ (calculated): 524.35, $[M+5H]^+/4$ (observed): 524.35, $[M+6H]^+/5$ (calculated): 419.68, $[M+6H]^+/5$ (observed): 419.69, $[M+7H]^+/6$ (calculated): 349.90, $[M+7H]^+/6$ (observed): 349.90.

4.3. Characterization of the PA/AON complexes

The secondary structure (Figure 4.10.), zeta potential (Table 4.1), morphology (Figure 4.11) and size (Figure 4.12) of PA and PA/AON complexes were characterized.

The secondary structure investigation was performed with circular dichroism (Figure 4.10.), all PAs and PA/AON complexes demonstrated a negative peak at 204 nm which is the characteristic peak of PPII secondary structure⁸⁷.

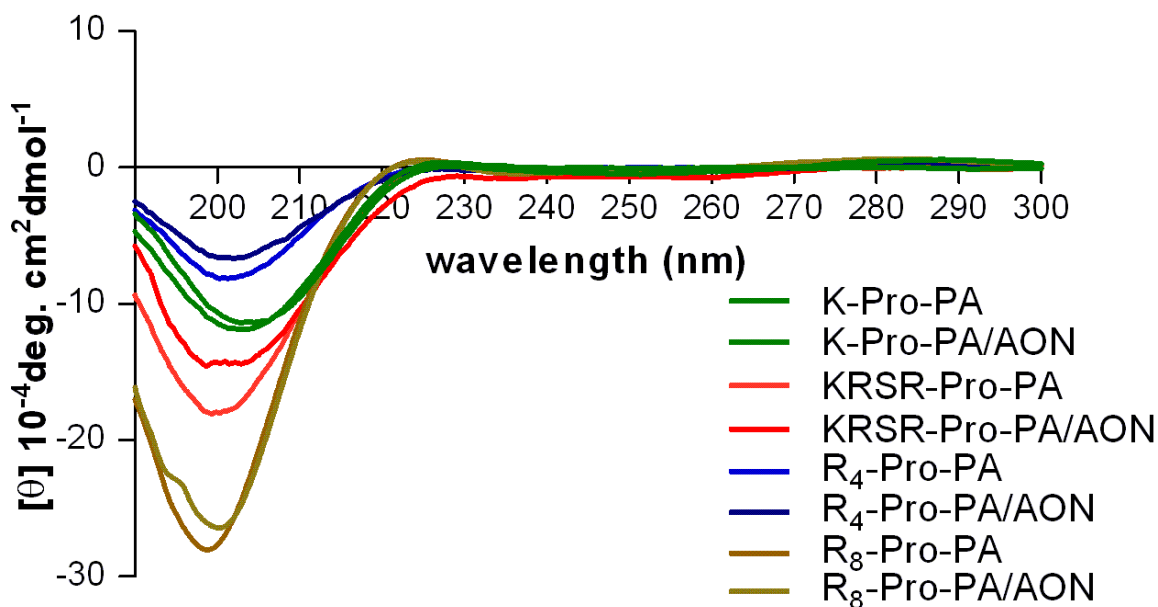


Figure 4.10. CD spectra of PAs and PA/AON complexes

In order to characterize the charge of the system, zeta potential of PA and PA/AON complexes at different molar ratio were investigated. (Table 4.1.) Bare PAs were positively charged due to the presence of lysine and arginine residues. When PAs were complexed with AON, the negativity was increased resulting from the negativity of AON itself. At the molar ratio used in *in vitro* experiments, K-Pro-PA was slightly negative, R₄-Pro-PA/AON and KRSR-Pro-PA/AON were neutral. R₈-Pro-PA/AON was positive at all molar ratios that were used.

Table 4.1. Charges of PAs and PA/AON complexes. (n=3)

	Only PA	30:1 PA/AON	100:1 PA/AON	300:1 PA/AON
K-Pro-PA	1.1 ± 2.1 mV	(-) 8.1 ± 3.2 mV	(-) 6.3 ± 2.4 mV	(-) 12.8 ± 1.1 mV
KRSR-Pro-PA	8.2 ± 1.0 mV	1.4 ± 0.5 mV	9.7 ± 0.2 mV	15.1 ± 0.9 mV
	Only PA	10:1 PA/AON	30:1 PA/AON	100:1 PA/AON
R₄-Pro-PA	15.1 ± 0.6 mV	(-) 7.1 ± 0.8 mV	2.5 ± 0.2 mV	10.8 ± 0.7 mV
	Only PA	3:1 PA/AON	10:1 PA/AON	30:1 PA/AON
R₈-Pro-PA	22.6 ± 3.1 mV	29.1 ± 2.0 mV	31.4 ± 2.4 mV	29.6 ± 3.6 mV

Table 4.2. Charges of KRSR-Pro-PA/R₈-Pro-PA/AON complexes. (n=3)

3:1:1 KRSR/R₈/AON	(-) 18.4 ± 0.3 mV
30:10:1 KRSR/R₈/AON	36.1 ± 1.4 mV

Morphologies of PA and PA/AON complexes were characterized with transmission electron microscopy. (Figure 4.11.) Bare PAs formed nanospheres with a diameter around 20 nm. R₈/KRSR-Pro-PA nanospheres, which are the combination of two PAs, were smaller compared to other PAs with a diameter between 5-10 nm.

The morphologies and sizes of PA/AON complexes were also investigated and an increase in size of the nanospheres was observed upon complexation with AON. R₄-Pro-PA/AON and KRSR-Pro-PA/AON nanospheres were especially bigger compared to bare PAs. R₄-Pro-PA/AON formed elongated spheres that tended to stick together. The increase in size might stem from the fusion of PA micelles with AON, or PAs form micellar aggregates in the presence of

oligonucleotide. Although size of KRSR-Pro-PA/AON was increased, the morphology was different from R₄-Pro-PA/AON. R₈-Pro-PA, as an exception, formed smaller spheres in the presence of AON. In general, a difference in size and morphology of nanospheres occurred when AON was complexed with different PAs. The formation of different morphologies might stem from the differences in the sequence of the PAs.

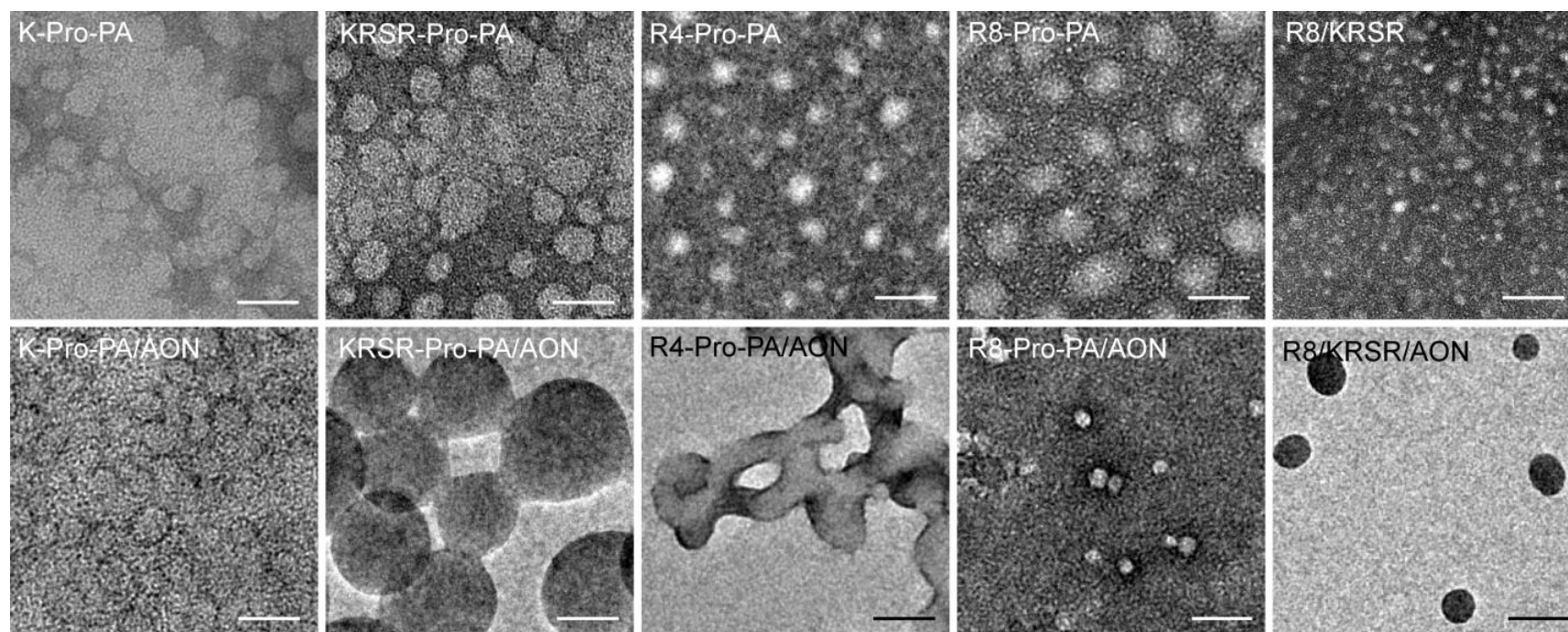


Figure 4.11. TEM images of PAs and PA/AON complexes. (Scale bar: 50 nm)

Hydrodynamic sizes of PAs were investigated with DLS and size of bare PAs was found to be around 70 nm, which is greater than the sizes in TEM images. (Figure 4.12) The difference in TEM size and DLS size results from the difference between the size of the dried sample and hydrodynamic size that was observed in several studies^{101,102}. Upon complexation with AON, KR SR-Pro-PA and R₄-Pro-PA/AON formed nanospheres with increased size which was parallel to TEM images; whereas, K-Pro-PA/AON, R₈-Pro-PA/AON and R₈/KR SR/AON formed similar sized small nanospheres around 70 nm.

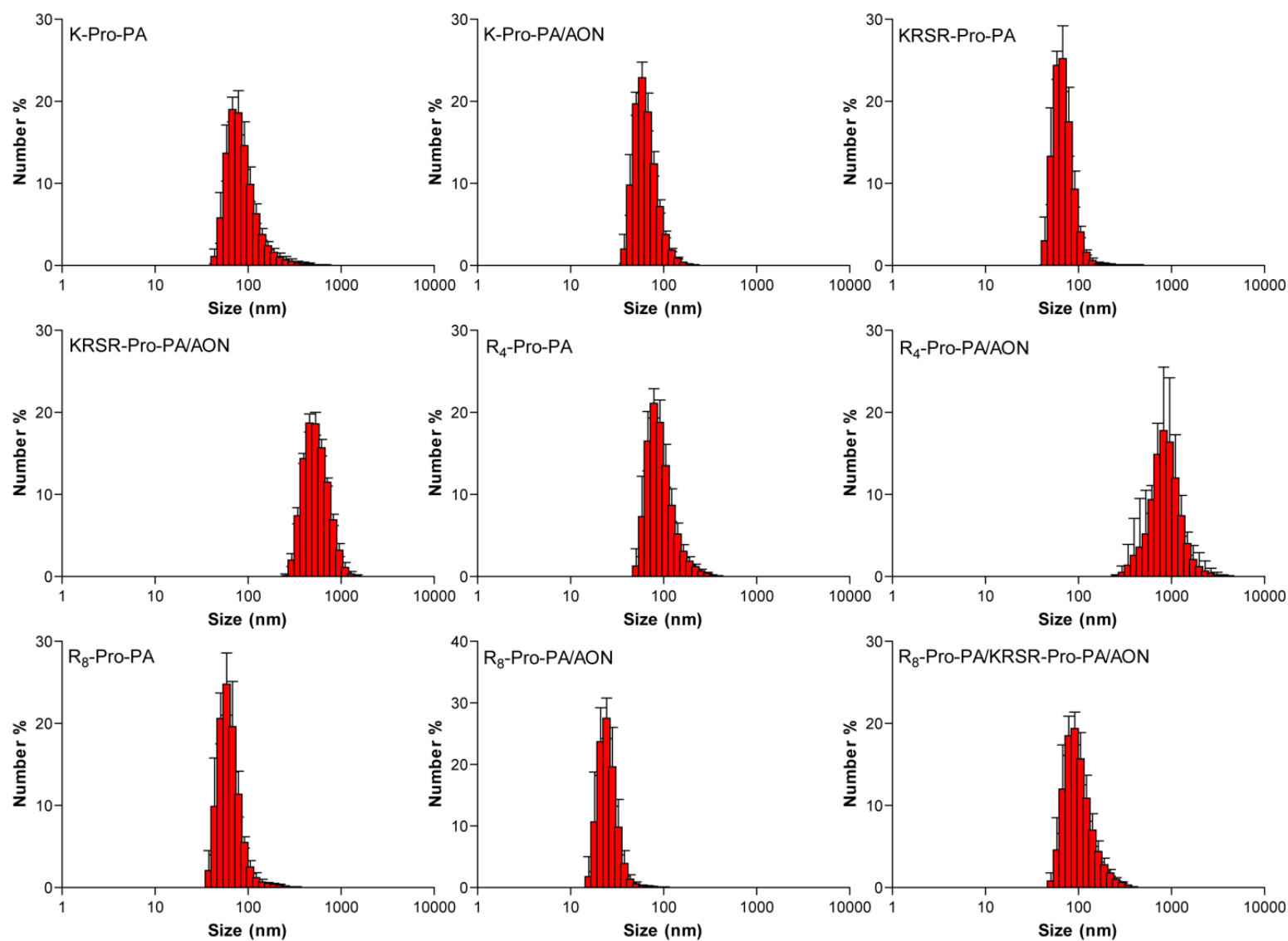


Figure 4.12. Size distribution of PA and PA/AON nanospheres measured with DLS (dynamic light scattering) part of zetasizer.

4.4. Cytotoxicity of PAs

In order to investigate the toxic dose of peptide amphiphiles, viability of MCF-7 cells treated with PAs with different concentrations was studied. (Figure 4.13.) K-Pro-PA, KRSR-Pro-PA and R₄-Pro-PA were found to be biocompatible at the concentrations (30-60 μ M) that were used *in vitro*. The 24 h viability of cells treated with R₈-Pro-PA were found to be 50% at 50 μ M concentration. The cytotoxicity of R₈-Pro-PA stems from the arginine rich cell penetrating peptide R8. Previously, polyarginine CPP toxicity was found to be increased with increasing arginine number in the sequence^{103,104}.

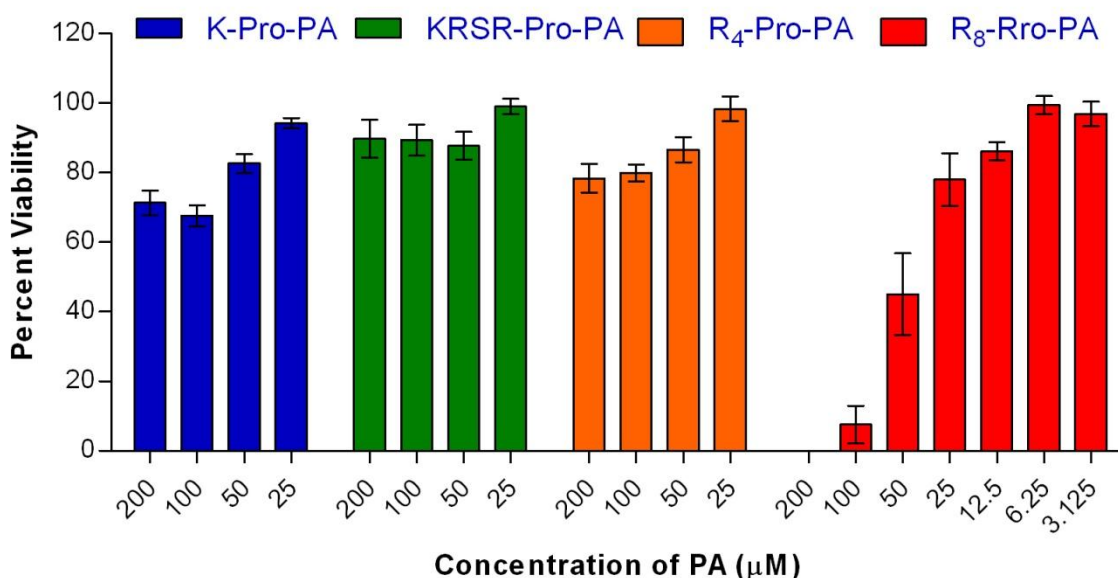


Figure 4.13. Cytotoxicity of PAs administered to MCF-7 cells for 24 h. (Error bars show SEM, n=4)

4.5. Cellular internalization of PA/AON complexes

Internalization of PA/AON complexes was illustrated in Figure 4.14. KRSR-Pro-PA/AON and R₄-Pro-PA/AON were found to be internalized more compared to K-Pro-PA/AON. Uptake of R₈ was prominent even at low concentrations. Combination of KRSR-Pro-PA and R₈-Pro-PA was

also efficient in internalization of AON. The quantification of internalized PA/AON complexes with flow cytometry was demonstrated in Figure 4.15. At the same concentrations, uptake of R₈-Pro-PA/AON and the combination of KRSR-Pro-PA and R₈-Pro-PA was higher than other PA/AON complexes.

4.6. Cellular internalization mechanisms of PA/AON complexes

Internalization mechanisms of PA/AON complexes were investigated with endocytosis pathway inhibitors. (Figure 4.16.) Internalization of K-Pro-PA/AON, KRSR-Pro-PA/AON and R₄-Pro-PA/AON was found to be via non-endocytic pathway. They might enter cells via direct transduction. R₈-Pro-PA internalization was found to depend on dynamin mediated pathway. R₈/KRSR-Pro-PA/AON internalization was found to be via dynamin, clathrin and caveolae dependent pathway.

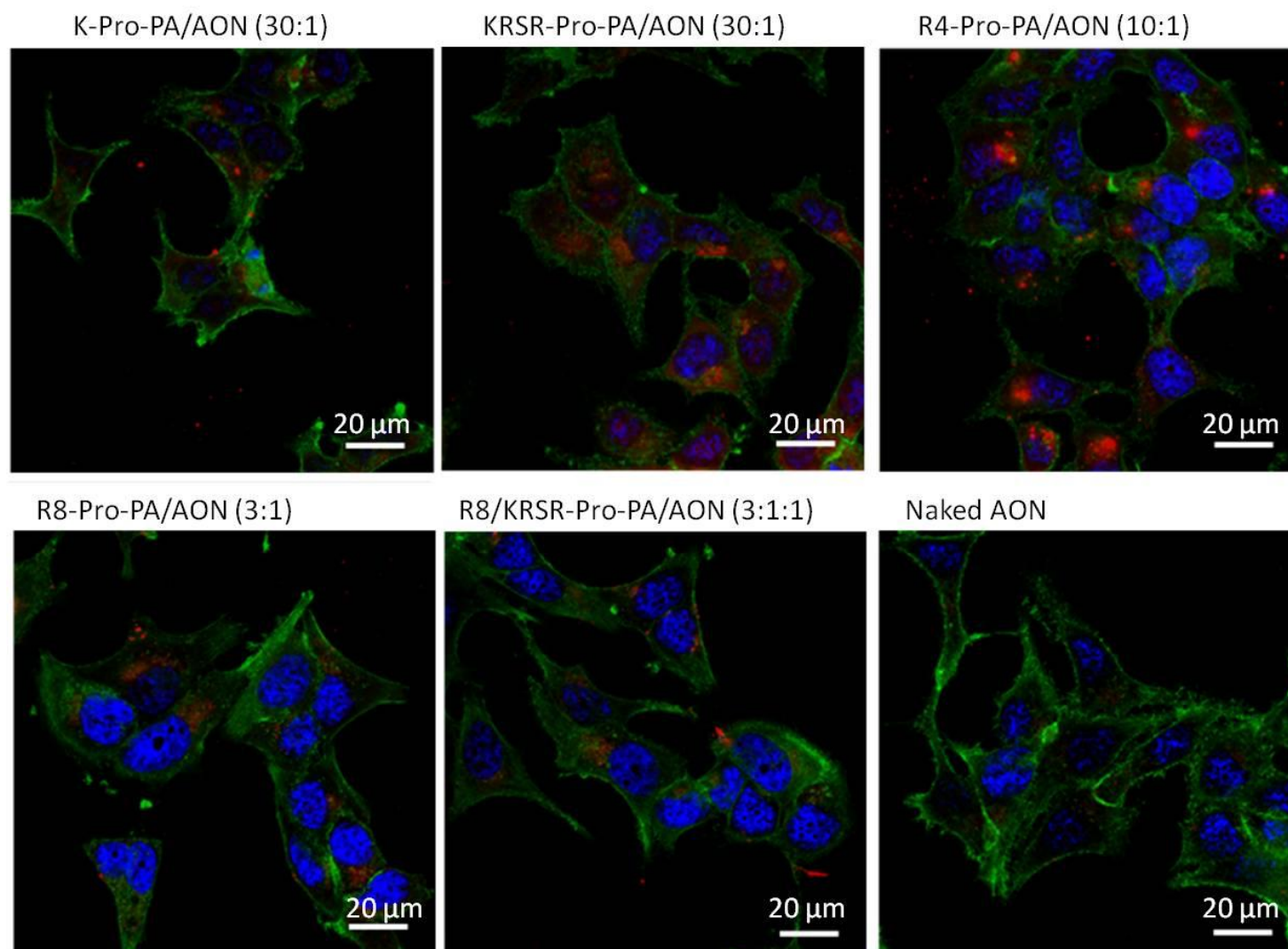


Figure 4.14. Cellular internalization of PA/AON complexes in MCF-7 cells after 24 h. FAM-AON (red), phalloidin (green), TO-PRO-3 (blue). (Scale bar: 20μm.)

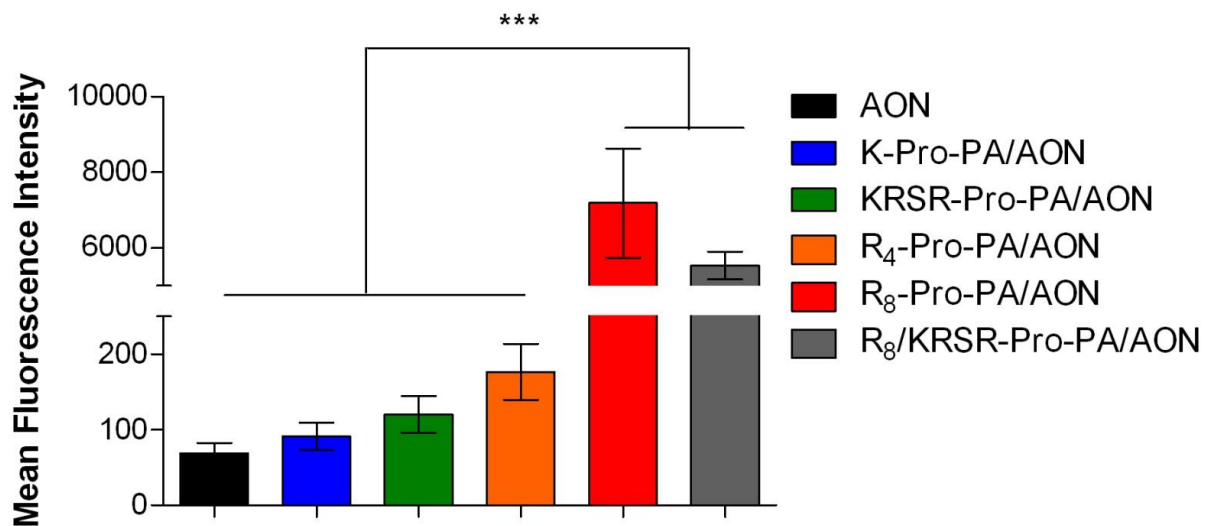


Figure 4.15 Uptake of PA/AON complexes by MCF-7 cells quantified with flow cytometry. (Error bars show SEM, two independent experiments were repeated with n=3 in each experiment.)

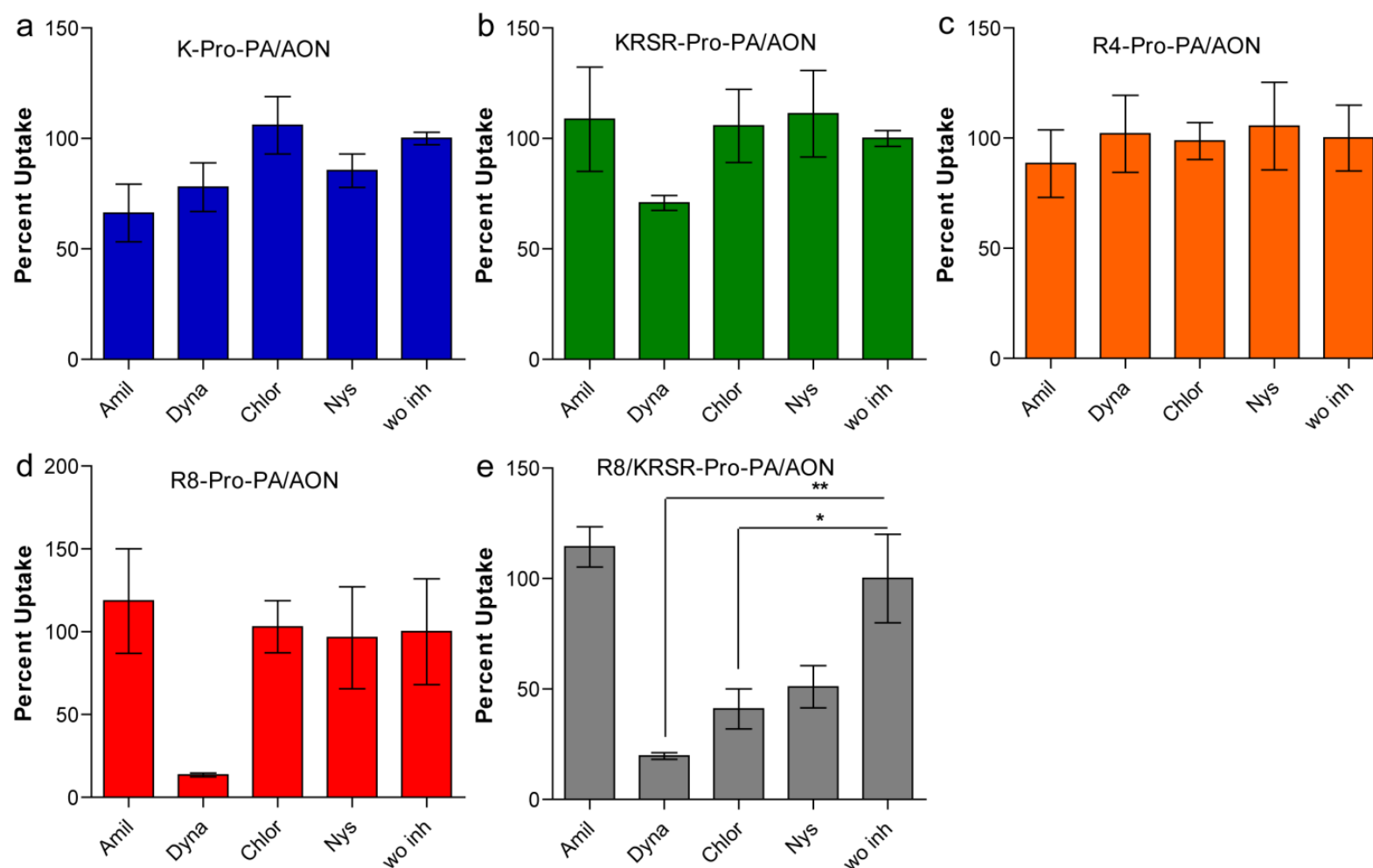


Figure 4.16. Mechanisms of cellular internalization of PA/AON complexes in MCF-7 cells. Amiloride (Amil), Dynasore (Dyna), Chlorpromazine (Chlor), Nystatin (Nys) were administered to cells to inhibit endocytosis pathways, uptake was normalized cells without inhibitor (wo inh). (Error bars show SEM, two independent experiments were repeated with n=3 in each experiment. One way ANOVA shows statistical significance with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

4.7. Expression of Bcl-2

To be an efficient delivery system, internalized AON must bind to its target mRNA and decrease the expression of target gene. In Fig 4.17, target gene (Bcl-2) expression in MCF-7 cells treated with PA/AON or PA/MM complexes was analyzed. A mismatch oligonucleotide was used as a control and gene expressions were normalized to PA/MM treated MCF-7 cells. KRSR-Pro-PA, R₈-Pro-PA and the combination of these two PAs were shown to be effective in delivery of AON and decreasing the expression of target gene. Also, the efficiency in silencing of PAs was comparable to Lipofectamine, which is a commercial transfection reagent composed of cationic lipids.

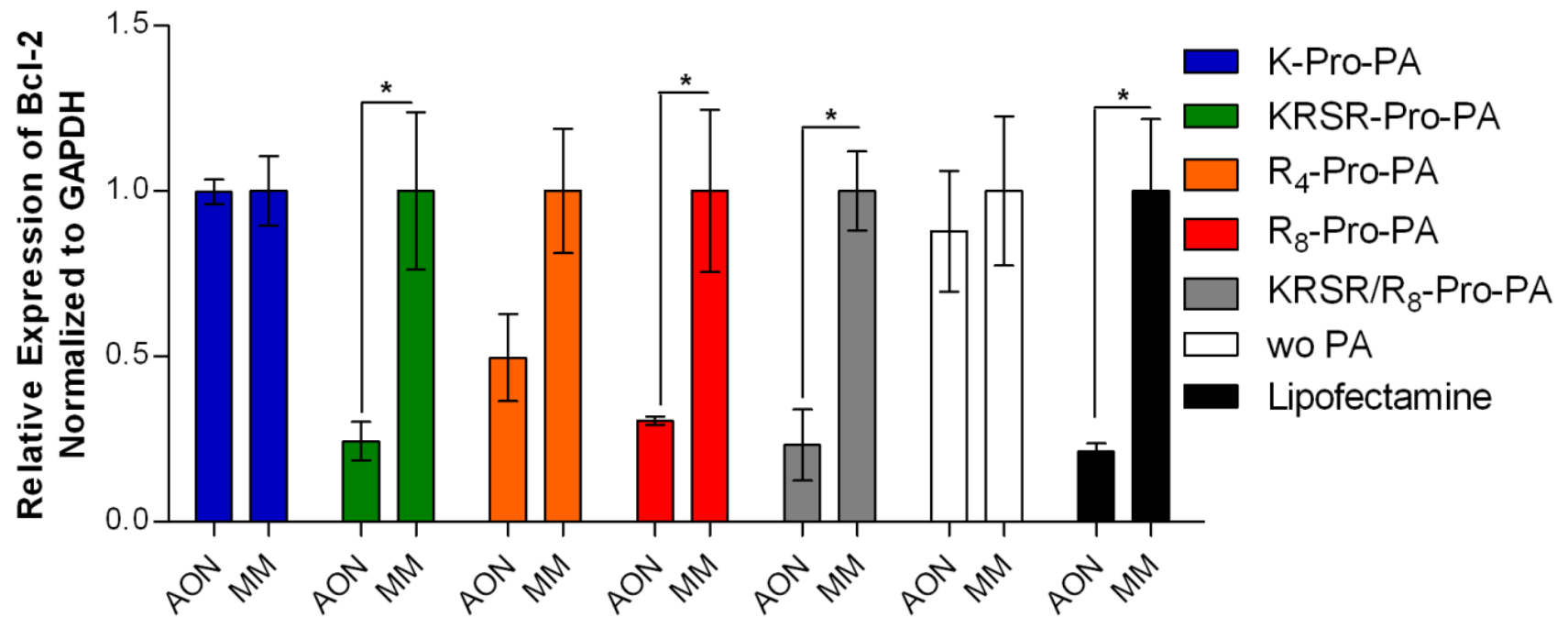


Figure 4.17. Bcl-2 expression in MCF-7 cells treated with PA/AON or PA/MM complexes. Gene expression is normalized to GAPDH. AON: antisense oligonucleotide MM: mismatch control. (Error bars show SEM, two independent experiments were repeated with n=3 in each experiment. Student's t-test shows statistical significance with * p<0.05, ** p<0.01, *** p<0.001)

CHAPTER 5. OLIGONUCLEOTIDE DELIVERY WITH BIOACTIVE NANOFIBER FORMING PEPTIDE AMPHIPHILES

5.1. Background

Targeting of the delivery system is important to increase the local concentration of the drug in the target tissue and to decrease the side effects of the drug. A targeting peptide (RGD) was used for the design of nanofiber forming PAs to explore the complexation of AON with PA for a possible application in *in vivo* targeting.

To perform its activity, after internalization of the delivery system via endocytosis, AONs need to escape from endosome. To provide endosomal escape properties to the PA delivery system, it was decorated with histidines that can be protonated at acidic pH in the late endosome and provide endosomal escape of the cargo.

5.2. Design and synthesis of peptide amphiphiles

Fiber forming peptide amphiphiles were designed with a sequence VVA that can induce β -sheet formation and nanofiber formation through self-assembly in the presence of oligonucleotide. The nanofiber forming PA was decorated with bioactive peptide epitopes to introduce targeting and endosomal escape properties to the delivery system. RGD peptide epitope has been previously studied for oligonucleotide delivery to target tumor tissue based on the knowledge that RGD binds to $\alpha(v)\beta(3)$ integrins that are overexpressed in cancer cells^{52,105,106}. Therefore, we designed cationic peptide amphiphile with an RGD targeting peptide. In addition, histidine rich peptides have been shown to be effective in endosomal escape¹⁰⁷. To overcome endosomal entrapment of peptide amphiphiles and enhance the delivery system, we decorated this PA with two histidines. Peptide amphiphiles (Figure 5.1.) were characterized with LC-MS. (between Figure 5.2. and 5.5.)

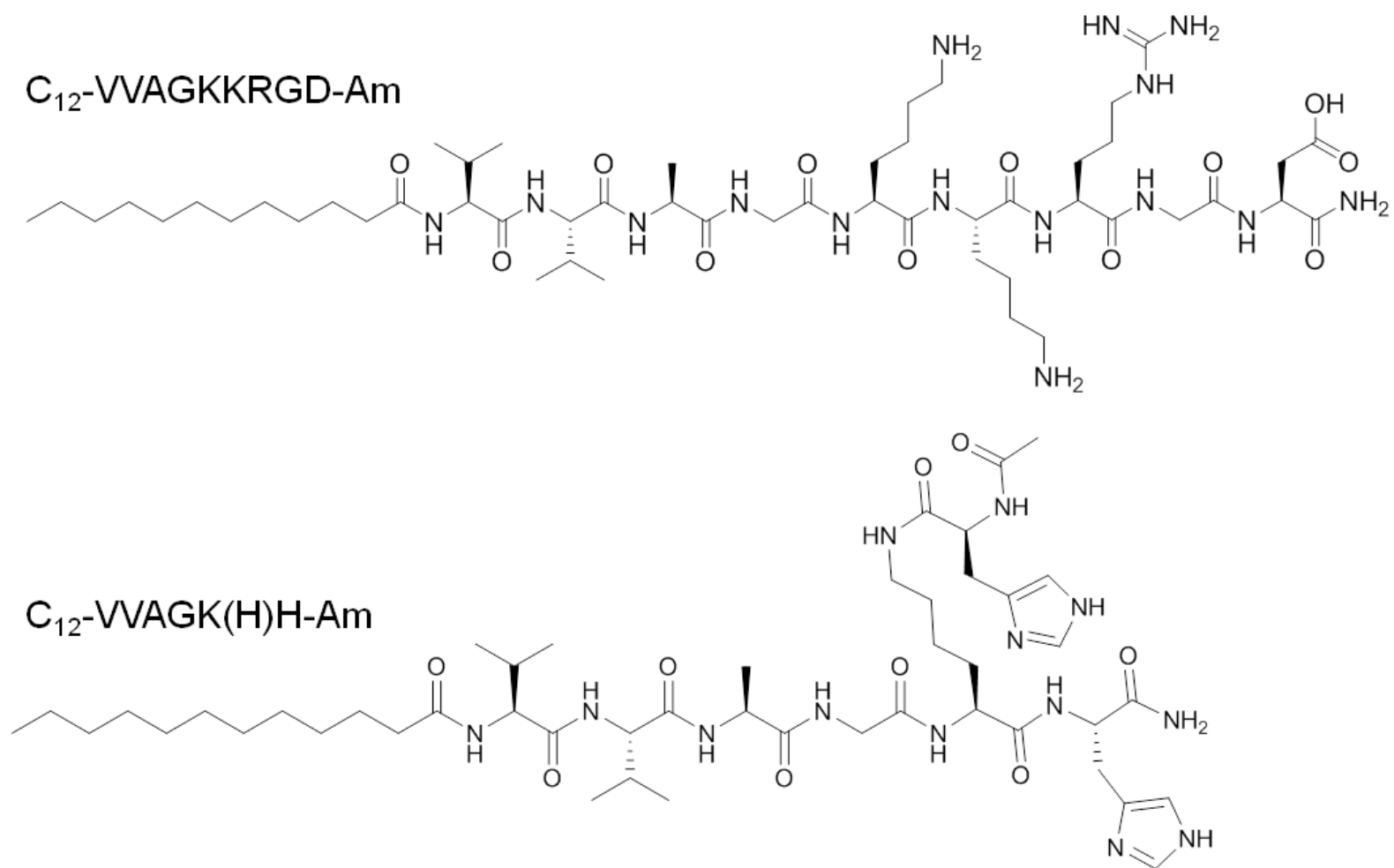


Figure 5.1. Bioactive fiber forming peptide amphiphiles that are used in this study: VVAGKKRGD (C₁₂-VVAGKKRGD-Am) and VVAGK(H)H (C₁₂-VVAGK(H)H-Am).

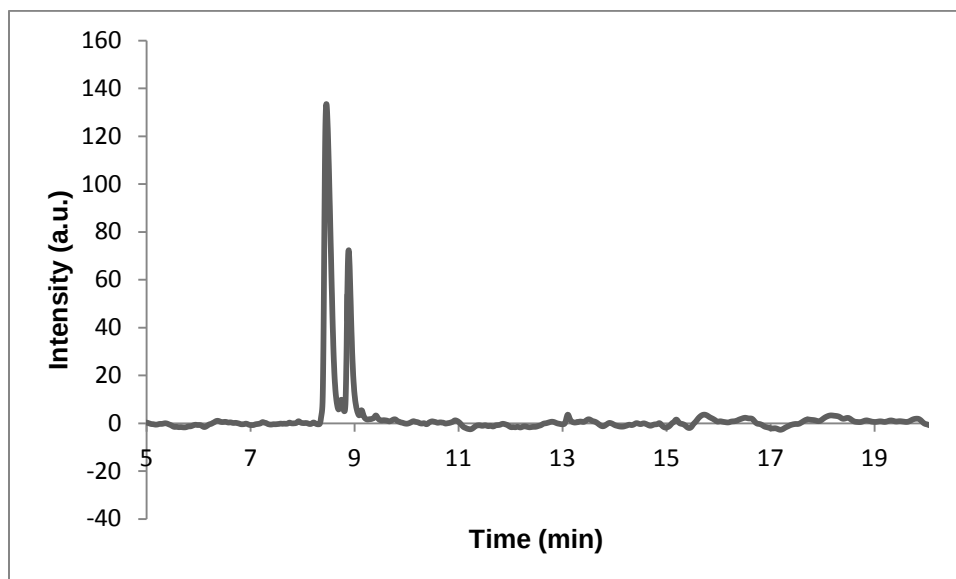


Figure 5.2. Liquid chromatogram of C₁₂-VVAGKKKRGD-Am after subtraction of water, the change of response units with respect to time at 220 nm. Two peaks demonstrated the same product with same mass. Purity is calculated as 90%.

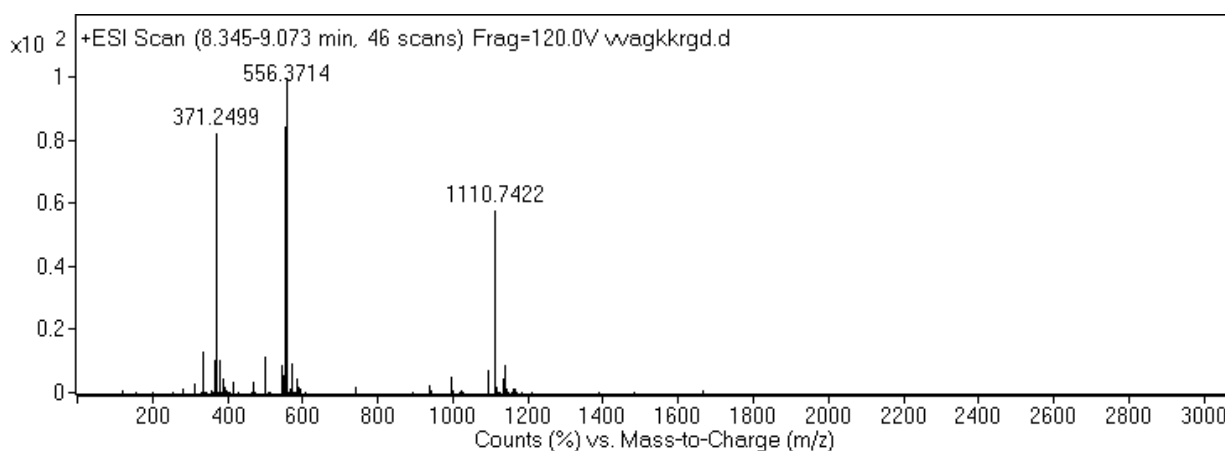


Figure 5.3. Mass spectrum of C₁₂-VVAGKKKRGD-Am. Mass data [M+H]⁺ (calculated): 1110.73, [M+H]⁺ (observed): 1110.74, [M+3H]⁺/2 (calculated): 556.36, [M+3H]⁺/2 (observed): 556.37, [M+4H]⁺/3 (calculated): 371.24, [M+4H]⁺/3 (observed): 371.25.

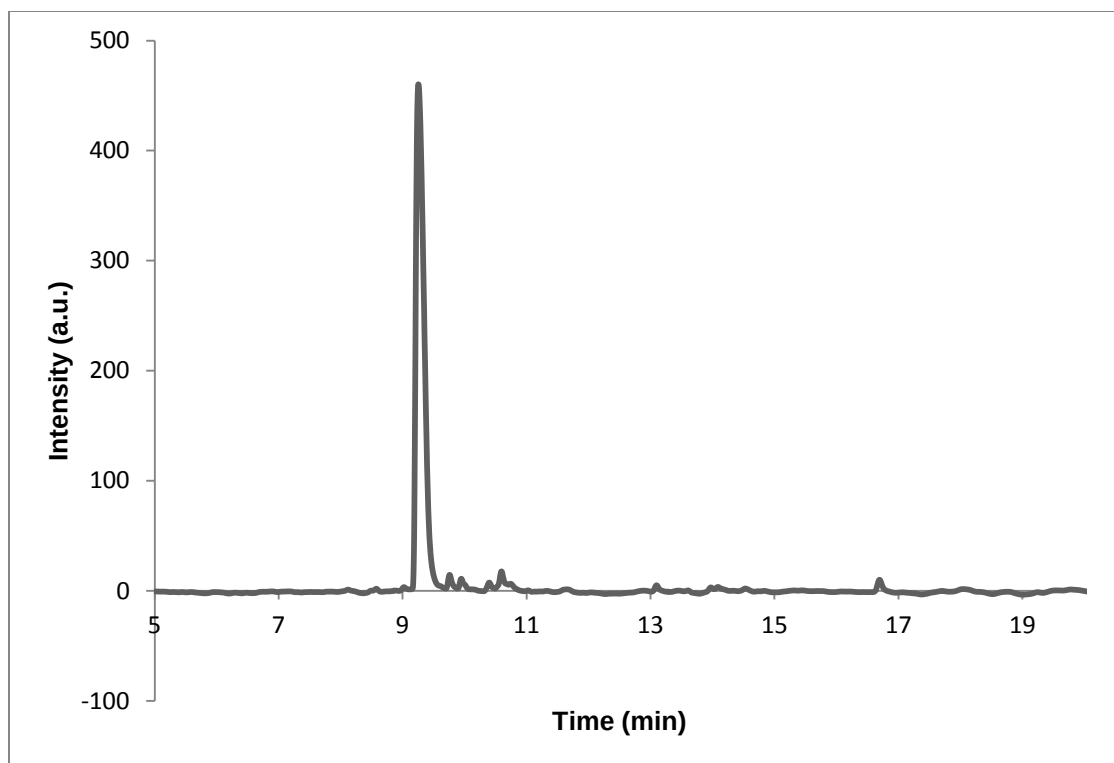


Figure 5.4. Liquid chromatogram of C₁₂-VVAGK(H)H-Am after subtraction of water, the change of response units with respect to time at 220 nm. Purity is calculated as 94%.

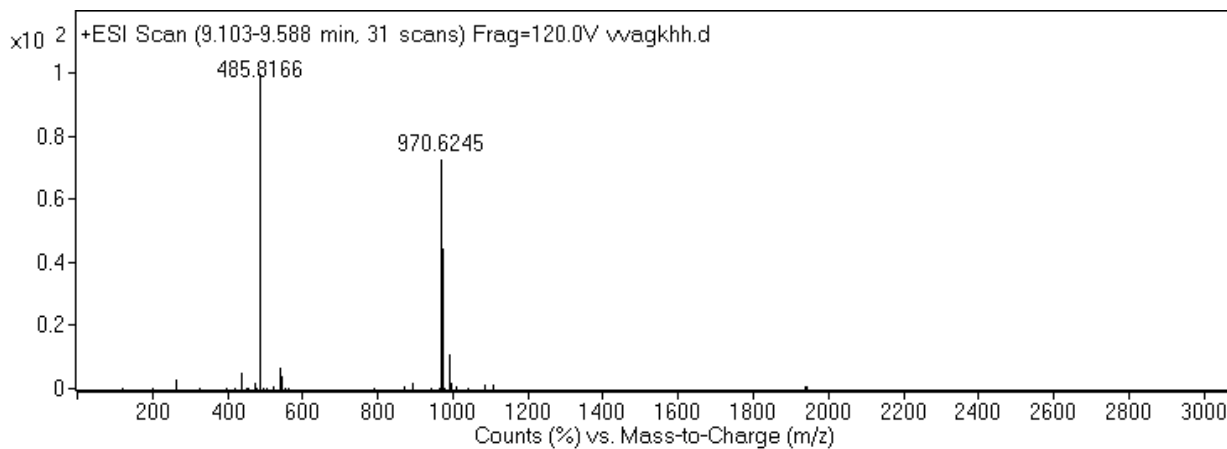


Figure 5.5. Mass spectrum of C₁₂VVAGK(H)H-Am. Mass data [M+H]⁺ (calculated): 970.61, [M+H]⁺ (observed): 970.62, [M+2H]⁺/2 (calculated): 485.80, [M+2H]⁺/2 (observed): 485.82.

5.3. Characterization of PAs and PA/AON complexes

Secondary structure of PAs and PA/AON complexes was determined with circular dichroism (Figure 5.6.) PAs formed random coil structure in the absence of AON with a negative peak at 198 nm. Upon complexation with AON, VVAGK(H)H formed β -sheet structure with a negative peak at 204 nm and a positive peak at 224 nm. On the other hand, VVAGKKRGD did not form a complete β -sheet structure in the presence of oligonucleotide. Indeed, the complex formed demonstrated a heterogeneous secondary structure containing β -sheet and random coil. A negative peak at 196 nm demonstrated the random coil structure and a negative peak at 226 nm stems from the β -sheet structure.

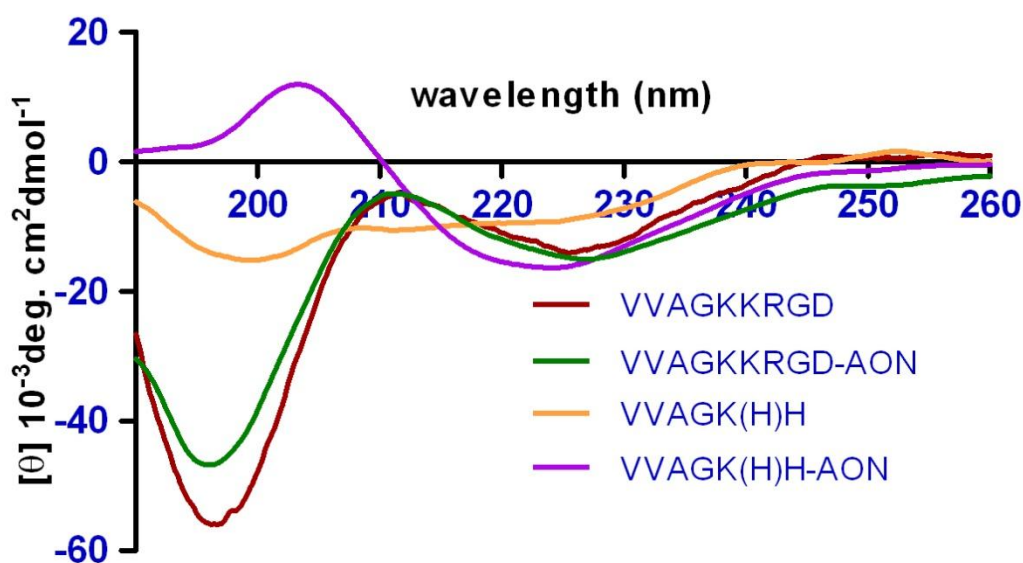


Figure 5.6. Circular dichroism spectra of PA and PA/AON complexes at pH 7.

The zeta potentials of the system were characterized with a Zeta Sizer (Table 5.1.) Only peptide amphiphiles were positively charged due to the presence of lysine residues. At 30:1 molar ratio, PA/AON complexes presented negative charge resulting from the complexation with oligonucleotide.

Table 5.1. Zeta Potentials of PAs and PA/AON complexes at pH 7.

	Only PA	30:1 PA/AON	100:1 PA/AON	300:1 PA/AON
VVAGKKRGD	15.1 ± 1.8 mV	(-)12.5 ± 0.3 mV	5.0 ± 1.0 mV	13.8 ± 0.9 mV
VVAGK(H)H	18.6 ± 2.7 mV	(-)10.1 ± 0.5 mV	16.4 ± 0.2 mV	26.0 ± 2.0 mV

5.4. Cytotoxicity of peptide amphiphiles

Viability of MCF-7 cells was studied after 48 h of administration of peptide amphiphiles. VVAGK(H)H did not induce any cell death at 48 h. VVAGKKRGD was demonstrated to be cytotoxic at 100 and 200 μ M concentrations, yet it was biocompatible at the concentrations that were used *in vitro*.

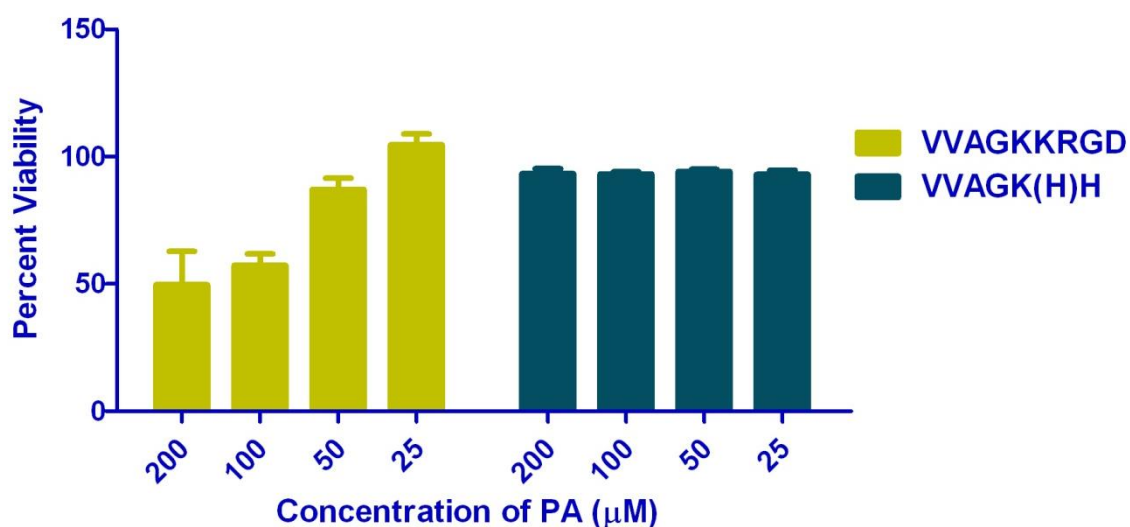


Figure 5.7. 48 h viability of MCF-7 treated with VVAGKKRGD or VVAGK(H)H in serum free media. (Error bars show SEM, n=5).

5.5. Cellular internalization of PA/AON complexes

PA/AON complexes were shown to bind to cell membrane at 4 h, and they were internalized more at 24 h. The combination of VVAGK and VVAGK(H)H demonstrated a better cellular uptake of AON compared to VVAGK(H)H alone. Therefore, the combination was used in gene expression study.

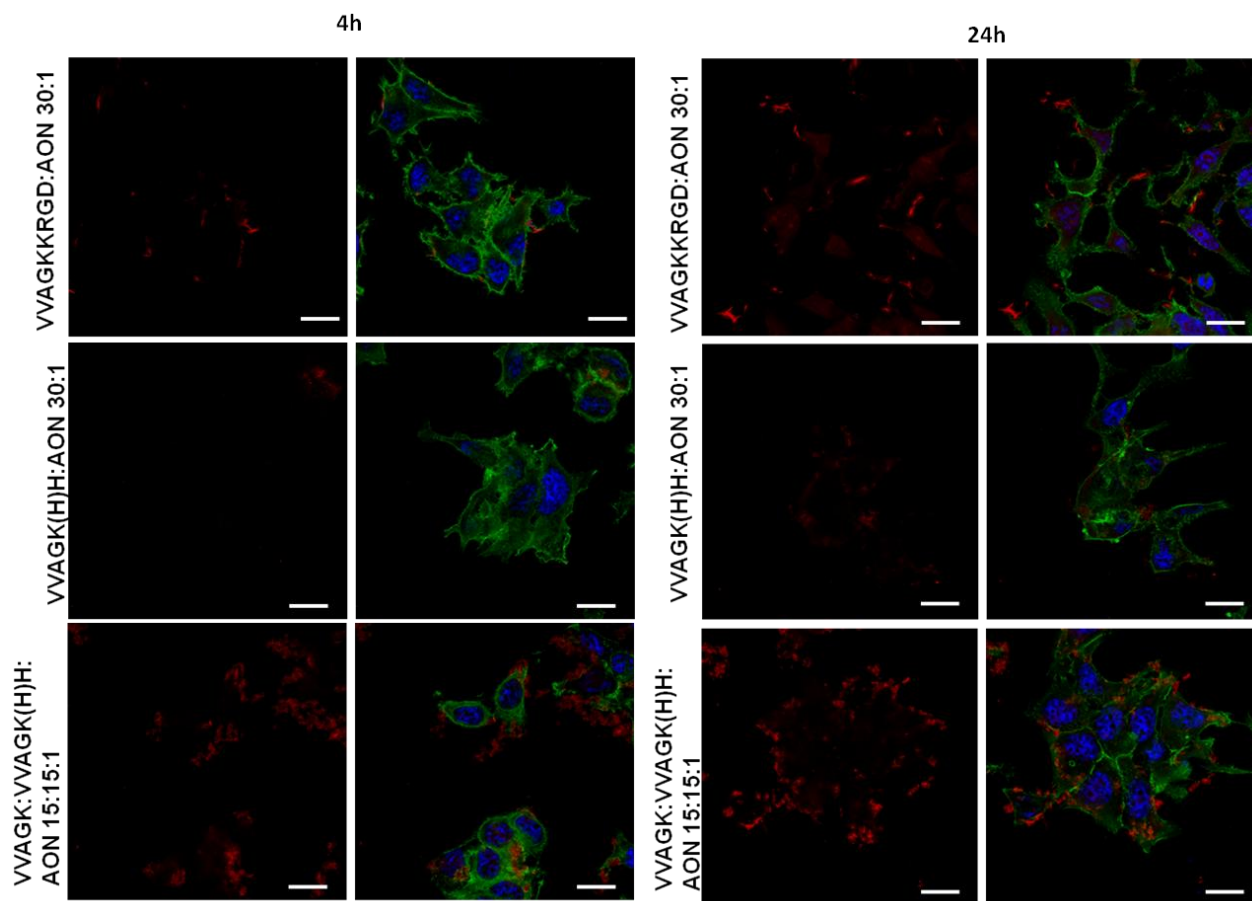


Figure 5.8. 4 h and 24 h uptake of PA/AON complexes in MCF-7 cells. FAM-AON (red), phalloidin (green), TO-PRO-3 (blue). (Scale bar: 20 μ m.)

5.6. Expression of Bcl-2 in MCF-7 cells treated with PA/AON complexes

MCF-7 cells were treated with peptide amphiphile / Bcl-2 AON complexes in order to decrease the gene expression of Bcl-2 gene. A mismatch oligonucleotide was used in this study as a control. VVAGKKRGD/AON complex did not decrease the expression of Bcl-2 gene, and thus VVAGKKRGD PA was inefficient in silencing the target gene. The combination of VVAGK

and VVAGK(H)H did not decrease the gene expression significantly, therefore the delivery system was also ineffective in antisense oligonucleotide delivery.

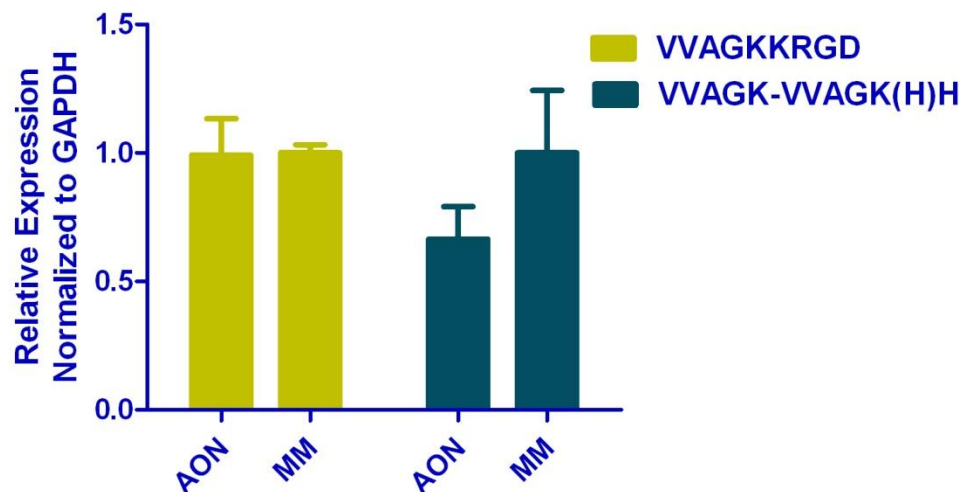


Figure 5.9. Bcl-2 expression of MCF-7 cells transfected with fiber forming PA/AON or PA/MM complexes for 48 h. Gene expression is normalized to GAPDH. (Error bars show SEM, n=3.)

CHAPTER 6. CONCLUSION AND FUTURE PERSPECTIVES

We have heretofore described the development of a self-assembled peptide amphiphile delivery system for oligonucleotide therapy. Peptide amphiphiles are considered to be ideal materials for the delivery of oligonucleotide drugs, due to their biocompatibility, functionalization capacity and ability to self-organize into structurally distinct nanoscale assemblies. The former part of this thesis concerns the effects of nanostructure geometry on the efficiency of oligonucleotide delivery. Taking advantage of the structural versatility inherent to PA-based systems, we have designed peptide amphiphile carriers displaying two distinct geometries, and demonstrated that the morphology of the delivery system is a key determinant in the cellular internalization of cargo molecules. Nanofiber-forming peptide amphiphiles were observed to perform better at oligonucleotide delivery within cells compared to nanospheres. This effect was further demonstrated to result from a fundamental difference between the internalization mechanisms of fibrous and spherical PA delivery systems: While the internalization of the nanofibers was observed to be mediated by endocytosis, the uptake of nanospheres was independent of endocytic pathways. In addition, nanofiber- and nanosphere-forming peptide amphiphiles were functionalized with glucose to provide them with tumor-targeting properties. The cellular internalizations of glucose-decorated, nanofiber-forming PAs were enhanced compared to non-glucose bearing PAs, and the internalization of glyco-PAs were observed to depend on the presence of glucose transporters on the membrane surface.

Our nanosphere-forming peptide amphiphiles were further decorated with several bioactive peptides to enhance their cell penetration capacities, the evaluation of which comprises the latter part of the present thesis. A cell surface binding peptide (KRSR) and two polyarginine CPPs (tetraarginine/R4 and octoarginine/R8) were found to increase the transfection efficiency of antisense oligonucleotides when used in the functionalization of sphere-forming PA assemblies. Octaarginine PAs were effective in the cellular internalization of oligonucleotide even at low concentrations, but displayed dose-dependent toxicity due to their strongly positive charge and pore-forming properties, which are also expected to create non-selective internalization issues under *in vivo* conditions. KRSR decorated PA/AON complex, on the other hand, were neutral and non-toxic under *in vitro* experimental conditions, and despite this lack of a strong positive charge, the transfection efficiency of this system was comparable to lipofectamine control. The

good biocompatibility and high silencing efficiency of KRSR-decorated PAs had made this combination especially effective in the delivery of oligonucleotides.

We have therefore shown that peptide amphiphile assemblies are efficient vectors for oligonucleotide drugs, and that these assemblies can be further decorated with cell-penetrating peptides and targeting moieties. In addition to conventional functionalization methods, a system displaying two or more distinct functionalities can also be achieved by the co-assembly of multiple PA molecules, which renders PA-based systems advantageous in relation to other delivery vectors. In addition, PAs and AONs were shown to readily form complexes in the presence of each other, without requiring additional steps to effect the creation of a PA/AON assembly. Taking into consideration the versatility of the above-described system and the ease at which the PA/AON complexation occurs, we conclude that peptide amphiphile-based delivery systems are highly promising for further development efforts involving the use of targeting peptides for the selective delivery of AONs or siRNAs to specific tissues or cell types.

For the improvement of developed delivery system, nanospheres can be functionalized with the integrin-binding peptide RGD for selective localization in tumor tissues, and with the cell surface binding peptide KRSR for the enhancement of internalization efficiency. Two or more functionalities (*e.g.* targeting and cellular internalization) can therefore be combined in one delivery system for effective oligonucleotide delivery. Such targeted carrier systems should first be tested *in vivo* to investigate whether they are capable of localizing in tumor tissues and decreasing tumor volumes. Targeting delivery is expected to increase drug efficiency, lower the effective dose and prevent the accumulation of the oligonucleotide in the liver and spleen.

A glucose targeting approach, while effective in enhancing the delivery AONs to their target cells, was not successful in decreasing Bcl-2 expression and is therefore more suited to other carrier/AON systems. A different anticancer AON may be a better candidate for use with glyco-functionalized PA carriers, as the presence of glucose did increase delivery system uptake, and the use of another AON may more effectively silence of the target gene without suffering from the unforeseen consequences of glucose functionalization (*i.e.* increased Bcl-2 expression) observed in this work. If successful under *in vitro* conditions, this new PA/AON complex would also be tested in an *in vivo* model to investigate its therapeutic efficiency. Glucose

functionalization is also expected to increase the localization of the oligonucleotide in the tumor site, and therefore prevent the accumulation of the AON in the liver and the spleen.

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