MULTI-GENE REGULATION USING DNA-ORIGAMI-AuNPs NANOSTRUCTURES IN BREAST CANCER CELLS

by
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MULTI-GENE REGULATION USING DNA-ORIGAMI-AUNPS NANOSTRUCTURES IN BREAST CANCER CELLS

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Dedicated to my son

May you never stop learning…

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ABSTRACT

MULTI-GENE REGULATION USING DNA-ORIGAMI-AUNPS NANOSTRUCTURES IN BREAST CANCER CELLS

Gene regulation is a novel approach to cure a gene related disease. Thus, there is an ongoing effort to increase the efficiency of gene delivery. In this thesis, we developed a DNA based nanostructure to deliver gene regulation elements for the up-regulated genes in breast cancer as alternative to the currently used non-viral delivery systems. A tile shaped DNA origami nanostructure is constructed by including morpholino antisense oligonucleotides targeting the HER2, ERα, EGFR, and Ki-67 genes. Then, the sticky ends of the DNA origami nanostructures were hybridized to the complementary oligonucleotide sequence attached to gold nanoparticles (AuNPs) to enhance their cellular uptake. The constructed nanostructure was characterized using agarose gel electrophoresis, AFM, DLS and UV/Vis spectroscopy. Following the stability and toxicity analysis of the structure, the nanocarrier system was used for silencing of the mentioned genes in their overexpressing breast cancer cell lines. It was found that the antisense oligonucleotide embedded DNA origami-AuNPs structure was highly effective for inducing single and multiple-gene silencing in breast cancer cells. The prepared nanocarrier inhibited the expression of target genes approximately 50 per cent at 30 nM morpholino concentration. For multi-gene silencing, more than one morpholino oligos targeting different genes were embedded into DNA nanocarrier and the silencing efficiency increased to about 80 per cent by synergistic effect. After silencing of the target genes alone and in combination, the effects of the gene regulation on breast cancer cell proliferation and cell cycle phase distributions were investigated. It was showed that gene silencing using DNA origami-AuNPs nanostructures inhibited proliferation of the breast cancer cells and altered their cell cycle phase distributions. Silencing efficiency of DNA origami-AuNPs nanostructures was compared with commercial transfection agents and found to be more effective. Furthermore, DNA origami-AuNPs nanostructures did not affect viability of cancer and healthy cells, while the tested commercial systems were highly toxic. The proposed novel nanostructure provides an effective and biocompatible carrier for gene silencing studies.

ÖZET

DNA-ORIGAMI-AUNPS NANOYAPILARI KULLANILARAK MEME KANSERI HÜCRELERINDE ÇOKLU GEN ANLATIMININ DÜZENLENMESI

Gen anlatımının düzenlenmesi, genetik temelli olan hastalıkların tedavisinde özgün bir yol olarak görülmektedir. Bu sebeple gen taşınması ve düzenlenmesinin verimini artırmak amacıyla yoğun olarak çalışmalar yürütülmektedir. Bu tez çalışmasında hâlihazırda var olan taşıma sistemlerine alternatif olarak morfolino antisens oligonükleotidlerinin gömülü olduğu DNA tabanlı bir nanoyapı oluşturulmuştur. Karo şeklinde tasarlanan DNA origami yapısı hazırlanarak hücre içi alımı artırmak amacıyla altın nanopartiküller ile modifiye edilmiş ve meme kanseri hücrelerinde oldukça yüksek anlatımı bulunan HER2, ERα, EGFR ve Ki-67 genlerinin baskılanmasında kullanılmıştır. Hazırlanan yapı agaroz jel elektroforezi, AFM, DLS ve UV/Vis spekroskopi teknikleri kullanılarak karakterize edilmiştir. Stabilite ve toksisite analizlerinin ardından nano yapı bahsi geçen genlerin bu genler bakımından yüksek anlatıma sahip olan meme kanseri hücre hatlarında susturulması amacı ile kullanılmıştır. Morfolino gömülü DNA origami-AuNPs nanoyapılarının tekli ve çoklu gen susturmada etkin yapılar oldukları belilenmiştir. 30 nM morfolino konsantrasyonunda hedef gen anlatımını yüzde 50 oranında baskıladığı gösterilmiştir. Çoklu gen susturma çalışmaları kapsamında birden fazla morfolino oligosu yapıya gömülmüş ve gen susturma veriminin sinerjitik etki ile yüzde 80'e yükseldiği belirlenmiştir. Hedef genlerin tekli ve çoklu düzenlenmesinin ardından gen susturmanın kanser hücre çoğalması ve hücre döngüsü fazları üzerindeki etkileri araştırılmıştır. DNA origami-AuNPs kullanılarak gerçekleştirilen gen susturmanın kanser hücre çoğalmasını baskıladığı ve hücre döngüsünde farklılıklara yol açtığı belirlenmiştir. DNA origami-AuNPs nanoyapılarının susturma verimliliği ticari transfeksiyon ajanlarının verimlilikleri ile kıyaslanmış ve hazırlanan yapının daha etkili olduğu gösterilmiştir. Diğer yandan DNA origami-AuNPs nanoyapıları kanserli ve sağlıklı hücreler üzerinde herhangi bir toksik etki göstermezken test edilen ticari ajanların hücre canlılıklarını etkiledikleri görülmüştür. Hazırlanan nanoyapının gen düzenlenmesi çalışmaları için etkili ve biyouyumlu bir taşıyıcı system olduğu görülmektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

mg	milligram
ml	milliliter
nm	nanometer
2D	2 Dimensional
3D	3 Dimensional
A	Adenine
AFM	Atomic force microscopy
AuNPs	Gold nanoparticles
C	Cytosine
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELISA plate reader	Enzyme-linked immunosorbent assay plate reader
ERα	Estrogen receptor alpha
G	Guanine
G_0	Gap 0
G_1	Gap 1
G_2	Gap 2
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
HER2	Human epidermal growth factor receptor II
M	Mitosis phase
M1	1 Morpholino embedded DNA origami-AuNPs
M2	2 Morpholinos embedded DNA origami-AuNPs
M3	3 Morpholinos embedded DNA origami-AuNPs
M4	4 Morpholinos embedded DNA origami-AuNPs
NA	Nucleic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PI	Propidium iodine
RNA	Ribonucleic acid
RNAi	RNA interference
S	Synthesis phase
SDS	Sodium dodecyl sulfate
T	Thymine
TAE/Mg^{2+}	Trizma base-acetic acid-ethylenediaminetetraacetic acid/magnesium acetate
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline, 0.1 per cent Tween 20
UTR	Untranslated region
UV/Vis spectroscopy	Ultraviolet/Visible spectroscopy
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate

1. INTRODUCTION

Biomacromolecules such as oligonucleotides and peptides as building blocks of living systems are sought to use constructing novel nanostructures for solution of variety of problems in medicine. The use of oligonucleotides to prepare well-defined nanostructures utilizing Watson-Crick base paring was proposed over two decades ago [1, 2]. Since then, the concept has evolved into an exciting field demonstrating the preparation of several novel 2D and 3D structures in a wide range of applications including delivery, sensing, imaging, assembly, and immunostimulation [3-9]. Gene and drug delivery studies using DNA origami were successfully also demonstrated *in vitro* and *in vivo* [10-16].

Transfection methods for gene regulation were widely investigated after the discovery of RNAi, which allows silencing of specific genes [17]. The DNA nanotechnology concept has showed promising results for the transfection of siRNA and antisense oligonucleotides by embedding them into DNA based structures. For example, phosphorothioate antisense oligonucleotides were incorporated into triangular prism shaped DNA cages by stapling oligonucleotides complementary to the antisense oligonucleotides via hybridization [14]. 3D DNA cages were generated folding of three unmodified DNA strands, which have single stranded regions complementary to antisense oligonucleotides. This simple strategy allowed incorporation of 1, 2, 4, or 6 antisense oligonucleotides into the DNA cages. Then, the whole structure was delivered into Hela cells using liposomes for silencing of luciferase expression. In another study, one dimensional periodic DNA structures called nanoribbons were constructed and their surface was modified with siRNA for targeting survivin mRNA in human ovarian cancer cells [15]. Product of rolling circle amplification used as scaffold and this long strand had periodic repeats so that it could be folded by using the same staple strands set. By folding of the scaffold and three small oligonucleotides, nanoribbon structures were generated. The DNA nanoribbons had SPDP-modified strands and thiol modified siRNAs were conjugated to those SPDP-modified staple strands through thiol exchange. The siRNA conjugated nanoribbons were successfully silenced the survivin expression. *In vivo* gene silencing using DNA nanostructures were also reported [16]. Prism shaped tetrahedral DNA nanostructures modified with three folate molecules were used for in vivo siRNA delivery. The tetrahedral DNA nanoparticles were formed by assembling of six complementary DNA strands, which

have complementary overhangs at the 3’ ends providing siRNA binding. DNA nanoparticles were designed with nicks in the middle of oligonucleotides where the 5’ and 3’ ends of sequences meet at each edge. Using externally labeled DNA nanoparticles with siRNA and folate, longer blood circulation time and efficient knockdown of GFP and luciferase genes were achieved.

In order to increase target specificity and affinity in gene regulation studies, oligonucleotide backbone is modified with certain chemical groups to generate synthetic antisense oligonucleotides. These oligonucleotides include morpholinos, methylphosphonates, phosphorothioate NAs, peptide NAs, and a number of others [18]. Morpholino, an antisense oligonucleotide synthesized by linking morpholine rings through phosphorodiamidates as a substitute of phosphate groups, is preferred in silencing studies since it resists to enzyme digestion and has high sequence specificity [19]. These antisense oligonucleotides show high stablity in biological systems. Together with enhanced stability and high specificity, morpholinos overcome many limitations of other antisense agents [20].

Among the non-viral delivery vehicles, cationic polymers [21] and cationic lipids [22] are more widely used for siRNA delivery. Positively charged groups of the cationic delivery vehicles naturally interact with negative phosphate backbone of NAs electrostatically [23, 24]. Since morpholino antisense oligonucleotides do not carry charge, their packing with positively charged carriers may not be efficient [25]. However, DNA nanotechnology approach allows to incorporation of any type of artificial oligonucleotides into DNA based nanostructure through complementary base pairing.

Since the cellular uptake of negatively charged species is highly restricted, DNA tiles are not effectively uptaken by cells. Thus, we incorporated the AuNPs with an average size of 13 nm into the DNA origami structure due to the fact that oligonucleotide coated AuNPs are effectively uptaken by cells [26]. The AuNPs coated with oligonucleotides complementary to the sticky ends of the origami hybridized to the three sticky ends of the origami. Note that the safety and biocompatibility of oligonucleotide coated AuNPs was studied and found that they caused no toxic cellular responses [27].

In this study, the morpholinos were specifically designed to regulate human breast cancer genes HER2, ERα, Ki-67, and EGFR. In the proof of concept experiments, each

morpholino antisense oligonucleotide was embedded into DNA origami-AuNPs carriers separately and also in combination. Expressions of the mammalian genes were successfully inhibited individually and simultaneously in human cancer cells. First, the effective DNA origami concentration was determined. Then, the transfection of the cells with morpholino embedded DNA origami-AuNPs was studied by evaluating the expression of targeted genes. The effects of single and multiple gene silencing on cellular proliferation and cell cycle were also investigated. The efficacy of DNA origami-AuNPs were compared to a cationic lipid based commercial carrier, non-liposomal lipid based carrier, and a polymer based agent. The results showed that DNA origami-AuNPs more effectively down-regulated the genes. The nanostructure was able to transfer at higher antisense concentrations at which all the commercial carriers were toxic.

2. THEORETICAL BACKROUND

2.1. DEOXYRIBONUCLEIC ACID (DNA)

Nucleic acids were discovered by a physician Friedrich Miescher in 1868. He isolated the new compound from nuclei of white blood cells, which today is known as deoxyribonucleic acid (DNA) [28]. In biology: DNA contains the information required for development, maintenance and reproduction of an organism. Each cell in the organism contains this information and transmits to daughter cells. Nucleic acids are biomacromolecules consist of smaller units called nucleotides. The nucleotides contain a sugar moiety, a phosphate group and a nitrogenous base (Figure 2.1a). There are 4 types of bases; A, T, G, and C. Combination of the order of these letters determines the instructions of DNA; in other words; genetic code. The whole genetic data is written with this 4-letter-alphabet. The nucleotides form long chains by phosphodiester linkage between their sugar and phosphate moieties, which is called a DNA strand (Figure 2.1b). The phospahe backbone of DNA provides negative charge to the molecule.

The molecular shape of DNA was revealed by Francis Crick and James Watson in 1953 [29]. Two antiparallel DNA chains are linked through hydrogen bonding between bases (Figure 2.1c) and form DNA helix (Figure 2.1d). This hydrogen bonding between the bases of nucleotides are called Whatson-Crick base pairing. The only types of base pairing are G-C and A-T. C always pairs with G and A always pairs with T, which assures transfer of exact same DNA to new cells in cell division. The structure of DNA defined more than sixty years ago and this characteristic ladder like helical shape became one of the most famous icons.

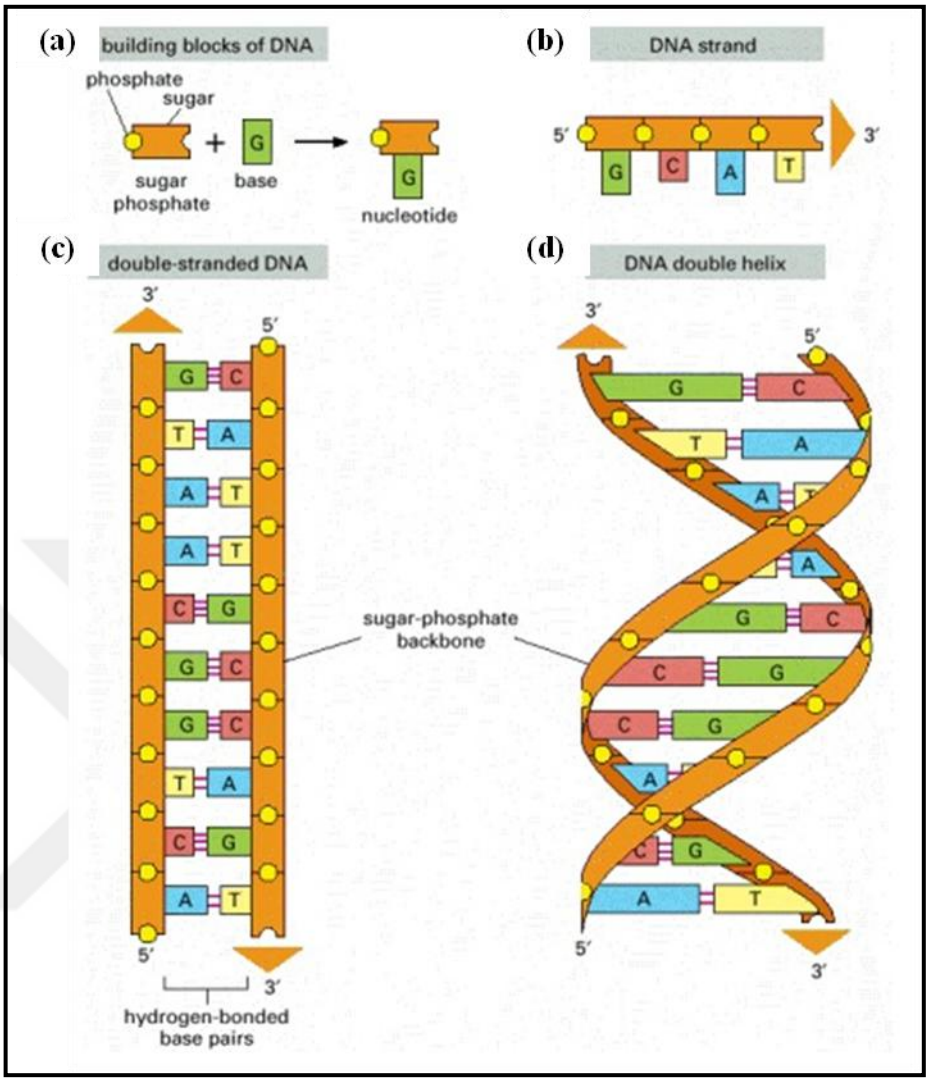


Figure 2.1. Structure of DNA. (a) Subunits of DNA molecule, (b) Single DNA strand, (c) double stranded DNA consists of base pairs, (d) double helix [29].

After discovery of this biomacromolecule and determination of its function as genetic material, DNA has been center of researchers' interest in biology. However, for the last 3 decades, we have been utilizing DNA as buiding material in the field of nanotechnology.

Another interesting application area of DNA is digital data storage [30-32]. Together with the growing digital technologies, need for storage of data is greater than before and DNA became a suitable material due to its dense structure and long half-life [31].

2.2. DNA NANOTECHNOLOGY

DNA is a hereditary material that is responsible for the preservation of genetic information in all organisms. Besides the genetic information it carries, its programmability, structural stability and self-assemling properties make DNA attractive for different applications. The development of functional and dynamic structures using the properties of DNA and the use of these structures in many different fields such as medicine, biotechnology and computation has provided the rising and development of DNA nanotechnology concept which is a sub-branch of nanotechnology. Development of DNA nanotechnology and the selected evolutionary DNA nanostructures are shown in Figure 2.2.

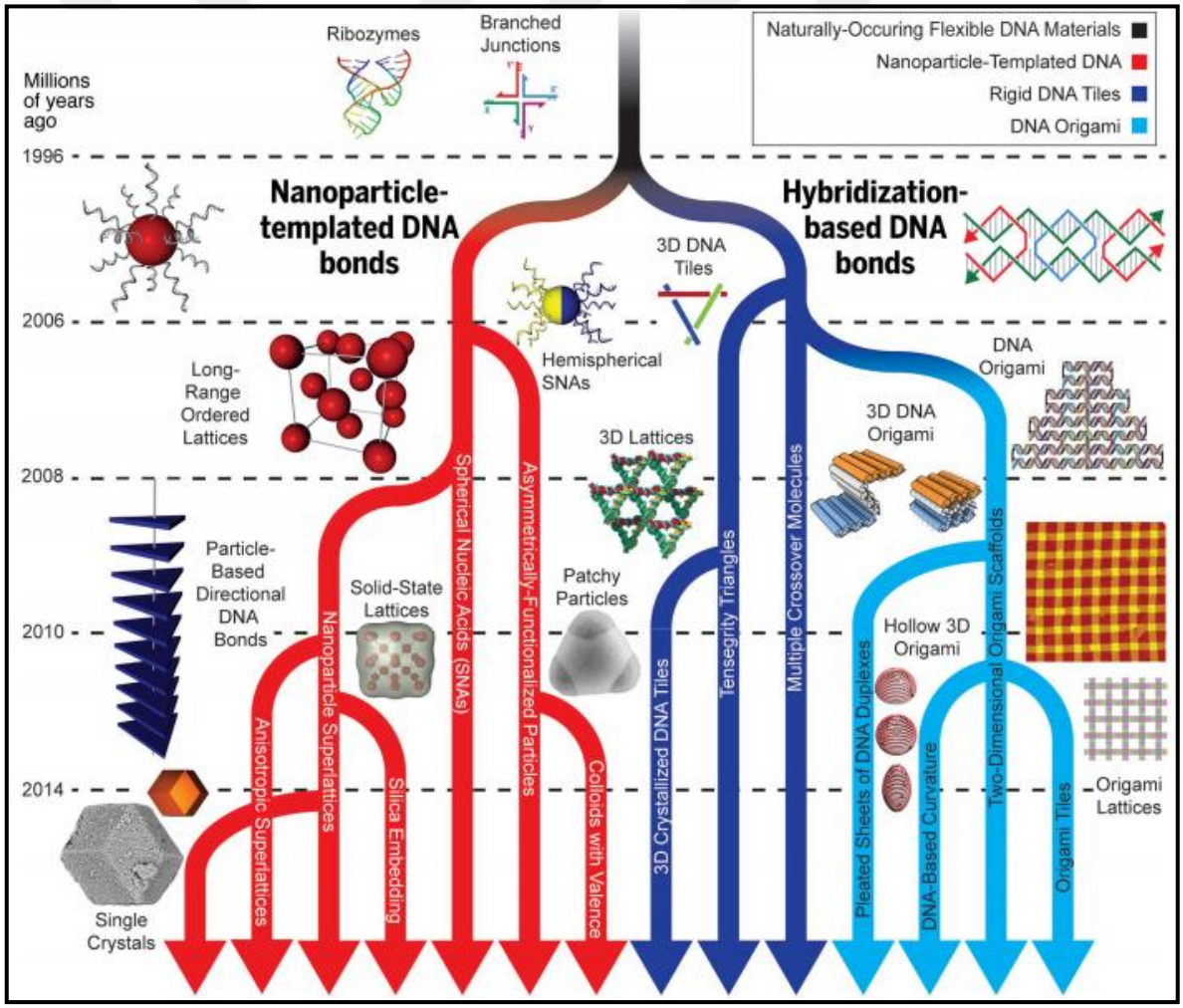


Figure 2.2. Development of DNA nanotechnology and evolutionary DNA nanostructures [33].

Hybridization of nucleic acids is very important in biology. It is essential for many biological processes such as gene silencing, replication, transcription and translation, which are crucial for transmission and expression of genetic information. DNA molecule has been in the limelight of scientists as it encodes the genetic information. However, DNA nanotechnology utilizes DNA as a building material of any desired shaped 2D and 3D architectures. This branch of nanotechnology uses DNA hybridization to assemble DNA into specific arrangements rather than using its biological properties. DNA nanotechnology is an interdisciplinary branch of nanotechnology with collobration of biology, chemistry, computational and material sciences. This technology allows formation of well-defined nanostructures with controlled size and shape by DNA hybridization, which depend on the sequence of DNA strands and highly selective. Using specific base-pairing of complementary DNA bases, many unique DNA constructs are prepared [34]. The formation of various structures in nanoscale using DNA molecules has created structural DNA nanotechnology and this technology has become a rapidly growing research field. The transformation predispositions of the synthesized DNA structures which are produced with this approach, into multifunctional structures make the DNA origami approach important.

In 1980s, the studies of Seeman et al, before Rothemund introduced his DNA origami approach, was the first examples of this approach which has not yet been named [35]. Seeman was inspired by a byproduct occurs during genetic recombination; the four-armed “Holliday junction” and from this point; Seeman demonstrated that different DNA patterns could be prepared using different DNA motifs such as double crossovers. It has been shown that the double crossover motifs have the required rigidity and stability to create a controlled geometry and connection [36].

In later studies, double-crossover structures were utilized in the formation of 2D DNA crystal structures [36]. Double crossover tile motifs were followed by the use of different DNA motifs, such as the triple crossover, which were interconnected by chain exchange [37]. The building blocks, called DNA tiles, consist of double crossover molecules with sticky ends and can grow independently to form 2D crystal structures such as lattices. 2D DNA tile lattices assembly by hybridization of sticky ends of one DNA tile structure with sticky ends of other DNA tiles particularly [38]. The lattices were used for assembly of other materials such as ligand - protein assembly or AuNPs arrays [39, 40].

The first DNA nanomechanical device was developed in 1999, taking advantage of the rigidity of the double crossover motifs [41]. DNA tile has the simple shape, which any desired sequence can easily incorporated into the structure for silencing studies and cost effective with the decreased number of oligonucleotides. Until 1998, design of numerous 2D and 3D DNA structures have been carried out by researchers [42]. Within that period, it has been shown that DNA-based structures can be an active part of molecular machinery.

In 2006, P. Rothemund brought a new dimension to DNA nanotechnology with his new study [2]. In this study, Rothemund showed that the long viral single-stranded DNA molecule (scaffold chain) could be folded over itself to form a structure as desired. He performed this with the help of short helper chains (staple strands) that brought together certain parts of the scaffold chain. The use of staple strands without purification simplifies the assembly formation. The folding of scaffold chain with staple chains during origami formation schematized in Figure 2.3 [43].

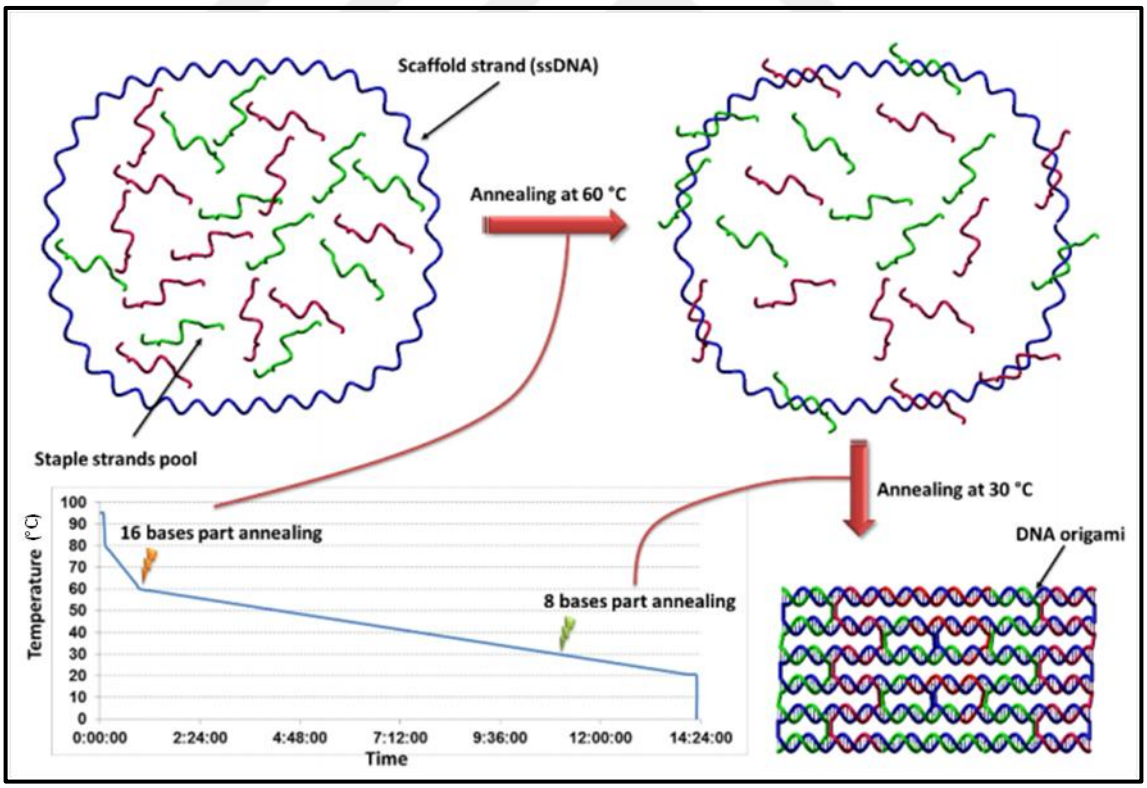


Figure 2.3. Folding of scaffold chain with staple chains during origami formation [43].

Rothemund named his approach as DNA origami inspired by the origami of Japanese paper folding. It can be said that Rothemund could be inspired by the works of Yan et al. and Shih et al. (Figure 2.4) and the studies shed light on Rothemund before revealing the concept of DNA origami [44, 45].

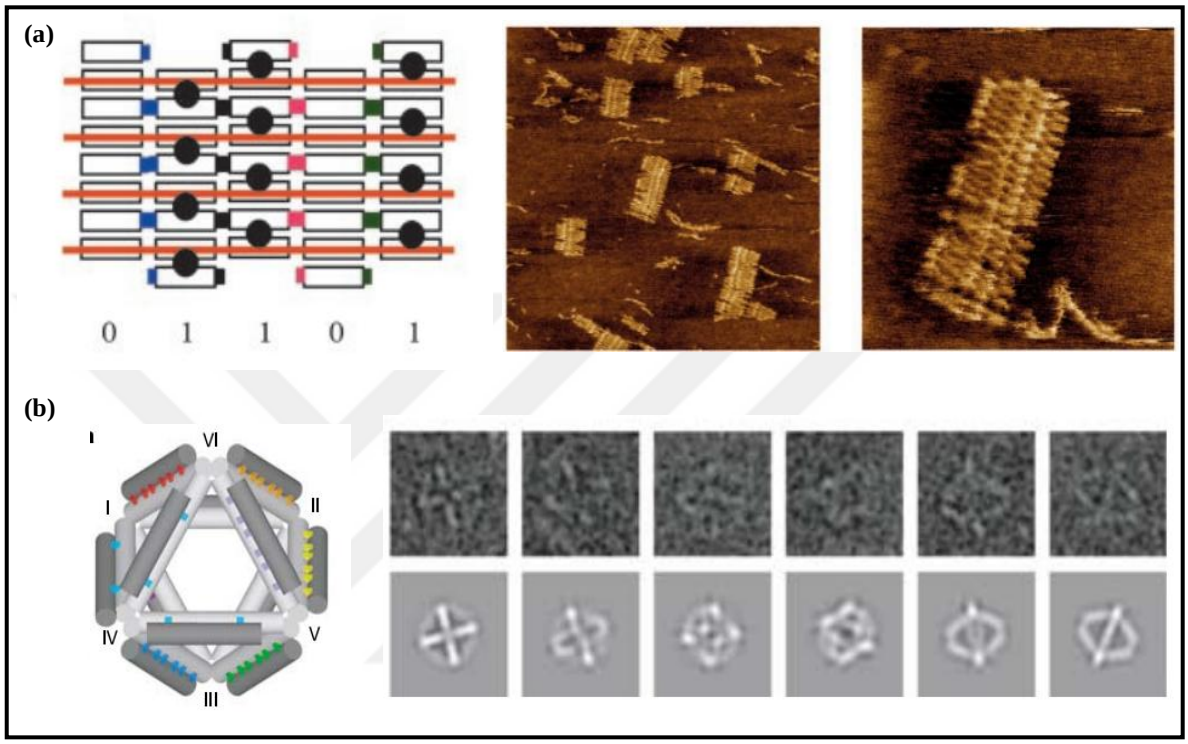


Figure 2.4. (a) The creation of 01101 barcode DNA lattice by Yan et al. and (b) DNA octahedron formation by Shih et al. [44, 45].

The first use of the scaffold chain, which can be folded on itself, in the structural DNA technology, was described in the study of Yan et al. The first use of the scaffold chain, which can be folded on itself, in the structural DNA technology was described in the study of Yan et al [44]. Yan et al used the scaffold chain to make barcode array. In the later study, Shih et al. created the octahedron structure using the scaffold chain and five helper chains [45]. Rothemund most likely developed the DNA origami approach, inspired by the works of Yan et al and Shih et al.

Later on, Rothemund reported the formation of many complex regular and irregular 2D structures with DNA origami approach (Figure 2.5). America map and smiley face are the most famous among these structures [2].

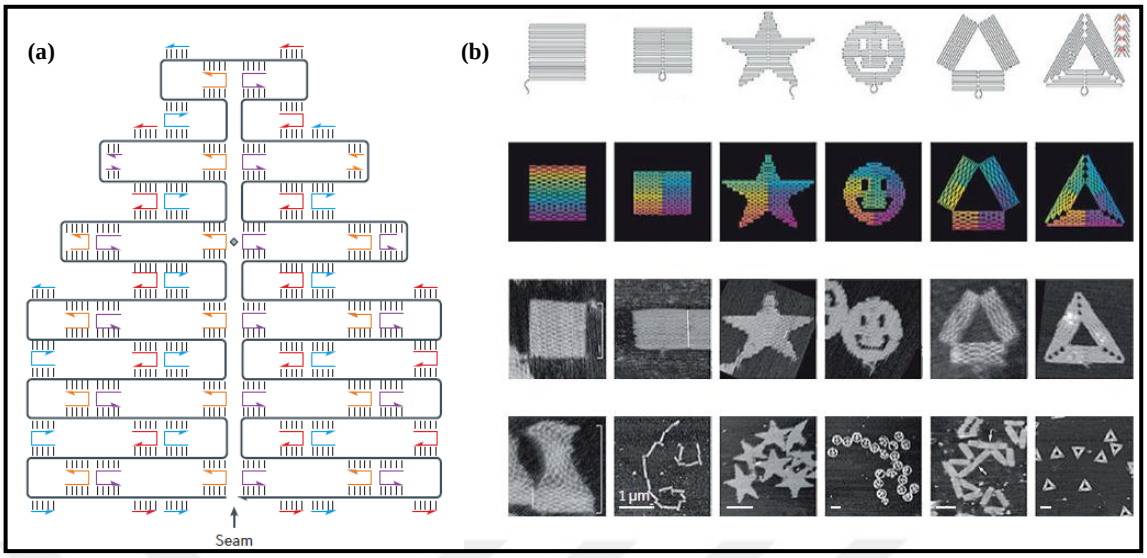


Figure 2.5. (a) The DNA origami approach and (b) images of the structures created scaffolded DNA origami [2].

Recently, brick assembly or single-stranded tile approach, which is similar to DNA origami but not requiring long scaffold chain, has been also reported [46]. In this approach called as brick assembly or single-stranded tile, the computer-aided design of the structures is realized and the construction of the target structure is constructed by the use of hundreds of single-stranded DNAs that form interconnected duplexes. In Figure 2.6, The single-stranded DNA tile or Brick assembly diagram and AFM images of the structures created with this method are shown [46].

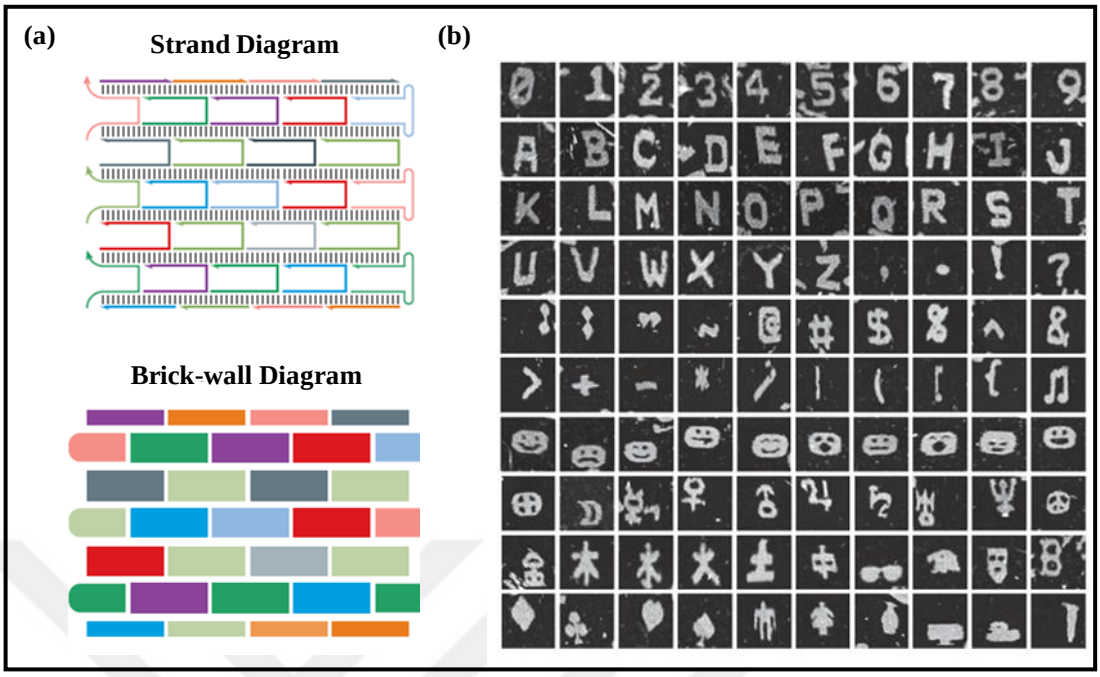


Figure 2.6. (a) The single-stranded DNA tile and brick assembly diagram and (b) AFM images of the structures created with this method [46].

Apart from utilizing only base pairing, there are also 2D and 3D DNA nanostructures generated by using metallic nanoparticle based templates [47]. The most important feature of this approach, which is termed as particle-templated DNA construction, is that the role that DNA plays in sequence-specific interactions is different from the rigid scaffold role played by nanoparticles to create the desired structures efficiently. In Figure 2.7, examples of the nanoparticle-templated DNA nanoconstructs are shown.

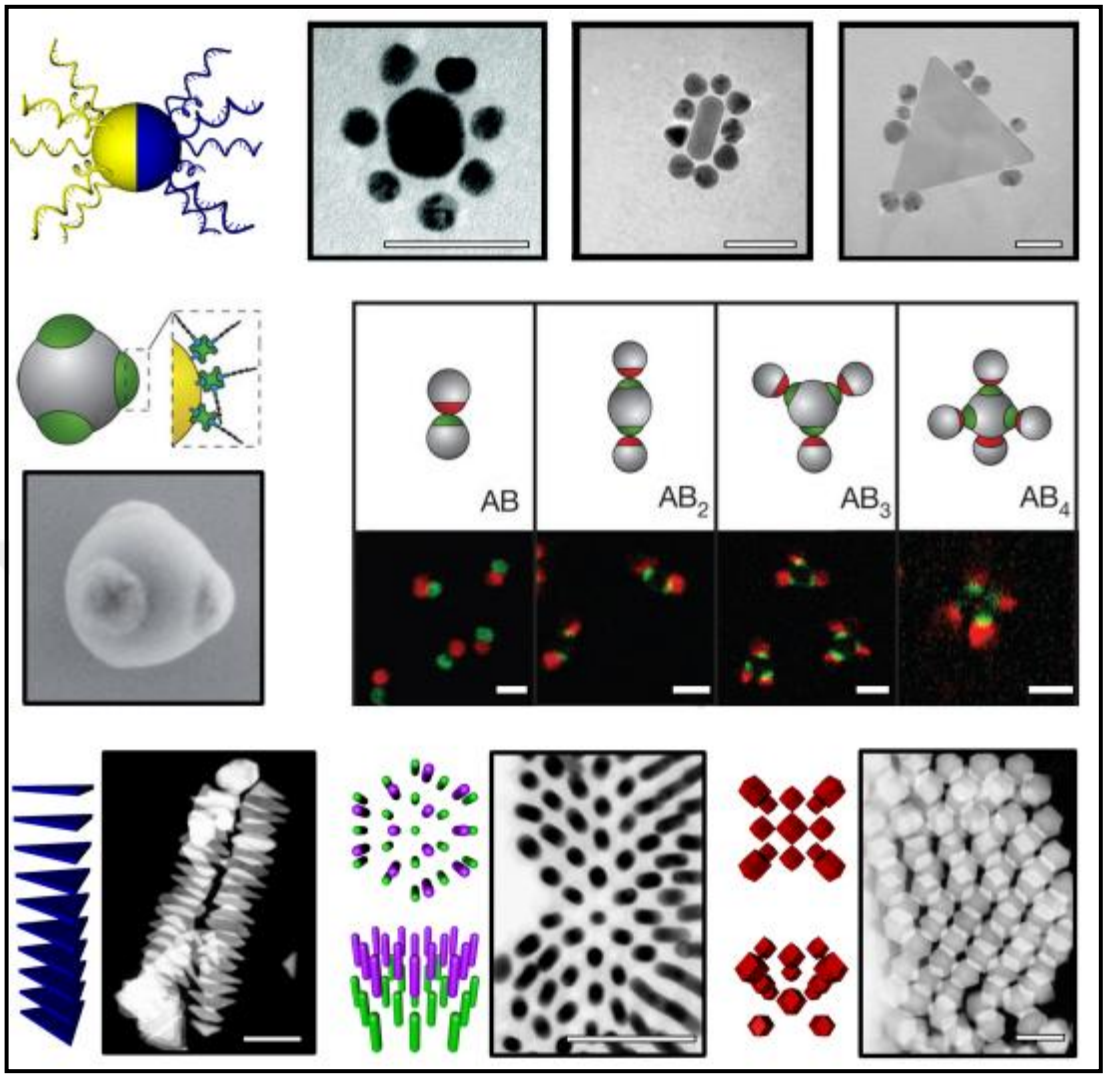


Figure 2.7. DNA nanoconstructs formed with nanoparticle templating [48-51].

In the formation of nanoparticle-templated structures, ionic or metallic bonds, which form the crystalline cage of the inorganic core structure, have replaced the use of DNA hybridization or the use of different motifs. In this case, unlike the approaches based on hybridization for structure formation many challenging structural requirements can be ignored as an advantage when functionalizing the inorganic scaffold material with an intense oligonucleotide ligand layer. The rigidity of the inorganic part and the intense and directed orientation of the oligonucleotides are sufficient for the formation of directional DNA bonds [33]. Surface of spherical nanoparticles were modified with appropriate DNA sequences and these nanoparticles were used to prepare 3D DNA nanostructures via DNA hybridization. In Figure 2.8, 3D cubical nanostructures are shown prepared by assembling AuNPs by thiol modified oligonucleotides.

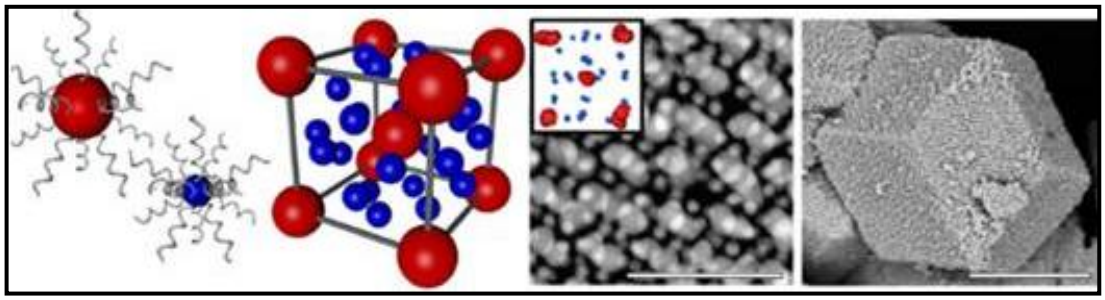


Figure 2.8. Nanoparticle based 3D DNA nanostructures [33].

2.2.1. Preparation of DNA Based Nanostructures

Introduction of DNA origami approach by Rothemund is a significant milestone in DNA nanotechnology. It has been reported that recently, nucleic acids have been used in the formation of nanoscale complex shapes and devices extensively using DNA origami approach. Different preparation methods have been used in the actualization of these studies. There are three types of DNA origami structure preparation strategies including multi-stranded, scaffolded, and single-stranded approaches [52, 53]. These strategies are schematized in Figure 2.9.

In multi-stranded strategy, short complementary oligonucleotides are used for the formation of desired shaped DNA origami structures [52]. This strategy has the disadvantages of being inefficient. Also it strongly depends on critical concentrations of oligonucleotides, needs extra purification steps, and is limited with simple geometric shapes [52]. Single-stranded and scaffolded origami approaches are more suitable than the multi-stranded approach for the synthesis of complex and large DNA constructs.

In the scaffolded approach, a number of helper oligonucleotides bind to the long, scaffold DNA to construct an origami structure [2]. Excessive use of helper chains eliminates the problem of concentration equalization of oligonucleotides. Being efficient, making the preparation duration shorter, possibility of formation of complex geometries and lack of necessity of extra purification steps make the scaffolded approach favorable [2, 53].

In single-stranded approach, it has been utilized from the folding capability of biological molecules [54]. Using these features, compact and well-defined structures can be formed by using a single DNA chain.

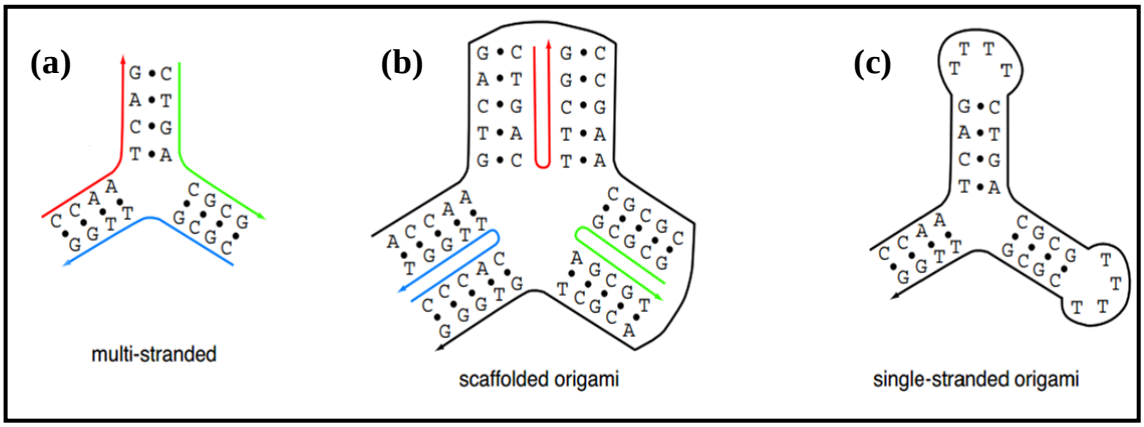


Figure 2.9. Preparation ways of DNA origami constructs. (a) Multi-Stranded, (b) scaffolded and (c) single-stranded origami approach [52, 53].

Design tools such as computer-aided software and programs are utilized for the precise and well-defined creation of DNA structures independent of the selected preparation method [55-58]. With the help of such design tools, it is possible to reduce and prevent any kind of errors that may occur in the desired structure by preventing the unwanted base interactions. CADNAno is the most widely used DNA origami design program by researchers in this sense [59].

Furthermore, wide variety of DNA nano objects were prepared by using other approaches such as sticky-ended cohesion, which allows formation of larger structures by hybridization of 2 different sticky-end containing motifs, or tile-based construction of 2D DNA lattices and preparation of DNA bricks using single stranded DNA tiles [60]. By using DNA bricks like legos, the highest complexity was achieved [61]. A recent strategy was reported to assembly of DNA nanotube by using a long DNA, which was prepared with an enzymatic approach called temporal growth [62].

2.2.2. Applications of DNA Nanotechnology

The assembly of different molecules including organic or inorganic structures is the most well-known and widespread applications of the DNA nanotechnology. The first one-dimensional AuNP regulation was reported in 1996 [63]. This arrangement was accomplished by functionalizing AuNPs with a single DNA chain. One of the first studies on the arrangement of nanoparticles is also the work reported by Mirkin [64]. This study is

the first example of the development of DNA nanoparticle hybrid materials that can be useful in terms of optical, electronic and structural properties and contains the controlled organization of AuNPs. The first arrangement studies of the nanoparticles performed by Mirkin and Alivisatos using AuNPs are shown in Figure 2.10.

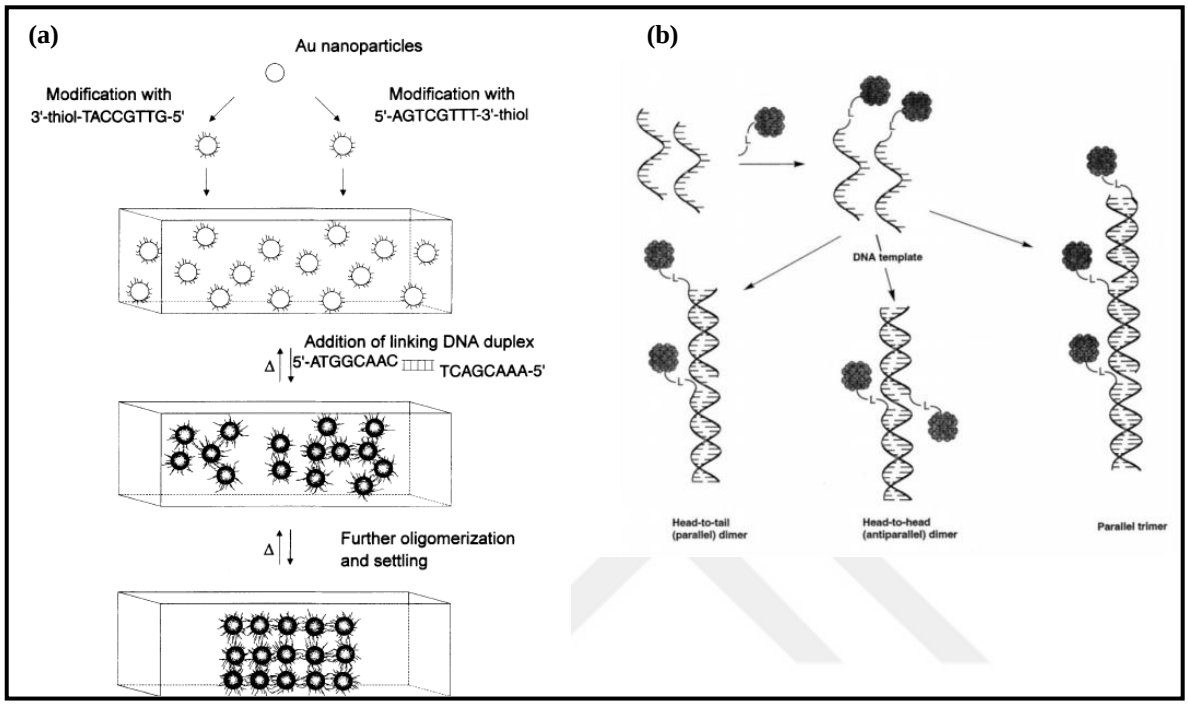


Figure 2.10. The first arrangement studies of the nanoparticles carried out (a) by Mirkin and (b) by Alivisatos using AuNPs [63, 64].

In subsequent studies, different sizes of AuNPs were assembled according to the checkerboard model using DNA tile structure and then 3D tetrahedral AuNP arrangements were reported in literature [7, 65-67]. As shown in Figure 2.11, apart from the gold nanoparticles, Also arrangement of silver nanoparticles (AgNPs) and single-walled carbon nanotubes was provided by DNA origami approach.

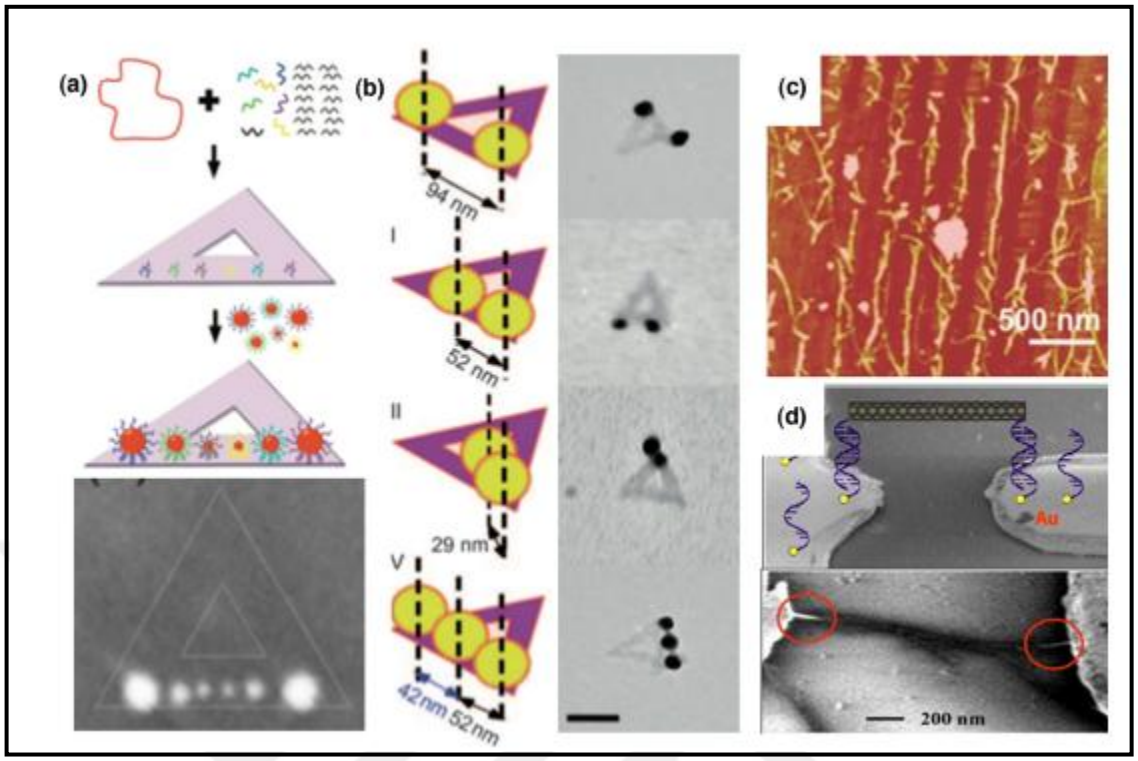


Figure 2.11. Assembly of nanomaterials by DNA origami approach. Arrangement of (a) AuNPs (b), AgNPs (c), single-walled carbon nanotubes, and (d) reciprocal placement of AuNP electrodes [7, 65-67].

DNA origami constructs are used to arrange the inorganic nanomaterials and metals for their activity in optical, electronic and sensor applications. Since the optical and electronic properties of inorganic materials are influenced by the geometrical arrangement of these materials, the arrangement of these materials to obtain the desired function or property is very important. In this sense, the use of DNA origami-based structures is being studied extensively [68].

There are also studies including utilization of DNA constructs in the organization of structures such as polymers, lipids, liposomes, and proteins (Figure 2.12) [69-73]. The combination of DNA molecules and polymers enables the DNA structure to achieve a new structure with properties that can be used for different applications [68] . It has also been reported that molecules such as lipid chains and cholesterol direct their assembly properties with DNA scaffold structures.

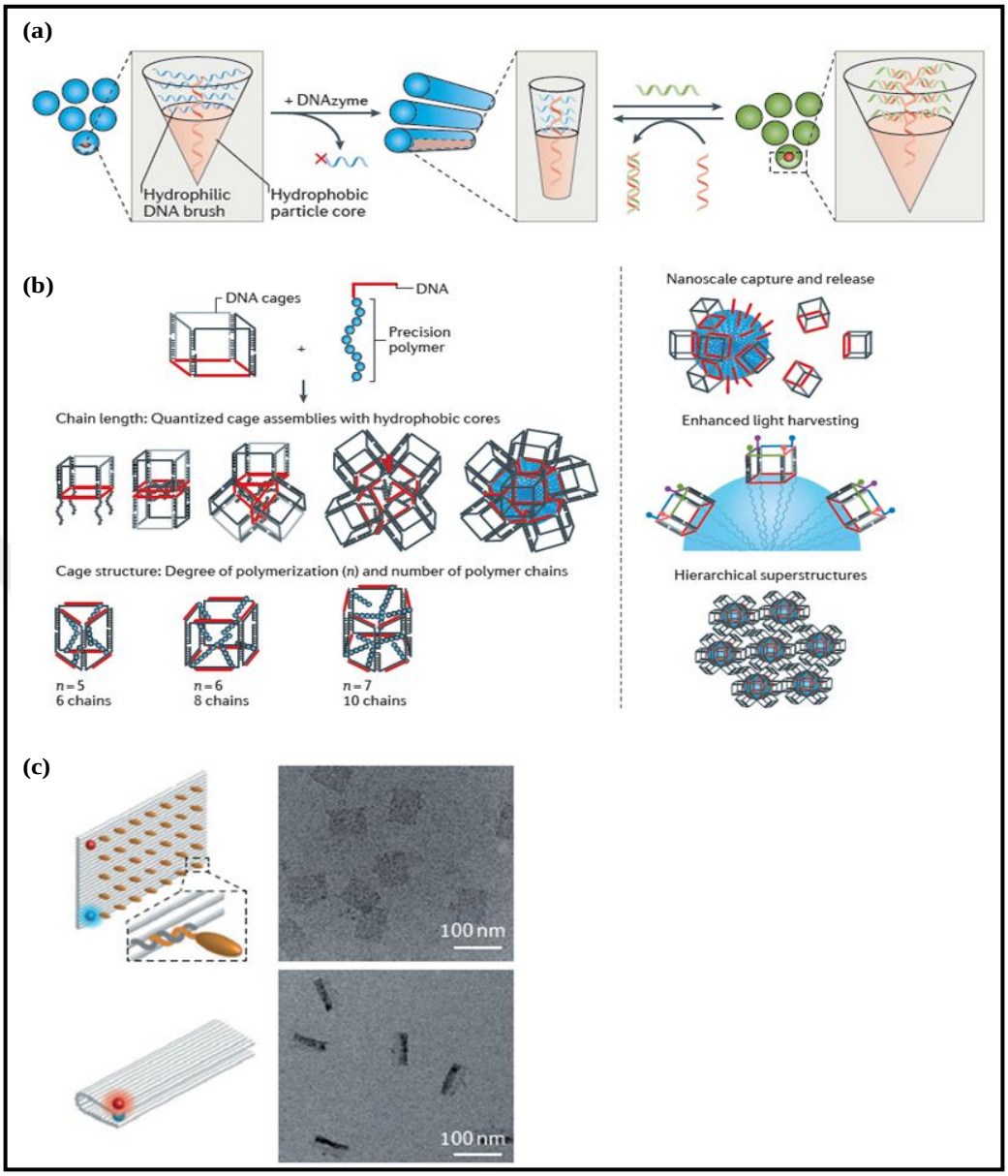


Figure 2.12. Interactions of DNA nanoconstructs with lipid and polymer molecules. (a) The arrangement of DNA–polymer structures forming spherical micelles [70], (b) DNA cage structures modified with hydrophobic polymers [71, 73], and (c) Reactangle DNA origami structure combined with cholesterol molecules [72].

DNA-directed regulation of liposomes is also studied extensively [74-82]. The fusion of liposomes including fusogenic lipid molecules [77, 79, 80, 83, 84], formation of two-dimensional liposomal structures [74, 78, 84] can be carried out using DNA hybridization. The examples of the DNA-directed arrangement of DNA-functionalized liposomes are shown in Figure 2.13.

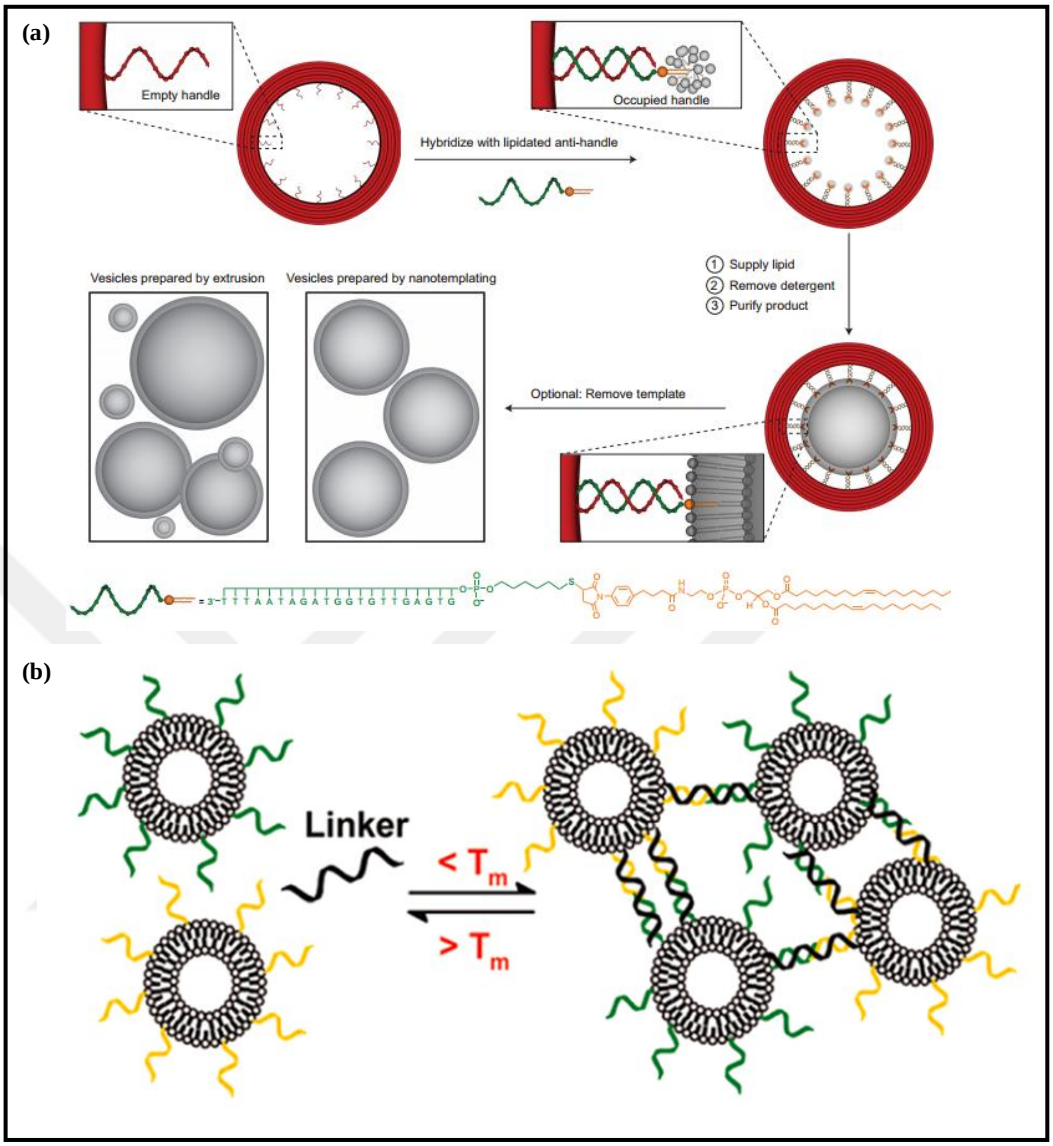


Figure 2.13. Examples of the DNA-directed arrangement of DNA-functionalized liposomes. (a) The size-controlled vesicle formation by nanotemplating and (b) liposome assembly formation with hybridization [85, 86].

DNA nanotechnology has been shown to be an effective strategy for the elucidation of protein structures, protein-protein relationships. At the same time, studies including the controlled regulation of proteins have been reported extensively [69]. Structures formed by DNA origami approach are utilized as templates for the regulation of protein molecules. DNA template structure was used to assemble dimer and trimer membrane proteins on lipid bilayer nanodiscs. In that study, it has been reported that the formation of such a structure is more difficult when the DNA template is not used and the formation percentage of this arrangement is lower [87]. Using DNA nanotechnology to study the

behavior of motor proteins and arrangement of the enzyme structures on DNA templates are the other studies, which performed using DNA hybridization [88]. In Figure 2.14, The arrangement of membrane proteins and enzymes using DNA hybridization is shown.

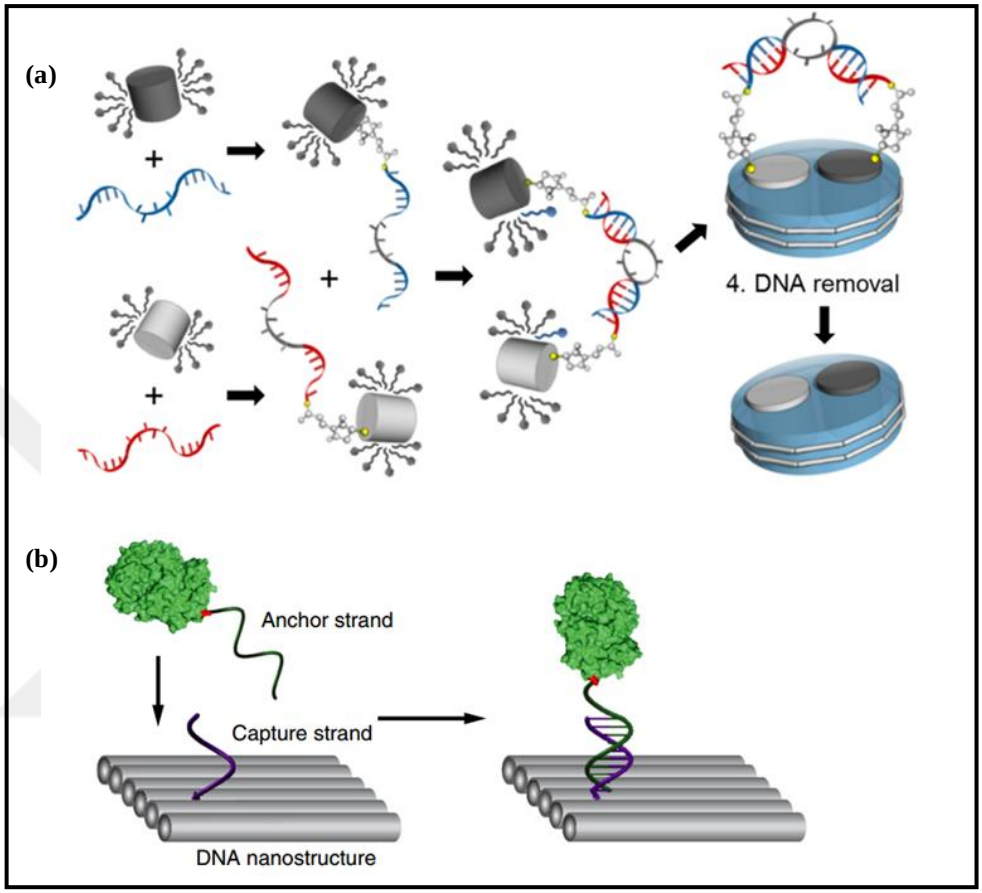


Figure 2.14. The arrangement of (a) membrane proteins [87] and (b) enzymes [88] using DNA.

For effective treatment of diseases, it is essential for increased efficiency of therapeutic agents and decreased side effects. To that end, well designed delivery vehicles, which effectively carry and release the agents to the target site are necessary. Most of the carrier systems generate biological responses like generation of immune response, effecting gene expression, causing oxidative stress, or toxicity. The biocompatibility is one of the major issues for disease treatment in clinical studies. Biomacromolecular structures, which are naturally present in cells, are thought to be solution of biocompatibility problems. From this viewpoint, DNA has started to come into prominence as a building block of a delivery vehicle.

Fate of the drug carrier in the organism is determined by many factors such as size and shape of the structure, molecule and position of the drug and other ligands on the vehicle. Therefore, nanostructures prepared using DNA origami approach has gained importance for the improvement of delivery systems. In Figure 2.15, Types of DNA nanostructures used for delivery applications and their cargos are shown.

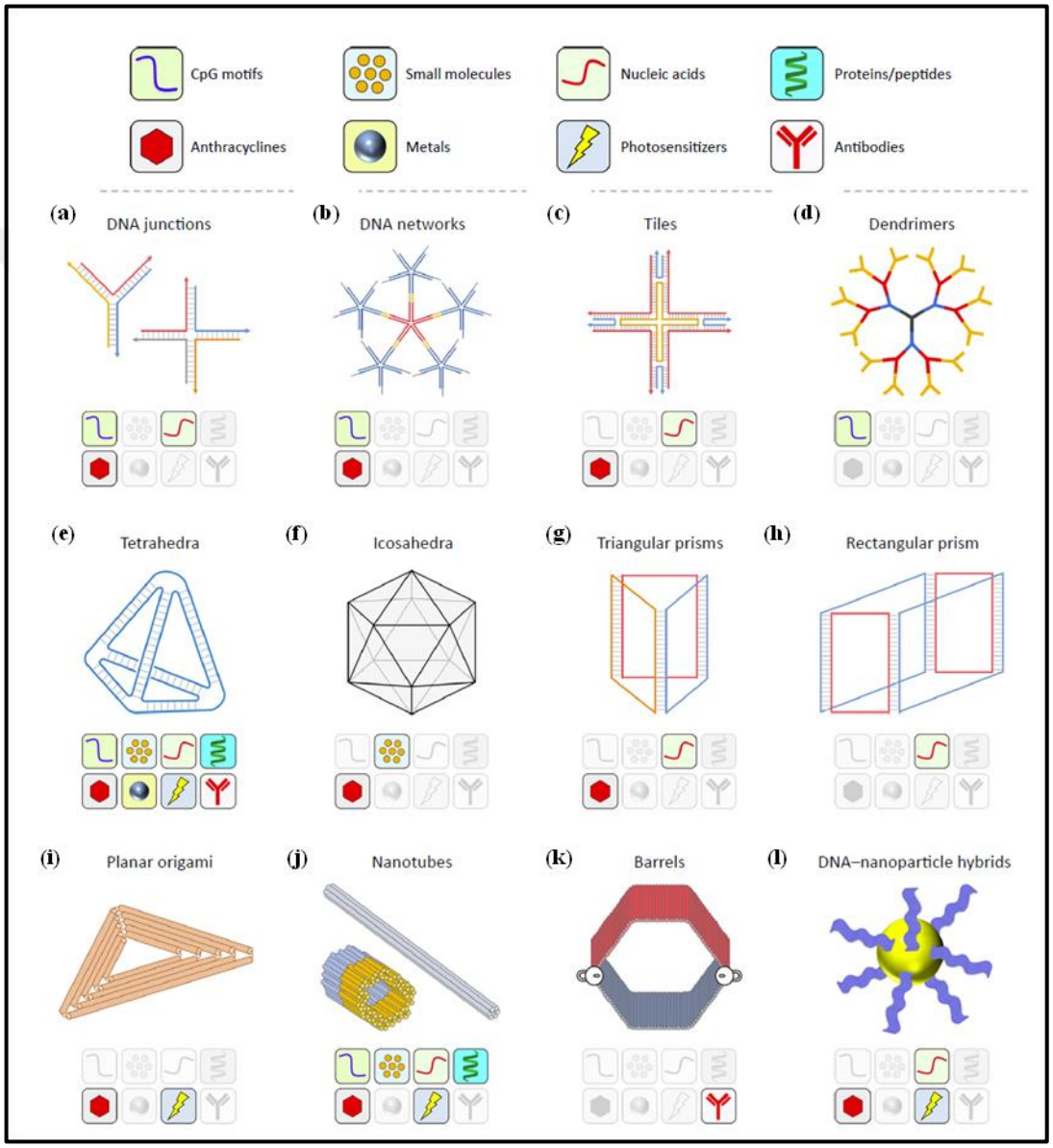


Figure 2.15. Types of DNA nanostructures used for delivery applications and their cargos [60].

Successful *in vivo* and *in vitro* applications of carrier systems, which are generated using DNA origami structures, are reported in the literature [10-12]. DNA wireframe cage structures have been reported to have more cellular penetration than single and double-

stranded DNA constructs without a transfection agent. This feature increases the use of cage structure as a drug carrier structure.

The use of DNA nano-constructs in the delivery of cancer drugs, which can intercalate into the DNA structure, to cancer cells is quite common [10]. In the literature, DNA origami structures, which are designed in different shapes such as triangular, square, tube and tetrahedron have been reported for their use in delivery of chemotherapy drug doxorubicin to cancer cells. In these studies, the internalization of more drugs into the cell with the use of DNA origami structure designed in the form of triangles has shown that the shape of the carrier system is an important factor to be considered in the drug carrier design to obtain a maximum therapeutic effect.

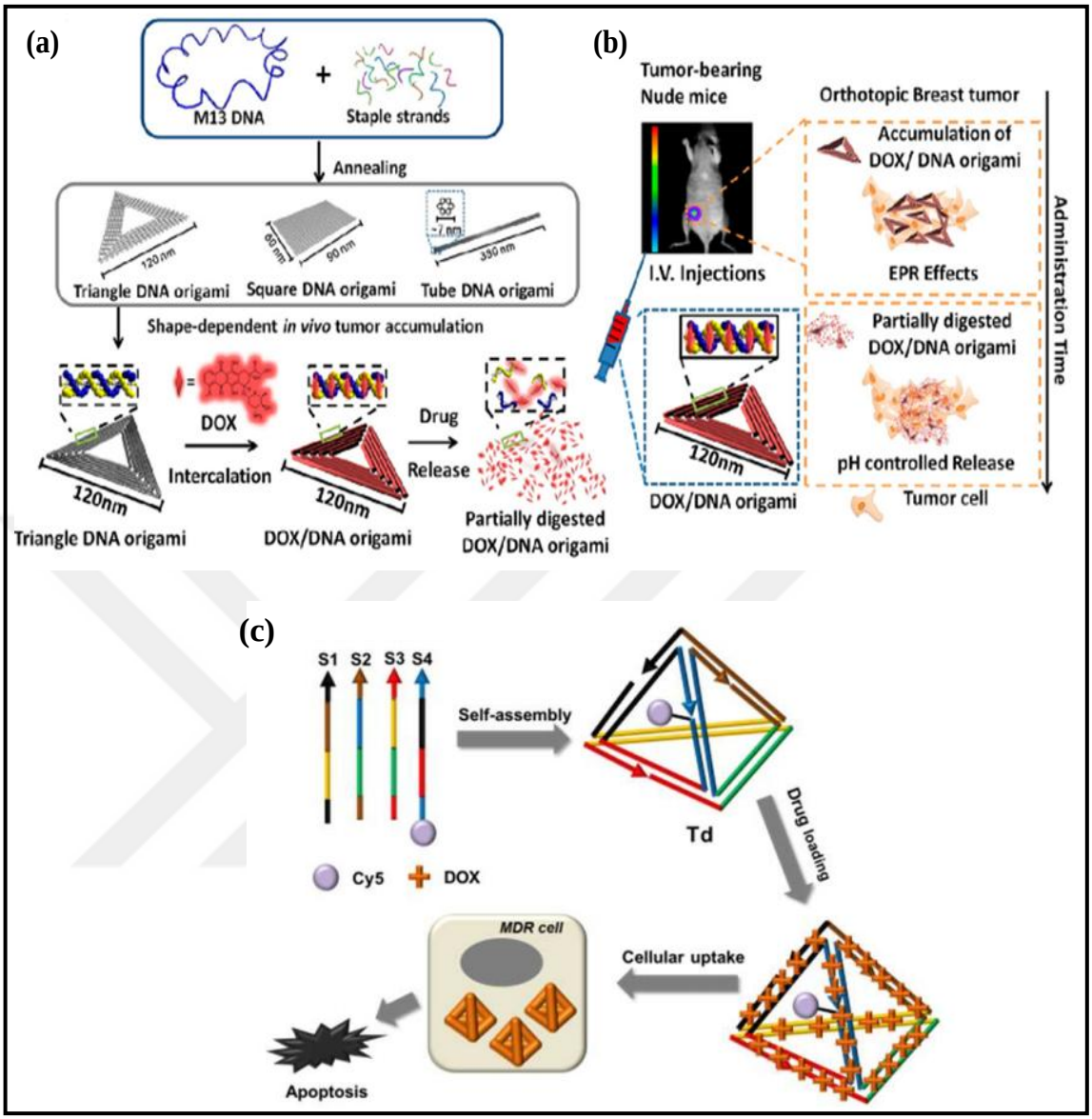


Figure 2.16. Docorubicin delivery with different DNA origami structures. The studies of the triangle, tube and square shaped DNA origamis (a) *in vitro*, (b) *in vivo*, and (c) DNA tetrahedron structure in doxorubicin delivery.

Moreover, the functionalization of the prepared structures with targeting molecules makes them capable of targeting cancer cells. It was reported that when the doxorubicin intercalated DNA cage structures were functionalized with MUC-1 aptamers, functionalized DNA structure targeted to MUC-1 cancer cells [89]. The cellular entry of MUC-1 aptamer modified DNA structure and possible internalization pathway for this structure is shown in Figure 2.17.

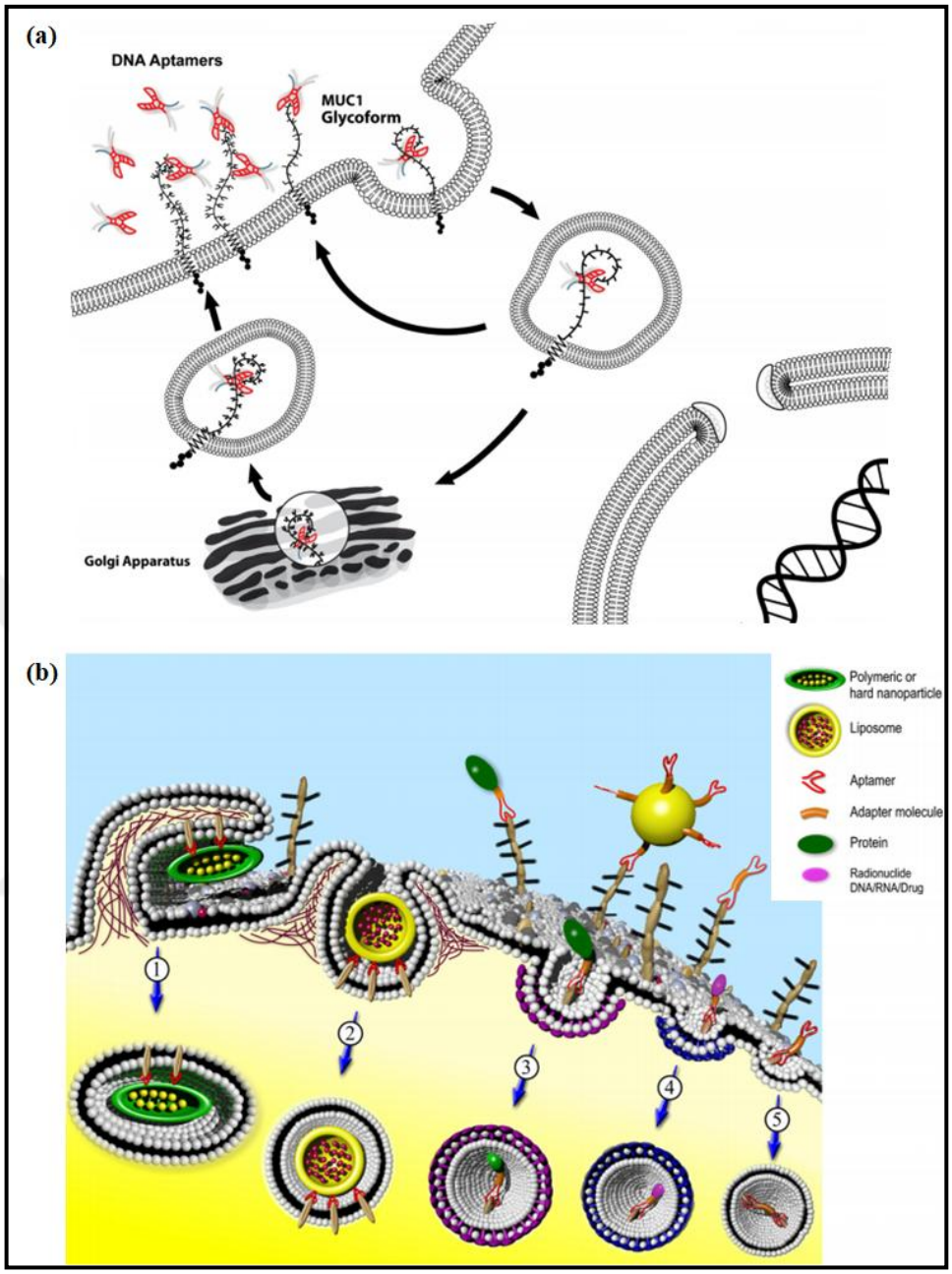


Figure 2.17. (a) The cellular entry of DNA-aptamers and (b) endocytic pathways for the aptamer-cargoes [89].

The DNA tetrahedron structure, functionalized with folate molecules and carrying siRNA, has been reported to be successful for gene silencing application *in vivo* without a transfection agent [90].

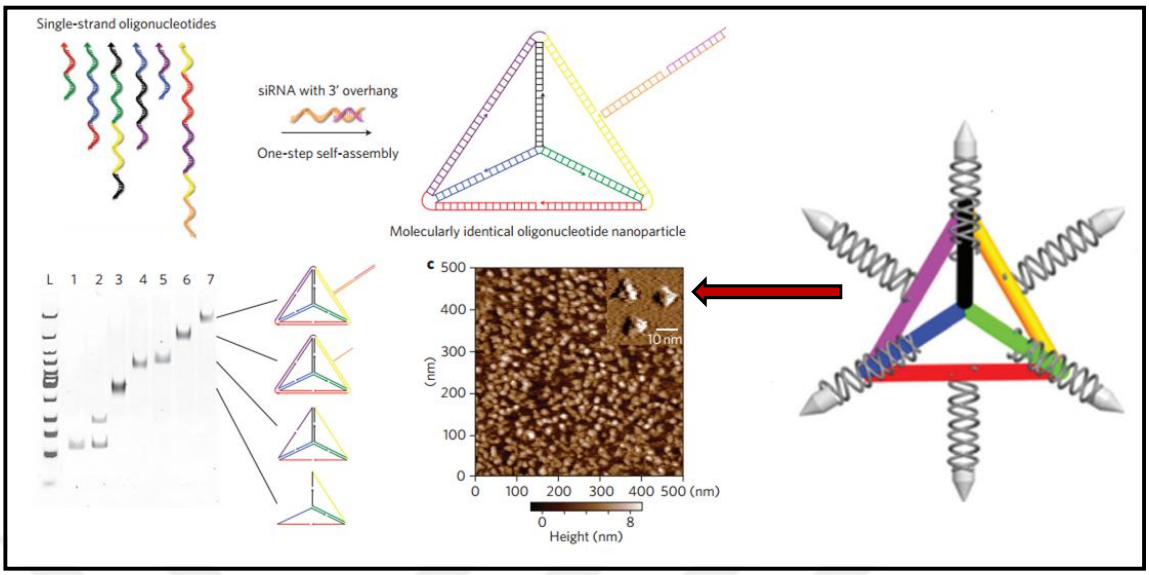


Figure 2.18. Preparation of DNA tetrahedral nanoparticles [90].

In the regenerative medicine and tissue engineering applications, DNA nanostructures allow for the placement of differentiation and growth factors. Thus, suitable media for pre-designed tissue growth can be obtained [91]. It has been reported that the DNA nanotube structures functionalized using the RGD peptide increase the transformation of neural stem cells (Figure 2.19) [92].

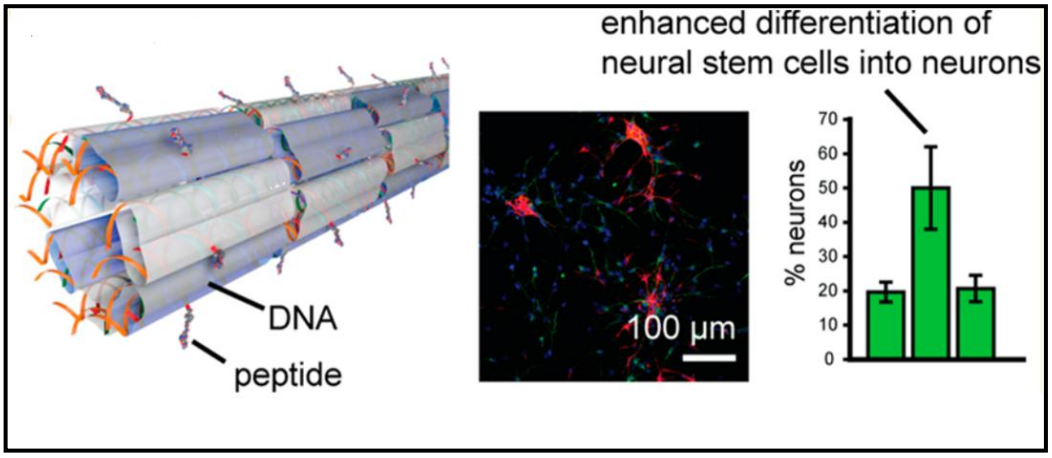


Figure 2.19. RGDS peptide modified DNA nanotubes and neural stem cell differentiation [92].

2.3. GOLD NANOPARTICLES (AuNPS)

There are few nanoparticles that have had the attention of researchers as much as gold nanoparticles (AuNPs). Its physicochemical properties such as scattering and absorption of light in visible region of the spectrum make AuNPs popular in medical applications; imaging, therapeutics, and diagnostics. AuNPs is used as agent in imaging applications including X-ray, fluorescence, photoacoustic, optical imaging and surface enhance Raman spectroscopy [93-95]. Inert nature of gold core makes AuNPs biocompatible and nontoxic. Also easy surface modifications make drug loading, ligand binding or coating possible for targeting therapies [96]. Surface of gold nanoparticles stably interact with thiol (SH) groups and can be easily modified through Au-thiol bond [97]. Without any targeting ligands, AuNPs can accumulate in tumor by long circulation and penetration to tumor vasculature [98]. With the benefits of accumulation in tumor, AuNPs are widely used as drug carriers. Drugs such as doxorubicin, cisplatin, and capecitabine can be bound to gold nanoparticle surfaces non-covalently [99]. In addition to drug delivery, biomacromolecules like proteins, DNA, and RNA are potential cargos of AuNPs [100].

Due to their many features such as non-toxic and inert nature, high cellular internalization and easy modification, AuNPs are suitable nanoparticles and have been widely utilized as gene and drug carriers [101-104].

2.4. INHIBITION OF GENE EXPRESSION

Silencing of a specific gene is a widely studied research area that holds promise for therapy of diseases. Inhibition of a disease related genes besides being an alternative of traditional treatments; improves the efficiency when combined with them. Gene silencing has the potential of contribution to genome based personalized treatment strategies or gene function studies. Post-transcriptional inhibition of gene expression comprises RNA interference (RNAi) and antisense oligonucleotides. RNAi refers double stranded small RNAs including small interfering RNA (siRNA) [105]. Both RNAi and antisense oligonucleotides have the same mechanism of action. A nucleotide sequence binds to its target mRNA sequence through hybridization and provides the degradation of the mRNA or blocking binding of proteins, which are necessary for translation. RNAi is a double

stranded oligonucleotide and when it couples with RNA-induces silencing complex (RISC), it becomes single stranded. RNAi is always connected with a complementary sequence or a partner protein, while an antisense oligonucleotide always acts and stay as single strand. Their blind sides and strong sides originate from this difference [106].

2.4.1. Antisense Oligonucleotides

Development of antisense oligonucleotides dates late 1960s [107-110]. Inhibition of pathogen replication with antisense mechanism using sentetic oligonucletides was reported by Zamecnik and Stephensen in 1978 [108]. This and following studies showed that gene specific silencing was a promising therapeutic application.

Gene silencing agents should be highly specific for their target sequence and avoid off-target effect. Target selectivity indicates binding of antisence oligonucleotide to only its target sequence selectively, which prevent non-spesific inhibition of cellular proteins. Off-target effect of antisense oligos is derived from interactions of antisense oligos with intracellular, surface and extra cellular proteins [111]. To obtain increased stability in biological systems and target specificity, different types of antisense oligos were developed with different chemistries rather than using miRNA and siRNA molecules. Mostly used antisense oligonucleotides and their structures are shown in Figure 2.20.

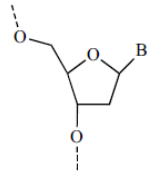
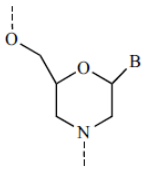
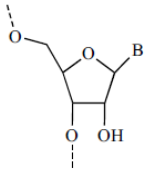
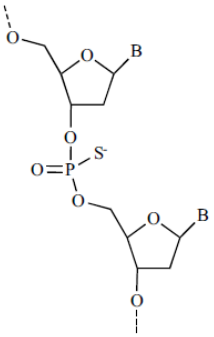
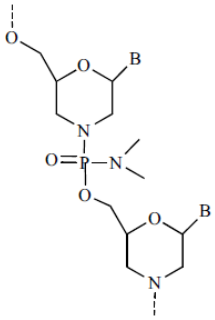
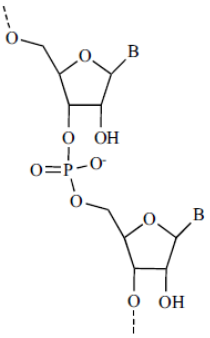
	(a)	(b)	(c)
Class	RNase H-dependent	Steric block	Interfering RNA
Type	Phosphorothioate	Morpholino	Short Interfering RNA
Subunit structure B= A, C, G, T(U)	 deoxyribose ring	 morpholine ring	 ribose ring
Backbone structure	 S-DNA	 Morpholino	 siRNA
Principle sites of action	nucleus	nucleus and cytosol	cytosol

Figure 2.20. Types of gene silencing oligos [111].

Oligonucleotides are about 13-25 nucleotides long, single stranded, modified or unmodified DNA sequences, which are designed to hybridize only their target within the cells containing many other DNA molecules. Antisense oligonucleotides are powerful tools for gene silencing and widely used as therapeutic agents or in gene function studies. After development of antisense oligonucleotides for therapy, oligonucleotides has been approved by Food and Drug Administration as therapeutic agents and the first commercial antisense oligonucleotide, Vitravene, has taken place on the market [112]. And many antisense oligonucleotides are at different clinical development phases [113]. Progressions in the understanding the fundamental principle of silencing mechanism, low cost of synthetic oligonucleotides, and knowledge about sequence of human genome make the antisense technology to be widespread in laboratory and clinic.

Although how the oligonucleotides act in cell is complex and is not fully understood, basic principle of mechanism of action includes two types of antisense oligonucleotides: first class is RNase-dependent oligonucleotides and second class of oligonucleotides are steric-blokers. As shown in Figure 2.21, RNase-dependent antisense oligonucleotides cause

mRNA degradation, while steric-blockers prevent translation by inhibiting the binding of translational factors or splicers to mRNA [18].

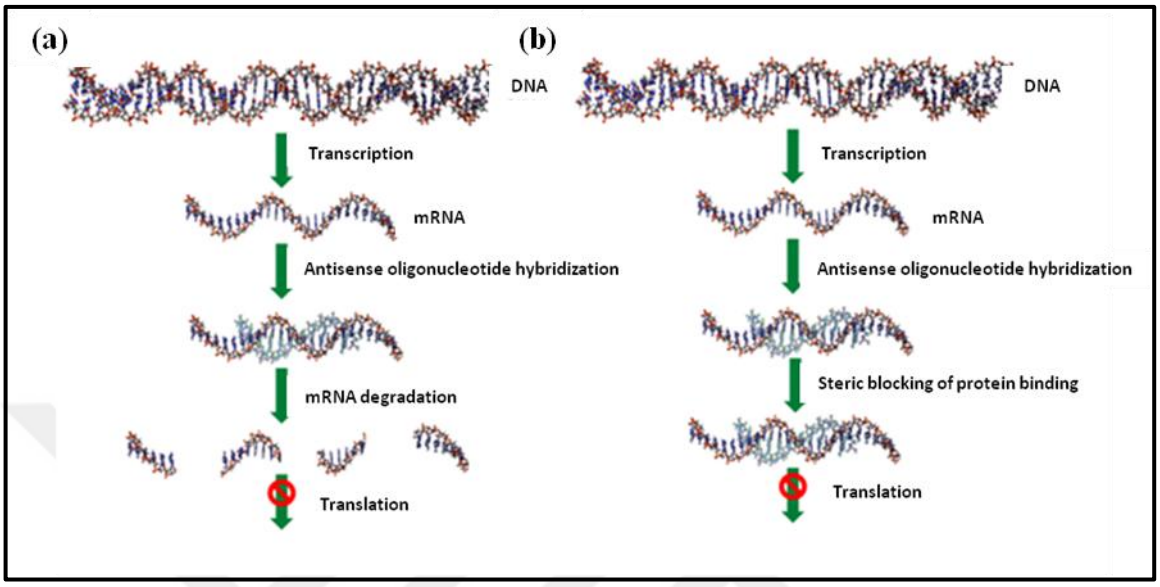


Figure 2.21. Mechanism of antisense oligonucleotides. (a) RNase H-dependent oligonucleotide mechanism and (b) steric-blocker oligonucleotide mechanism.

The most of the antisense drugs are RNase H-dependent oligonucleotides. RNase H is an enzyme carries on degradation of RNA/DNA duplex. RNase H enzyme-dependent gene silencing mechanism shows high efficiency of inhibition of expression up to 80-95 per cent. Moreover, RNase H-based oligonucleotides inhibit the expression of mRNA when they bind any region of the target sequence, while steric-blockers work only when they bind start codon of target mRNA [114]. On the other hand, steric blockers, morpholinos and peptide nucleic acids (PNAs) are the widely used oligonucleotides in this class, are highly stable, easy to synthesize, and have high affinity binding to nucleic acids due to lack of steric inhibition caused by negative charge. They can form stable complexes with DNA or RNA.

2.4.1.1. Morpholinos

Living organisms contain a lot of genes and the function of many of the encoding genes remains unknown. Clarifying of function of these genes is important to understand the genome. Phosphorodiamidate morpholino oligonucleotides provide researchers to identify

the gene function on whole-genome scale by silencing studies [115]. RNAi has been effectively used for determination of function but this approach inapplicable for large-scale studies [116]. In mammals, the use of RNAi structures has been associated with non-specific effects by activating some pathways, which cause non-specific inhibition of expression [117]. Also siRNAs may generate immune response and have off-target effect [118]. The specificity of silencing tools and biological response are important for predictable and trustworthy outcomes of studies. Synthetic antisense oligonucleotides have been preferred and studied commonly for silencing. For the minimization of toxicity, and increasing the target specificity and affinity, certain chemistries were applied on antisense oligonucleotides backbone [119].

Morpholinos are nuclease digestion resistant and water soluble synthetic DNAs, which contains neutral charged phosphorodiamidate inter-subunit linkages and 6-membered morpholine ring (Figure 2.22). The backbone structure of morpholino provides nuclease resistance and effective binding to complementary sequence, especially for RNA [19]. And water solubility of morpholino is one of the bright sides. It is essential for accession to the target in the cytoplasm. Structural properties of morpholino oligonucleotides provides quite advantages.

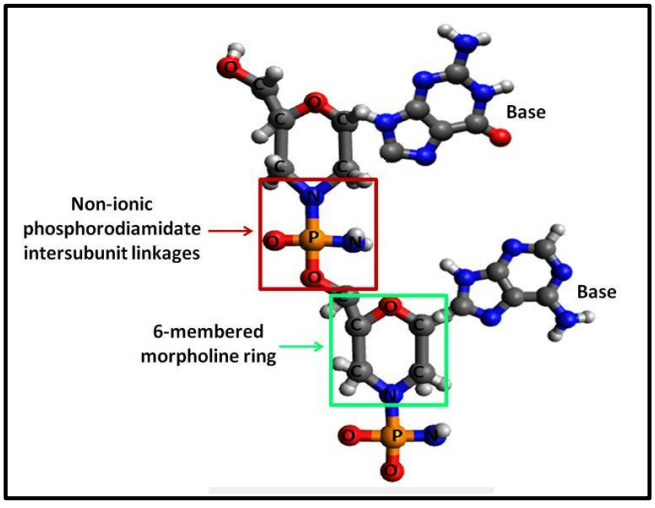


Figure 2.22. Morpholino chain containing non-ionic phosphorodiamidate intersubunit linkages and 6-membered morpholine ring instead of deoxyribose ring.

Together with enhanced stability, relatively low cost, and easy synthesis, morpholino oligonucleotides overcome many limitations of conventional antisense tools and provide a rapid and effective method for gene silencing experiments [120]. Morpholino antisense

reagents can modulate gene regulation with different ways depend on the oligonucleotide sequences; they can inhibit the initiation of translation or block mRNA splicing to modify mRNA maturation [18]. They inhibit gene expression whether in the presence of RNase H or not. Morpholino mechanism relies on the inhibition of translation. They target the complementary sequence in the cell by Watson-Crick base pairing. As shown in Figure 2.23, morpholinos block mRNA slicing; target sequences of antisense oligonucleotide are specific junctions or prevent ribosomal assembly; antisense oligonucleotide binds to 5'-untranslated region (5'-UTR) of gene transcript. The mRNA position, where the morpholino hybridizes, determines the mechanism of action. Both mechanisms inhibit formation of a functional protein [121].

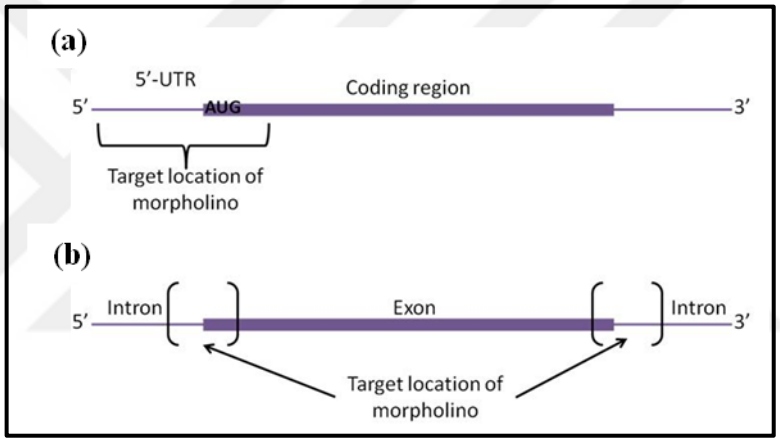


Figure 2.23. Target mRNA location of morpholino oligonucleotides. (a) Target position of morpholino, which blocks the ribosomal assembly. The morpholino sequence targets the 5'-UTR of mRNA including AUG start codon and (b) mRNA binding sides of morpholino that blocks splicing. The antisense oligonucleotide binds less than 10 bases of exon sequences [122].

2.4.2. Delivery of Oligonucleotides

To fulfill their biological activity, antisense oligonucleotides must be able to enter the cell compartments, where their target sequence is expressed or acts in. antisense oligonucleotides and RNAi are promising tools for regulation of gene expression for therapeutic purposes and also there are ongoing clinical trials [123]. Oligonucleotides, in almost all cases, can enter the cell by endocytosis. It is a general term of a variety of entrance pathways. Understanding the mechanism of endocytosis is important for effective

enter to site of action. For reaching the site, some targeting ligands and cell penetrating peptides are used. Beyond the cellular level, biological barriers are important for *in vivo* studies [124].

Although gene regulation is a powerful strategy, there have been problems; maybe the most important one is gaining entry to the nucleus or cytoplasm, where they are acted in the cell. Cationic delivery agents are used in *in vitro* studies. Liposomes [22] and cationic polymer nanoparticles [125] have been used for delivery but these cationic polymers or lipids may be disputable for *in vivo* because of their large size and toxicity [126]. There are other polymeric particles as nanocarrier of oligonucleotides for various cells [127]. But it should be taken in consideration that all of the delivery vehicles have their own biological effects on gene expression. Other traditional ways for introduction of DNA into cells like electroporation or microinjection have disadvantages of being limited with *in vitro* studies and they can transfect limited number of cells [128].

2.4.2.1. Nanotechnology for Gene Delivery

RNAi is a primitive immunity of cells against to the exogenous genetic materials in biological systems. After the discovery of RNAi, synthetic nucleic acids were produced for gene silencing. The ability of modulating gene regulation led many studies about treatment of gene related diseases such as cancer and infectious diseases. To overcome the limitations of RNAi, many antisense oligonucleotides with high sequence specificity were developed. The major problems of the silencing tools are poor bioavailability and short half life in biological fluids. The oligonucleotides are barely uptaken by cells and the negligible amount of them can escape from endosomes. Nanotechnological approaches were developed for delivery of oligonucleotides using non-viral systems. The viral systems are efficient but cause immune response and unpredictable toxicity so that they are involved in safety issues. Here, liposomes and inorganic nanoparticle based systems were discussed as non-viral delivery vehicles.

2.4.2.1.1. Liposomes

Among the variety of non viral delivery systems, liposomes draw great interest from researchers for delivery of nucleic acids and are well-established for drug delivery [129]. The biocompatible nature and enhanced cellular permeation make the liposomes convenient for delivery studies [130]. Together with all the advantages they have, lipid structures provide rational design of novel carrier compounds.

Basically cationic transfection lipid structures consist of a hydrophobic domain, a linker, and a cationic head group [131]. The hydrophobic domain consists of carbon chains or steroids. The head group derived from various moieties such as polyamines and quaternary ammonium groups. Also amino acids can be head domain of a liposome. While there are various hydrophilic head groups, nitrogen based domains are the most widely used motifs [132]. The linkers are also variable and the most common linkers include glycerol, ethers, esters, and amides. All this chemical nature of the liposomes affects the biodegradability, stability, flexibility, and transfecion efficiency of the structure.

There are many commercial lipid based agents for delivery of nucleic acid molecules. Most frequently used liposomes including N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), Dioleoylphosphatidylethanolamine (DOPE) dioctadecylamidoglycylspermine (DOGS), 3β[N-(N_,N_-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol), [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane] (DOTAP), and 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-l-propanaminium trifluoroacetate (DOSPA) are shown in Figure 2.24 [133]. All the commonly used and well established liposomes for nucleic acid delivery have distinctive structures, which effects their stability, toxicity, and transfection efficiency.

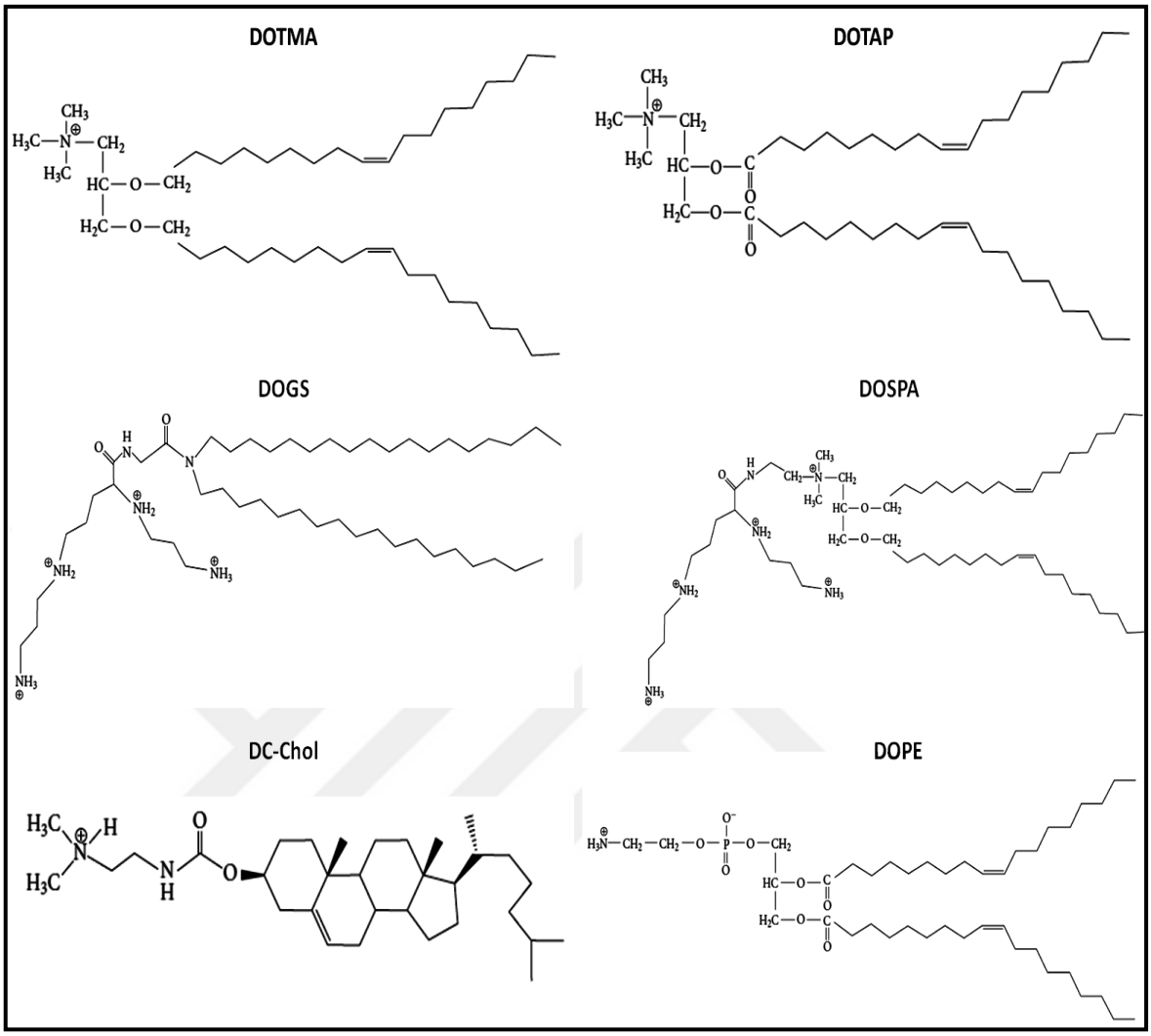


Figure 2.24. The mostly used lipids in gene delivery [133].

Cationic lipids naturally interact with polyanionic molecules such as DNA and RNA. The cationic lipid structures form complexes through interaction of positive groups of lipids with negatively charged phosphate backbone of NA molecules [131]. Lipid – DNA complexes are called as lipoplex. The overall positive charge of lipoplex complex enables the adsorption of the complex onto cells, which have glycoproteins and glycolipids provide negatively charged surface. Evidence indicates that cationic liposomes deliver their nucleic acid cargos through clathrin-mediated endocytosis pathway rather than cell membrane fusion [134, 135]. The formation of liposome-DNA complex and intracellular uptake of the complex are shown in Figure 2.25. The polyanionic nucleic acid structures interacted with cationic head groups of liposomes electrostatically resulted with the attachment of DNA or RNA to the liposomes. The strength of the interaction increases proportionally number of the positive groups of liposome. Lipofection occurs through endosomal internalization.

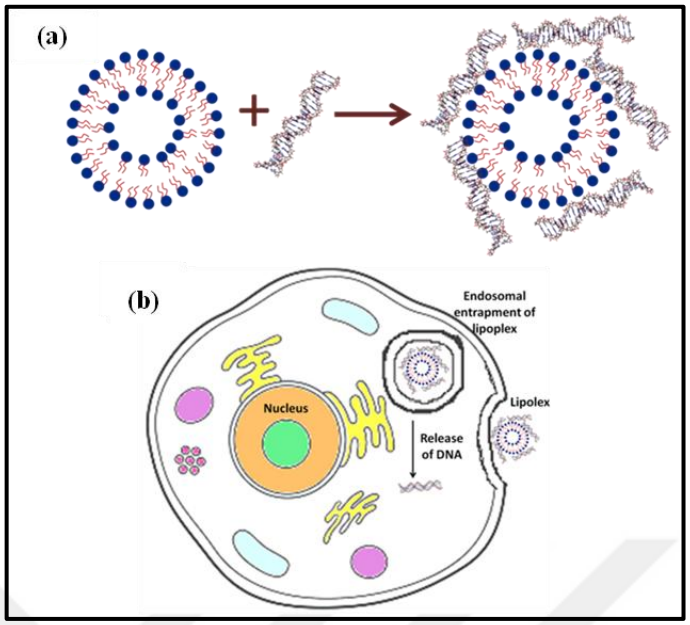


Figure 2.25. (a) Interaction of liposome with nucleic acid molecules and formation of lipoplex and (b) intracellular uptake mechanism of lipoplex.

Liposomes are extensively investigated and most efficient non-viral delivery systems. However, liposomes involve toxicological concerns. The use of liposomes effects cell viability due to the interaction of these cationic structures to intracellular anionic lipids such as cardiolipin, which is a mitochondrial membrane lipid, so that metabolic pathways of the cell can be negatively influenced by these interactions [136]. Also transfection efficiency is effected by a number of parameters such as size of the liposomes [137].

Gene silencing studies using liposomes were demonstrated *in vitro* and *in vivo* [138-142]. Palliser *et al*. reported that delivery of virus targeted siRNAs prevented viral infection in animal models [138]. siRNAs were designed and delivered using liposomes by local administration for targeting herpes simplex virus 2 genes. Intra-vaginal application of siRNA-liposome complex targeted UL27 gene encoding a virus envelop glycoprotein, and UL29, which is a DNA binding protein gene, protected mice from infection without causing any undesirable immune response. Rather than local administration, gene silencing were achieved also by systemic delivery of siRNA-liposome complexes [139]. Sorensen *et al.* showed that green fluorescence protein (GFP) expression was silenced in spleen and liver following intravenous injection of the lipoplexes in GFP expressing adult mice. In another study, galactosylated liposomes were utilized for targeted delivery of siRNA to liver cells by intravenous administration in mice [143]. The galactosylated liposome-

siRNA complexes effectively inhibited Ubc13 gene expression in liver. Moreover, galaycosylated liposome-siRNA complex did not cause any liver toxicity.

Many studies using different liposome formulations were promoted to clinical phases [144]. RNAi-lipid nanoparticles have been assessed in clinical trials for a variation of cancer diseases such as myeloma, lymphoma, and leukemia. Lipid-based gene silencing drugs have been also exploited for diseases other than cancer in clinic such as hepatitis B, hepatic fibrosis, and transthyretin-mediated amyloidosis. The clinical studies based on RNAi-lipid therapeutics were shown in Table 2.1.

Table 2.1**.** Lipid-based drugs for gene silencing in clinical trials [144].

Target gene	**Therapeutic**	**Disease**	**ClinicalTrials.gov ID (phase)**
PLK1	siRNA-liposomes	Hepatocellular carcinoma	NCT02191878 (Ph I/II)
BCL2	Lipid nanoparticles encapsulating a single stranded DNAi	Lymphomas	NCT02378038 (Ph II) NCT02226965 (Ph II) NCT01733238 (Ph II)
EphA2	siRNA-liposome	Solid tumors	NCT01591356 (Ph I)
Grb-2	Neutral liposomes encapsulating antisense oligo	Leukemias	NCT01159028 (Ph I)
NYC	DsiRNA-lipid nanoparticles	Lymphoma, hepatocellular carcinoma solid tumors, multiple myeloma, or	NCT02110563 (Ph I) NCT02314052 (Ph I/II)
PKN3	AtuRNAi-liposomes	Pancreatic cancer	NCT01808638 (Ph I/II)
HSP47	Vitamin A-siRNA lipid nanoparticle conjugate	Hepatic fibrosis	NCT02227459 (Ph I)
Target three sites on the HBV genome	Lipid particle containing three RNAi therapeutics	Hepatitis B	NCT02631096 (Ph II)
Disease-causing TTR	RNAi-Lipid nanoparticles	Transthyretin-mediated amyloidosis	NCT02510261 (Ph III) NCT01961921 (Ph II) NCT01960348 (Ph III)

2.4.2.1.2. Inorganic Nanoparticle Based Delivery Systems

Gene silencing is a powerful technology for altering of gene expression in diseased cells. However, there are some obstacles to overcome for being a serious clinical practice rather than laboratory research. The primary problem of nucleic acid therapeutics is enterance to target cells both *in vitro* and *in vivo*. Depends on the administration, the nucleic acid cargo should pass through many biological barriers and exposed to nuclease degradation in biological fluids. They may also stimulate immune system in living organisms and vulnerable to be eliminated from the body. Beyond reaching to the target side in the organism, the nucleic acid molecules cannot penetrate cell membrane because of their negative charge. Therefore, delivery vehicles, which carry the nucleic acids to target cells and provide both protection from undesirable degradation and elimination and penetration into cells are needed. There have been developed many strategies for nucleic acid delivery. A number of parameters such as surface chemistry, size and shape determine the success of the delivery vehicles. Over the many delivery structures, nanoparticle-based systems are one of the most promising vehicles. Besides providing delivery and intracellular uptake, nanoparticles offer multi functional systems for the purposes of targeting, labeling, imaging, and control with magnetic field.

Nanoparticles are used in wide variety of applications due to their distinctive chemical and physical properties. They are capable of interacting with biomolecules. Nanoparticles have unique features such as high surface area and reduced size. Surface functionality properties also make them favorable for delivery studies combined with specific targeting.

One of the most popular nanostructures is AuNPs. Another emerging therapeutic potential of AuNPs is intracellular gene regulation. AuNPs have quite advantageous properties such as biocompatibility, easy synthesis in different sizes, and easy modification through interaction with thiol groups. AuNPs can be easily synthesized in small sizes to increase the surface area and maximize the nucleic acid loading onto the surface. Thus they are remarkable candidates for gene delivery. Multifunctional AuNPs platforms were reported for specific and targeted *in vitro* and *in vivo* down-regulation of c-myc protooncogene [103]. The AuNPs surface was modified with thiolated PEG with carboxyl end and thiol-PEG-azide polymers, TAT peptides, RGD peptides, and siRNAs in order to increase the solubility and provide cellular penetration, cell recognition, and gene silencing. siRNAs for

silencing of c-myc gene were loaded onto the surface of AuNPs either covalent and ionic approach. In covalent approach the thiolated siRNAs were attached to the surface of AuNPs using strong thiol:Au binding. In ionic way, the surface of AuNPs was modified with quaternary ammonium groups and the polyanionic siRNAs were attached to the nanostructure through electrostatic interactions. The siRNA loaded vehicles prepared in both covalent and ionic approach were tested on *in vitro* and *in vivo* studies and significant silencing of gene expression was monitored.

In another study, gold nanorods were used for a drug addiction therapy by gene silencing in neurons [104]. Gold nanoshell structures coated with antisense oligonucleotides and siRNAs against green fluorescence protein (GFP) were designed [101, 102]. The siRNA molecules attached to the surface of Au-nanoshel by electrostatic interaction and release of oligonucleotides was triggered by near-IR laser. The NIR radiation induced controlled release of oligonucleotides caused effective silencing of GFP expression. The surface of the Au-nanoshell was modified with a cationic peptide with the sequence of cysteine, tyrosine, Serine, and poly lysine. siRNAs were attached to the surface through electrostatic interaction with positive lysine residue. To monitor the radiation triggered siRNA release, fluorescent labeled oligonucleotides were interacted to the surface and release of oligos was detected after exposure to 800nm laser. The nanostructures were delivered to human lung cells and the radiation controlled treatment resulted in down-regulation of targeted GFP expression.

High dense oligonucleotide coated polyvalent AuNPs were used for gene silencing studies with the properties of resistance to nuclease degradation and increased intracellular uptake in various cell types [145]. Poly-L-lysine (PLL) and siRNA are compexed with AuNPs layer by layer and multi-layered AuNPs were used for gene silencing studies. siRNA was released from AuNPs complex gradually by degradation of PLL and led knockdown of targeted gene [146]. There are also successful examples of AuNPs-polymer conjugates for siRNA delivery studies [147-149].

AuNPs modified with P12 and CGDK peptides were used for receptor mediated intracellular uptake and upregulation of p53 tumor suppressor gene [150]. A study shows that by the modifications of AuNPs with major domains of a transcription factor, AuNPs can act as a transcription factor and regulate gene expression itself [151]. Another gene regulation, silencing, is also studied with AuNPs. Nanoparticles were coated with

complementary oligonucleotides to target mRNA sequences, for down-regulation of a specific gene. Tetrathiol-modified oligonucleotides were bound to 13 nm AuNPs and highly stable and selective oligonucleotide delivery platforms were formed. Although this delivery platform was coated with negatively charged DNA, AuNPs-oligonucleotide conjugates could enter the cells directly while most of the transfection agents need positively charged materials to manage the intracellular uptake [145]. In another study of the group, siRNA-AuNPs conjugates were designed for silencing of firefly luciferase gene. By using polyvalent RNA-nanoparticle conjugates, cellular uptake and specific gene silencing were provided efficiently without any chemical modification. Impressively, the optical and physical properties of AuNPs remain unchanged [152].

One of the other attractive nanoparticles in research is Quantum dots (QDs) for their physicochemical properties. QDs are used as luminescence probes in many applications and have important advantages over organic dyes such as considerable brightness and superior photostability [153]. QDs at different sizes have different emission wavelengths and single light source can excite all the QDs with different size. Rely on the features of QDs, they have part in detection and imaging applications of biology and medicine. Many multifunctional QDs based platforms for targeting, diagnosis, and treatments were developed [27-29]. Both siRNA delivery and cellular imaging were carried out by using polymer encapsulated QDs nanoparticles in breast cancer cells [154]. Yezhelev et al. reported an important study by using a novel nanostructure based on proton-sponge coated QDs for knockdown of cyclophilin b in breast cancer cells. By balancing carboxylic acid and tertiary amine groups on the surface of QDs, proton-sponge effect was constituted and used for silencing of cyclophilin b expression. After delivery of the nanostructure, proton-sponge on the surface causes absorption of protons by acidic organelles leading osmotic pressure increase through the organelle membrane. This increase in osmotic pressure provides leak of entrapped materials. The proton-sponge coated siRNA modified QDs were uptaken by cells through endocytosis pathway and siRNAs were released from endosomes due to proton-sponge effect.

Another example of multifunctional studies are using PEGlated QDs complexed with siRNA and tumor targeting molecule for inhibition of enhanced green fluorescence protein and tumor recognition [155]. Specific and efficient gene silencing using QDs

functionalized with siRNA was also reported for maintaining the integrity of blood brain barrier [156].

The idea of controlling the carrier system externally generated the usage of iron oxide nanoparticles (IONs). IONs are non toxic, tunable magnetic nanoparticles. Most of the delivery studies of IONs integrated with imaging or control by magnetic field. Medarova et al. developed a multifunctional magnetic nanoparticles for both siRNA transfer and the monitoring its accumulation in tumor tissue using magnetic resonance imaging (MRI) [157]. Simultaneous siRNA delivery and its accumulation in tumor tissue were showed using the same delivery system at ones. Superparaagnetic nanoparticles were modified with siRNAs, Cy5.5 dye, and membrane translocation peptide for silencing of GFP, near-infrared *in vivo* optical imaging, and intracellular uptake, respectively. The dextran coated nanoparticles were labeled with average three Cy5.5 optical dye, four myristoylated poly arginine peptides (MPAP), and five siRNAs per particle. *In vivo* silencing efficiency of the system was determined using *in vivo* imaging on bilateral tumor bearing mice. A decrease in GFP-associated fluorescence was observed, while there was no change in RFP-associated fluorescence. The results indicated specific silencing of GFP by magnetic prob. The GFP silencing was confirmed independently by quantitative RT-PCR. The decreased GFP mRNA levels indicated effective *in vivo* silencing of the system compared to saline and mismatch control.

Delivery of siRNAs was displayed using IONs coated with lipid-like molecules for gene silencing. It is also shown that, the efficiency of nucleic acid delivery was increased by external magnetic field [158].

High surface area, controllable pore size, easy modification, and being non toxic make mesopourus silica nanoparticles (MSNs) one of the mostly used nanoparticles for encapsulation of drugs and other molecules [159]. Delivery of siRNAs against EGFP and Akt genes using MSNs led attenuated expression of the genes in mammalian cells [160]. To deliver siRNAs to PANC-1 cells, the MSNs were functionalized with polyethylene imine and due to the electrostatic interaction of cationic polymer with oligonucleotides; siRNAs were loaded to the nanoparticles. After proving that MSNs can deliver and silenced EGFP gene, an endogenous human gene Akt were targeted and attenuated expression of the gene was monitored in human pancreas cancer cells.

Also simultaneous transport of siRNA and doxorubicin drug using mesoporous silica nanoparticles enhanced the response of drug resistant cancer cells to chemotherapy [161].

Moreover, there are studies using SWCNTs coated with siRNA noncovalently for targeting HIF-1α gene *in vivo* [162]. Both sensing and gene silencing using graphene oxides were reported for hepatitis C virus gene in liver cells [163].

2.4.2.2. Commercially Available Transfection Reagents

Transfection of NAs has been studied extensively due to the growing attention to NA-based therapeutics. Wide range of transfection methods exist in laboratory practices, including physical, chemical and biological methods. Electroporation, calcium phosphate precipitation and viral delivery can be given as examples to these methods. However, it is not always easy to follow a long procedure or obtain many types of chemicals and equipments during the experiments. Furthermore, these methods do not enable large-scale applications. Fortunately, companies provide reagents for easy/rapid transfection of NAs. Commercially available reagents dominate the transfection applications in laboratory practices due to the simplicity of their methods. The most important features of a transfection reagent are to be applicable for many types of cells, having high transfection efficiency-low toxicity, and providing consistent outcomes. One transfection reagent may fulfill all or some of these requirements and it is important to choose the true transfection reagent for each cases.

Viral vectors offer the most efficient delivery but they have safety issues such as provocation of mutagenesis or immune responses and problems such as NA size limitation. Commercially available trasfection reagents have been developed to also overcome these problems. These reagents are mostly lipid-based and polymer-based formulas. In here, the structure and transfection efficiency of the most commonly used commercially available transfection reagents were discussed.

2.4.2.2.1. Lipid Based Transfection Reagents

The mechanism of lipid based transfection depends on electrostatic interactions between cationic lipids and negatively charged phosphate backbone of DNA then the DNA-lipid complex is internalized by cells through endocytosis. The earliest synthesized cationic lipid was DOTMA [164]. After introduction of this cationic lipid formulation, many other types

of cationic lipids have been synthesized and become available on market. Lipofectamine, Lipofectin, DreamFect Gold and Fugene are the examples of the mostly studied cationic lipid-based transfection reagents. Lipofectamine, Lipofectine and DreamFect Gold are liposomal transansfection reagents and Fugene is a non-liposomal lipid based system. The formulations of the reagents are different combinations of the cationic lipid structures discussed previously in Figure 2.24 at various ratios. Lipofectin consists of monocationic lipids with the composition of DOTMA/DOPE (1:1 w/w), while Lipofectamine contains polycationic lipid structures [165]. DreamFect Gold and Fugene reagents contain cationic lipids and other components.

Highly efficient transfection of NA molecules using Lipofectin [164, 166, 167] and Lipofectamine [168] was reported in the literature. It was shown that the transfection efficiency of adenovirus was increased after complexing with Lipofectamine in human hematopoietic cells [169]. DreamFect Gold reagent has been widely used for magnetofection experiments [170-172]. Mixture of magnetic nanoparticles and DreamFect Gold was used to obtain magnetic lipoplexes for successful gene delivery to human corneal cells [171]. The reagent was also used for delivery of Foxp1 vector to investigate possible functions of the gene on spinal cord neurons during embryogenesis [173].

All of the transfection systems have been used in many studies for successful gene delivery. However, FuGene seems to be the most efficient among all these reagents. In comparison of the transfection efficiency of nine different cationic lipid-based commercial reagents including lipofectin, lipofectamine and Fugene, Fugene was shown to be slightly more efficient than all in mouse embrio cells. In another study, Fugene provided yet again the most efficient transfection efficiency in Huh-7 cells among five commercial reagents including lipofectamine and lipofectin [174]. However, its success in transfection efficiency was detracted by high cytotoxicity. Fugene was also compared with a wide range of commercial transceftion systems including both cationic lipids and cationic polymers and it was suggested that Fugene was the most effective reagent for a number of cell lines [175]. In another study, condrocyte cells were transfected for luciferase expression using eight different transfection reagents including Lipofectamine, FuGene and DreamFect Gold. DreamFect Gold provided the maximum expression of luciferase but FuGene caused minimum toxicity on cells [176]. On the other hand, lipofectamine was found to be the most effective transfection reagent out of many commercial transfection

systems including cationic lipids and cationic polymers for delivery of EGFP to HUVEC cells, which are tricky to transfect [177].

Each commercial transfection reagent has superior sides to one another depending on cell types, type and the size of the NA cargos and transfection conditions. Mostly the reagents are successful to deliver negatively charged NAs, while their biggest limitation is toxicity [178]. Reseachers have been investigating strategies to increase the transfection efficiency and decrease the toxicity.

2.4.2.2.2. Polymer Based Transfection Reagents

The ease of synthesis and modification of polymers has led to the use of polymers in gene therapy applications. Many of the polymers used in such applications have a net positive charge and can be modified to posses targeting residues. The cationic structure assists the escape of DNA from endosomes during endocytosis by efect of a proton sponge [179, 180]. Size and chain length of a polymer effects its internalization, escape from endosomes, release of DNA cargo, and entry into nucleus [179].

The most commonly used cationic polymers as transfection vectors are poly-L-lysine (PLL), Polyethyleneimine (PEI) and poly(2-*N*,*N*-dimethylaminoethylmethacrylate) (PMA) [181]. Different structures of these polymers exist such as branched, linear or dendrimer-like architectures. There are also copolymers developed for NA transfection [182]. Structures of the mostly used polymers are shown in Figure 2.26.

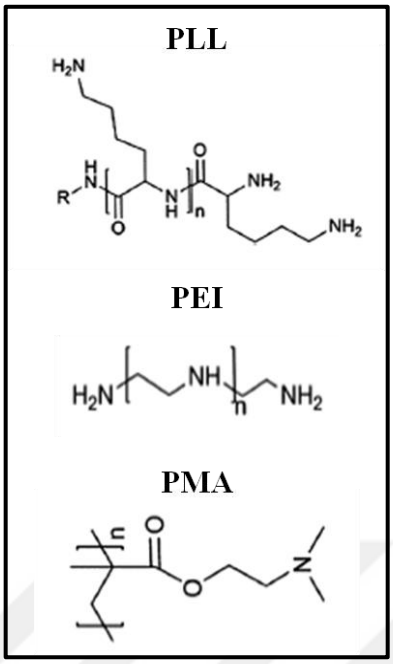


Figure 2.26. The most commonly used cationic polymer structures in gene delivery [183].

PLL is polymer of L-lysine amino acid, which effectively interacts with negatively charged DNA through amine groups as they are protonated at physiological pH. However, PLL has decreased transfection efficiency as a consequence of limited buffering capacity; they show toxic effect and tend to precipitate [184].

PEI is one of the mostly used cationic polymers in gene delivery applications. It is an inexpensive polymer with long shelf life and has advantages due to its chemical structure, which allows chemical modifications for biochemical applications including gene delivery applications.Their branched and linear forms exist. It has high transfection efficiency. Although their transfection effectiveness are compromised by toxicicity and being non-degradable [185].

PMA is a pH responsive cationic polymer. Being water soluble and protonated under physiological conditions makes it a good candidate as a transfection system [186]. The polymer has also modest toxicity upon cell culture systems [181]. Furthermore, its success on delivering DNA to cytosol through eascape from endosomal entrapment promotes efficiency of PMA in NA transfection applications [187].

Clinical studies of DNA delivery using polymer structures are very limited. There are only a few clinical trials exist in polymer based transfection, which only involves delivery of DNA vaccines [188].

Efficient *in vitro* gene delivery studies were reported in the literature using the most widely prefered commercially available cationic polymer structures: Express Fect [189], JetPEI [190], Xfect [191-193] and an activated-dendrimer reagent Superfect [194]. There are also reagents, which contains both polymers and lipids in their formulation. Arrest-In is an example of liposome-polymer composite structure.

In a study, transfection efficiencies of Express Fect, JetPEI, SuperFect, Arrest-In, FuGene and Lipofectamine were compared in nine different cell lines. The findings showed that FuGene was effective on the majority of tested cell lines and Arrest-In and JetPEI followed it [175]. In another study, a number of polymer based commercial reagents including TransIT-TKO, TransIT-siQUEST, X-tremeGENE, Xfect and Nanofectin were compared in terms of gene silencing efficiency in mouse embryonic stem cells [195]. The maximum inhibition rate in Oct3/4 gene obtained with TransIT reagents and X-tremeGENE system with 80 per cent silencing. Nanofectin fallowed these reagents with 70 per cent efficiency and Xfect induced 50 per cent silencing of the target gene.

The transfection efficiency of a delivery system depends on the cell type, passage number of cells, cell confluency, type of NA, mycoplasma contamination and cell medium conditions. Also size of the NA-transfection reagent complex is very important for systemic delivery to pass through biological barriers. There are many parameters for selection of appropriate transfection reagents to obtain satisfactory results in experiments or successful therapeutic effect.

2.5. BREAST CANCER

Female breast consists of ducts and lobules. About 80 per cent of the cancers initiate in ductal area and the rest occurs in lobules [196]. In Figure 2.27, normal female breast and formation of ductal and lobular carcinomas are shown. Transformation of normal breast tissue to cancer can be induced by some factors such as environmental and genetic factors. Today, many people suffer from breast cancer.

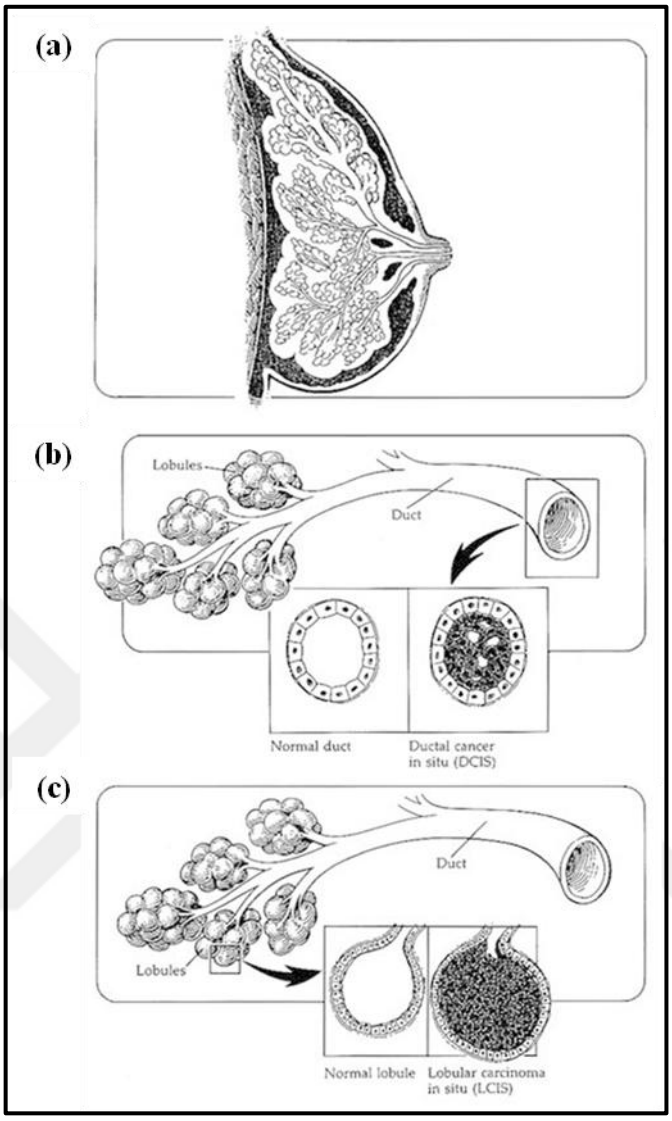


Figure 2.27. (a) Structure of female breast, (b) ductal carcinoma, and (c) lobular carcinoma [196].

According to the Globocan 2018 data of International Agency for Research on Cancer, breast cancer remains the most common cancer type in females worldwide (Figure 2.28a) [197]. Also in Turkey, the mostly diagnosed malignancy in women is breast cancer (Figure 2.28b); one of the every four women, who were diagnosed with cancer, was suffering from breast cancer. Approximately 17.000 women were diagnosed with breast cancer in single year in the country according to the statistics of ministry of health [198].

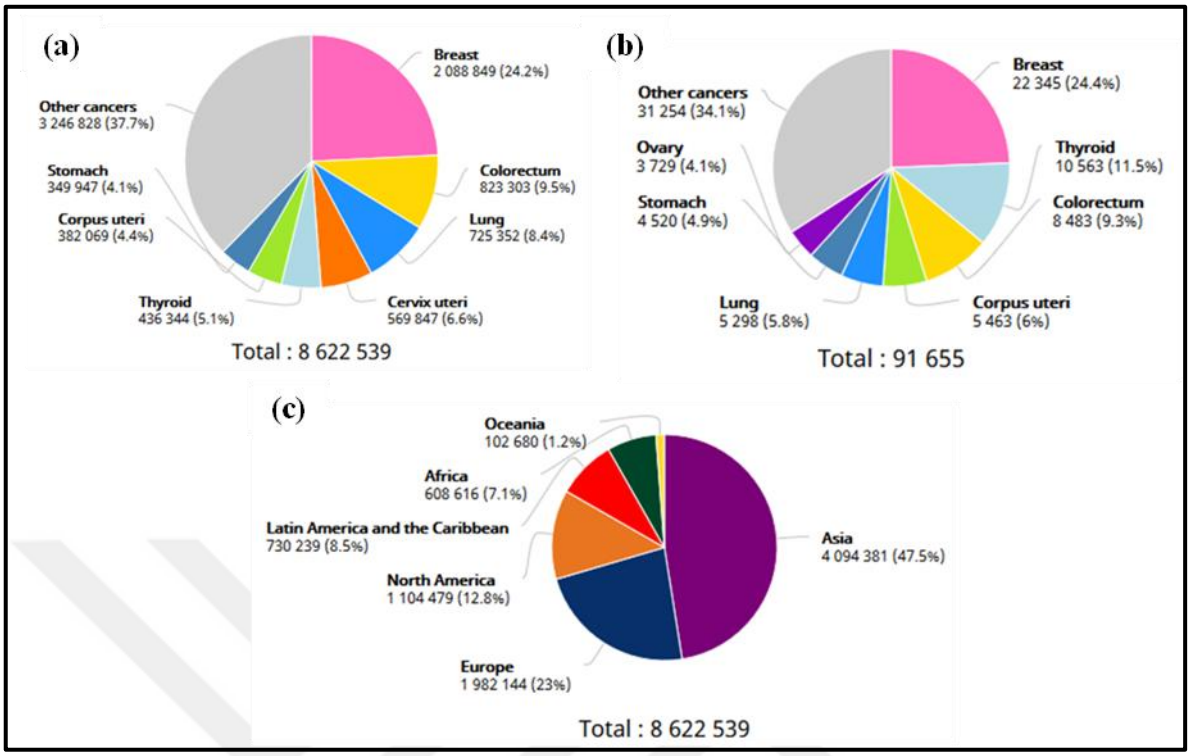


Figure 2.28. Estimated number of new cases in 2018 for females in all ages (a) worldwide, (b) in Turkey, and (c) breast cancer cases in continents [197].

For decades, researchers have developed new treatment strategies targeting the molecules that promote tumor growth and proliferation. Specific targeting of cancer cells has always been the main objective of cancer therapy. The ideal system is thought to reach specifically target side without effecting healthy cells in the body and remove all the side effects of drugs. There is no such system that works 100 per cent efficiently but with the effort of studies, targeting therapies is getting closer to the goal of being more effective than standard cures. For the treatment of breast cancer, therapies targeting the molecules, which is expressed in higher levels in cancer than healthy tissue, has been designed. Especially growth factors; EGFR, ERα and HER2 are included [199].

Through the knowledge has been gained about breast cancer, targeting the genes that have overexpression is promising strategy for cancer therapy. HER2, ER, EGFR, and Ki-67 are the genes that expressed in high levels in breast cancer cells and related with cancer proliferation and growth.

2.5.1. Human Epidermal Growth Factor Type 2 (HER2)

Breast cancer has a number of characteristics in case of expression status of genes like p53, Estrogen and Progesterone receptors, HER2, Ki-67, EGFR and Androgen receptor (AR) [200]. These expression profiles are used as immunohistochemstry (IHC) markers for classification of breast cancer cell lines. It is important to determine the cell lines, which are used in clinical studies according to their subtypes for the outcome of the studies.

The ER, PR, and p53 genotype of the cells and expression level of HER2 is highly related to the cancer disease. 20 to 30 per cent of invasive breast cancers show overexpression of HER2 [201]. In most of the patients, high expression of this gene or higher number of copies indicates decreased survival and differential responses to treatment. So targeting to HER2 (also known as HER2/neu or ErbB2) looks like an important strategy for cancer treatment [202]. Overexpression of this gene becomes a signal, which causes unregulated growth of cells and metastasis, and also resistance to chemotherapy [203]. The underlying mechanism of HER2 in cancer involves complex networks. ErbB2 or HER2, which has extracellular, transmembrane, and intracellular domains, is a member of tyrosine kinase receptor family. It is related with a number of signaling pathways. Presence of HER2 affects the cell cycle through upregulation of D-type cyclins expression; causes reducing of dependence to mitogenic stimulation. HER2 overexpressing breast cancer is very aggressive type with poor prognosis.

A monoclonal antibody drug Trastuzumab (Herceptin), targeting the HER2, is successfully used for treatment but not all cases and development of resistance, which is the most common problem, ends up with failure of treatment [204]. Herceptin treatment combined with chemotherapy is reported to be one of the most helpful treatments that improve patient survival [205]. Polymalic acid-based nanopolymers, which carry both morpholinos against HER2 mRNA and trastuzumab (anti-HER2 antibody), were resulted with simultaneous inhibition of both activation of already existing receptors and new synthesis of her2 receptors, and enhanced apoptisos [206]. In another study, HER2/neu was targeted with short interfering RNA (siRNA) in HER2 overexpressing breast cancer cells and they have observed arrested cell cycle at G0/G1 phases, inhibited proliferation rate, and induced apoptosis [207]. For the examination of silencing of cancer related genes on different cell

types, HER2, cyclin B, and protein kinase C were transfected with siRNA. Results confirmed the previous studies that HER2 silencing leads to cell death [208].

2.5.2. Ki-67

Ki-67 is another gene that has role in identification of breast cancer type. It is seen that this protein has an important role in cell proliferation but function of it could not identified exactly [209]. Targeting Ki-67 for breast cancer therapy is an attractive strategy. By inhibiting the expression of Ki-67 in the breast cancer cells, inhibition of cell proliferation is intended. For this purpose, a various types of breast cancer were transfected with siRNAs targeting Ki-67, decreased cell proliferation and increased controlled cell death were observed [210]. Inhibition of cell growth in renal cancer has also been studied with short hairpin RNAs (shRNA) and the same results were received with breast cancer in both *in vitro* and *in vivo* studies [211].

2.5.3. Epidermal Growth Factor Receptor (EGFR)

EGFR is a receptor tyrosine kinase (RTK) family member, which is necessary for cellular growth [212]. When epidermal growth factor binds to the receptor, phosphorylation occurs that allows a series of proteins binding to receptor [213]. A molecular cascade follows the binding of these proteins and ends up with entry to S phase of the cell. EGFR action is related not only cell proliferation but also differentiation and tissue generation [214]. Most of the basal-type breast cancer cases have overexpressed and/or increased copy of EGFR gene [215]. Because of the reason that most of the triple negative breast cancer cases related with EGFR expression, it has been one of the most significant targets for treatment of breast cancer. Recent studies reveal that targeting EGFR increases the sensitivity to chemotherapy of triple negative breast cancer cells so EGFR-targeted strategies might be promising [216].

2.5.4. Estrogen Receptor Alpha (ERα)

Estrogen has an important function in normal sexual development and maintenance the function. It affects also other biological systems like immune or cardiovascular systems [217]. The action of estrogen depends on the estrogen receptors. Estrogen receptors does not act themselves, they have correlation between a numbers of proteins[218]. Estrogen molecule and receptors estrogen are responsible for regulation of breast epithelial proliferation and apoptosis. Their action is related with cellular proliferation, so understanding the mechanism is important to understand the both normal and cancer growth [219]. In 1950s, an estrogen binding protein was discovered, today known as estrogen receptor alpha (ERα) by Elwood Jensen [220]. It is a nuclear protein activated by binding of estrogen. It works as a transcription factor, activates the estrogen-responsive genes and it is thought to be related with breast cancer development and growth [221]. More than half of breast cancers (~ 70 per cent) show over expression of ERα, and high expression of this gene in breast epithelium is being concern as high risk of breast cancer [222].

ERα effects the cell cycle through inhibiting the p53/p21 expression and enhancing the expression of proliferating cell nuclear antigen (PCNA) to induce cellular proliferation of breast cancer cells [223]. There are hormonal therapies, like tamoxifen, targeting the inhibition of ERα [224]. Stable transfection of MCF-7 breast cancer cells by siRNA against ERα were carried out to establish an endocrine resistance cell line [225]. It is seen that lock of ERα expression change expression of a lot of apoptosis and cell proliferation related genes, in the mean of both up-regulation and down-regulation. Downregulation of ERα expression by using a vector-based RNAi resulted in considerable decrease in cell division rates of MCF-7 and Bcap-37 cells [226]. And also total inhibition of tumor size was demonstrated following gene silencing in nude mice. Silencing of ERα is not a persistent mechanism so its reversible nature may provide researchers to develop new therapeutic strategies for breast cancer.

2.6. AIM OF THE STUDY

The aim of the study is to prepare DNA origami-AuNPs nano-carriers by embeding morpholino sequences into the structure for the purpose of silencing specific genes, which are highly expressed in breast cancer cells. Morpholino antisense oligonucleotides are effective gene silencing agent with high efficiency and target specificity. However, delivery of the antisense oligos is insufficient due to their neural charge since most of the carriers interact with their cargos through electrostatic interactions. On the other hand, DNA nanostructures are very suitable for delivery of morpholinos as complementary base pairing is the only necessary condition for packing.

It is aimed to incorporate morpholino oligonucleotides into the prepared DNA origami-AuNPs nanostructures and to silence spesicif genes alone and simultaneously. Breast cancer cells were used for proof of concept experiments. Four different cancer proliferation genes were selected and morpholinos were designed to target these genes. *In vivo* knockdown effect of gene silencing system was investigated systematically starting from one target gene to four targets. Effects of single and simultaneous silencing of the target genes were determined. And the efficiency of DNA origami-AuNPs nanostructures was compared with other carrier systems.

3. MATERIALS AND METHODS

3.1. CHEMICALS AND MATERIALS

Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), Trypsin-EDTA (0.25 per cent) and Ribonuclease A were obtained from Sigma-Aldrich (Germany). Phosphate Buffered Saline (PBS) and Penicillin-Streptomycin (10,000 U/mL) were purchased from Gibco (UK). WST-1 Assay (Roche Diagnostic GmbH, Germany), Annexin V-FITC-/PI (Calbiochem, Germany) were purchased to monitor viable and death of cells. RIPA buffer was purchased from Santa Cruz Biotechnology (USA). Tissue culture flasks (25 cm^2, 75 cm^2), 12 and 96 well plates, Falcon tubes (15 ml), serological pipettes, were purchased from TPP (Switzerland).

Magnesium acetate was purchased from Applichem (Germany). Ethidium bromide was purchased from Fisher Scientific. Glutaraldeyde, sodium azide (NaN3) and tri-sodium citrate ($C_6H_5Na_3O_7$) were purchased from Merck Millipore (Germany). Chloroauric acid ($HauCl_4•3H_2O$), trizma base, acetic acid, hydrochloric acid, dimethyl sulfoxide (DMSO), ethanol, isopropanol and agarose were purchased from Sigma-Aldrich (Germany). Ethylenediaminetetraacetic acid and sodium dodecyl sulfate (SDS) were purchased from Bio Basic (Canada). Oligonucleotides and morpholino antisense oligonucleotides were purchased from Alpha DNA (Montreal, Canada). EGFR, HER2, GAPDH, Cyclin B1 primary antibodies were purchased from Cell Signaling Technology (USA), Ki-67 antibody was purchased from Santa Cruz (USA). Erα antibody was purchased from Merck Milipore (USA).

3.1.1. Cell Lines and Cell Culture

BT-474, MCF7, and MDA-MB-231 breast cancer cell lines were cultured in DMEM high glucose cell medium supplemented with 10 per cent FBS and 1 per cent PS, MCF-10A cells were cultured with Mammary Epithelial Cell Growth Medium (MEGM) at 37°C under 5 per cent CO2 atmosphere. Trysin-EDTA solution (0.25 per cent) was used to detach cells and PBS solution was used for washing steps. BT-474, MCF7 and MDA-MB-

231 breast cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA), MCF-10A cells were kindly provided by Fikrettin Şahin Lab.

3.2. METHODS

3.2.1. Preparation of DNA Origami Nanostructures

There has been an ongoing effort on designing wide range of carrier systems to provide delivery of therapeutic agents to the target site in order to treat diseases effectively. Most of these systems cause biological responses. Biomacromolecular systems have been solution to this problem. 2D and 3D nanostructures with controlled size and shape have been demonstrated by using DNA as building block through adenine-tymine, guanine-cytocine base pairing [1, 2, 33].

In this thesis, scaffolded DNA approach was used to prepare morpholino embedded DNA origami structures. Helper oligonucleotides, which are complementary to long DNA in different positions, are hybridized to the scaffold chain at their annealing temperature during the temperature decrease. The DNA origami nanostructure was prepared using 9 oligonucleotides including scaffold and morpholinos. The oligonucleotide sequences are shown in Table 3.1. Reference sequence numbers of target mRNAs and their targeted sequences are shown in Table 3.2. The primer numbers of oligonucleotides used for preparation of 1, 2, 3, and 4 morpholinos containing DNA origami nanostructures are shown in Table 3.3. Although all the prepared DNA origami structures had tile shape, the number of morpholinos and the targeted genes differed.

Table 3.1. Primer sequences used for DNA origami construction.

Primer numbers	**Oligonükleotide sequences**
P1	AGGCACCATCGTAGGAAAATCTGCGTCAGCTCTCCGTACACCAGTGCTTCCATGCGAAGTAAAACGTTCCGATCACCAACGGAGTAAAACGATCTAACTGATAACTAGCACCTCTTGCTCACGTGAGGAGTAAAACTTGCC
P2	TTTAGAAATGTACGGAGAGCTGACGCAGACCTACGATGGATGGAGCT
P3	TGAGCACCTGCCTGGCAAGACTCCTCACGTGAGCAGAGGGCAGCGAT
P4	TTCGGGGATGCTAGTTATCAGTTAGATCGACTCCGTTGGTTTTCCAG
P5	TGTGCTCTTGATCGGAACGACTTCGCATGGAAGCACTGGATGTGGCC
P6	GTCGCATCGCTGCTCCCCGAAGAGC
P7	TCGTGGGCCACATTTTCTAAACAGT
P8	GCCACCTGGAAAAAGAGCACAGCCC
P9	CCGCCAGCTCCATGGTGCTCACTGC
P10	AAAAAGGCCACATTTTCTAAATTTT
P11	AAAAAGTGCTCACTGCGGCTCTTTT
P12	AAAAAATCGCTGCTCCCCGAATTTT
P13	AAAAACTGGAAAAAGAGCACATTTT

Table 3.2. Reference sequence numbers of target mRNA in GenBank and target sequences of each morpholino oligonucleotide

	Ref. Seq.	**Target mRNA sequence**
HER2	NM_004448	gccggagcc**[g cagtgagcac c(atg)gagctg gcgg]**ccttgt gccg
EGFR	NM_005228	cggagcga**[gc tcttcgggga gcagcg(atg)c gac]**cctccgg
Ki-67	NM_002417	ttatactcaactt**[actgtttagaaa(atg)tggcccacga]**ga cgcctggtta
ERα	NM_000125	tcgcctctaa cctc**[gggctg tgctcttttt ccaggtggc]**c cgccggtttc

Table 3.3. Primer numbers used for DNA origami construction

$\mathbf{M_{EGFR}}$	P1+P2+P3+P4+P5+P6+P11+P12+P13
$\mathbf{M_{Ki\text{-}67}}$	P1+P2+P3+P4+P5+P7+P10+P12+P13
$\mathbf{M_{ERα}}$	P1+P2+P3+P4+P5+P8+P10+P11+P13
$\mathbf{M_{HER2}}$	P1+P2+P3+P4+P5+P9+P10+P11+P12
M2	P1+P2+P3+P4+P5+P6+P7+P12+P13
M3	P1+P2+P3+P4+P5+P6+P7+P8+P13
M4	P1+P2+P3+P4+P5+P6+P7+P8+P9

Four different morpholinos were embedded into DNA origamis separately. DNA origami structures were prepared as explained in our previous study [227]. Briefly, DNA origami structures were constructed using 1 scaffold oligonucleotide and 8 complementary helper oligonucleotides including the morpholino sequences, which are provided in Table 3.1. Equimolar amounts of the oligonucleotides were added in TAE/Mg^{2+} hybridization buffer, which includes 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium

acetate pH 8.0. This mixture was heated to 95°C, and then slowly cooled to room temperature once. No further heating and cooling procedure was applied. DNA origami nanostructures were prepared upon bottom-up strategy by using self organization properties of nucleic acid molecules. The oligonucleotides were denatured at 95°C and cooled very slowly. During the temperature decrease the complementary oligonucleotides connected to the scaffold DNA at their annealing temperature and folded into a tile shaped DNA nanostructure. Firstly the four 47 base long oligonucleotides annealed and then the 25 base long oligonucleotides including morpholino incorporated to the structure by complementary base pairing resulting tile shaped DNA origami with a theoretical size of 28 nm and with 8 sticky ends. These sticky ends were further used for AuNPs modification.

3.2.2. Characterization of DNA Origami Nanostructures

3.2.2.1. Agarose Gel Electrophoresis

Formation of the DNA origami structure was monitored using agarose gel electrophoresis. The oligonucleotides and the whole contructed nanostructures were loaded into the 1 per cent agarose gel, which was prepared in 1X TAE buffer solution, and these samples were run at 90V for 45 min. The gel images were obtained using a gel imaging system (Bio-Rad, USA).

3.2.2.2. AFM analysis

For AFM analysis of DNA origami structures, the 1μM DNA origami solution was 100 times diluted and 10 μl of these diluted samples were subjected to freshly cut mica surface and air dried at room temperature. Images were taken using Park System XE AFM at non-contact mode at room temperature and analyzed using the XEI image processing program.

3.2.3. Preparation of DNA Origami-AuNPs Nanostructures

3.2.3.1. Synthesis of AuNPs

The AuNPs with an average size of 13 nm were synthesized with Turkevich method [228]. Briefly, 40 mg of gold chloride ($HAuCl_4.3H_2O$) was dissolved in 100 ml ddH2O. The prepared solution was heated and stirred until boiling. While heating, 10 ml of 38.8 mM sodium citrate ($Na_3C_6H_5O_7$) solution, which induces reduction of gold salt, was quickly added into the boiling solution. It was kept boiling for 15 minutes and left to cool at room temperature. The synthesized AuNPs were characterized using UV/Vis spectrometer (Lambda 25, Perkin Elmer) and dynamic light scattering (Nanozetasizer, Malvern).

3.2.3.2. Functionalization of AuNPs

Oligonucleotide modification of AuNPs (Oligo-AuNPs) was carried out according to salt aging method with a few modifications [229]. Briefly, 1 mL of 13 nm AuNPs (400 µg/mL) was mixed with 0.1 M potassium phosphate buffer at pH 7.4 and 0.125 mL of 0.1 per cent Sodium dodecyl sulfate (SDS) by bringing the final concentrations at 0.01 M phosphate buffer and 0.01 per cent SDS.

A 5 µl of 100 µM 5'-thiol modified 14-mer poly-thymine oligonucleotide (5'-SH-TTTTTTTTTTTTTT) was added to the mixture and the final mixture gently mixed for 30 min at room temperature. 0.1 mL of NaCl solution was added from 2M NaCl stock solution at three steps and the final NaCl concentration was brought to 0.15 M. At the every steps of NaCl addition, the suspension was sonicated for 20 seconds and mixed for min. After the third addition of NaCl solution, the suspension containing AuNPs was left shaking overnight. The process was followed by centrifugation of the suspension at 13.000 rpm for 20 min for the removal of excess oligonucleotides. The supernatant was replaced with ddH_2O. The washing step was repeated 3 times and the suspension was stored at 4°C. The suspension of Oligo-AuNPs was taken into 1X PBS before using in the cell culture studies.

3.2.3.3. Modification of DNA Origami Nanostructures with AuNPs

DNA origami structures were modified with AuNPs after their characterization with UV/Vis spectroscopy and DLS analysis. Approximately 5.4 x 10^{13} oligo-modified AuNPs were added to 1.8 x 10^{13} DNA origami structure and the mixture was shaked for 24h at room temperature for hybridization of the oligonucleotides with the sticky ends of the DNA origami structures. The preparation and modification of the DNA origami nanostructures are schematically shown in Figure 3.1.

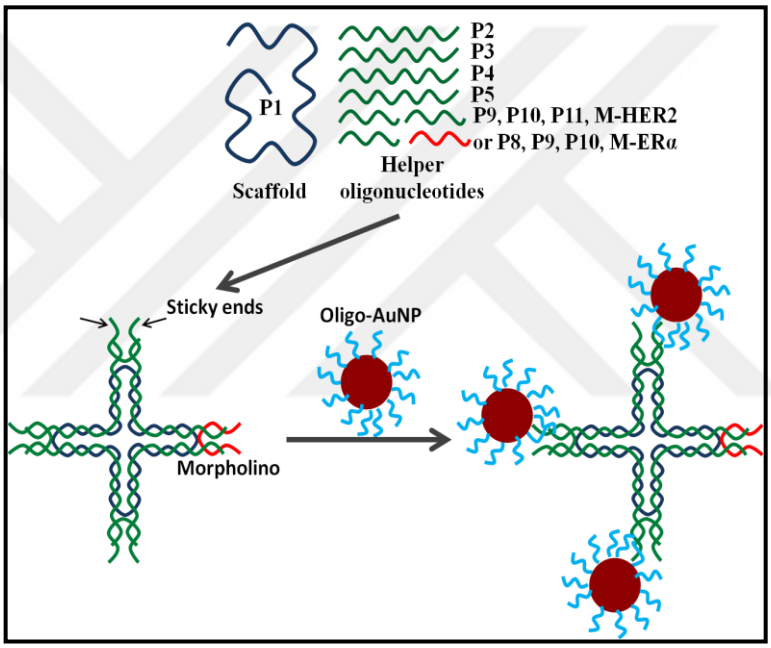


Figure 3.1. Preparation of DNA origami nanostructures and their modification with oligo-AuNPs.

3.2.4. Characterization of DNA origami-AuNPs nanostructures

3.2.4.1. UV/Vis Spectroscopy

Absorbance spectra of 1 nM of suspensions containing 13nm AuNPs, oligo-AuNPs and DNA origami-AuNPs were taken using UV/Vis spectrometer (PerkinElmer, USA).

3.2.4.2. Dynamic Light Scattering (DLS)

Hydrodynamic sizes of 13 nm AuNPs, oligo-AuNPs and DNA origami-AuNPs nanostructures were determined using Nanozetasizer (Malvern Instruments) at 25°C with a 173° scattering angle using 4mW He-Ne laser conditions DLS measurements were repeated three times.

3.2.5. Determination of Stability of DNA Origami-AuNPs Nanostructures

Stability of the prepared nanocarriers were investigated under physiological and cell culture conditions. To that end, DNA origami structures were incubated in PBS and serum containing cell culture medium for different time intervals. After incubations, stability of the DNA constructs was analyzed applying the agarose gel electrophoresis. 1 g agarose dissolved in 100 mL TAE buffer solution. The samples were subjected into the 1 per cent agarose gel and run at 90 V for 1h. The gel images were obtained using a gel imaging system (Bio-Rad, USA).

3.2.6. Determination of Cytotixicity of DNA Origami-AuNPs Nanostructures

Breast cancer cells were treated with morpholino containing and bare DNA origami nanostructures at different concentrations. Cells were seeded into the ELISA reader plate at cellular density of 5.000 cells per well and left for attachment for 24h. The cells were treated with the nanostructures and viability of the cells was determined using WST1 assay.

3.2.7. Cellular Internalization and Localization of DNA Origami-AuNPs Nanostructures

Internalization and localization of the gene silencing nanostructure was determined using confocal microscopy. The primer number P5 was ordered with a 5’-Cy5 modification. For internalization experiments, the dye-modified oligonucleotide was used for construction of the DNA origami structure. Glass cover slips were sterilized with 70 per cent ethanol and

placed in 6-well plates and 100.000 BT-474 cells were seeded in each well. Attached cells were treated with Cy5-labeled DNA origami nanocarriers and incubated for 30 min. The cells were fixed using 2 per cent paraformaldehyde solution for 20 min at +4°C and stained with lysotracker green at room temperature. The samples were washed with PBS 3 times for 5 min and immediately analyzed with confocal microscopy.

3.2.8. Gene Silencing Studies

For gene silencing studies, morpholino oligonucleotides were designed for specific inhibition of HER2, ERα, Ki-67, and EGFR genes and these morpholinos were embedded into DNA origami-AuNPs nanostructures separately or in combination. Effectiveness of the prepared gene silencing system was determined on breast cancer cells, which overexpresses the selected genes. Silencing efficiency of the designed nanostructure was evaluated comparatively with commercial transfection systems including cationic liposome, a non-liposomal lipid-base delivery system and a polymeric tranfection reagent. The expression rates of the genes were determined using Western blotting technique, which provides to monitor cellular protein levels.

Breast cancer cells were seeded in 6-well plates at 150.000 cells per well. After the attachment at the end of 24h, the cells were washed with PBS and treated with morpholino embedded DNA origami-AuNPs nanostrcutres in FBS free DMEM at the concentrations between 20 nM and 40 nM and effective concentration was determined. To assess silencing efficiency of the silencing system, a routinely used liposome system, DreamFect Gold (OzBiosciences, Marseille, France) was employed. The cells were treated with 30 nM DNA nanostructure in a serum-free medium. The morpholino containing liposome treatments were performed using the reagent according to the instructions of manufacturer. Briefly, 5µl of DreamFect Gold was dissolved in 0.5 mL of FBS free media, and then combined with 0.5 mL of morpholino containing media at a final morpholino concentration of 30 nM and the mixture incubated for 20 min at room temperature.For validation of silencing using DNA origami-AuNPs nanostructures, two other commercial reagents: A polymer based delivery system Xfect (Clontech, Palo Alto, USA) and a non liposomal lipid based delivery agent FuGene HD (Promega, Madison, USA) were also used. Treatments were performed according to instructions. For Xfect; 1.5µl reagent for

every 5µg nucleic acid in 100µl of reaction buffer was mixed and incubated for 10 min at RT prior to treatment. For FuGene; 3µl reagent for every 1µg nucleic acid was mixed gently and incubated for 15 min at room temperature prior to treatment. The cells were treated with morpholino embedded DNA origami-AuNPs and morpholino containing commercial reagents for 4h. The serum-free medium was replaced with FBS enriched DMEM medium and incubated for another 24h. Cell lysates were prepared using RIPA buffer. Protein concentration of each sample was determined using Bradford Assay according to the instructions reported in the reference [230]. A 40 µg of total protein was subjected to SDS-PAGE. The proteins were transferred to PVDF membranes and placed into blocking solution (3 per cent bovine serum albumin (w/v) in TBS-T) at room temperature for 1h. The membranes were incubated with rabbit anti-HER2/neu primary antibody, rabbit anti-ERα primary antibody, rabbit anti-EGFR primary antibody, Ki-67 (Santa Cruz, USA) and Cyclin B1 primary antibody (all 1:1000 dilutions as indicated) overnight at 4 °C. GAPDH expression was used as control of the equal amounts protein loading. The Western, blotting images were taken using a ChemiDoc MP imaging system (BioRad, USA). The analyses were performed with ImageJ software and the band intensities were normalized to GAPDH and quantified with respective to controls.

Alternative to Western blotting, decrease in the expression level of HER2, which is a cell membrane receptor, was determined also using confocal microscopy. Cover slips were sterilized using 70 per cent ethanol and placed in 6-well plates. BT-474 cells were seeded at 100.000 cells per well. The cells were treated with 30nM morpholino packed liposome and morpholino embedded DNA origami-AuNPs and incubated for 48h. The cells were fixed with cold-methanol at -20°C for 15 min. After washing steps, primary antibody solution specific to HER2 protein was added and incubated at 4°C overnight. The cells were washed with PBS 3 times and incubated with fluorescence labeled secondary antibody Alexa fluor 647 for 2h. Nucleus of the cells was stained with DAPI and the samples were examined using confocal microscopy under 63X objective.

3.2.9. Inhibition of Cancer Cell Proliferation

Breast cancer cells were seeded in 6-well plates at density of 20.000 cells per well. After 24 h attachment, the cells were treated with morpholino embedded gene silencing systems

and expressions of target genes were inhibited. Following the knockdown of the genes, the cells were left for 72 h for proliferation. At the end of the incubation time, the cells were detached from plates and living cells were counted. To eliminate the death cells, trypan blue dye was used.

3.2.10. Determination of Cell Cycle Phase Distributions

After treatment of breast cancer cells with DNA origami-AuNPs nanostructures, the cell cycle phase distributions were determined using flow cytometry. BT-474, MCF7, and MDA-MB-231 cells were treated with morpholino embedded DNA origami-AuNPs silencing system designed for silencing of HER2, ERα, and Ki-67 genes respectively. The cell cycle phase distributions of the cells were determined after treatment of different time intervals according to cell cycle duration of the treated cell line. The cells were seeded in 6 well plates at 100.000 cells per well. Following surface attachment, the cells were treated with 30 nM of gene silencing system in FBS free cell medium and incubate for 4 h. Starvation provided synchronization of cell cycles of the cultured cells. FBS enriched cell medium was added to the samples. BT-474 cells were incubated for 120h, while MCF7 and MDA-MB-231 cells were incubated for 48h post-transfection. The cells were detached using trypsin-EDTA solution and collected with centrifugation at 2.500 rpm for 5 min. after washing 3 times with PBS, the cells were dissolved in 70 per cent ice-cold ethanol and left for fixation at +4°C overnight. DNA content of the cells was stained using PI solution after RNase A (300 µg/mL) treatment at 37°C for 30 min. The cell cycle phase distributions were determined 5 min after staining according to their DNA content using Guava EasyCyte flow cytometer (MerkMilipore, USA).

3.2.11. Determination of Apoptosis

As indicated above, the selected genes were related with cancer proliferation and progression. After silencing of these genes using DNA origami-AuNPs nanostructures, it was investigated whether the breast cancer cells entered apoptosis pathway using Annexin V-FITC assay. The cells were seeded into 6-well plates at 100.000 cells/well density and left for 24h. After attachment, the cells were treated with 30 nM morpholino embedded

DNA origami-AuNPs gene silencing structures and incubated for 48 and 72 h. At the end of the incubation times, the cells were detached with trypsin and collected with centrifugation at 1500 rpm for 5 min. Following washing steps with cold PBS, the samples were stained with Annexin and PI dyes in binding buffer for 5 min and analyzed with flow cytometry using red and green filters.

4. RESULTS AND DISCUSSION

4.1. PREPARATION AND CHARACTERIZATION OF DNA ORIGAMI NANOSTRUCTURES

4.1.1. Construction of Morpholino Embedded DNA Origami Nanostructures

For preparation of DNA origami structures, mostly a viral genome and hundreds of staple strands are needed. The complicated formation of the structures makes DNA origami expensive and impractical for large-scale potential applications. In this thesis, we constructed a tile shaped DNA nanostructure by using only nine complementary oligonucleotides (see Table 3.1) including a morpholino sequence with one-step hybridization as schematically represented in Figure 3.1. Up to 4 copies of antisense oligonucleotides can be incorporated into one DNA origami structure from the four arms. It is also possible to embed morpholino into the origami body but it may take longer time for release due to the required longer time for degradation of the origami. Thus, we preferred to place the morpholino to the arms. The constructed DNA origami structure is shown in Figure 4.1.

In this thesis, scaffolded DNA approach was used to prepare morpholino embedded DNA origami structures. In this approach, which allows the successful formation of complex DNA constructs, a long scaffold strain and the shorter helper oligonucleotides are used. Helper oligonucleotides, which are complementary to the long scaffold DNA chain in different positions, are hybridized to the scaffold chain at their annealing temperature during the temperature decrease. The process of hybridization of complementary oligonucleotides due to the gradual reduction of temperature is called thermal annealing, and the thermal annealing process is important for the proper forming of the desired DNA structure. The thermal annealing process can be carried out in a controlled manner using water bath or PCR devices.

Origami formation is carried out in the appropriate buffer solution medium. By altering the buffer solution composition, the efficacy of the oligonucleotide hybridization can be increased. TAE/Mg^{2+} buffer solution is the most commonly used buffer solution for the DNA origami formation and Mg^{2+} ions, which are added into it, prevent electrostatic interactions between neighboring double chains to construct desired DNA structure. When the appropriate buffer solution is not used, this situation makes difficult the acquisition of the desired structure and negatively affects the preservation of the structural integrity of the structure obtained.

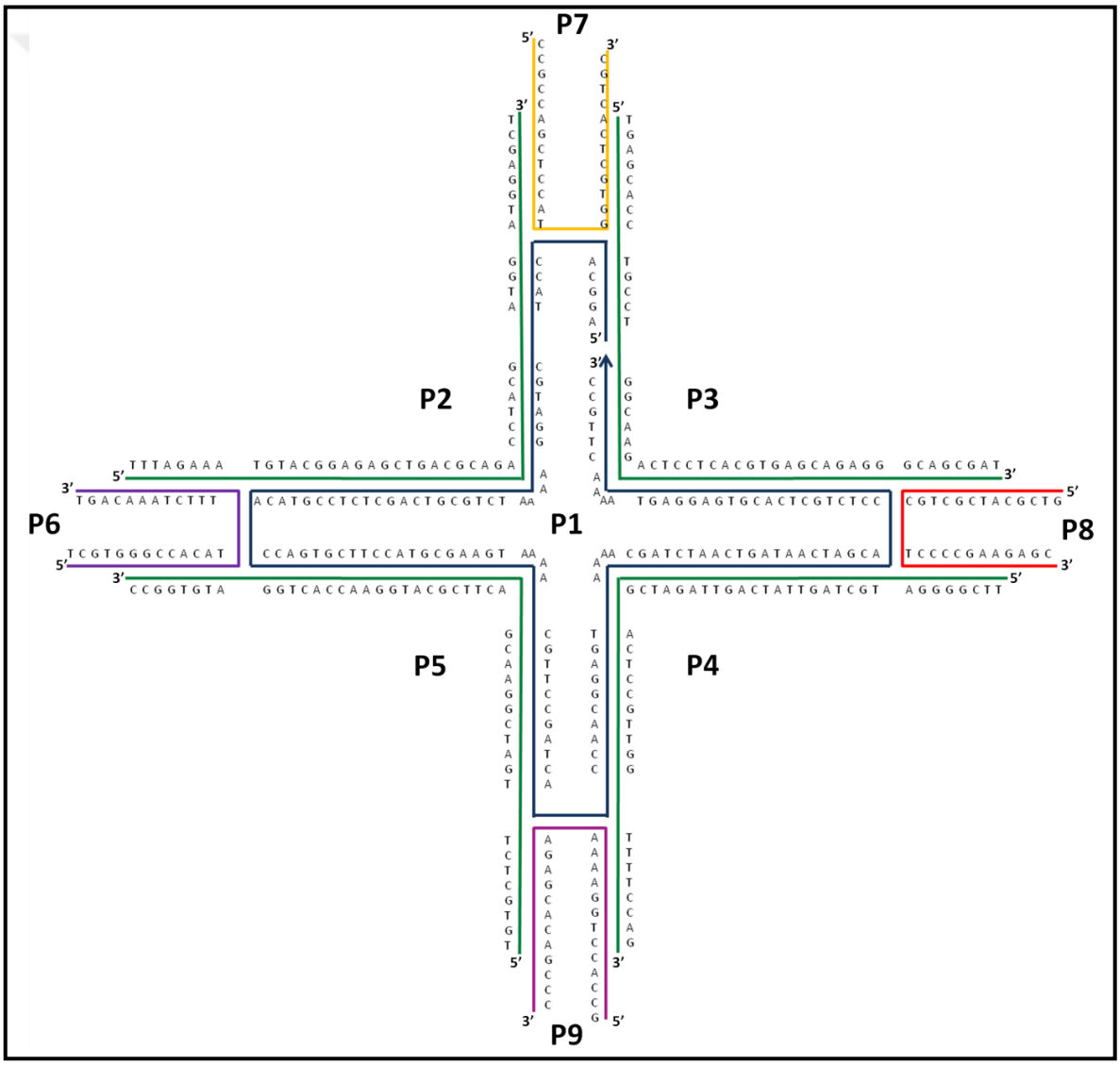


Figure 4.1. Structure of DNA origami

4.1.2. Characterization of the Morpholino Embedded DNA Origami Structures

The methods commonly used in the characterization of DNA constructs are microscopic and electrophoretic techniques. Using these techniques, formation of the newly prepared structures can be monitored. In addition to that, shapes and sizes can be analyzed in detail. Although each technique has superiority over another technique, they also exhibits some disadvantages. The necessity of fixing the structures to the analysis surface and high volume sample requirement are the main disadvantages, which are observed in these analysis methods. Atomic force microscopy, transmission electron microscopy, agarose and polyacrylamide gel electrophoresis are the common analyse techniques used for the structure confirmation and monitoring. To characterize the morpholino embedded DNA origami structures, agarose gel electrophoresis and AFM techniques were performed in this thesis.

4.1.2.1. Agarose Gel Electrophoresis

Agarose gel electrophoresis is one of the most preferred methods for characterization of DNA constructs because of the small amount sample requirement and its easy applicability. The formation of the desired DNA structure can be easily monitored on the agarose gel. Due to the increase in molecular weight by hybridization of oligonucleotides, the DNA structures migrating at different rates in the agarose gel can be distinguished easily.

The formation of the DNA origami structures was monitored using agarose gel electrophoresis. In Figure 4.2, the agarose gel electrophoresis image of the sequentially hybridized oligonucleotides is shown. The wells numbered from 1 to 9 contain the increasing number of complementary oligonucleotides to form the origami structure. The first well has only one oligonucleotide and the others were the samples prepared by adding sequential complementary oligonucleotides. The last sample was complete DNA origami structure. As seen, the molecular weight of the hybridized structures gradually increases with the addition of sequential complementary oligonucleotides, which indicates formation of the origami structure.

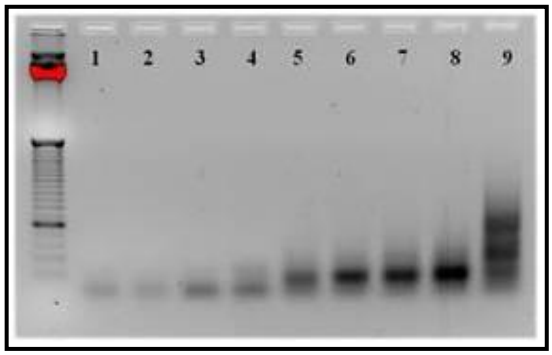


Figure 4.2. Agarose gel electrophoresis of increasing number of complementary oligonucleotides forming DNA origami. The “1” is the smallest oligonucleotide itself, others are the structures formed with the addition of complementary oligonucleotides sequentially numbered as 2, 3, 4, 5, 6, 7, 8, and 9.

Antisense oligos can be perfectly integrated into DNA nanostructures due to their similar chemistry [60]. The morpholino oligos were embedded into the DNA origami structures simply by just using complementary base pairing. Unlike other antisense oligos, morpholino/DNA hybridization occurs efficiently independent from salt concentration [231] indicating DNA nanostructures as perfect candidate for delivery of morpholinos. Morpholino embedded DNA origami structures were prepared systematically. 1, 2, 3, and 4 morpholino oligos embedded DNA origami structures were formed and characterized using agarose gel electrophoresis. Figure 4.3 shows the gel image of the DNA origami without morpholino that is labeled as Origami, and 1 morpholino, 2, 3, and 4 morpholinos embedded DNA origami that is labeled as M1, M2, M3 and M4, respectively. Morpholino oligos did not run through gel matrix as they are neutral. However, when the morpholino oligos incorporate into the origami structure, they run together with origami but slower that DNA origami without morpholino. So the increasing number of morpholinos embedded to the structures seen like increase in size. The gel electrophoresis image showed that morpholino oligos efficiently integrated to DNA origami nanostructures.

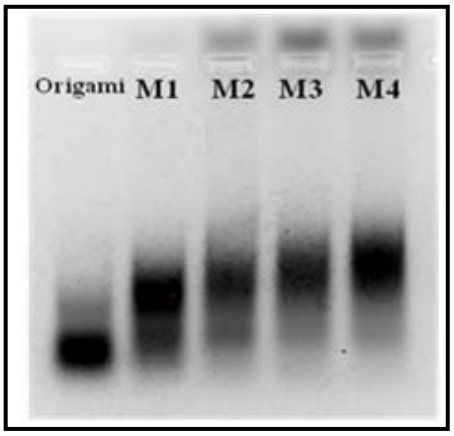


Figure 4.3. DNA origami nanostructures with and without morpholino. The first sample is the DNA origami without morpholinos. M1, M2, M3, and M4 are 1, 2, 3, and 4 morpholinos embedded DNA origami respectively.

4.1.2.2. Atomic Force Microscopy

To determine the formation of DNA origami structures, the prepared samples were analyzed using AFM. For AFM analysis, DNA origami samples were diluted 100 times to prevent formation of salt crystals on mica surface. Theoretically one edge of the tile shaped origami is 30 nm. In AFM images, size of some spots was consistent with the theoretical size. However, some of the particles had higher sizes than theoretical size. During sample preparation for AFM analysis (air-drying on mica surface), structures in bigger size than expected could occur because of aggregation of DNA origami samples. AFM images are shown in Figure 4.4, 4.5, and 4.6. The AFM analysis supported the agarose gel electrophoresis results and so formation of DNA origami structures.

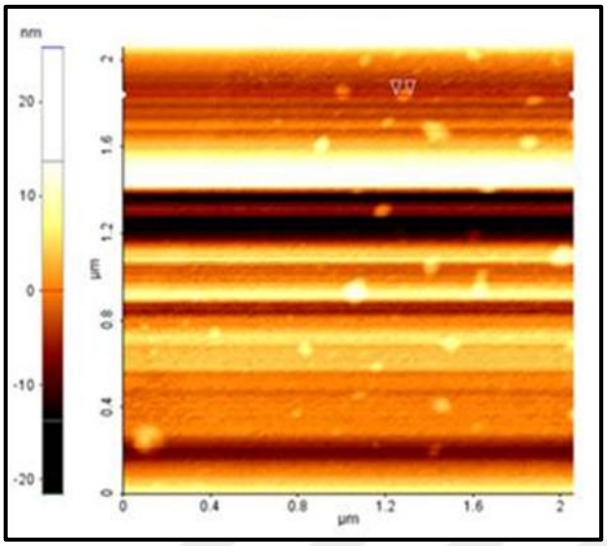


Figure 4.4. AFM image of droplet area belongs to suspension containing DNA origami (2 x 2 μm^2).

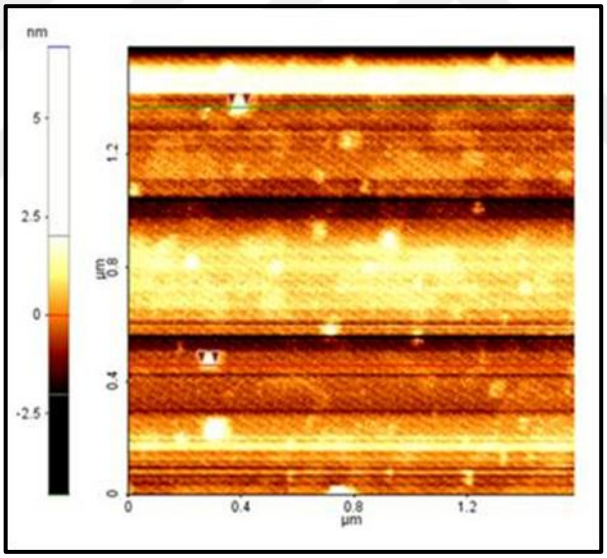


Figure 4.5. AFM image of droplet area belongs to suspension containing DNA origami (2 μm^2).

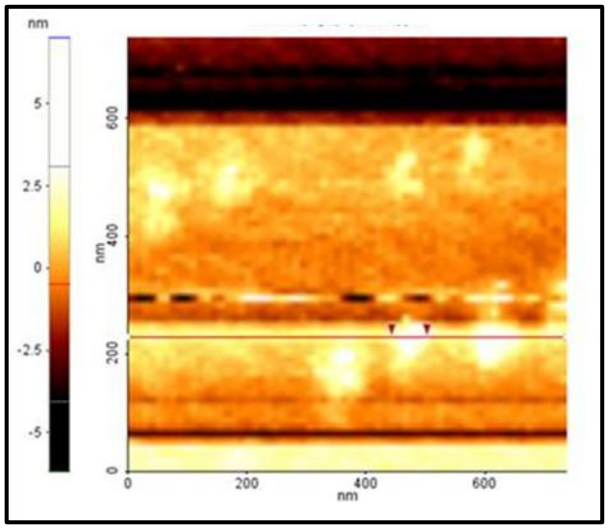


Figure 4.6. AFM image of droplet area belongs to suspension containing DNA origami (600 x 600 nm2).

4.2. PREPARATION OF DNA ORIGAMI-AuNPs NANOSTRUCTURES

It was reported that DNA modification of AuNPs increased internalization of the particles and provided the DNA higher resistance to nuclease digestion in serum [47]. In addition, when DNA delivered to cells using liposome, interferon-β level was pretty high, while AuNPs-DNA conjugates showed no immunological response. To increase intracellular uptake and for being biocompatible, DNA origami structures were functionalized with AuNPs. After the preparation and characterization of the DNA origami structures, they were modified with the Oligo-AuNPs through the sticky ends of the origamis. The AuNPs with 13 nm of average size were coated with thiol modified oligonucleotides, which are complementary to sticky ends of the origamis.

Size and concentration of AuNPs can be determined using UV/Vis spectroscopy [232]. Synthesized spherical AuNPs from 5 to 100 nm size were analyzed using TEM and UV/Vis spectroscopy and found that theoretical calculations from the spectra agrees with TEM results. Due to optical properties of AuNPs, spectra of these nanoparticles depends on their size and aggregation [233, 234]. UV/Vis spectroscopy allows fast and easy determination of size of AuNPs. Synthesized AuNPs, modification of the nanoparticles with oligonucleotides (Oligo-AuNPs) and preparation of DNA origami-AuNPs nanostructures were characterized using UV/Vis spectroscopy. UV/Vis spectra of prepared

nanostructures are shown in Figure 4.7. The maximum absorbance wavelength of 13 nm AuNPs containing suspension was 520 nm. Depend on the size increase, maximum absorbance of the oligonucleotide modified AuNPs and DNA origami-AuNPs nanostructures were shifted to longer wavelengths. With the oligonucleotide modification, the absorption maximum increased to 529 nm. When the absorbance spectrum of DNA origami-AuNPs was measured, it was found as at around 538 nm suggesting the formation of the targeted structure.

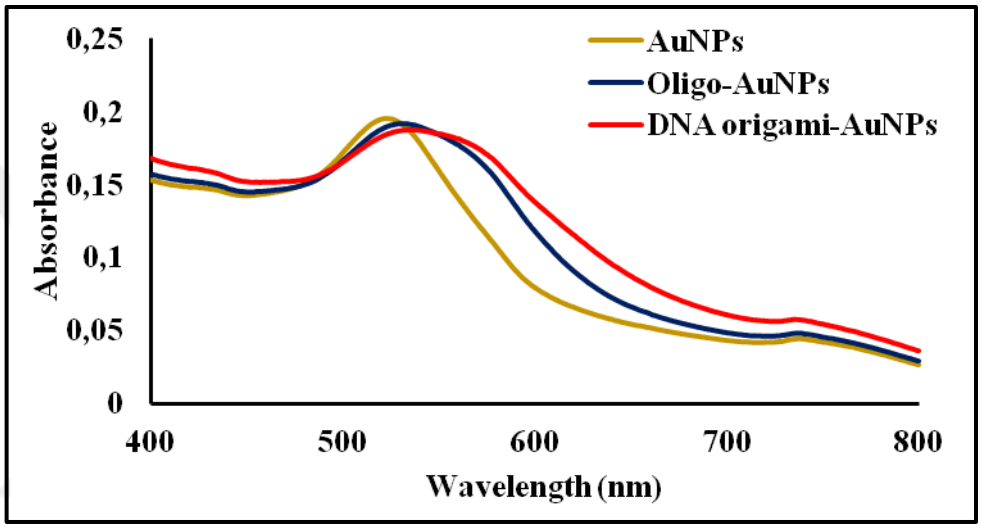


Figure 4.7. UV/Vis spectra of colloidal suspension of 13 nm AuNPs, Oligo-AuNPs, and DNA origami-AuNPs.

DLS is an analytical technique used for determination of hydrodynamic size of nanoparticles in aqueous media. DLS is highly convenient for determination of hydrodynamic size of AuNPs and its conjugates [235]. The hydrodynamic sizes of the AuNPs, Oligo-AuNPs and DNA origami-AuNPs nanostructures determined using DLS and are shown in Figure 4.8. The average hydrodynamic size of bare AuNPs was 13 nm. The average sizes of the Oligo-AuNPs and DNA origami-AuNPs observed with DLS were about 24 nm and 51 nm, respectively. As seen, an increase in hydrodynamic radius was observed from bare AuNPs to DNA origami-AuNPs supporting the results of UV/Vis spectroscopy and indicating the formation of aimed nanostructure.

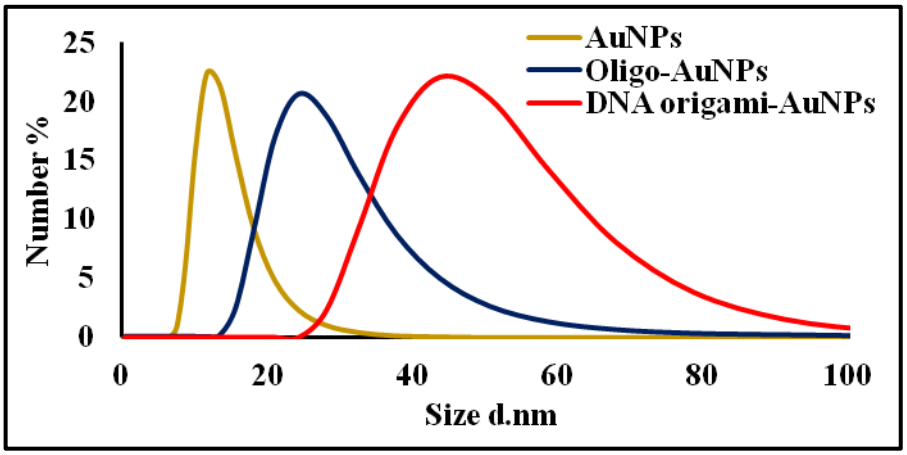


Figure 4.8. DLS spectra of AuNPs, Oligo-AuNPs, and DNA origami-AuNPs.

4.2.1. Stability Analysis of DNA Origami-AuNPs Nanostructures

Delivery systems are susceptible to dissociation in the extracellular environment. One of the major issues about a gene delivery system is to retain structural integrity until their cellular internalization to protect the silencing oligonucleotides from external effects. The success of a therapeutic depends on many factors including stability of delivery vehicles [236]. Thus, the stability of the DNA origami structures in PBS and in cell culture medium was studied. Figure 4.9 shows the gel image of DNA origami structures incubated in PBS. As it is seen from the image, the nanostructures stayed stable after the incubation intervals.

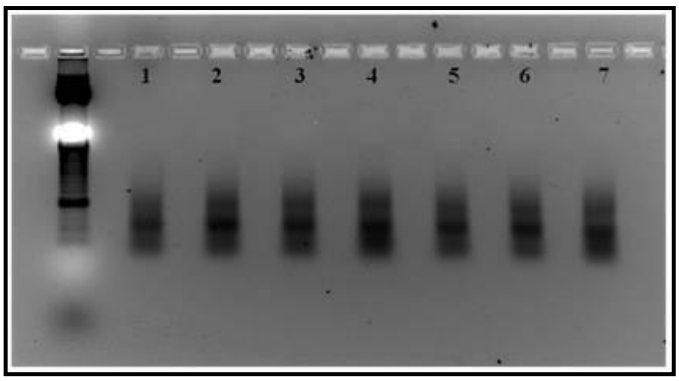


Figure 4.9. Agarose gel electrophoresis image of DNA origami incubated in PBS. The “1” is the control sample without incubation, 2, 3, 4, 5, 6, and 7 represent the origamis incubated for 4, 6, 8, 12, 24, and 48 hours.

After demonstrating DNA origami structures are stable in isotonic environment, their stability in blood serum containing medium was investigated. For this, the DNA origamis were incubated in a cell medium supplemented with 10 per cent FBS for 6, 24, 36, and 48 hours. After the indicated time intervals, the samples directly taken from the incubated environment and loaded onto agarose gel. Figure 4.10 shows the gel electrophoresis of the origamis recovered from the cell culture medium. As seen, there is no indication that the origamis are disassembled in cell culture medium as indicated by intact spots on the gel electrophoresis image. One can easily conclude that the origamis are stable in cell culture medium and structural integrity of DNA origami was remained up to 48 hours.

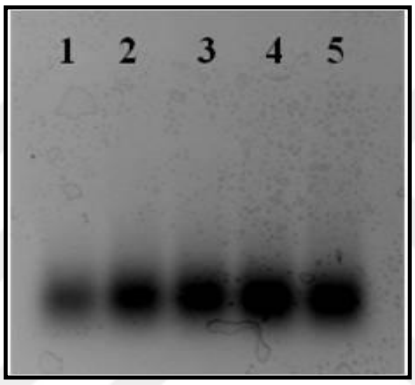


Figure 4.10. Agarose gel electrophoresis image of DNA origamis incubated in cell medium. The “1” is the control sample without incubation, 2, 3, 4, and 5 represent the origamis incubated for 6, 24, 36, and 48 hours.

4.3. CELL CULTURE EXPERIMENTS

For gene silencing studies, breast cancer was selected as model and 4 different genes related to breast cancer cell proliferation were determined. These are HER2, EGFR, Ki-67, and ERα genes, which are highly expressed in many cancer cells. In scope of cell culture studies, 3 different breast cancer cell lines were purchased; BT-474, MDA-MB-231 and MCF7. BT-474 cell line has high expression levels of all the 4 selected genes and especially HER2 is overexpressed in this cell line. The BT-474 cell line was used in the silencing of the HER2 oncogene, which had the highest expression, was also used in multi-gene silencing studies. Other cell lines MDA-MB-231 and MCF7 were used in the suppression of Ki-67 and ERα genes in which they had the highest expression levels, respectively. On the other hand, MDA-MB-231 is a triple negative resistant cell line and

was used as negative control in some experiments. Expression profiles of the cell lines in terms of the selected genes are shown in Table 4.1.

Table 4.1. Expression levels of HER2, EGFR, Ki-67, and ERα genes in MDA_MB_231, MCF-7 and BT-474 cell lines [237].

	HER2	**EGFR**	**Ki-67 (%)**	**ERα**
MDA_MB_231	Low	Positive (+1)	100	Negative(0)
BT-474	High	Positive (+1)	70	Positive(+1)
MCF-7	Low	Positive (+1)	90	Positive(+6)

4.3.1. Toxicity of DNA Origami-AuNPs Nanostructures

There have been many studies to provide effective delivery of antisense oligos into cells. Most the delivery vehicles have been successful although their biggest limitation was toxicity [231]. Safety and bioavailability of a delivery vehicle is essential for its success. Thus, toxicity of our proposed nanocarrier was investigated on healthy and cancer cells. The effect of morpholino embedded DNA origami-AuNPs nanostructures on cell viability was determined using WST-1 test. Morpholino oligos were designed against HER2, EGFR, Ki-67 and ERα genes and custom made by Gene Tools. The purchased morpholinos were integrated into DNA origami structure. The toxicities of DNA origami structures with and without morpholinos were determined. Figure 4.11 shows the viability of MDA-MB-231 breast cancer cells treated with DNA origami structures without any silencing oligo. It was seen that DNA origami structures, which did not contain morpholino oligos, did not have any effect on cell viability. The prepared nanosturctures were non-toxic and biocompatible.

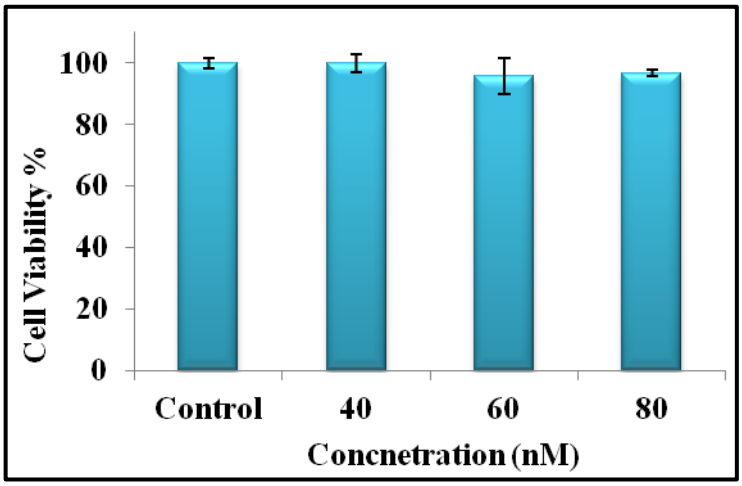


Figure 4.11. Viability of MDA-MB-231 cells treated with DNA origami-AuNPs structures without morpholino oligos.

The toxicity of morpholino embedded DNA origami-AuNPs nanostructures were investigated on both breast cancer and healthy breast cells. For this, MDA-MB-231 cells, which are HER2 negative, were treated with morpholino embedded DNA orgami-AuNPs designed against HER2. The viability of the cells was determined using WST-1. As it is seen in Figure 4.12, the viability of cells treated with the gene silencing system did not decrease and no toxic effect was observed.

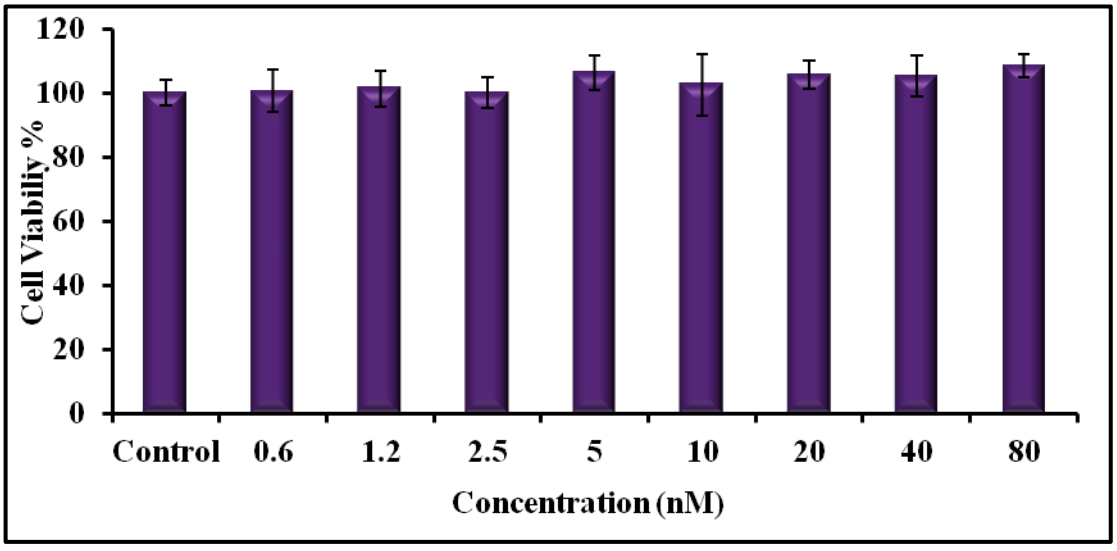


Figure 4.12. Viability of MDA-MB-231 breast cancer cells treated with morpholino embedded DNA origami-AuNPs against HER2 oncogene.

The toxicity of morpholino embedded DNA origami-AuNPs nanostructures was also investigated on healthy cells. For this, non-tumorigenic breast tissue cells were used. After treatment with morpholino embedded DNA origami-AuNPs against ERα gene, viability of MCF-10A healthy cells were determined using WST-1 and showed in Figure 4.13. It was demonstrated that the designed gene silencing system did not have any toxic effect on the tested health cells.

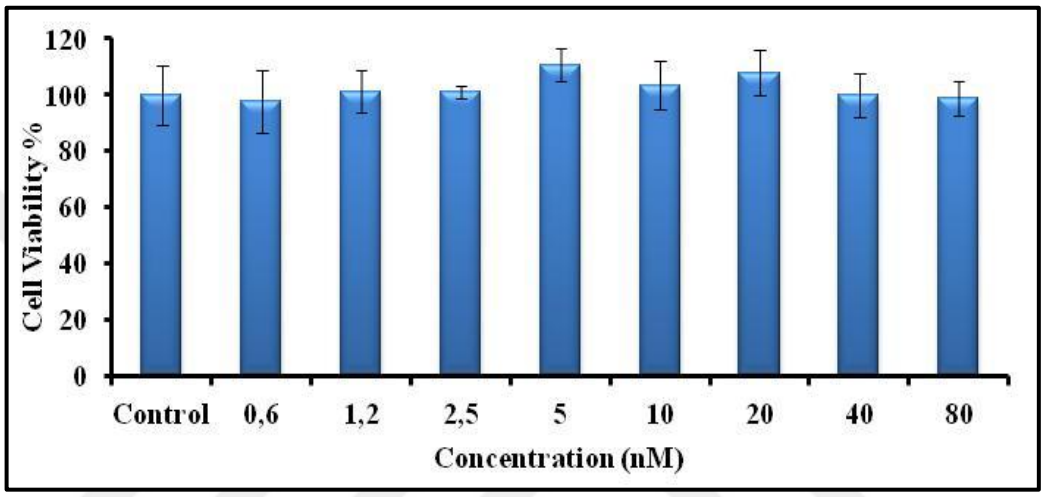


Figure 4.13. Viability of MCF-10A healthy breast cells treated with morholino embedded DNA origami-AuNPs against ERα gene.

4.3.2. Cellular Internalization and Localization of DNA Origami-AuNPs

Cellular internalization of a delivery system is important for function of their cargos at target location. The studies showed that DNA nanostructures managed to enter cells through endosytosis without requirement of any transfection reagents [238]. There are some studies demonstrating DNA nanostructures internalized via spesific receptor mediated endocytosis [239-241]. The internalization and intracellular localization of DNA origami-AuNPs nanostructures were investigated by confocal microscopy. For this, one of staple strands (P10) used for DNA origami construction was provided with Cy5 modification. The DNA origami structures were prepared with Cy5 modified oligonucleotides and the breast cancer cells were treated with fluorescence labeled DNA origami-AuNPs. After 30 min incubation with DNA nanostructures, endosomes and lysosomes were stained with lysotracker green, a dye molecule. Following the washing steps, the cells were analyzed with confocal microscopy and the images are shown in

Figure 4.14. The endosomes are seen as green and the DNA origami-AuNPs nanostructures are seen as blue. In the merged images, it can be seen that some of the DNA origami-AuNPs carriers were inside of the endosomes (the spots blue and green colors overlap) and some of them located in cytoplasm (the blue spots shown with arrow mark). The images showed that DNA origami-AuNPs were internalized through endocytosis and some of the nanostructures managed to escape from endosomes and reach to cytoplasm, where their site of action.

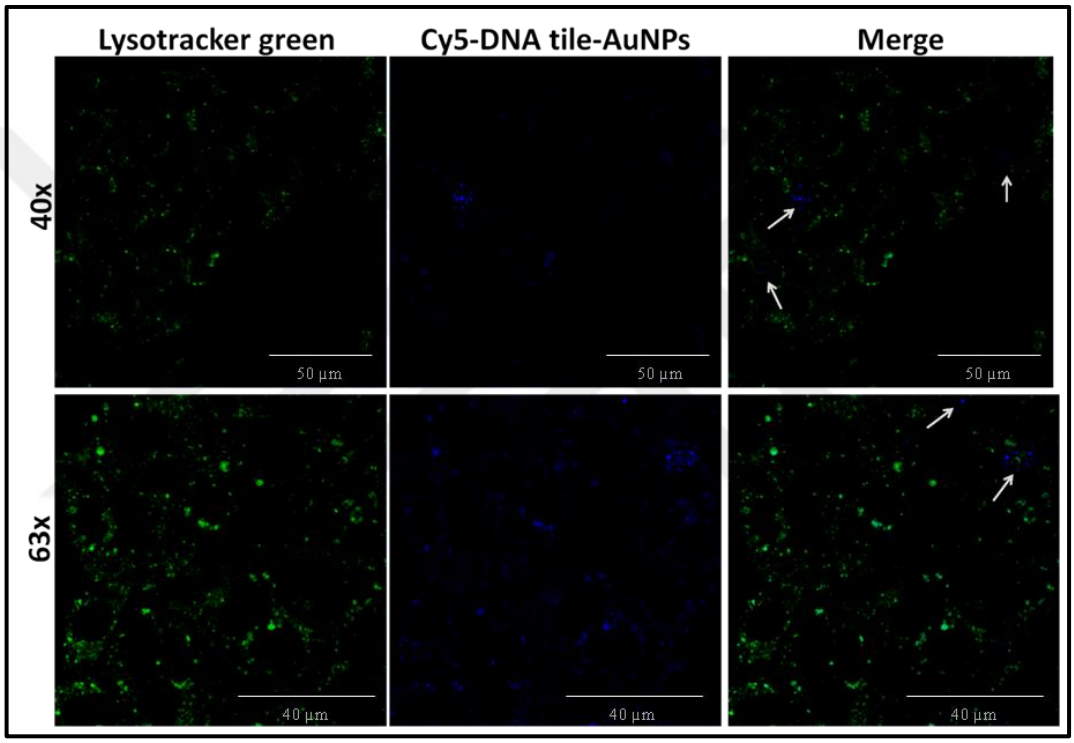


Figure 4.14. Intracellular localization and endosomal escape of DNA origami-AuNPs nanostructures.

4.3.3. Gene Silencing Studies

Alteration in genetic status of cells has been an effective approach for treatment of genetic-based or infectious-triggered diseases and makes possible personalized treatment. Many types of antisense oligonucleotides were developed for regulation of target genes in cells [242]. However, under normal conditions these oligonucleotides are unsuccessful to enter into cytosol or nucleus [231]. Delivery of the antisense oligonucleotides, which are analogs of DNA or RNA, is limited with many delivery systems. On the other hand, these

oligonucleotides can be easily integrated into DNA nanostructure simply through hybridization for delivery. The DNA origami-AuNPs nanostructures were proposed for delivery of morpholino oligos to breast cancer cells. A systematic study was designed to investigate the gene silencing features of DNA origami-AuNPs nanostructures. First, nanocarriers were prepared for silencing of single gene. Morpholino sequences specific for four target genes were embedded to DNA origami structures individually. The prepared carriers for single gene silencing were represented schematically in Figure 4.15.

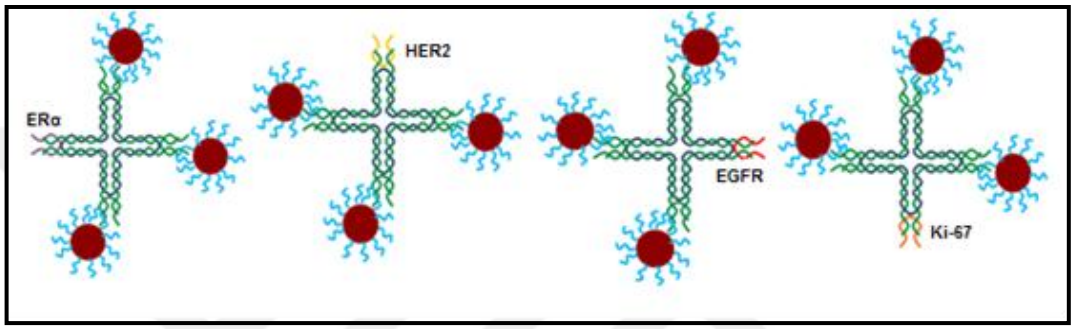


Figure 4.15. Schematic representation of DNA origami-AuNPs nanostructures designed for single gene silencing.

Breast cancer cells were treated with different concentrations of DNA origami-AuNPs nanostructures and an appropriate concentration was determined for further experiments. GAPDH, a house keeping gene, was used as control of equal protein loading. Expression levels of HER2 gene in BT-474 cells and level of ERα gene in MCF7 cells were determined after treatment with DNA origami-AuNPs nanostructures targeting these genes. The immunoblot results are shown in Figure 4.16. The data showed that even in very low morpholino concentrations such as 20 nM, DNA origami-AuNPs carriers effectively decreased the target gene expressions. In addition, the gene silencing efficiency was increased in a dose dependent manner. The designed nanocarriers can be used for silencing of target genes to obtain high silencing rates as the nanostructure is non-toxic. Further experiments were conducted at 30 nM concentration of morpholino, which provided approximately 50 per cent gene silencing.

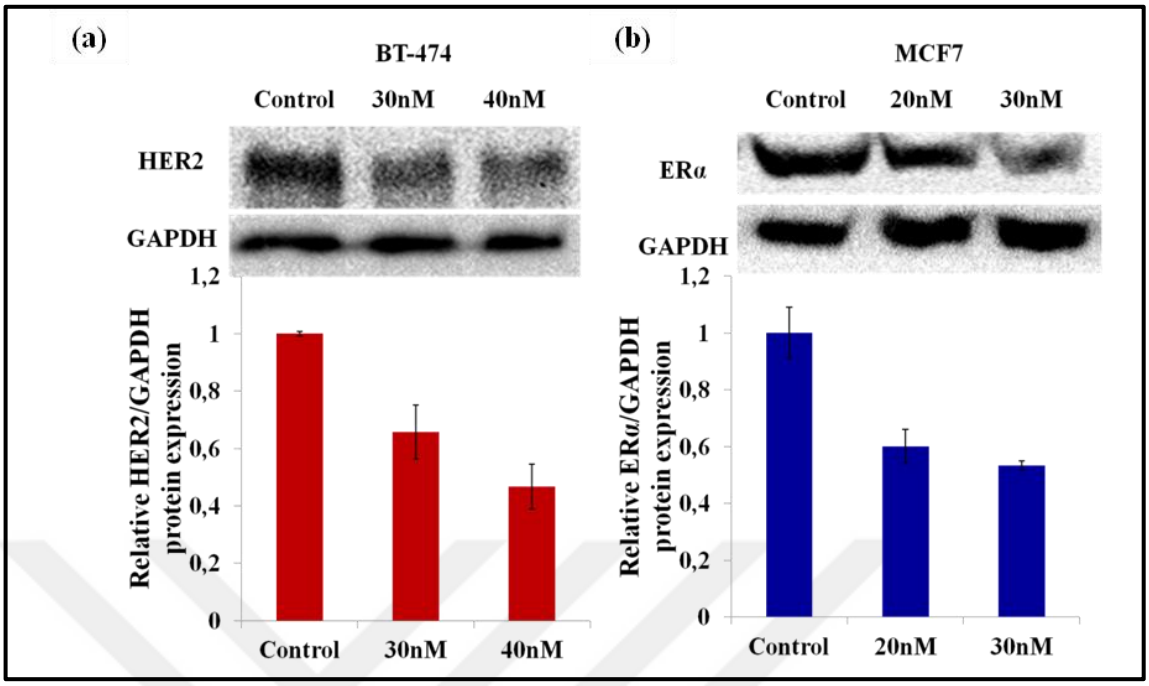


Figure 4.16. Relative protein expression levels of (a) HER2 and (b) ERα genes after treatment with morpholino embdedded DNA origami-AuNPs nanostructures in BT-474 and MCF7 cells, respectively.

To understand if morpholino oligonucleotides have any effect on gene expression without any carrier system, MCF7 cells were treated with bare morpholinos against ERα gene and the intracellular protein level was determined using immunoblotting. The Western Blot image is shown in Figure 4.17. It was determined that morpholino embedded DNA origami-AuNPs carrier system decreased the target gene expression level considerably while the target protein level remain unchanged when the cells were treated with bare morpholino oligos. The results showed that morpholinos had no effect on gene expression without any delivery system.

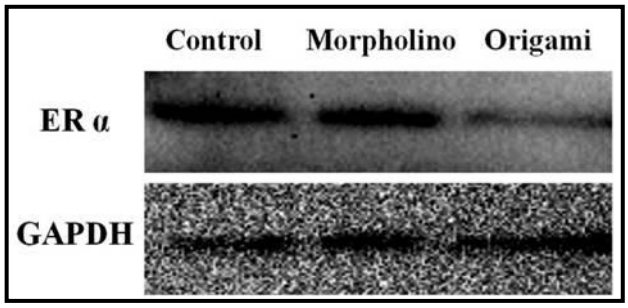


Figure 4.17. ERα protein level in MCF7 cells treated with bare morpholino and morpholino embedded DNA origami-AuNPs.

The gene silencing efficiency of the designed DNA origami-AuNPs nanostructures, which showed gene silencing activity even at low concentrations, were evaluated comparatively with the silencing efficiency of a liposomal delivery system. Morpholino antisense oligos were packed with a liposome system commercially available in the market and the cells were treated with liposome-packed morpholinos in parallel with morpholino embedded DNA origami-AuNPs at same concentrations. HER2 and ERα were targeted in BT-474 and MCF7 cells, respectively. Morpholino oligos specific for the target genes were delivered using both liposome and DNA origami-AuNPs systems and target protein levels were determined after 24h treatment. Western blotting images and the gene expression analysis are shown in Figure 4.18. It was observed that silencing efficiency of the cationic liposome was variable and DNA origami-AuNPs carriers inhibited the targeted protein expressions approximately 30 per cent more effective than the liposome.

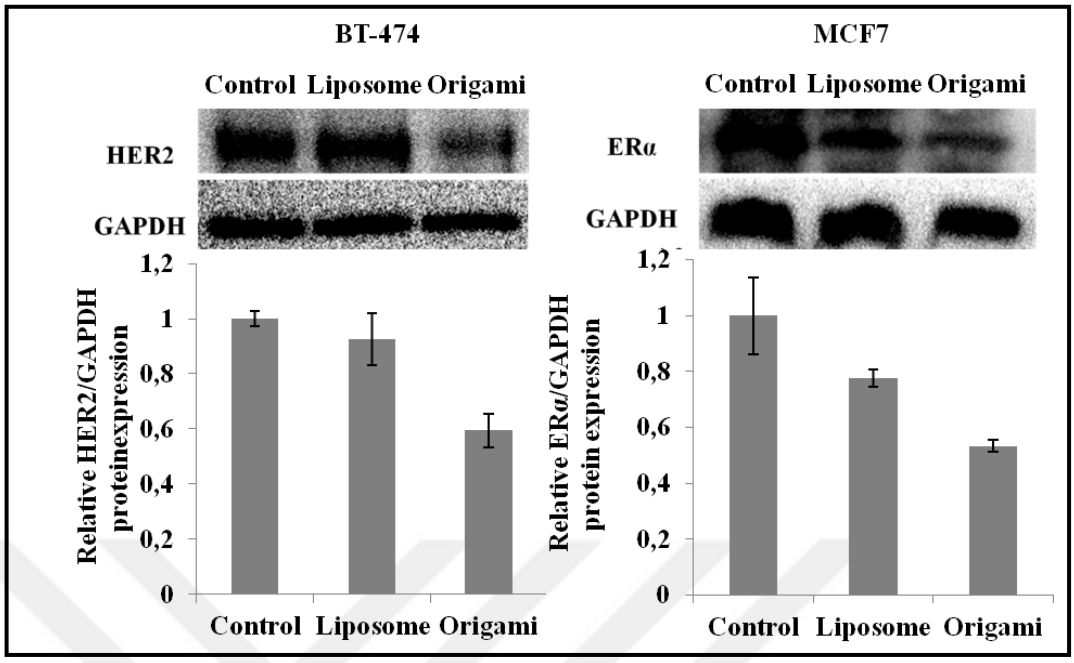


Figure 4.18. Changes in HER2 expression in BT-474 cells and ERα expression in MCF7cells after silencing using liposome and DNA origami-AuNPs at 30nM morpholino concentration.

Cationic liposomes have hydrophilic head groups containing one or more amine (NH_2) groups, which provide cationic character [133]. These kinds of cationic delivery systems interact with their DNA or RNA cargos electrostatically through negatively charged phosphate groups of the nucleic acid molecules [131]. Morpholino oligonucletides are nuclease resistant, neutral oligonucleotides, which contain phosphorodiamidate groups in their backbone instead of phosphate groups [19]. Because of their neutral structure, interaction of morpholinos with cationic liposomes and polymers are insufficient [25]. Thus, delivery of morpholino oligos with cationic delivery systems is unsatisfactory. In this thesis, DNA origami-AuNPs nanostructures were used for delivery of morpholinos and efficient internalization and gene silencing were obtained.

Gene silencing using morpholino embedded DNA origami-AuNPs nanostructures was demonstrated also by using confocal microscopy. BT-474 cells were treated with the nanocarriers for silencing of HER2, a surface receptor protein. After inhibition of the gene expression, the cells were incubated with fluorescence-labeled antibodies and analyzed with confocal microscopy. In Figure 4.19, confocal image of BT-474 cells after 48h incubation is shown. HER2 proteins are labeled with Alexafluor 647 antibody (red), and

cell nucleus was labeled with DAPI (blue). Control is the cells incubated with only cell medium, morpholino is only morpholino oligos without any carrier. The controls showed high luminicence while morpholino delivered cells using liposome and DNA origami showed slightly decreased brightness in red fluorescence indicating gene silencing.

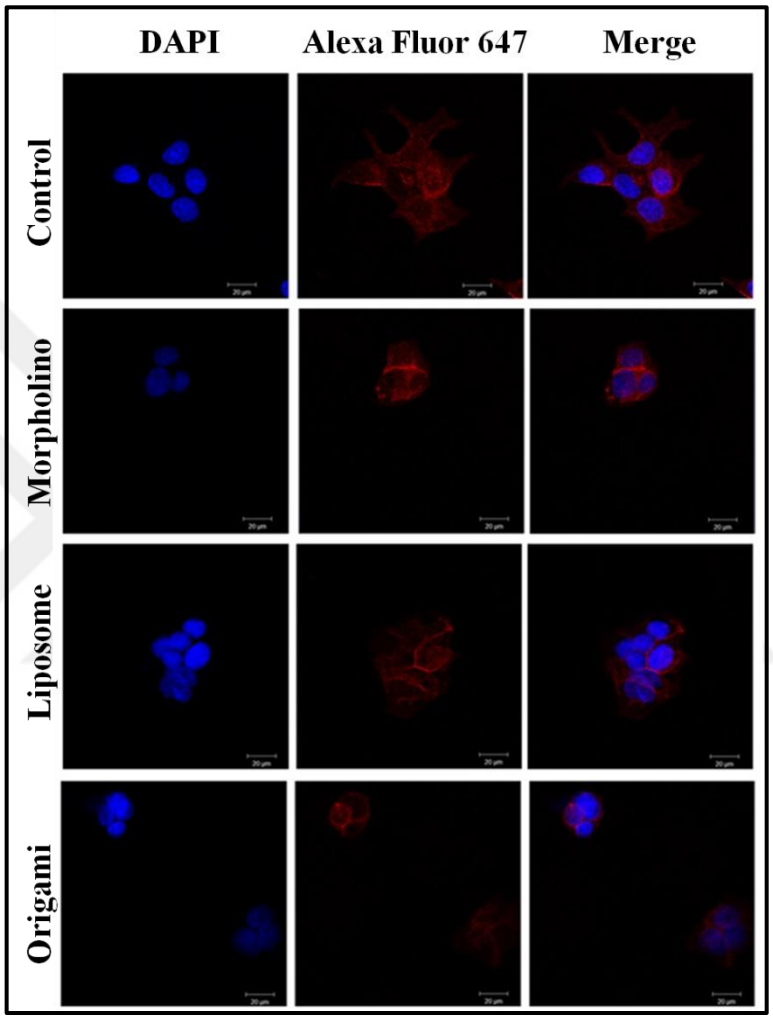


Figure 4.19. Confocal images of BT-474 cells after silencing of HER2 gene.

Targeted genes have more than one transcript variants and the morholino oligonucleotides were designed against the mostly produced transcript variant and complementary to sequences common in all mRNAs if it is possible. The designed morpholino oligos effectively silenced the HER2 and ERα genes, To determine the effectiveness of morholino sequences designed for silencing of EGFR and Ki-67 genes, BT-474 and MDA-MB-231 cells were treated with morpholino embedded DNA origami-AuNPs, respectively and the decrease in the target protein expression was showed using immunoblotting. The Western blot images are shown in Figure 4.20. According to the findings, all 4 morpholino

sequences designed to use in this thesis were effective for silencing of their target genes in the selected cell lines and specific for their targets indicating the successful design of morpholino antisense oligonucleotides.

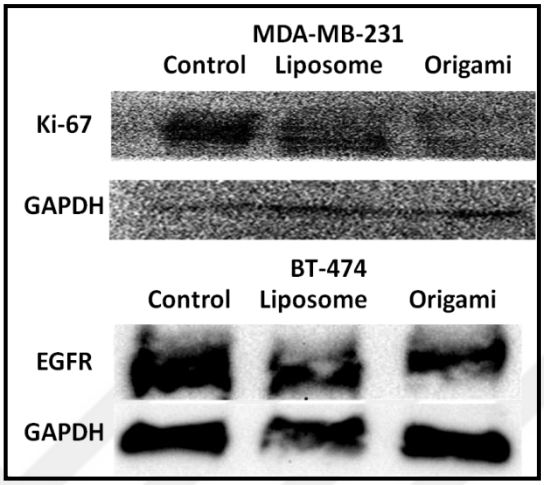


Figure 4.20. Changes in Ki-67 expression in MDA-MB-231 cells and EGFR expression in BT-474 cells after silencing using liposome and DNA origami-AuNPs at 30nM morpholino concentration.

4.3.4. Inhibiton of Cancer Cell Proliferation

For the proof of concept experiments, breast cancer was selected as model and 4 different genes, which are related with breast cancer growth and proliferation, were chosen for regulation study. Aberrant expression of ER is a predictive marker for breast cancer. Although hormone receptor positive cancer types are mostly sensitive to hormone therapies, 25-50 per cent of these tumors show resistance to the hormone treatment [243]. Estrogen hormone triggers mitosis in breast cancers, which contains ER [244, 245]. Most of the primary breast cancers are ERα positive and needs estrogens for tumor growth. Estrogens act through ERs and increase cell division. As a result, estrogens shows mitogenic activity in ER+ cells [246] and ERs has an significant role in cancer progression. Inhibiting ERα expression in breast cancer cells possibly will inhibit the mitogenic activity of estrogen hormone.

One of the other predictive markers of breast cancer is HER2. About 25-30 per cent of breast cancers were determined to have abnormal HER2 expression [247]. The expression

of these biomarkers determines personalized treatment practices in clinic. A patient with HER2 positive tumor usually respond to HER2 targeted therapy trastuzumab, which decrease proliferation of HER2 positive cancer cells through degradation of the protein [248]. However HER2 overexpressing tumors are not always sensitive to trastuzumab and some tumors develop resistance to this therapy. Breast cancer cells manage to develop a mechanism against the antiproliferative effect of the drug [249] so new strategies are needed to inhibit excessive cell division of HER2 positive breast cancer cells.

Ki-67 is a proliferation marker and studies with invasive ductal carcinoma patients showed that Ki-67 expression signifies poor prognosis and associated with expression of some predictive molecular markers such as estrogen receptors, HER2 and EGFR [250]. The majority of the markers, including Ki-67, determine malignant grow characteristic of cells [251]. Expression of Ki-67 in breast cancer malignancies refers poor outcome in patients [252]. Inhibition of Ki-67 gene caused inhibition of cancer cell proliferation and growth, increase of apoptotic cell rates in both *in vitro* and *in vivo* studies [211, 253, 254].

Effect of suppression of these genes in their overexpressing cell lines on cell proliferation was investigated. Morpholinos designed against HER2, ERα and Ki-67 genes were delivered to breast cancer cells using DNA origami-AuNPs nanostructures and effect of gene silencing on proliferation rates of the cells were determined. For this, treated cells were cultured for 72h for proliferation. At the end of this time, cells were detached, living cells were counted and the proliferation rates of the cells after gene silencing were determined. As seen in Figures 4.21, 4.22 and 4.23, after silencing of HER2 gene in BT-474 cells, ERα gene in MCF7 cells, and Ki-67 gene in MDA-MB-231 cells using DNA origami-AuNPs, cancer cell proliferation rates decreased. Silencing of HER2, ERα and Ki-67 genes led approximately 55 per cent, 30 per cent and 45 per cent inhibition in cancer cell proliferation, respectively.

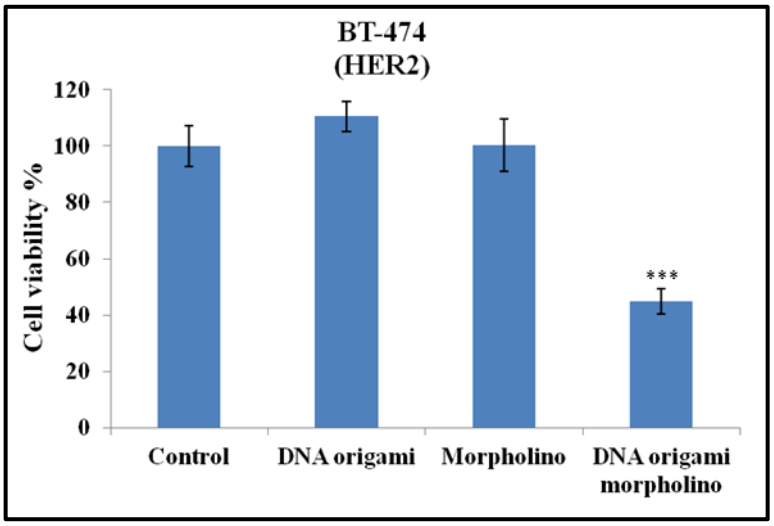


Figure 4.21. Effect of silencing of HER2 gene on proliferation of BT-474 cells. P value represents ***P<0.001.

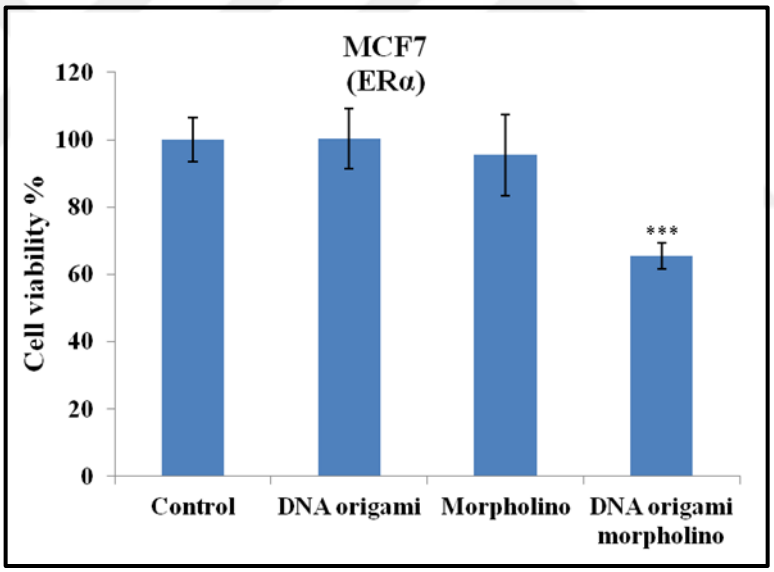


Figure 4.22. Effect of silencing of ERα gene on proliferation of MCF7 cells. P value represents ***P<0.001.

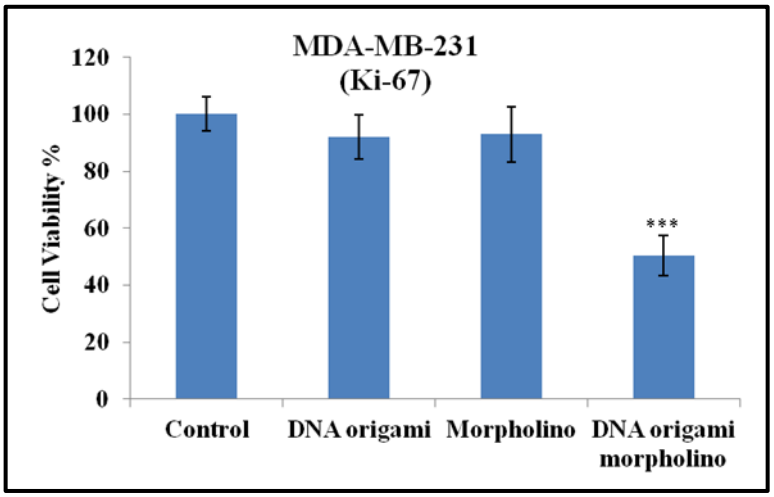


Figure 4.23. Effect of silencing of Ki-67 gene on proliferation of MDA-MB-231 cells. P value represents ***P<0.001.

4.3.5. Cell Cycle Phase Distribution Analysis

After determining effect of gene silencing on cell proliferation, changes in cell cycle phase distribution of cells were investigated. In previous studies, proportion of cells at G_0/G_1 phases was increase as a result of HER2 silencing [255]. Overexpression of ERα gene in breast cancer cells resulted increase number of cells in S and G_2/M phases. [256]. Estrogens act through ERs and increase cell division. This hormone drive inactive (G_0) cells into active cell cycle, accelerate G_1 phase span so reduce the total cell cycle duration [257, 258]. The effect of estrogen on cell cycle also reported *in vivo* [259]. A decrease in cell proportion at these phases was expected after silencing of ERα. Ki-67 is a proliferation marker and expressed in all phases of cell cycle but G_0. The knowledge about exact function of Ki-67 is scarce but it is thought to has a role in couple of cellular functions including cell cycle progression [260]. It is known that the protein has a role in cancer proliferation and exists in all active cell cycle phases [261]. It is likely for cacer cells to enter G_0 phase after silencing of this gene. After delivery of morholinos designed for silencing of these genes using DNA origami-AuNPs nanostructures, cell cycle phase distribusions of each overexpressing cell line were determined.

Compared to the untreated control cells, an increase in G_0/G_1 phase was observed in all treatment groups. The cell cycle phase distributions of breast cancer cells after silencing of HER2, ERα and Ki-67 genes are shown in Figures 4.24, 4.25 and 4.26, respectively. The

suppression of HER2 gene caused a decrease mostly at G_2/M phases in BT-474 cells. A proportion of cells at S phase was decreased after the silencing of ERα gene in MCF7 cells while silencing of Ki-67 led to a decrease at both S and G_2/M phases in MDA-MB-231 cells.

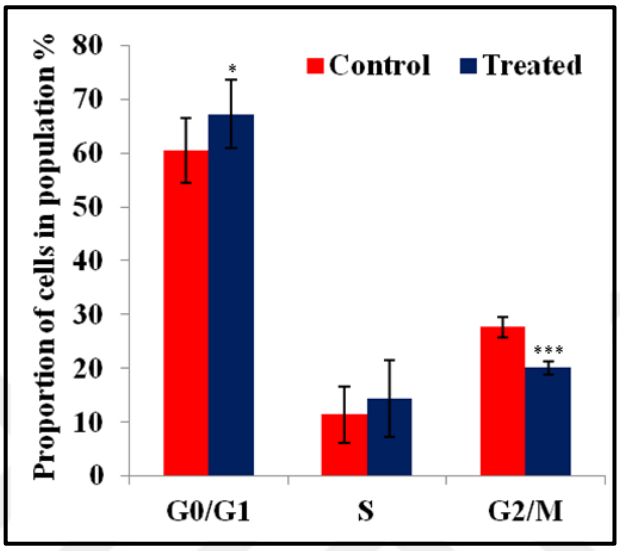


Figure 4.24. Cell cycle phase distributions of BT-474 cells. Control is untreated cancer cells, Treated is the cells treated with morpholino embedded DNA origami-AuNPs nanostructures against HER2 gene. P value represents *P<0.05; ***P<0.001.

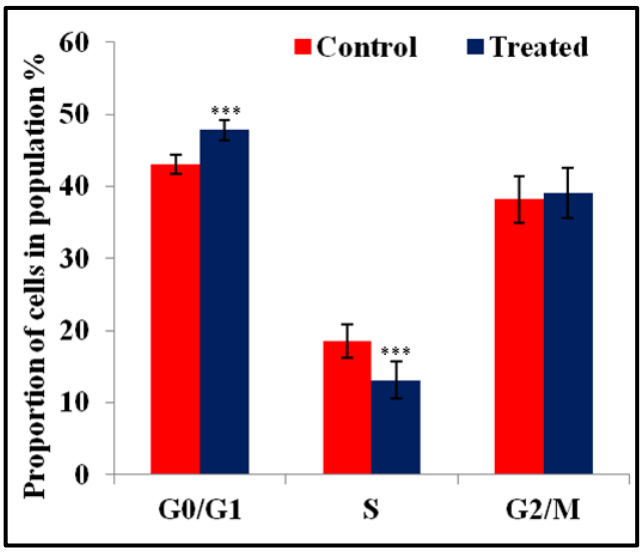


Figure 4.25. Cell cycle phase distributions of MCF7 cells. Control is untreated cancer cells, Treated is the cells treated with morpholino embedded DNA origami-AuNPs nanostructures against ERα gene. P value represents ***P<0.001.

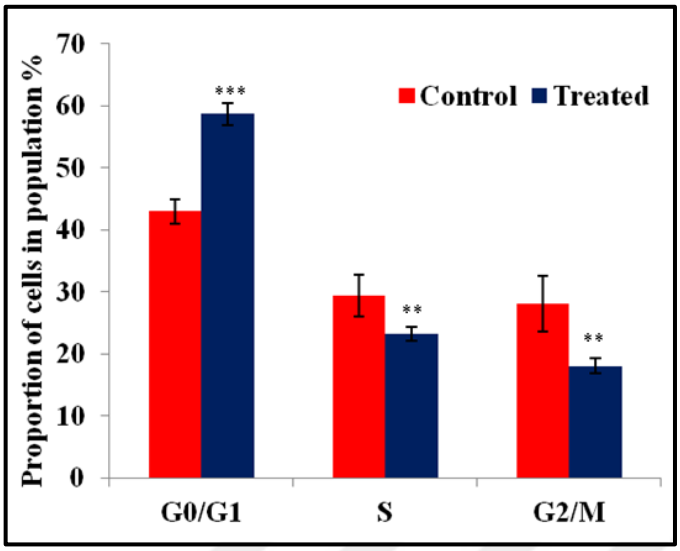


Figure 4.26. Cell cycle phase distributions of MDA-MB-231 cells. Control is untreated cancer cells, Treated is the cells treated with morpholino embedded DNA origami-AuNPs nanostructures against Ki-67 gene. P value represents **P<0.01; ***P<0.001.

The results of the cell cycle analysis showed that silencing of the target genes induced changes in phase distributions and the obtained data was consistent with the cancer cell proliferation analysis results. The decrease in S and G2/M phases was obtained following the silencing of HER2 and ERα genes and the results of flow cytometry analysis were confirmed by determination of cyclin B1 expression level by Western blotting. Cyclin B1 is a protein, which has a role in cell cycle progression. The Western, blotting image is shown in Figure 4.27. The decrease in cyclin B1 expression after silencing of HER2 and ERα genes confirmed the cell cycle analysis by flow cytometry.

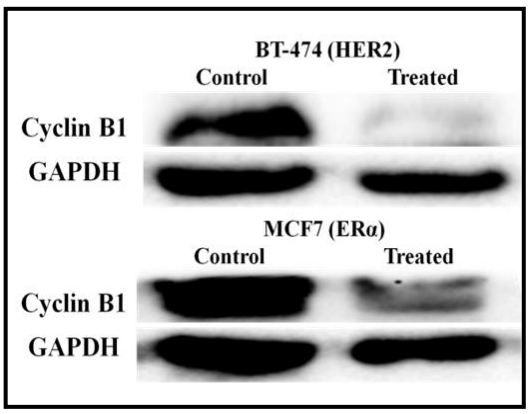


Figure 4.27. Expression level of cyclin B1 protein in BT-474 and MCF7 cells after silencing of HER2 and ERα genes, respectively.

4.3.6. Multi-Gene Regulation

Gene silencing is a sophisticated strategy for treatment of gene-related diseases. There have been many clinical trials of gene silencing therapeutics targeting cancer and viruses [262]. Inhibiting more than one gene at the same time was demonstrated as an effective cancer treatment strategy due to simultaneous inhibition of multiple pathways [263]. Also multiple locations in virus genome was targeted with RNAi for treatment of infectious caused diseases [264, 265] In gene silencing therapies, it is preferred to target multiple genes for synergistic effect and prevention of resistance to therapeutics [266]. Delivery of multiple siRNAs is mostly a requirement as viruses could develop rapid mutations for resistance when single gene was targeted by RNAi [267]. On the other hand, multiple gene-targeting is necessary for treatment of cancer types caused by abnormal expression of more than one gene. In multi-gene regulation studies, DNA origami structures harboring more than one morpholino oligos were used for silencing of multiple genes simultaneously. Silencing of 2, 3 and 4 different genes were attempted. Initially, simultaneous silencing of 2 genes was achieved. For this, DNA origami structures containing two morpholinos were prepared. In these experiments, HER2-ERα and EGFR-Ki-67 couples were targeted. The DNA origami-AuNPs nanostructures designed and prepared for 2-gene targeting are schematically shown in Figure 4.28. Breast cancer cells were treated with the multi-gene regulating DNA orgami-AuNPs nanosystems and the expression levels of the silenced genes were determined using Western blotting after 24h incubation.

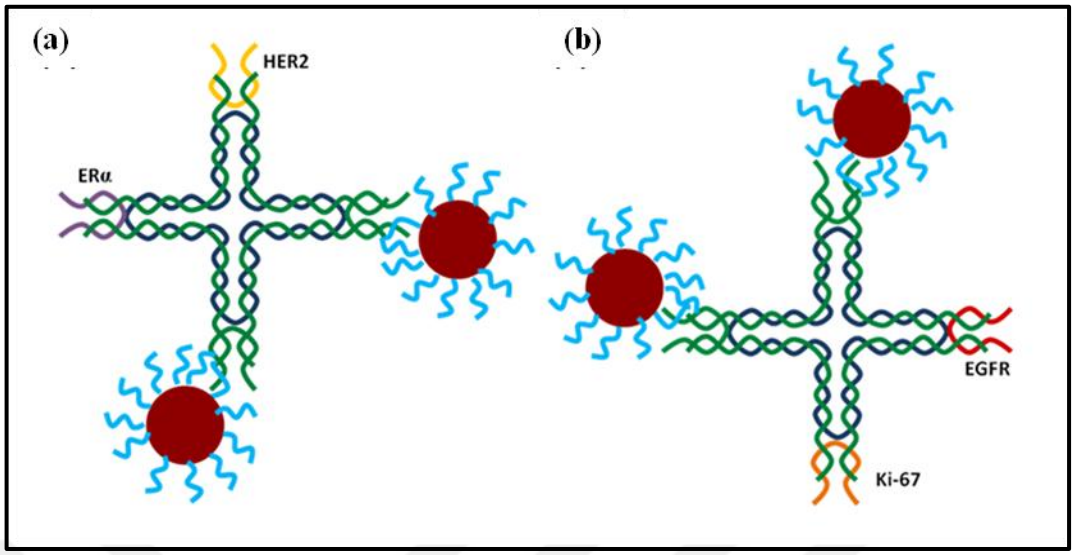


Figure 4.28. DNA origami-AuNPs nanostructures used for multi-gene regulation studies (a) the nanostructure used for simultaneous silencing of ERα and HER2 genesand (b) the nanostructure used for simultaneous silencing of EGFR and Ki-67 genes.

The expression levels of ERα and HER2 genes following their simultaneous silencing in MCF7 and BT-474 cells are shown in Figure 4.29. DNA origami-AuNPs carriers containing both HER2 targeting morholino and ERα targeting morpholino were used for gene silencing. Together with that, these genes were targeted individually by using DNA origami-AuNPs and liposome. Single morpholino containing nanocarrier was named as M1, 2 morpholinos containing nanocarrier was called M2. In multi-gene regulation strudies, control cells were treated with DNA origami structures containing morpholinos, which are non-complementary to human genome. The results after silencing the target genes alone and in combination showed that simultaneous silencing increased the efficiency of inhibition of gene expression in both genes.

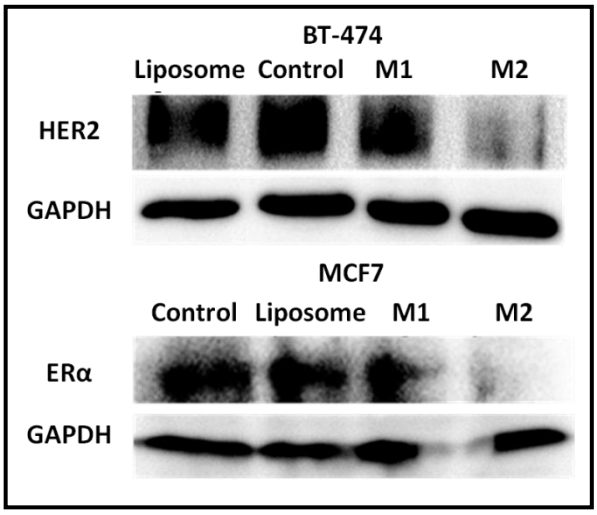


Figure 4.29. Protein expression levels of HER2 and ERα in BT-474 and MCF7 cells, respectively.

Also EGFR and Ki-67 genes were simultaneously targeted using DNA origami-AuNPs nanostructures. BT-474 cells were treated with the nanocarrier system for inhibition of these genes. The protein expression analyses after silencing of EGFR and Ki-67 simultaneously are shown in Figure 4.30. At the beginning of the study, DNA origami-AuNPs nanostructures inhibited target gene expressions approximately 50 per cent. The Western, blotting analysis showed that multiple silencing of genes induced about 80 per cent decrease in protein levels. It was demonstrated that multi-gene regulation increased gene silencing efficiency of all targeted genes in all tested cells and DNA origami-AuNPs nanostructures are very suitable systems for multi-gene regulation.

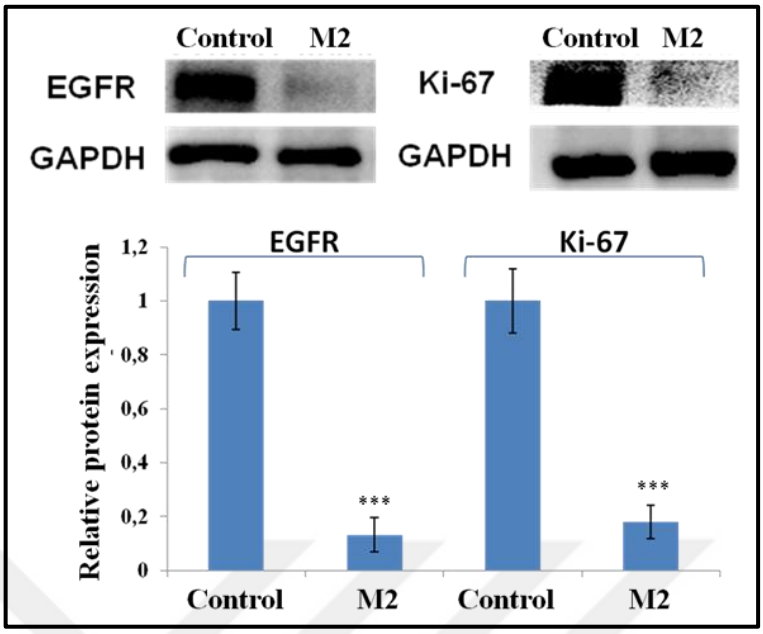


Figure 4.30. Expression levels of EGFR and Ki-67 genes in BT-474 cells after multi-gene regulation. P value represents ***P<0.001.

Two genes were targeted at the same time for the multi-gene regulation studies and simultaneous silencing of 3 and 4 genes were carried out afterward. Successful integration of 1, 2, 3 and 4 morpholinos into DNA origami structure was demonstrated by agarose gel electrophoresis and effective silencing of multiple genes by using these DNA origami structures was showed with immunoblotting. Expression levels of EGFR protein after silencing only EGFR (M1), simultaneous silencing of EGFR and Ki-67 (M2), and silencing of EGFR, Ki-67 and ERα genes simultaneously (M3) are shown in Figure 4.31. The data showed that regulation of multiple genes synergistically increased the silencing efficiency.

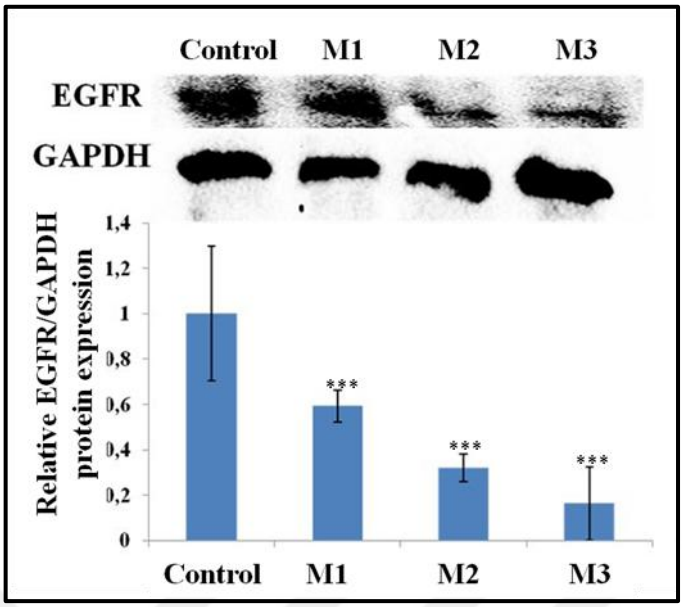


Figure 4.31. Expression levels of EGFR gene after silencing of 1, 2 and 3 different genes simultaneously. P value represents *P<0.05; **P<0.01; ***P<0.001.

4.3.6.1. Inhibition of Proliferation after Multi-Gene Regulation

The delivey of more than one silencing agents resulted in enhanced inhibition on target genes expressions [266]. When it is compared to single gene silencing, multi-targeted inhibiton had increased therapeutic efficiency in cancer therapy. Silencing of the target genes were determined to decrease proliferation of the breast cancer cells. To understand effect of multi-gene regulation on cancer cell proliferation, proliferation rates of BT-474 cells were determined following simultaneous knockdown of EGFR and Ki-67 genes. The cells were treated with single morpholino embedded DNA origami-AuNPs against EGFR, single morpholino embedded DNA origami-AuNPs against Ki-67, and DNA origami-AuNPs containing 2 morpholinos for silencing of both genes. At the end of 72h incubation, the cells were counted and the proliferation rates were determined according to control group. The viability of the cells treated with DNA origami-AuNPs gene regulating systems was shown in Figure 4.32.

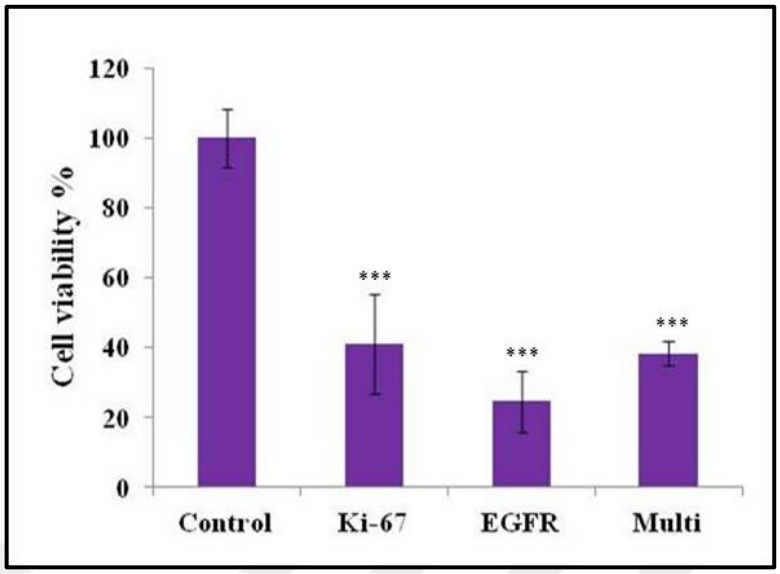


Figure 4.32. Viability of BT-474 cells after multi-regulation of Ki-67 and EGFR genes. P value represents ***P<0.001.

To understand the effects of multi-gene regulation, 1, 2, 3, and 4 morpholinos designed for silencing of different genes were incorporated into DNA origami-AuNPs nanostructures and BT-474 cells, which is positive for all the target genes, were treated with single and multiple morpholino embedded nanocarriers then their proliferation rates were determined. M1 contains EGFR morpholino, M2 and M3 contains EGFR+Ki-67 and EGFR+Ki-67+ERα, respectively. M4 harbors all EGFR, Ki-67, ERα and HER2 morpholinos. Viability of the cells is shown in Figure 4.33. In Western, blotting results, it was showed that multi-gene silencing increased silencing efficiency synergically but did not significantly differ than single gene silencing in cancer cell proliferation.

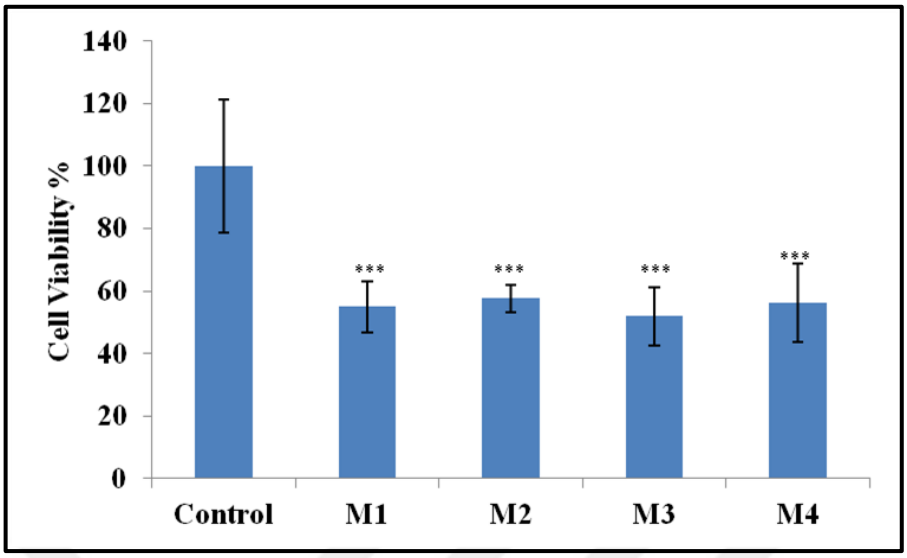


Figure 4.33. Proliferation rates of BT-474 cells treated with 1, 2, 3, and 4 morpholino embedded DNA origami-AuNPs. P value represents ***P<0.001.

4.3.6.2. Cell Cycle Analysis after Multi Gene Silencing

It was determined in single-gene silencing studies that inhibition of the target genes altered in cell cycle phase distributions of breast cancer cells. For the investigation of the effect of simultaneous gene regulation on cycle of cancer cells, the expression of EGFR and Ki-67 genes were inhibited simultaneously and cell cycle phase distributions of BT-474 cells were determined following the gene silencing. The cell cycle analyses are shown Figure 4.34. 48h after silencing of EGFR and Ki-67 genes decrease in mitosis phase was observed. Multi-gene silencing also led decrease in mitosis. However, there was no significant difference on cell cycle phase distributions between single and multi-gene silencing, which is consistent with proliferation results.

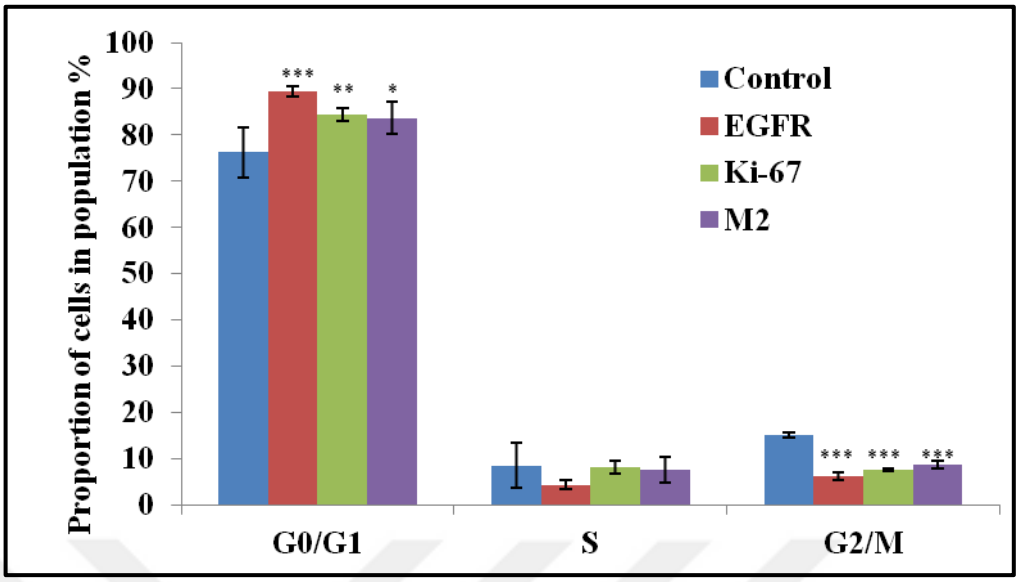


Figure 4.34. Cell cycle phase distributions of BT-474 cells after silencing of EGFR and Ki-67 genes. P value represents *P<0.05; **P<0.01; ***P<0.001.

Effect of multi-gene silencing on cell cycle phases were investigated also by silencing of EGFR, Ki-67 and ERα genes. Figure 4.35 shows the cell cycle phase distributions of BT-474 cells 72h after silencing of the genes alone and simultaneously.

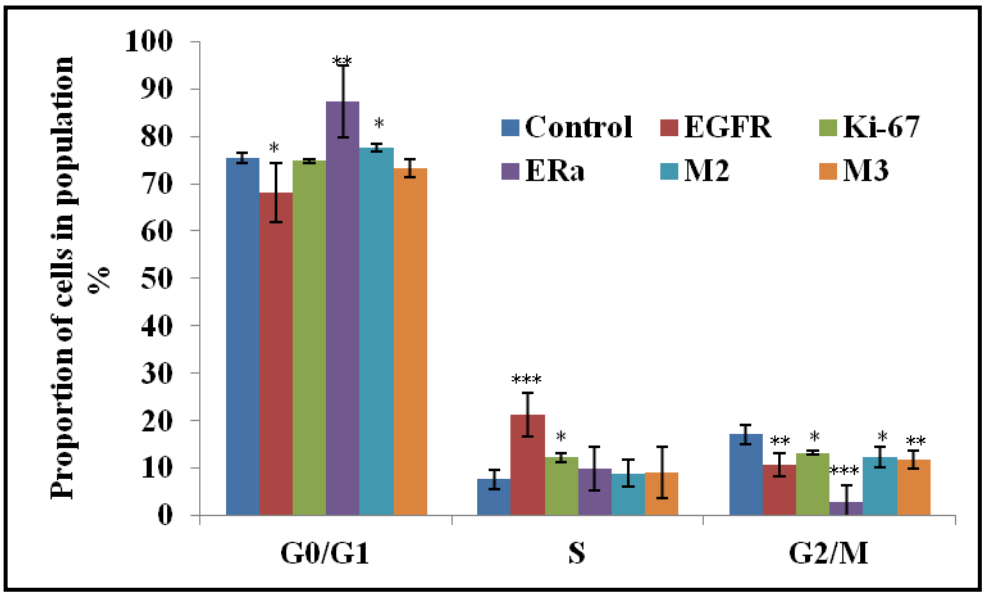


Figure 4.35. Effect of multi-gene silencing on cell cycle phase distributions of BT-474 cells. P value represents *P<0.05; **P<0.01; ***P<0.001.

4.3.6.3. Apoptosis Analysis after Multi-Gene Regulation

Apoptosis is controlled cell death mechanism and in some diseases suc as cancer, promotion of apoptosis is required for treatment [268, 269]. It was determined that single and multiple silencing of the target genes caused inhibition of cancer cell proliferation. Whether gene silencing using DNA origami-AuNPs nanostructures induced apoptosis was investigated with Annexin V-FITC assay. In Figure 4.36 and 4.37, apoptosis analysis of BT-474 cells after multi-silencing of HER2 – ERα and EGFR – Ki-67 genes are shown, respectively. It was also investigated whether the simultaneous suppression of 2, 3 and 4 different genes starting from a single gene had an effect on entrance to apoptosis pathway and the flow cytometry results are shown in Figure 4.38. After regulation of the genes, the mortality of the breast cancer cells was around between 30 per cent and 40 per cent. The obtained data showed that gene regulation using DNA origami-AuNPs nanocarriers resulted in apoptosis in cancer cells and increase the death rate.

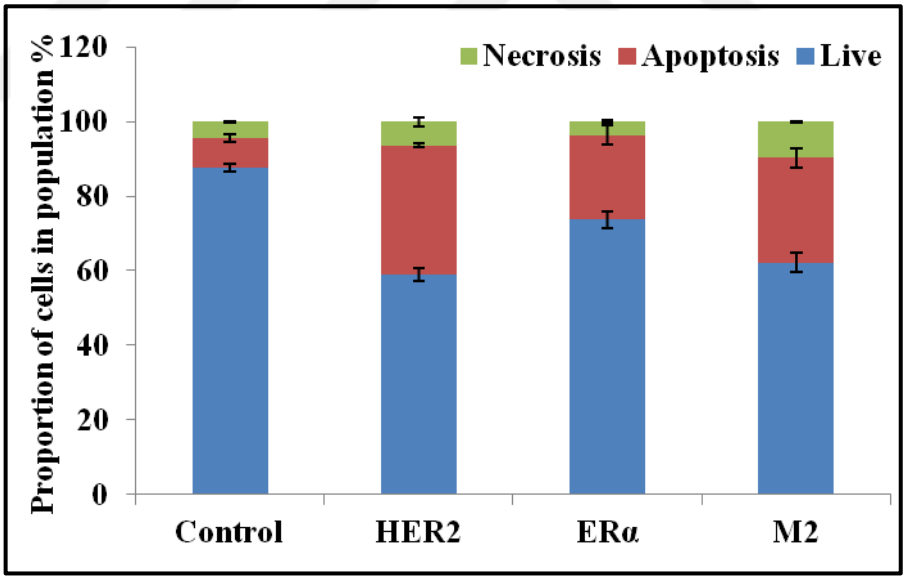


Figure 4.36. Apoptosis analysis of BT-474 cells after silencing of HER2 and ERα genes alone and simultaneously.

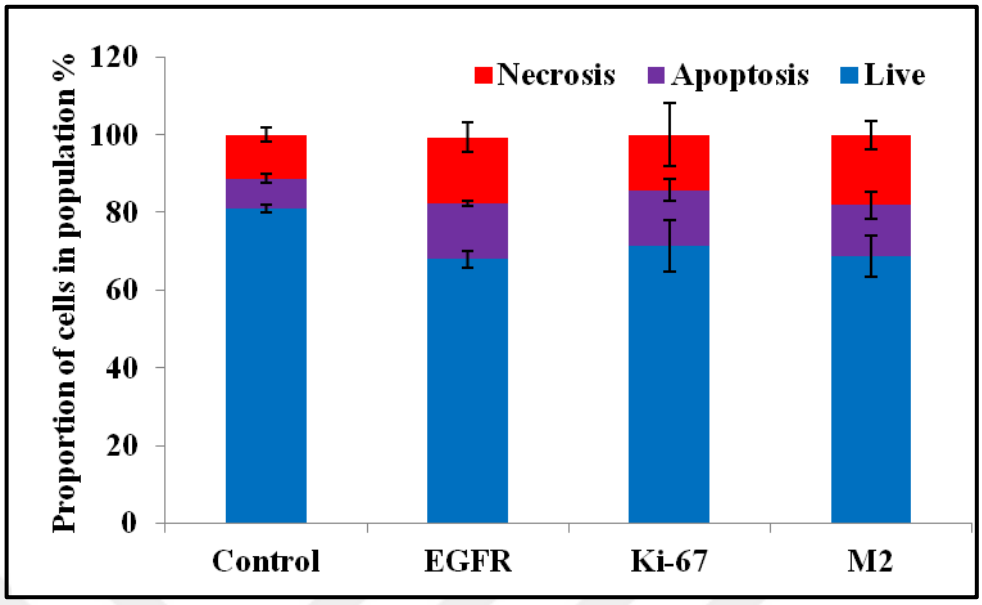


Figure 4.37. Apoptosis analysis of BT-474 cells after silencing of EGFR and Ki-67 genes alone and simultaneously.

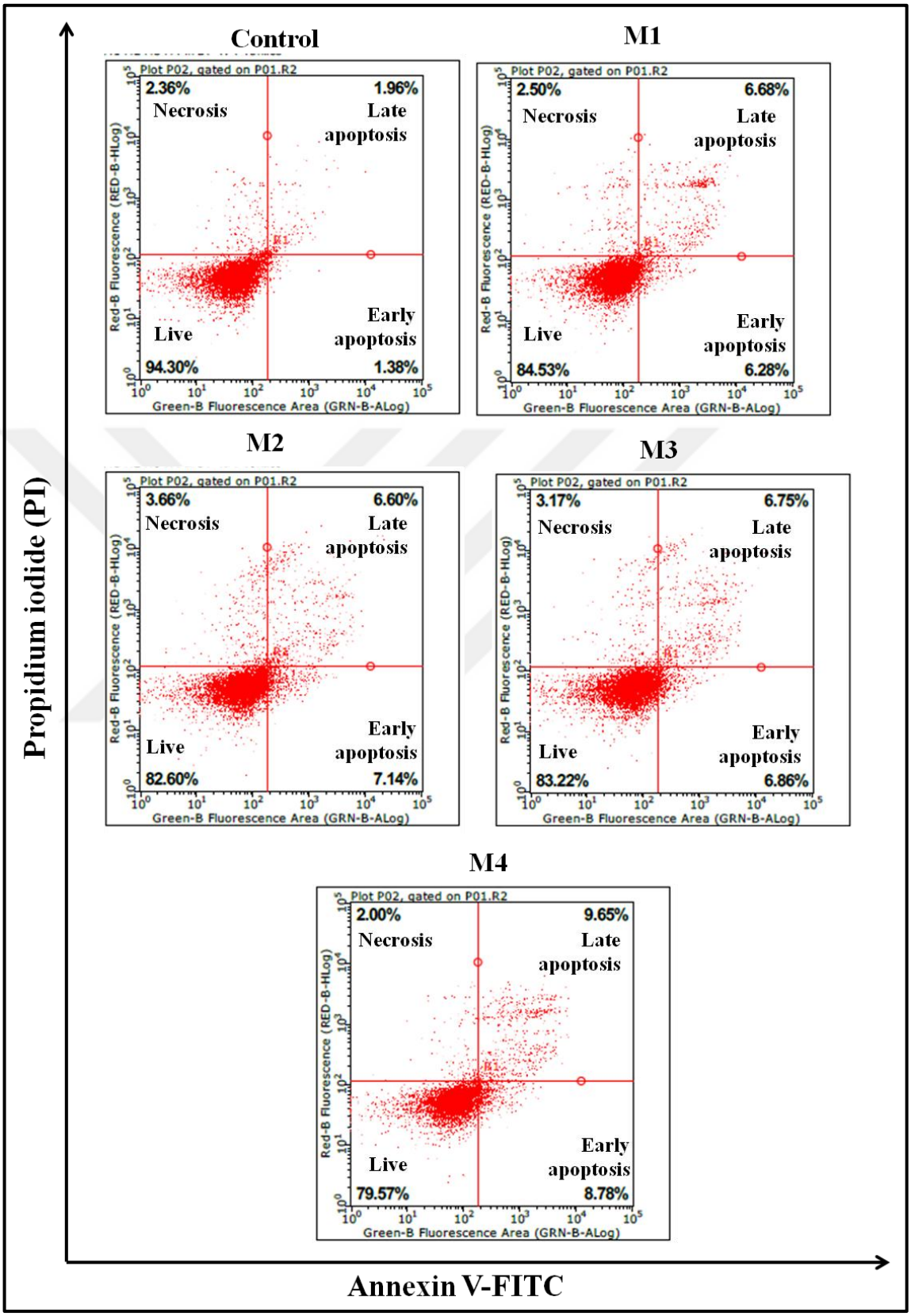


Figure 4.38 Apoptosis rates of BT-474 cells after simultaneous silencing of EGFR, Ki-67, ERα and HER2 genes.

4.3.7. Validation of Gene Silencing

For effective treatment of diseases, it is necessary to increase the efficiency of therapeutic agents and decrease the side effects. To that end, well-designed delivery vehicles, which effectively carry and release the agents to the target site have been investigated by researchers. Viruses are efficient delivery options however, they have safety issues. Nanostructures as non-viral delivery sistems such as dendrimers [270], metallic nanoparticles [271], and carbon nanotubes [272], lipid-based [273] and polymer-based nanoparticles [274] have been studied in cancer therapy. The most of the carrier systems generate biological responses such as immune response, effecting gene expression, causing oxidative stress, or toxicity. The biocompatibility is one of the major issues for disease treatment in clinical studies.

DNA nanostructures have superior features compare to other non-viral delivery systems. DNA, which is building block of these nanostructures, can be easily modified with other molecules and materials for multiple functions such as targetin, imaging, and sensing. Moreover, DNA nanomaterials are stable and biocompatible. In this thesis, a morpholino embedded nanostructure was designed and prepared using DNA nanotechnology approach. It was shown that this nanostructure managed to inhibit target gene expressions effectively and regulated multiple genes simultaneously by embedding more than one morpholino oligos into the structure. To validate the efficiency of the designed nanostructure, inhibition in target protein expression after treatment with DNA origami-AuNPs was compared with some of commercially available transfection reagents. In single and multiple gene regulation experiments, silencing rates of DNA origami-AuNPs were determined in parallel with liposomes and according to Western blotting results, the liposomes were not sufficient for delivery of morpholino oligos and decreased the target gene expressions approximately 30 per cent less than DNA origami-AuNPs nanostructures.

In addition to liposome, 2 other transfection reagents were used for validation of DNA origami-AuNPs. Gene silencing rates of DNA origami-AuNPs carriers were compared with a non-liposomal lipid based transfection system FuGene and a polymer based reagent XFect. Western blotting images were shown in Figure 4.39. Silencing efficiency of DNA origami-AuNPs structures was higher than both reagents.

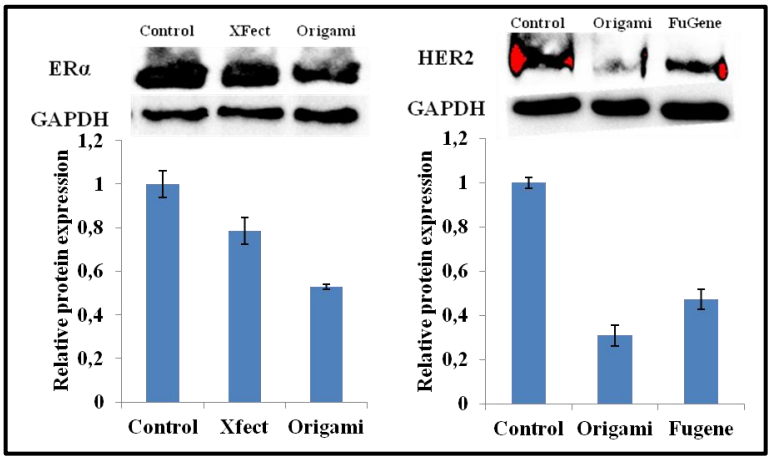


Figure 4.39. Expression levels of ERα ve HER2 proteins after gene silencing using DNA origami-AuNPs, XFect and FuGene delivery systems.

Gene and drug delivery systems are preferred to be biocompatible. There should not be any effect of the delivery system itself o cells rather than the cargo it carries. However, biocompatible delivery systems are studied by researchers due to toxicity of commercially available and widely used carriers. It was showed that the designed and prepared DNA origami-AuNPs delivery system in this thesis was non-toxic on both cancer and healthy cells. On the other hand, the transfection reagents used for validation of gene silencing efficiency of DNA origami-AuNPs nanostructures were determined as highly toxic. This makes impossible to deliver high concentrations of DNA into cells. Toxicity of the commercial delivery systems used for validation of the DNA origami-AuNPs carriers are shown in Figures 4.40, 4.41 and 4.42. Viability of BT-474 cells was determined after 24h treatment with necessary amounts of delivery agents for transfection of DNA concentrations indicated on graphs.

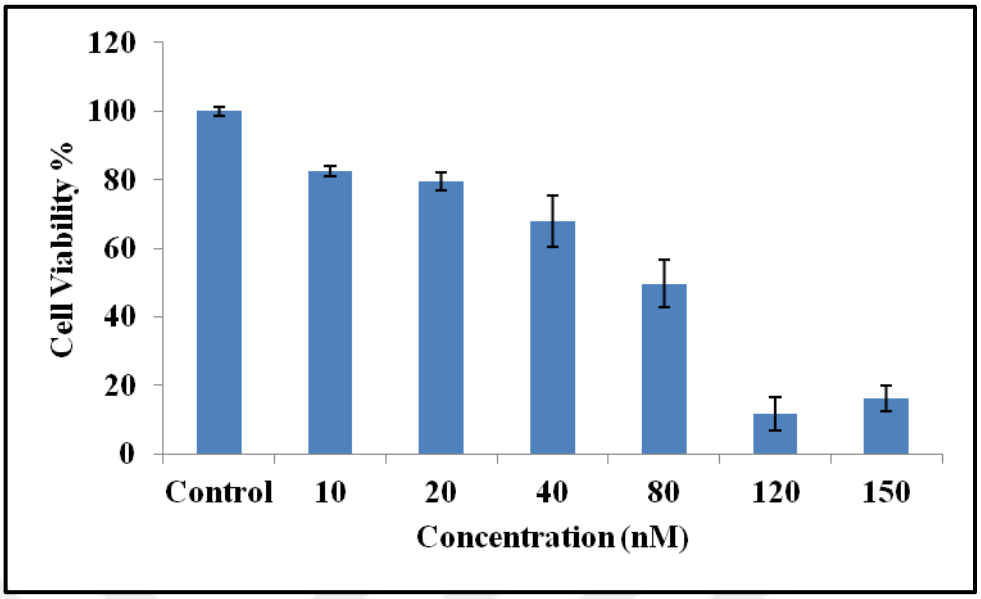


Figure 4.40. Viability of BT-474 cells treated with liposomal delivery system DreamFect.

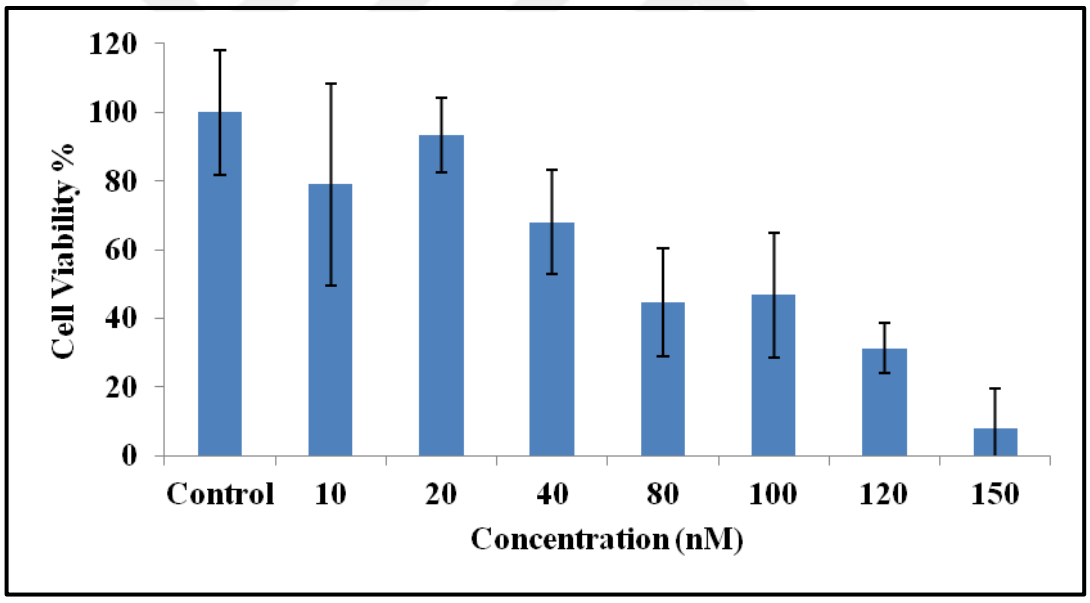


Figure 4.41. Viability of BT-474 cells treated with non-liposomal lipid based delivery system FuGene.

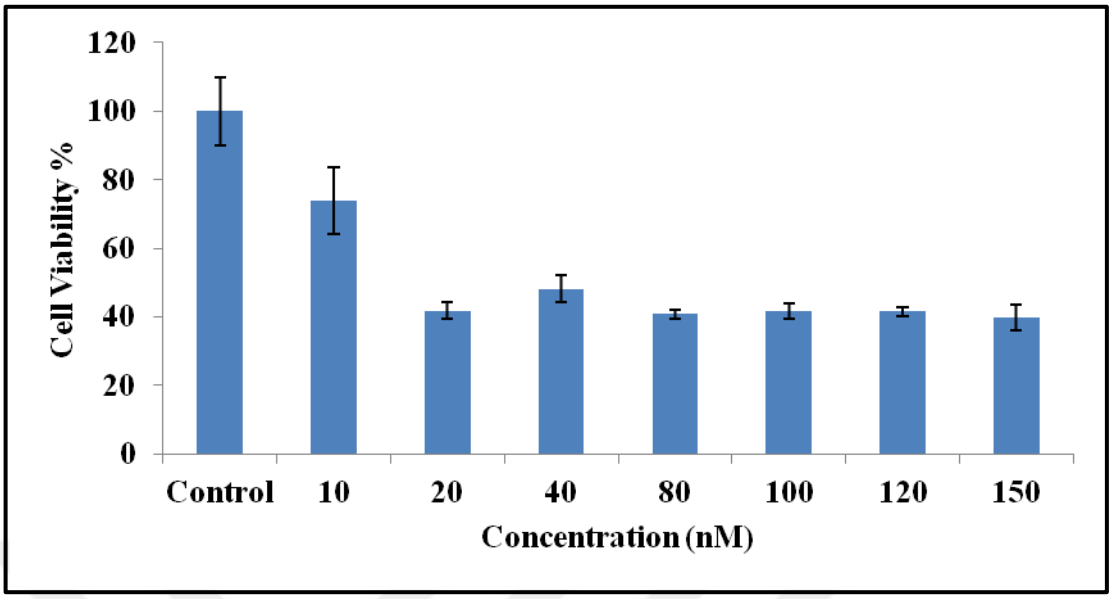


Figure 4.42. Viability of BT-474 cells treated with polymer based transfection system XFect.

5. CONCLUSIONS AND FUTURE PERSPECTIVE

In this thesis, DNA based delivery systems, which contain morpholino antisense oligonucleotides in their structure, were designed for regulation of multiple genes alone and in combination. These DNA nanostructures were successfully used for silencing of 4 different cancer proliferation related genes in breast cancer cells as model. The designed DNA origami-AuNPs nanostructures were prepared and the 1, 2, 3, and 4 different morpholino oligos embedded, tile shaped delivery systems were characterized. It was determined that the structures were stable both in isotonic solutions at physiological pH and in serum containing cell medium. In the scope of cell culture studies, 3 different breast cancer cell lines were cultured and used for proof of concept. Gene silencing studies were started with using single morpholino embedded DNA origami-AuNPs and effective knockdown was observed even in very low concentrations. Decrease in the expression of all individually targeted genes was determined using Western blotting technique. Suppression of these cancer proliferation related genes were led inhibition of cellular proliferation and alteration on cell cycle phase distributions of breast cancer cells. The obtained data after silencing of these genes were corresponding with the previous studies published in literature [210, 216, 226, 275].

Efficiency of DNA origami-AuNPs nanostructures was evaluated comparatively with liposome, most commonly used transfection system. It was seen that DNA origami-AuNPs had no toxic effect and led 30 per cent more decrease in the target gene expressions. In addition, due to their high toxicity, DNA concentration that can be sent to cells with the liposome is limited while the cells can be treated with any desired concentration of silencing agent using DNA origami.

For gene silencing studies, morpholinos against HER2, ERα and Ki-67 genes were embedded to DNA origami-AuNPs nanosturctures separately and their expressions were inhibited in breast cancer cell lines, which were overexpressed in terms of these genes. After silencing of each gene, proliferation rates of breast cancer cells were decreased and rates of G_0/G_1 phase were increased in cell cycle phase distributions. It was determined that the designed morpholinos and morpholino embedded DNA origami-AuNPs nanostructures were successful for regulation of selected genes and the gene regulation studies were carried further with simultaneous silencing of 2 genes. For this, 2 different

morholinos were embedded into DNA origami structures. HER2- ERα genes and EGFR-Ki-67 genes were silenced simultaneously and the effect of multi-gene silencing was investigated by determining cell proliferation rates and cell cycle phase distributions. The multi-gene regulation studies continued with silencing of 3 and 4 genes at the same time. It was showed that inhibiting more than one gene simultaneously increased the rate of silencing. By using 30 nM morpholino embedded DNA origami-AuNPs, approximately expression of target gene decreased 50 per cent in single gene silencing, while multi-gene regulation increased the gene silencing rate to about 80 per cent. After determination of expression levels of the target genes, effect of multi-gene regulation of cell proliferation and cell cycle was investigated. It was observed that also simultaneous silencing of target genes decreased the proliferation rates and changed the cell cycle phase distributions but not significantly different from single gene regulation.

Gene silencing efficiency of DNA origami-AuNPs nanosturctures was compared with 3 different commercial transfectin reagents. The gene silencing rates and toxicity of the transfection systems were determined in parallel with DNA origami-AuNPs and it was showed that our carrier system was more suitable for gene delivery compared to the performance of the other tested systems. The silencing rate of DNA origami-AuNPs was 30 per cent better than the liposomal delivery system, DreamFect Gold, 16 per cent better than non-liposomal lipid delivery system, FuGene, and 21 per cent better than a polymer based transfection system, XFect. Besides of being more effective in suppressing target protein expressions, DNA origami-AuNPs nanostructures were non-toxic to all types of cells used. However, the commercial reagents were highly adecreased the viability of cell.

It was demonstrated that morpholino embedded DNA origami-AuNPs nanostructures are effective gene silencing systems for single and multiple gene regulations. At the same time they are biocompatible structures and stable in blood serum. Although the proposed system has the advantages mentioned above, multi-model systems are required for enhanced treatment efficiency. Substantial exertion has been focused on fabrication of engideered delivery systems for multiple purposes to increase therapeutic effect of molecules. As antracyclines directly intercalate NA molecules, DNA origami structures are perfect candidates for delivery of chemotherapy drugs such as doxorubicin. Thus, DNA origami-AuNPs nanostructures can be loaded also with doxorubicin. Furhermore, the nanostructure enables photothermal therapy due to the AuNPs in the structure. For further studies, it is

possible to use the DNA origami-AuNPs nanostructures as multi-modal-systems for gene silencing, drug delivery and photothermal therapy. In our study, breast cancer releated genes were targeted to knockdown. However, there are many other disorders based on dysregulation of specific genes such as neurodegenerative diseases or infections diseases including Hepatitis-C and HIV. Moreover, a complex disease such as cancer is originated by not only overexpression but also suppression of genes. DNA origami-AuNPs nanostructures provide incorporation of more than one gene regulating elements into the structure. Therefore, different genes can be targeted for different types of diseases and also both upregulation and downregulation of genes can be studied using the proposed nanocarrier. The majority of the transfection reagents form complexes with their NA cargos through electrostatic interactions. However, morpholino antisende oligonucleotides are neutral and difficult to complex with. Thus *in vitro* delivery of them is very limited. In this study, effective and consistent delivery of morpholinos was demonstrated. There are many other efficient antisense oligonucleotides such as Phosphorothioate oligos and peptide nucleic acids (PNAs). Achiving delivery of these oligonucleotides in a non-toxic and biocompatible way remains a major challenge. One should note that the other types of antisense oligonucleotides rather than morpholinos can also be embedded into the DNA origami-AuNPs for non-toxic delivery. Althouh the prepared nanostructure is biocompatible and stable in blood serum; there are many other challenges for systemic delivery of therapeutic agents. It will be also appropriate to evaluate the nanostructures in *in vivo* studies.

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