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Toxicity studies of Bimetallic Nanoparticles encapsulated with Polymers on normal and cancerous cell lines

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Abbreviations

ADCs	antibody-drug conjugates
AgAu	silver-gold bimetallic nanoparticles
AgNPs	silver nanoparticles
AIBN	azobisisobutyronitrile
AuNPs	gold nanoparticles
CT	computed tomography
CTA	cellulose triacetate
DLS	dynamic light scattering
DMSO	dimethylsulfoxide
DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
DSPG	1,2-Distearoyl-sn-glycero-3-phosphoglycerol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EPR	enhanced permeation and retention
FBS	fetal bovine serum
FDA	Food and Drug Administration
GEMM	genetically engineered mouse model
HCT116	human colorectal cancer 116 cells
HEK293	human embryonic kidney 293 cells

HER2	human epidermal growth factor receptor 2
HPH	high pressure homogenization
HPMA	N-(2-Hydroxypropyl)methacrylamide
IFP	interstitial fluid pressure
LLC	Lewis lung carcinoma cell line
LSPR	localized surface plasmon resonance
Mal	maltose
MMP	matrix metalloproteinases
MPS	mononuclear phagocyte system
MRI	magnetic resonance imaging
NIR	near infrared
o/w	oil in water
PAMAM	poly(amidoamine)
PDMAEMA-b-POEGMA	poly[2-(dimethylamino)ethyl methacrylate-*b*-(oligoethylene glycol)methacrylate]
PEG	polyethylene glycol
PEI	polyethylenimine
PET	positron emission tomography
PLA	polylactic acid
PLGA	poly lactic-co-glycolic acid
QDs	quantum dots
ROS	reactive oxygen species

SLNs	solid lipid nanoparticles
SPARC	osteonectin
SPIONs	superparamagnetic iron oxide nanoparticles
TAMs	tumor-associated macrophages
THF	tetrahydrofuran
TRP	tryptophan
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
w/o/w	water in oil in water
YTS	NK lymphoma cell line

1.1. Abstract

Nanotechnology is a new field of science, which promises to revolutionize many aspects of human life as we know it until today. One of the cornerstones of nanotechnology are its applications in medicine. This field is called ***"Nanomedicine"*** and is rapidly expanding in the fields of diagnosis, treatment and personalized medicine. This work deals with nanomedical platforms and their utilization in cancer treatment. Two basic categories of nanocarriers are polymers and metallic nanoparticles, which when combined give birth to nanocomposites. AgAu bimetallic nanoparticles and PDMAEMA-b-POEGMA co-polymer are the two central components of this study. Apart from their manufacturing and characterization process, we present toxicity results of various combinations of them against HEK293 and HCT116 cell lines. The results show that the addition of the polymer does increase toxicity against both cell lines. Further studies need to be implemented in more cell lines and in mice in order to optimize the dosage, which achieves the highest antitumor effect, while at the same time systematic toxicity remains low.

1.2. Introduction

Cancer, along with cardiovascular problems, is the leading cause of morbidity and mortality in the developed world. The most prevalent malignancies in the United States are prostate cancer, colon cancer and melanoma in the male population, while uterine, colon and breast cancer are the most prevalent in women. Many treatment modes are applied, with the most radical being surgical treatment, which leads potentially to healing, when there is no distant tumor cell dissemination (metastatic disease). Other therapeutic modes include different regimes of chemotherapy and radiotherapy and novel agents, including hormone factors, monoclonal antibodies and protein inhibitors among others **[1].**

The evolution of drug therapies for the treatment of cancer in the last few decades has led to increased survival rates for patients (1-year survival rate for lung cancer increased from 34% in 1975 to more than 45% after 2010) and better quality of life **[1].** Still the complexity of cancer, as a multifactorial and multistage disease, with a different histological and genetic fingerprint among its various types and even during the course of the same type, diminishes the probability of total cure. As a result, the intrinsic limit of to-date therapies maintains scientific interest and prompts the introduction of nanotechnology in the field of cancer therapy **[2].**

The need for more effective and safer anticancer agents has led to the development of various nanotechnological platforms. Considerable progress has been achieved, but it is still mostly limited to the bench, due to several obstacles emerging from tumor heterogeneity, a lack of in-depth understanding of nanoparticle interactions with biological systems and inability for batch-to-batch reproducibility at an industrial level **[2].**

Nevertheless, oncological drug nanoplatforms share distinctive features, that make them promising novel therapeutic agents. These include a more targeted delivery of drugs in the desired site, leading to increased efficacy and reduced toxicity. Moreover, nanodrugs enable stimulus-triggered drug release and offer greater stability, solubility and drug half-life. Nanoparticles can be functionalized as carriers of one or more

anticancer drugs and are able to control precisely drug release in a spatiotemporal scale. Furthermore, they can be used in several diagnostic modes, in terms of imaging and biosensor development. This has led to a broader conception of nanoparticles as platforms that can be used simultaneously for diagnosis, treatment and follow-up, a field known as ***"Theranostics"*** **[2].**

One of the most promising categories of nanoparticles in the field of cancer theranostics are metallic nanoparticles. Metals, including gold (Au), silver (Ag), zinc (Zn), iron (Fe) and titanium (Ti), have great potential, either modified or even inherently, as anticancer agents. They have unique properties, such as large surface-to-volume ratio, surface plasmon resonance phenomenon, catalytic activity and superparamagnetism, which facilitate their application in imaging (contrast agents) and therapy (hyperthermia, drug delivery systems). Also, they are biocompatible and can be excreted easily from the body. The mostly studied ones are gold and silver metallic nanoparticles **[3].**

An interesting subcategory of metal nanoparticles are bimetallic ones. Their properties can be significantly different than elemental one-metal nanoparticles and depend on the distribution of different metallic atoms in their structure. Research is revolving mostly around ***AgAu bimetallic nanoparticles***. These show high antibacterial activity and can also be applied as photothermal anticancer agents **[4].**

Several studies have also suggested bimetallic nanoparticles as intrinsically highly cytotoxic against cancer cells and the distribution of the atoms in their structure alters their cytotoxic properties. Since a great advantage of nanotechnology is the fact that it allows the modification of nanoparticles' surface, thus enhancing the formation and study of various combinations, metallic nanonarticles can be easily encapsulated in many different natural or synthetic polymers, which confers them novel characteristics **[5].**

2. Theory

2.1. Nanomedicine and Cancer

During the past few decades, many steps have been taken towards the understanding and description of the mechanisms of carcinogenesis. Also, several diagnostic tools have been developed for tissue imaging and treatment. Despite this progress, cancer mortality worldwide remains one of the most prominent causes of death. The in-depth knowledge of genetic alterations that are responsible for cancer development has altered the therapeutic strategy. During the past few years, new diagnostic and therapeutic methods have been developed, which aim to diagnose and treat malignant diseases at an earlier stage (**Figure 1.**) **[6, 7].**

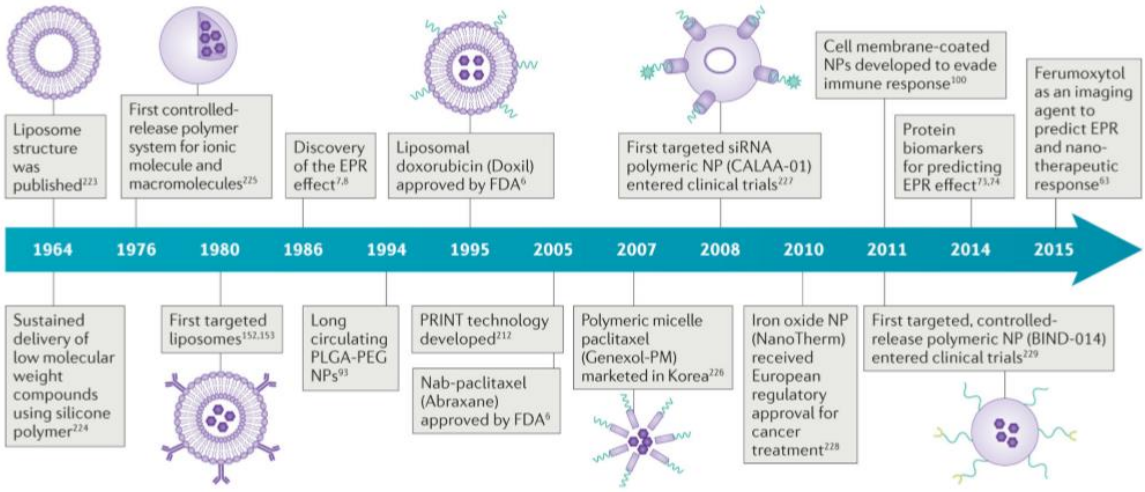


Figure 1: Timeline of historical evolution of Nanomedicine **[2].**

Scientists have also realized that the tumor microenvironment affects treatment and drug effectiveness. Thus, even if normal cells that neighbor a tumor do not display genetic changes, their physiological activity might still be changed, because they are surrounded by malignant cells. Understanding this microenvironment is crucial in order to understand cancer development and design a strategy to create effective anticancer drugs. This is a multidisciplinary task, that includes medicine, biology, chemistry, biochemistry, and biophysics. Their parameters should be evaluated in choosing the optimal therapeutic strategy. The factors and procedures of this multifunctional system are classified as follows:

• Factors related with the development and proliferation of cancer cells that should be controlled.

• Cancer cells do not follow the apoptotic procedure (programmed cell death).

• Development of a new vessel network (neoangiogenesis) around the tumor, which provides oxygen and nutrients.

• Cancer cell metastasis **[6, 7].**

The recognition of the complexity of cancer and the understanding of the related parameters and processes have created new trends regarding the development of innovative medicines. These medicines approach cancer as a "system" with many parameters and processes that need to be assessed. Nowadays, cancer therapeutics is oriented towards interdisciplinary cooperation and the application of new technologies, in terms of diagnosis, microenvironment understanding and drug design **(Figure 2) [6].**

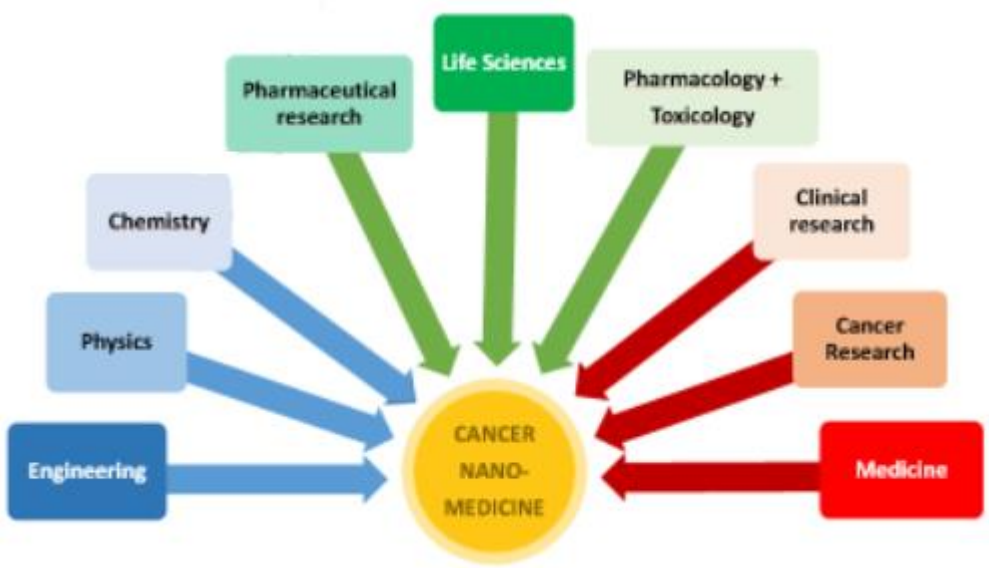


Figure 2: Cancer nanomedicine is a multidisciplinary field of science **[8].**

The following sectors are emphasized:

• *Cancer control and prevention.* It includes the development of nanodevices for the delivery of drug molecules to target tissues. Complex anticancer vaccines can be synthesized using nanodevices and be administered systemically.

• *Early diagnosis and proteomics.* Implanted biosensors, able to trace bio-indicators, are being developed. They can be evaluated ex vivo or in situ and they can send results through wireless technology to doctors and databases.

• *Imaging diagnostics* aim towards sensitive and precise imaging. The creation of "smart" injectable nanoparticles will allow early cancer detection.

• *Multifunctional therapies.* There is a great need for "theranostic" nanosystems; ones that incorporate both diagnostic and therapeutic functions. Controlling the nanomolecule release in a spatiotemporal and evaluation of its effectiveness at the same time.

• *Quality of life improvement* during therapy. Nanosystem utilization for the treatment of pain, nausea, fatigue and appetite loss.

• *Interdisciplinary education.* Interaction and effective communication between scientists of many fields. The interdisciplinary approach of nanotechnology in medical oncology has given birth to the term "nano-oncology" in the literature **[6].**

The design of efficient nanosystems that can deliver bioactive molecules is an important research field. It aims at developing "Trojan horses" that will avoid detection from the immune system and will not harm normal tissues. When it comes to cytostatic drugs, the following conditions must be fulfilled:

• Adequate bioactive molecule concentration is needed in biological fluids. This will enable the effective concentration in the tumor. For greater safety, there should be zero or minimal drug concentration outside the nanocarrier.

• The bioactive molecule should specifically target cancer cells and have a favorable therapeutic window **[6].**

Nanomedical research aims to the aforementioned points, using the special characteristics of each tumor. These characteristics allow passive and active targeting of cancer cells. Tumors exhibit high heterogeneity and neovascularization. The latter includes necrotic and highly perfused tumor areas. Cancer blood vessels exhibit an abnormal structure, regarding the proportion of endothelial cells and their cellular membrane, high blood vessel bending and defective pericytes. Cancer capillary vessels

are easily penetrated (***passive targeting***). Macromolecule transport in this case can happen through interendothelial connections and channels **[6].**

The lymph network of the tumor is also defective, which results in fluid retention and pressure increase in the tumor. The absence of normal lymphoid tissue results in the retention of nanocarriers in the intracellular space. Nanomedicine takes advantage of the abnormal blood and lymph vessels and enables passive cancer targeting. In this case a high nanosystem concentration is achieved into the cancer tissues. Higher concentration means higher drug release and subsequently increased toxicity against cancer cells. The nanocarrier surface can also be modified aiming the cancer tissue more specifically, using small molecules, antibodies or peptides that recognize special cancer antigens. This phenomenon is called ***active targeting***. Both passive and active targeting are discussed in more detail in the following sections of this work **[6, 7].**

During cancer treatment many questions arise:

• *Why are current treatment regimens not 100% effective?*

• *Is there a way to eliminate drug toxicity and side effects?*

• *How can we achieve greater drug efficacy?*

Current therapies fail to destroy solid tumors for three reasons:

• During diagnosis, the tumor is usually already developed. Even if we manage to destroy 99,9% of the tumor, the remaining 0,1% still contains millions of cancer cells.

• Most patients that undergo surgical removal of the tumor already have metastases. During metastasis, cancer cells circulate in the blood or lymphatic system and attach to other organs, creating new tumor lesions. Usually, metastases are not easily traceable due to their small size.

• The third and greatest hindrance in successful cancer treatment is tumor heterogeneity. Tumor cells contain different genetic material and different biological, biochemical and immune characteristics. Cells variability is high, regarding surface receptors, enzymes, morphology, karyotype, apoptosis, proliferation potential,

sensitivity in various drugs, and metastasis ability. This heterogeneity limits surgical options and initiates drug resistance **[6].**

After entering the body, the bioactive molecule does not exhibit selective accumulation in the cancer tissue. It is rather distributed in several organs and tissues. Furthermore, in order to reach its target tissue, it must surpass many biological barriers, where it can be destroyed or provoke side effects. For this reason, in order to achieve effective therapeutic concentration of the drug in the desired area we usually administer a high concentration of the drug, most of which will reach healthy tissues. Cytotoxic drugs unfortunately cannot discriminate between healthy and cancer cells, causing toxicity. During the last years, many efforts have been made to resolve this problem and achieve better targeting to cancer tissues. For target therapy, the following conditions should be taken into consideration:

• Drug administration protocols must be simple.

• The drug concentration should be as low as possible, to prevent toxicity to healthy cells.

• Bioactive molecule concentration in the tumor should be high enough, without causing severe side effects **[6].**

To summarize, cancer is a very heterogenous disease, with many aspects that are not yet fully explored. Many questions remain unanswered and many challenges still prevail. Nanomedicine can play a central role in expanding our diagnostic and therapeutic capability in terms of fighting this global health concern **[6].**

2.2. General classification of nanocarriers

2.2.1. Liposomes

Liposomes are one of the first classes of drug nanocarriers that have been reported in literature. They can be described as spherical vesicles, composed of a phospholipid bilayer that resembles the cell membrane **(Figure 3).** This unique structure potentiates the encapsulation of both water-soluble drugs (in the hydrophilic core) and lipid-soluble drugs (in the lipid bilayer) **[9].**

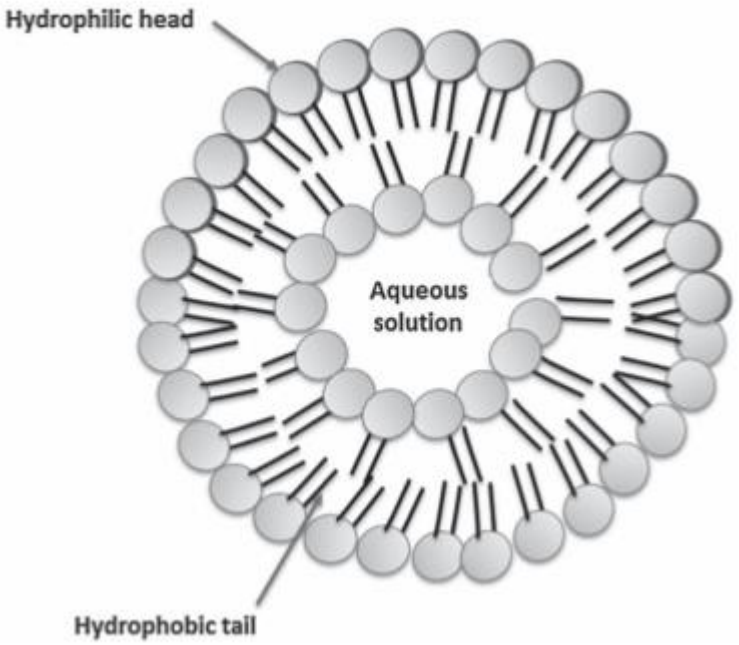


Figure 3: The structure of a liposome **[10].**

Several biocompatible phospholipids, such as 1,2-distearoyl-glycero-3-phosphocholine (DSPG) and 1,2-distearoyl- sn- glycero-3-phosphoethanolamine (DSPE), can already be found in products approved by the United States Food and Drug Administration (FDA) for medicinal use in humans **[11].** There are many different types of liposomes that also have different sizes. For example, multilamellar liposome size ranges between 500–5,000 nm, while unilamellar liposomes can be much smaller at 50–100 nm **[12].** The surface of the liposomes can be easily modified with polyethylene glycol (i.e., PEGylation) to prolong their blood circulation time, or with several other targeting moieties, such as small molecules or macromolecules (e.g., monoclonal antibodies) **[13-17].** This way they are able to bind to tumor cell targets that are exclusively or overly expressed by tumor cells. Several liposomal formulations of antitumor drugs already exist on the market, including PEGylated doxorubicin liposomes, non-PEGylated doxorubicin liposomes, and non-PEGylated daunorubicin liposomes **(Table 1) [18].**

2.2.2. Polymeric Nanoparticles

Various natural (e.g., chitosan and albumin) and synthetic polymers (e.g., poly-lactic acid (PLA) and poly-lactic-co- glycolic acid (PLGA)) have been used to manufacture

nanoparticles. PLA and PLGA specifically are widely used in nanoparticle preparation because they are biodegradable, biocompatible and safe **[19, 20].** PLA is a homopolymer and PLGA is a copolymer. Also, many block copolymers (e.g., PLGA-polyethylene glycol (PLGA-PEG)) have been used for the preparation of nanocarriers. Block copolymers that contain PEG offer ***'stealth'*** and 'long circulating' properties to the nanoparticles **[21].** Emulsion-based methods are very often used in order to prepare polymeric nanoparticles. Oil-in-water (o/w) emulsion is suitable to encapsulate lipophilic drugs in their structure (e.g., docetaxel **[22]**), whereas the double emulsion (w/o/w) method potentiates the encapsulation of hydrophobic drugs **[23].**

Other methods that are used to manufacture polymeric nanoparticles include spray drying **[24],** nanoprecipitation and supercritical fluid technology **[25].** In addition, several nanofabrication methods (e.g., PRINT) are developed in order to achieve the production of structures with a higher control level over size, shape and surface chemistry **[18, 26-28].**

Nanoparticulate-Based Products Approved For Commercial Use

	Drug	Product	Indication
Polymer	Abraxane®	Paclitaxel	Metastatic Breast Cancer
	SandostatinLAR®	Octreotide acetate	Carcinoid Tumors, Vasoactive Intestinal Peptide Tumors
	Lupron Depot®	Leuprolide acetate	Prostate Cancer, Endometriosis
	Bydureon®	Exenatide	Diabetes Mellitus Type 2
	Zevalin®	Ibritumomabtiuxetan	Non-Hodgkin Lymphoma
	Bexxar®	Tositumomab	Follicular Cancer
	Mylotarg®	Gemtuzumab	Acute myeloid Leukemia
Liposome	Ambisome™	Amphoteracin B	Fungal infection
	DaounoXome™	Daunorubicin	Kaposi's sarcoma
	Doxil™	Doxorubicin	Refractory Kaposi's sarcoma, recurrent breast cancer and ovarian cancer
	Visudyne®	Verteporfin	Age-related macular degeneration, pathologic myopia and ocular Histoplasmosis
	Myocet®	Doxorubicin	Recurrent breast cancer
	DepoCyt®	Cytarabine	Neoplastic meningitis and lymphomatous meningitis
	DepoDur	Morphine sulfate	Pain

Table 1: Nanoparticle-based products approved by the FDA **[10].**

2.2.3. Micelles

Micelles are self-assembled structures, made of amphiphilic block copolymers or lipidic molecules **(Figure 4).** They have a lipophilic core and a hydrophilic shell, so they can mainly solubilize lipophilic drugs. Various micelles made of PEGylated polyesters (e.g., PLA-PEG **[29]**) have been used to deliver antitumor drugs. A self-assembled nanostructure, which is composed of two or more block copolymers is called a ***mixed micelle***. In this case, the polymers are mixed together in order to confer the favorable features of each component to the final nanostructure **[18, 30].**

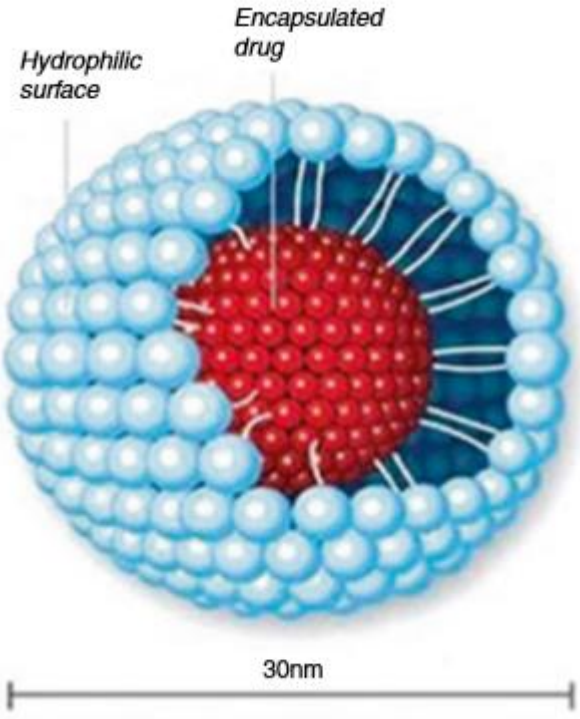


Figure 4. The structure of a polymeric micelle **[6].**

2.2.4. Solid lipid nanoparticles (SLNs)

SLNs are lipid-based nanoplatforms that have a solid form at room temperature and can successfully be manipulated to encapsulate lipophilic drugs. Many different lipidic materials are used to prepare them, for example include triglycerides, fatty acids (e.g., stearic acid), and several different phospholipids (e.g., phosphatidyl choline or lecithin). The most commonly used method to prepare SLNs is high pressure homogenization (HPH) **[31, 32].** Other methods that can be used include ultrasonication **[33],** emulsion-

based methods **[34],** and solvent injection method **[35].** SLNs preparation is cost-effective and can be achieved in a large scale **[18, 36].**

2.2.5. Dendrimers

Dendrimers are polymers consisting of long chains which are connected to a central core. The long chains consist of repeated structural units **(Figure 5).** They are attractive nanocarriers because they have a small size (<10 nm). Also, they exhibit many functional groups on their surface. Their interaction with their environment is dependent on their end groups. Dendrimers have unique properties due to their tree shape and inner cavities. Bioactive molecules can be trapped in these cavities or be conjugated to final surface groups. The mostly studied dendrimer group is PAMAM. It is also the most commercially used for research **[6].**

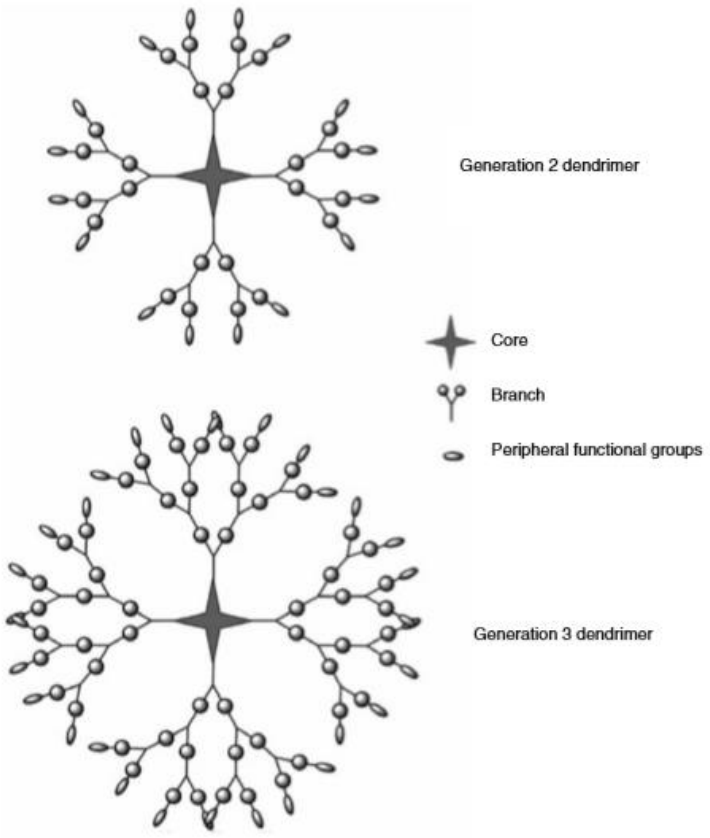


Figure 5. Dendrimer structure; core, branches and peripheral functional groups **[6].**

2.2.6. Drug-Polymer Conjugates

These conjugates are composed of a small molecular anticancer drug, which is covalently linked to a biocompatible polymer via a linker. ***Drug-polymer conjugates*** can improve the pharmacokinetics profile of the antitumor drug, enhance its stability, while simultaneously they increase drug accumulation in the tumor **[11, 37-40].** Several different drug-polymer conjugates have been investigated and proposed, such as the doxorubicin-(N-(2-hydroxypropyl) methacrylamide) copolymer conjugate (doxorubicin-HPMA). This is designed to unload the anticancer drug doxorubicin in tumor cells after the cleavage of the linker takes place by lysosomal enzymes **[41].** Other proposed drug conjugates include HPMA-doxorubicin-gemcitabine **[42],** and paclitaxel-polyglutamic acid (paclitaxel polyglumex) **[18, 37].**

2.2.7. Antibody-Drug Conjugates

Monoclonal antibody-drug conjugates (ADCs) are bifunctional molecules that benefit simultaneously from the targeting capabilities of the monoclonal antibody moiety and the cytotoxic activity of the conjugated drug. Candidate monoclonal antibodies should be chosen in a certain way, so that they target antigens exclusively expressed or overexpressed on the tumor site, but not on nearby healthy tissues **[43, 44].** ADCs offer various advantages, including the ability to achieve a high concentration of the cytotoxic agent in tumor cells (practically because of the targeting abilities of the antibody), lower toxicity **[45, 46],** and improved pharmacokinetics **[47].** ADCs are beneficial since monoclonal antibodies exhibit a long circulatory half-life **[48].** However, the same stability is a prerequisite for the linker between the antibody and the drug molecules **[18].**

An important clinical problem, which is associated with ADCs, is systemic toxicity, although it is smaller than with other drug categories. This can be attributed to unsuitable drug release and antigen leakage from the tumor tissues into the blood circulation. These leaked antigens can circulate and reside anywhere in the body, thus misleading the drug conjugate to a false target **[43, 49, 50]**. Other disadvantages can be observed as well, such as an unexpectedly low accumulation rate of the cytotoxic agent

in the tumor with values as low as 0.1 % **[49].** This necessitates the administration of large doses of the drug conjugates, which, in turn, stirs economic issues for pharmaceutical companies and health systems, especially when these drugs need to be administered for long-term therapy. Nevertheless, this class of nanodrugs is still regarded a very promising alternative compared to conventional chemotherapy **[18].**

2.2.8. Inorganic Nanoparticles

These include supraparamagnetic iron oxide nanoparticles, silica nanoparticles **[51],** metallic nanoparticles and quantum dots **[18, 52],** among others.

Inorganic nanoparticles have shown promising potential for both cancer diagnosis and treatment **[53],** but due to toxicity issues, organic nanoplatforms have been generally preferred **[54].** Nevertheless, their simple synthesis and versatility render them with great potential in theranostics. Some examples of inorganic nanoparticles are the following: iron oxide nanoparticles, mainly superparamagnetic ones ***(SPIONs),*** gold nanoparticles ***(AuNP)***, quantum dots ***(QD),*** and silica-based nanoparticles **[53].** Inorganic nanoparticles have been widely explored as imaging contrast agents, mainly for magnetic resonance imaging (MRI) and positron emission tomography (PET) scans and are also associated with other chemotherapeutic drugs for theranostic purposes. In addition, some of these nanoparticles can also be used as therapeutic agents by themselves.

In some therapy modes, such as hyperthermia and photothermal ablation, gold nanoparticles and SPIONs are utilized to enhance the imaging contrast and promote cell death simultaneously, upon application of an external stimulus, such as light, radiation or magnetic field **[55 -57].**

In recent studies, inorganic nanoparticles have been combined with organic ones, such as liposomes **[58, 89]** and polymeric nanoparticles **[60-62]** in order to achieve a theranostic outcome.

Silica-based nanoparticles are still in the preclinical stages of research, although they show promising results in drug delivery, exhibiting low toxicity and good

biocompatibility **[63].** Mesoporous silica nanoparticles are the most explored silica-based nanocarriers thanks to their high loading capacity. Their porous structure allows a high-yield encapsulation of both hydrophobic and hydrophilic drugs and imaging agents **[64].** Nonporous silica nanoparticles are also utilized in drug delivery and permit drug conjugation and surface functionalization **[65, 66].**

2.2.9. Metallic nanoparticles

***SPIONs* (Figure 6)** are some of the most studied inorganic nanoparticles in theranostics **[67, 68].** They are biocompatible, biodegradable and their superparamagnetic characteristics render them easily tunable **[69].** SPIONs become aligned when a magnetic field is present, but they exhibit no residual magnetism when it ceases, in contrast to other ferromagnetic materials. Magnetite (Fe3O4) and maghemite (Fe2O3) are used to manufacture iron oxide nanoparticles **[70].** They are currently used as MRI imaging agents **[71]** and as therapeutic drugs in hyperthermia **[72].** When we apply an alternating magnetic field to these nanoparticles, the temperature in the area where they are accumulated rises and heat is generated in the tumor, which kills the cancer cells **[66, 73].**

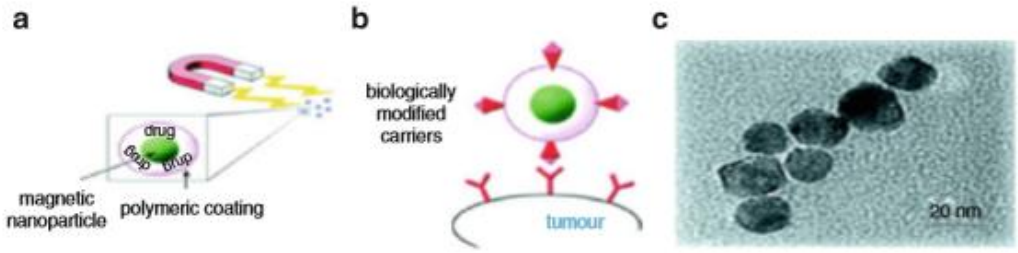


Figure 6: a) External magnetic field leading magnetic nanoparticles to the tumor site. b) Active targeting of the nanoparticles to specific tumor functional groups. c) Magnetic nanoparticles observed via electron microscopy **[66].**

***QDs* (Figure 7)** are semiconductor nanoparticles which have unique fluorescent, optical and electronic properties. They are small, ranging between only 3 and 10 nm, and can be utilized as imaging agents, since they display strong fluorescence and a wide emission spectrum (depending on their size), mostly in the near-infrared (NIR) region.

As fluorophors they are more stable than organic ones **[74]** and allow optical imaging. The quantum dot surfaces can be modified in order to promote their stability, solubility and biocompatibility, as well as in order to functionalize them so that they show better target specificity **[66].**

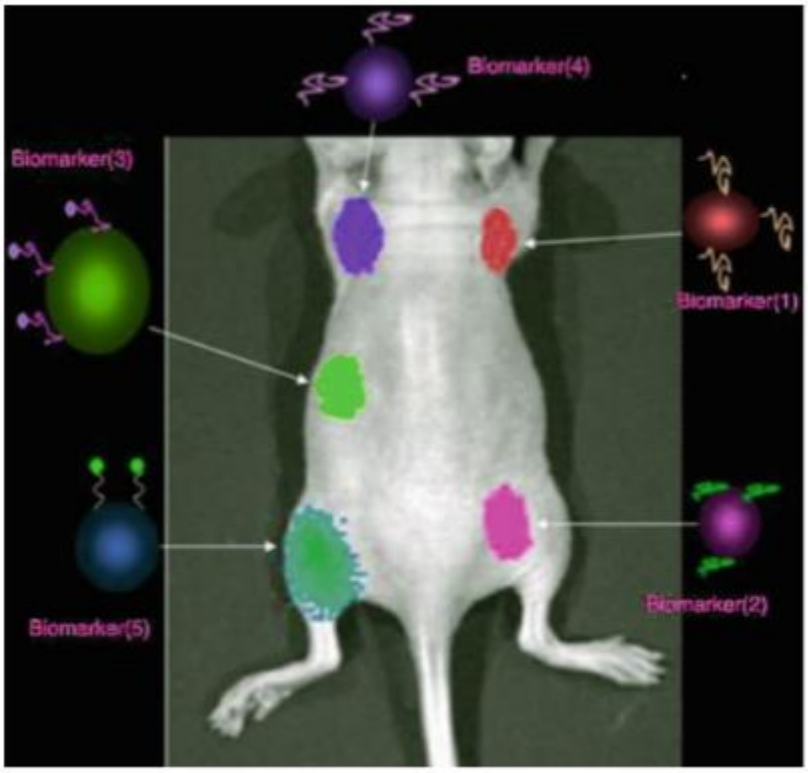
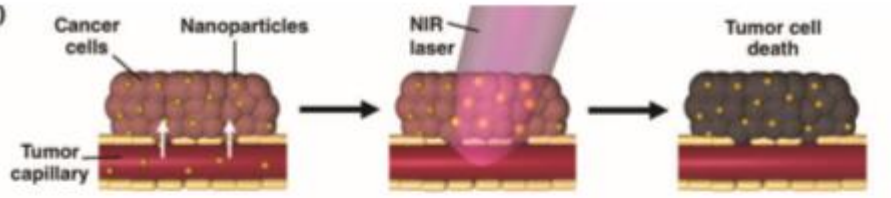


Figure 7. A hypothetical quantum dot system with different ligands, than can be applied for in vivo detection of different targets at the same time **[7].**

Gold nanoparticles have been used in cancer diagnosis and therapy for several years. They are biocompatible and have unique optical properties and small size. They are easily manufactured and functionalized, a fact that makes them very promising for oncologic therapeutic and theranostic applications. Gold forms a wide variety of nanoparticles, such as nanospheres, nanorods, nanocages and nanoshells **[55]**. For cancer therapy, they have been utilized as photothermal agents **(Figure 8).** Once excited by light of a certain wavelength, they generate heat and kill tumor cells **[75]**, in a process which is similar to hyperthermal ablation induced by SPIONs. Gold nanoparticles can be further functionalized to enhance passive and active targeting. They are also employed as X-ray contrast agents in computerized tomography (CT) in order to achieve higher attenuation **[66, 76].**

(a)



(b)

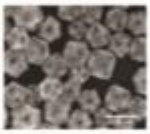
	Nanoshells	Nanorods	Nanocages	Nanostars
Schematic				
SEM				
Size for PTT	~150 nm diameter	~10 nm x 40 nm	~40-60 nm length	~45-120 nm
Stage of development	Clinical trials: lung, head & neck, and prostate cancer	Preclinical	Preclinical	Preclinical
Unique features	Core:shell structure	Two resonance peaks	Drug loading capabilities	Large surface area for bioconjugation

Figure 8: a) Presentation of photothermal therapy. b) The most commonly used Au nanoparticle forms in photothermal therapy and their properties **[77].**

Silver nanoparticles (AgNP) are plasmonic structures that absorb and scatter light. They are selectively uptaken into cancer cells, and as they absorb light, they can be used for hyperthermia, whereas the light they scatter makes them imaging agents, as well. Ag exhibits high efficiency of plasmon excitation and is the only metal whose plasmon resonance phenomenon can be tuned to any wavelength in the visible portion of the spectrum. As far as the therapeutic potential of Ag nanoparticles is concerned, they can affect the proteins, which are responsible for the neutralization of reactive oxygen species after interacting with cells. In this way they cause ROS accumulation since the antioxidant defense system of the cell is depleted. ROS accumulation triggers the inflammatory response and destroys the mitochondria **[3]. Table 2** shows other metallic nanoparticles that are investigated in nanomedicine.

Table 2. Other metals explored for use in the diagnosis and therapy of cancer

Metal	Formulation	Results
Barium	Barium titanate core-Au shell nanoparticles	NIR laser-induced thermoablation experiments revealed ability of nanoshells to produce hyperthermia and destroy human neuroblastoma cells
	Barium titanate nanoparticles	Optimal cytocompatibility of barium titanate nanoparticles was demonstrated based on noncovalent binding to glycol-chitosan; efficiency of DOX was highly enhanced
Bismuth	Bismuth selenide nanoparticles	Nanoparticles absorbed NIR laser light producing irreversible photothermal ablation of tumors; also exhibited strong X-ray attenuation that can be utilized for X-ray CT imaging
	Barium sulfide nanoparticles	Enhanced visualization of breast cancer using X-ray micro-CT achieved using Bi_2S_3 nanoparticles, modified to display a tumor-homing peptide (LyP-1, CGNKRTRGC); accumulation within tumor was increased by 260% over nonlabeled nanoparticles
Calcium	pH-sensitive calcium carbonate nanocrystals	CaCO□/DOX nanocrystals exhibited high uptake by breast cancer cells, producing greater reduction in growth compared with free DOX
	NIR emitting fluorophore-doped calcium phosphate nanoparticles	Nanoparticles accumulated in solid xenograft breast adenocarcinoma tumors via EPR; *ex situ* tissue imaging further verified facility of indocyanine green-embedded nanoparticles for deep-tissue imaging
Cu	PEG-coated Cu sulfide nanoparticles	Nanoparticles could be used both as contrast agent for PA tomographic imaging of mouse tumor vasculature and for confined photothermolysis of tumor cells
	Cu oxide nanoparticles	Apoptotic effect of Cu oxide nanoparticles mediated by ROS generation involving disruption of mitochondrial membrane potential
Magnesium	Magnesium silicate hollow nanospheres	Nanoparticles mostly accumulated in lysosome, which facilitated continual drug release and efficient cancer cell destruction
Nickel	PEGylated nickel carbide nanocrystals	Nanoparticles exhibited broad absorption from visible to NIR regions and rapid increase in temperature when irradiated with 808-nm laser, damaging cancer cells
	Nickel oxide nanoparticles	Reduced cell viability in a dose-dependent manner in Hep-2 and MCF-7 cells; oxidative stress indicated by depletion of glutathione and induction of ROS and lipid peroxidation; induction of caspase-3 enzyme activity and DNA fragmentation also observed
Titanium	Titanium dioxide nanoparticles	Significant induction in DNA damage in cells exposed to nanoparticles; expression of caspase-3 and caspase-9 mRNA increased

Table 2: Other metallic nanoparticles used for the diagnosis and treatment of cancer **[3].**

2.3. Bimetallic nanoparticles

According to Katifelis et al., ***bimetallic*** are the nanoparticles that have been constructed via the combination of two different metals (e.g. Ag-Au, Fe-Au nanoparticles). The basic characteristic of these nanoparticles is that they incorporate the different properties of each metal and therefore they exhibit multiple properties (catalytic, photocatalytic, novel electronic, and optical). Also, they can be applied in many different fields **[78].**

According to Srinoi et al bimetallic nanoparticles can be classified based on two distinct criteria:

- *structure:* mixed structures **(Figure 9a, b)** and segregated structures **(Figure 9c–f)**.
- *atomic ordering:* alloy, intermetallic, sub clusters, and core-shells **[79]**

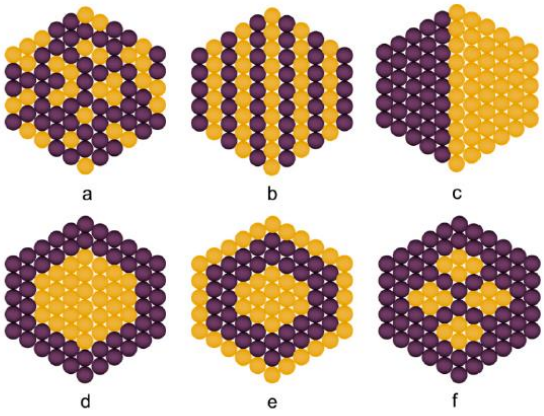


Figure 9: Types of bimetallic nanoparticles: a) alloy type, b) intermetallic, c) subclusters, d) core-shell, e) multi-shell core-shell, and f) multiple core materials coated by a single shell material. Yellow and purple spheres represent two different kinds of metal atoms **[80].**

The basic characteristic of the above categories of bimetallic nanoparticles is that the overall properties of alloy, intermetallic, subclusters, and core-shell nanoparticles depend on way of atomic configuration (random or ordered configuration). For example, a bimetallic nanoparticle with mixed structure and a random arrangement of atoms can be a bimetallic nanoparticle of allow type. If it has an ordered arrangement it can be as a bimetallic nanoparticle with intermetallic structure **[79].**

According to the Mazhar et al, bimetallic nanoparticles display more advantages compared to their monometallic counterparts, because of their mixing patterns and geometrical architecture. More specifically, bimetallic nanoparticles exhibit good stability, selectivity and catalytic activity. Also, they present novel properties, due to the synergistic effects of their metal components **[79].**

2.3.1. Methods of bimetallic nanoparticle synthesis

The exact composition of colloidal solutions is never easy to determine since even small changes in the synthesis process can lead to completely different results. Furthermore, the chemical process of preparing nanocolloid solutions can produce nanoparticles with a different crystalline structure. Metallic nanoparticles composed of noble metals are prepared naturally or chemically by noble metal atoms using a process of agglomeration

(methods of dispersing and condensing) in the presence or absence of protecting groups such as polymers, surfactants, or strong linking substituents.

Much of the research has been directed to the manufacturing of controlled-form metal nanoparticles.

In general, the synthesis methods of bimetallic nanoparticles are divided in three groups:

- *physical methods,*
- *chemical methods* and
- *biological methods* **(Figure 10) [81].**

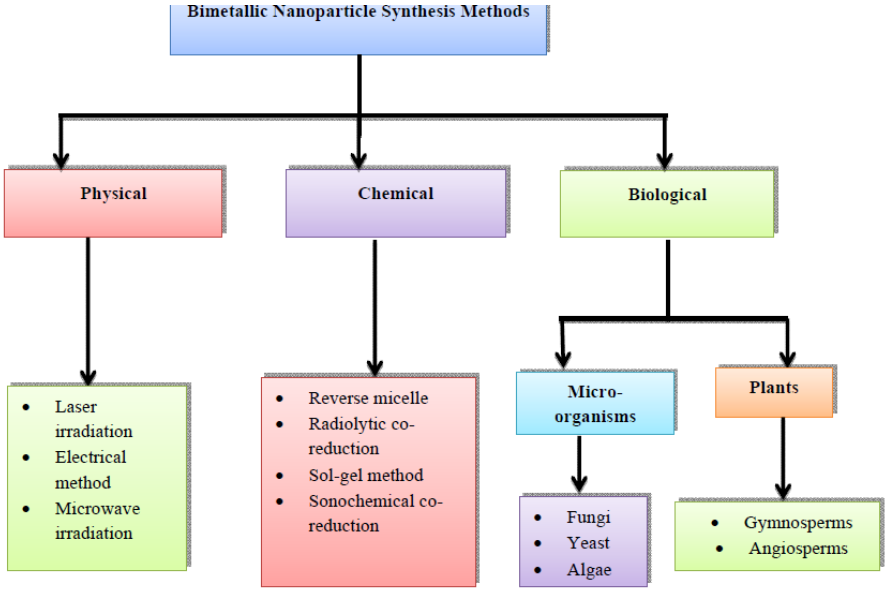


Figure 10: The methods of bimetallic nanoparticles synthesis **[81].**

In another classification, bimetallic nanoparticles can be synthesized using various methods **(Table 3).** These methods are distinguished in two categories: ***bottom-up*** methods and ***top-down*** methods **(Figure 11).**

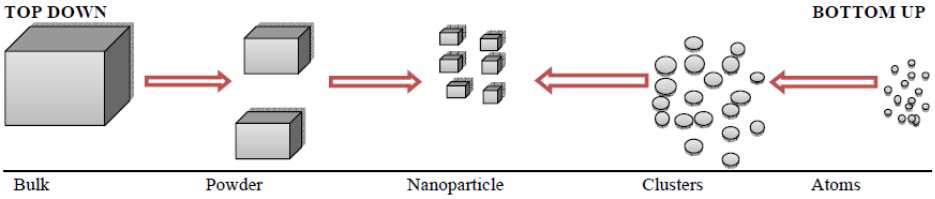


Figure 11: Schematic representation of nanoparticles synthesis **[79].**

The first category includes any method which starts with raw materials of macroscopic size. With appropriate processes their size is reduced up to the nanoscale. In the second category, the course is inverse, since the initiating materials are of atomic or molecular size and gradually "merge" into larger systems. Solid-phase methods belong to the top-down category, while those of liquid and gaseous phases belong to the bottom-up **[79-81].**

The top-down approach is based on natural microstructural processes, such as cutting, breaking, alloying, and finally sculpting the material in the nanoscale. This approach does not provide the desired control over the homogeneity of products and it does not always achieve as small dimensions as wished. Also, the top-down approach requires expensive high-tech devices which is a major disadvantage. Still top-down approaches are widespread as they produce large quantities of nanomaterials **[79-81].**

In contrast, the bottom up approach relies mainly on the physicochemical phenomena and the organization of the building blocks (atoms, molecules) in order to create nanoparticles. Applying a bottom-up strategy requires good understanding of the forces which develop between the building blocks, such as Van der Waals, electrostatic, intramolecular and intermolecular forces. Bottom-up processes exhibit several advantages:

- controlling the dimensions of the product is easy, through appropriate modification of the experimental conditions,
- they enable the preparation of various nanostructures that cannot be achieved through top-down approaches,
- the products have high homogeneity and good crystallinity
- most bottom-up methods are cheap, simple and environmentally friendly **[79-81].**

Table 3. shows the basic synthesis methods of many different types of bimetallic nanoparticles.

Material	Synthetic Method	Size
AuCu	Chemical reduction	8.3 nm, 20–25 nm
	Chemical reduction using green synthesis with Vitamin C	5–50 nm
AuPd	Hydrothermal conditions and microwave irradiation	40 to 65 nm, 150–200 nm
	Chemical reduction	1.6 nm
	Sonochemical	5–9 nm
	Microwave-assisted polyol	9 nm Au core, 3 nm Pd shell
	Seed-mediated growth	5 nm
		35–100 nm
	Green synthesis	7 nm
AuPt	Chemical reduction	3 nm, 6 nm
	Successive/reduction	30 nm
	Galvanic replacement	The apex–apex length of the concave nanocubes is 12.81 nm
	Transmetalation reaction/replacement	10–20 nm
	Seed-mediated growth	3–8 nm pearlike 5–8 nm peanutlike 7–10 nm cloverlike
	Ultrasonochemical reduction	39.5–101.6 nm
	Hydrothermal conditions and microwave irradiation	30 nm
PdCu	Chemical reduction using green synthesis with Vitamin C	5–50 nm
PtCu	Chemical reduction using green synthesis with Vitamin C	50–60 nm
PtRu	Hydrothermal (thermal decomposition)	2.5 nm
PtPd	Chemical reduction	5–8 nm, 13 nm
	Sonochemical	2–3.6 nm
PdAg	Galvanic replacement	Nanobox 63 nm in edge length
PtAg	Galvanic replacement	Nanobox 63 nm in edge length
	Radiolytic synthesis	3–20 nm
RhCu	Soft-templating strategy	74–135 nm
CuAg	Sono and electrochemical synthesis	10–80 nm
FePt	Chemical reduction	3 nm, 12 nm
	Pyrolysis (thermal decomposition)	9 nm
	Thermal decomposition	12 nm
	Polyol (chemical reduction)	3–12 nm
	Sonochemical	3–5 nm
	Hydrothermal	9.4 nm
FeCo	Chemical reduction	9 nm
	Physical vapor nanoparticle-deposition technique	3–100 nm
	Thermal decomposition	4,7 nm
	Sputtering gas condensation technique	12.8 nm

Material	Synthetic Method	Size
AuAg	Polyol reduction Galvanic replacement	50 nm
	Co-reduction Galvanic replacement	10.2 nm
	Chemical reduction	5 nm
		32 and 45 nm
	Radiolysis	1.5–12 nm
	Seed-mediated growth	10–16 nm
	Seed-mediated polyol process	52 nm
	In situ co-reduction method	2.6 nm
	Successive ionic layer absorption and reaction (SILAR)	10–20 nm
AuFe	Chemical reduction	15–30 nm
	Laser ablation of a bulk alloy	30–60 nm
AuCo	Chemical reduction	3–5 nm
		14.2 nm
		15–30 nm
PdCu	Chemical reduction using green synthesis with Vitamin C	5–50 nm
PtCu	Chemical reduction using green synthesis with Vitamin C	50–60 nm
PtRu	Hydrothermal (thermal decomposition)	2.5 nm
PtPd	Chemical reduction	5–8 nm, 13 nm
	Sonochemical	2–3.6 nm
PdAg	Galvanic replacement	Nanobox 63 nm in edge length
PtAg	Galvanic replacement	Nanobox 63 nm in edge length
	Radiolytic synthesis	3–20 nm
RhCu	Soft-templating strategy	74–135 nm
CuAg	Sono and electrochemical synthesis	10–80 nm
FePt	Chemical reduction	3 nm, 12 nm
	Pyrolysis (thermal decomposition)	9 nm
	Thermal decomposition	12 nm
	Polyol (chemical reduction)	3–12 nm
	Sonochemical	3–5 nm
	Hydrothermal	9.4 nm
FeCo	Chemical reduction	9 nm
	Physical vapor nanoparticle-deposition technique	3–100 nm
	Thermal decomposition	4,7 nm
	Sputtering gas condensation technique	12.8 nm

Table 3. The basic methods used for the synthesis of bimetallic nanoparticles **[79].**

2.3.1.1. Biological synthesis of bimetallic nanoparticles

Biological synthesis is characterized by Kuppusamy et al. as a green and environmentally friendly method that produces non-toxic and biodegradable metallic bimetallic nanoparticles. This approach uses bacteria, biomolecules and plant extracts for the synthesis of nanoparticles **(Figure 12). Table 4.** presents various types of nanoparticles synthesized by a variety of microorganisms. AgAu bimetallic nanoparticles can be biosynthesized by bacterial strains, such as Lactobacillus **[82, 83].**

Microorganism		Type of Nanoparticles	Size of Nanoparticles	Mode of Synthesis
BACTERIA	*Staphylococcus aureus*	Ag NPs	160-180 nm	Extracellular
	Bacillus cereus	Ag NPs	4-5 nm	Extracellular
	Marinobacter pelagius	Au NPs	2-10 nm	Extracellular
	Actinobacter sp.	Au NPs	50-500 nm	Extracellular
	Enterobacter sp.	Hg NPs	2–5 nm	Intracellular
	Lactobacillus	TiO_2 NPs	40-60 nm	Extracellular
	Escherichia coli	CdS QDs	2-5 nm	Intracellular
	Escherichia coli	CdTe QDs	2-3.2 nm	Extracellular
FUNGI	*Verticillium*	Ag NPs	25±12nm	Intracellular
	Aspergillus terreus	Ag NPs	1-20 nm	Extracellular
	Bryophilous Rhizoctoni	Ag NPs	20-50 nm	Extracellular
	Aspergillus flavus	Ag NPs	8.92 ± 1.61 nm	Extracellular
	Pleurotus ostreatus	Ag NPs	8-50 nm	Extracellular
	Fusarium oxysporum	Au NPs	8-40 nm	Extracellular
	Fusarium oxysporum	SiO_2 NPs	2-4 nm	Extracellular
	Fusarium oxysporum	Magnetite NPs	20-50 nm	Extracellular
	Fusarium oxysporum	TiO_2 NPs	6-13 nm	Extracellular
	Fusarium oxysporum	ZrO_2 NPs	7-8 nm	Extracellular
YEAST & ACTINOMYCETES	*MKY3*	Ag NPs	2-5 nm	Extracellular
	Candida albicans	Au NPs	20-40 nm	Extracellular
	Hansenula anomala	Au NPs	14 nm	Extracellular
	Yarrowia lipolytica NCIM 3589	Au NPs	9-23 nm	Extracellular
	Candida glabrata	CdS NPs	2 nm	Intracellular
	Rhodosporidium diobovatum	PbS NPs	2-5 nm	Intracellular
	Thermomonospora sp.	Au NPs	8 nm-40 nm	Extracellular
	Rhodococcus sp.	AuNPs	5-15 nm	Intracellular
Abbreviations: NPs- Nanoparticles, QDs- Quantum Dots				

Table 4. Various microorganisms reported to be involved in the synthesis of different types of metallic and bimetallic NPs **[81].**

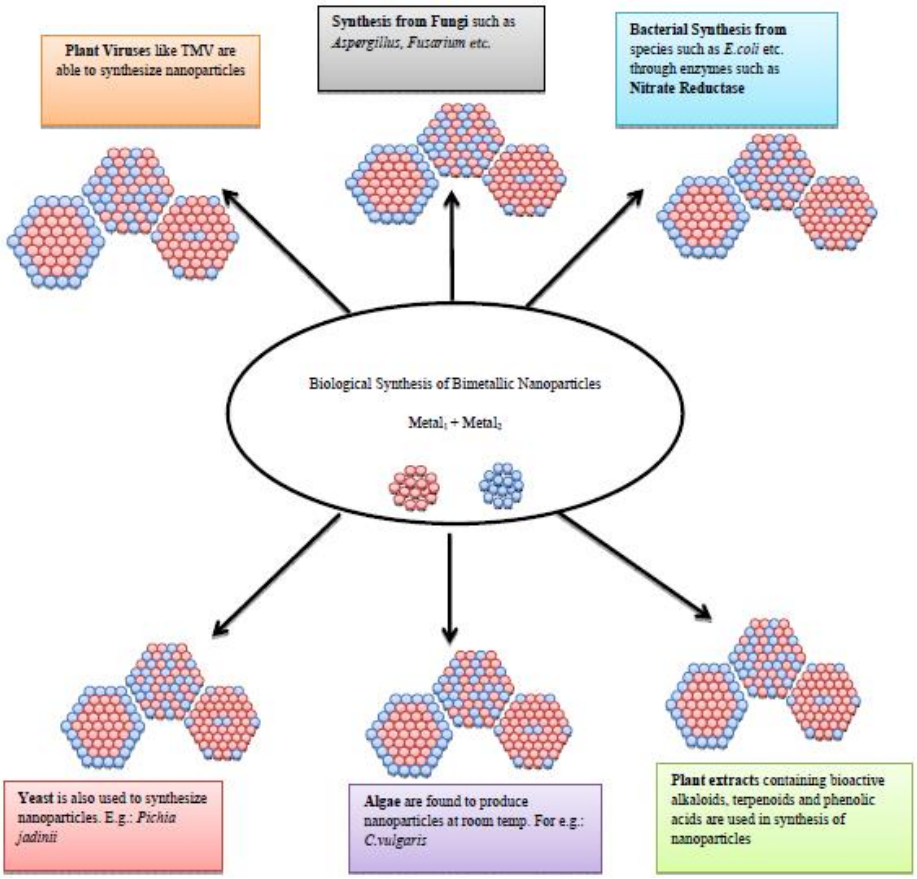


Figure 12. Representation of the biological synthetic process of bimetallic nanoparticles with different architectures, for example core-shell, alloy, and crown jewel **[79].**

The most commonly used biologic approach is bimetallic nanoparticle synthesis using plant extracts, because the method is effective, rapid, clean and non-toxic. The metabolites of various plants can be effectively used for the preparation of such nanoparticles **[81].**

In the literature various plant species are referred, regarding the biosythensis of AgAu nanoparticles. For example, Mondal et al manufactured Ag-Au bimetallic nanoparticles using the aqueous extract of mahogany (*Swietenia mahogani JACQ*.) leaves as a reducing agent. Also, the biologic synthesis of gold (Au), silver (Ag) and bimetallic alloy AgAu nanoparticles from aqueous solutions using *Cannabis sativa* as reducing agents has been reported **[84].**

Mazhar et al. displayed two more examples about bimetallic nanoparticles whose synthesis has been facilitated using plant extracts: leaf extract of Persimmon (*Diopyros kaki*) and leaf extract of *Piper pedicellatum* **[79].**

2.3.1.2. Physical methods for the synthesis of AgAu bimetallic nanoparticles

Laser-assisted synthesis of Ag–Au alloy NP

Since the cornerstone of this scientific work are the AgAu bimetallic nanoparticles, we describe their synthesis, using physical methods. Laser irradiation is a bottom-up method that can be applied for the synthesis of AgAu bimetallic nanoparticles. Bimetallic AgAu nanoparticles can be synthesized in one step with this method. More specifically the laser beam heats the solution of Au-Ag and as a result, Ag ions are reduced and alloying of Au and Ag occurs **[85].** Recently, Kuladeep et al. tried to synthesize AgAu bimetallic nanoalloys using a tunable laser frequency to cause localized surface plasmon resonance in the presence of polyvinyl alcohol as a reducing agent **[86].** Similar methods can be used to construct different types of nanoparticles, which consist of other metals, as well.

2.3.1.3. Chemical methods for the synthesis of AgAu bimetallic nanoparticles

Replacement reactions

Replacement reactions are a category of redox reactions in which one chemical replaces another in a compound, because of their different activity **(Table 4).** Once again, we describe the major chemical method for the manufacturing of AgAu bimetallic nanoparticles. The reaction that occurs between the metals for the creation of AgAu bimetallic nanoparticles is the following **(Figure 13) [87]:**

$$3Ag(s) + AuCl^{4}(aq) \rightarrow Au(s) + 3Ag + 4Cl^{-}(aq).$$

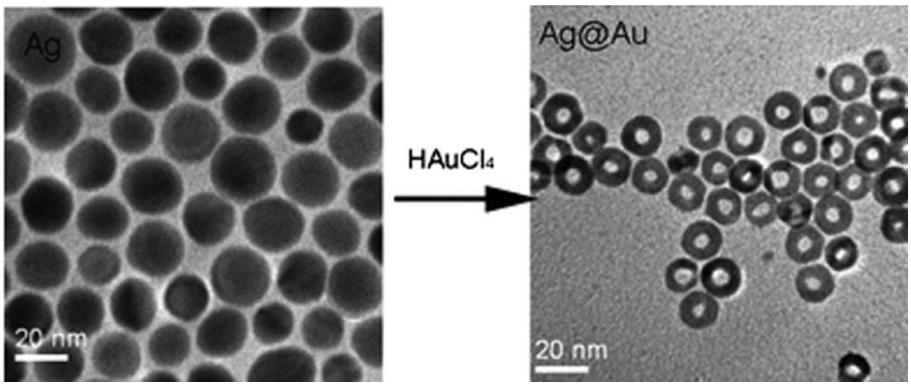


Figure 13. Synthesis of core/alloy shell NPs by the replacement reaction between Ag NPs and $HAuCl_4$ **[87].**

Advantages	**Disadvantages**
• rapid interdiffusion of Au and Ag atoms in the reduced dimension of NPs	• the size and composition of the alloy NPs are separately tunable
• high temperature of the process,	• the particles can be formed in high concentrations (good process scalability
• the plenty of interfacial vacancy defects created by the reaction	

Table 4. Advantages and Disadvantages of the replacement reaction **[4].**

2.3.2. Anticancer and other properties of bimetallic nanoparticles

Bimetallic nanoparticles and especially those which comprise of gold and silver have been explored for their possible antitumor activity. Such studies are based to a great extent on cell toxicity studies. Shmarakov et al noticed the cytotoxic effect of bimetallic AgAu nanoparticles on LLC cancer cell line. Three different molecular rations were used, namely AgAu(1:3), AgAu(1:1), AgAu(3:1) and different topology, as well (**Au**core**Ag**shell, **Ag**core**Au**shell). The team observed that all different nanoparticles exhibit cytotoxic effects. For most nanoparticles, cell viability was less than 70%. The maximum value for tumor inhibition (34%) was observed when the cells were incubated with AgAu nanoparticles of a molar ratio Ag:Au 1:3. In terms of topology, the highest antitumor effect was observed with **Ag**core**Au**shell nanoparticles **[88].**

In another study, Mittal et al explored the anticancer activity of AgSe nanoparticles on DL cell lines, using an MTT assay. The nanoparticles proved to be toxic in concentrations above 50μg/ml. At this concentration, viability stood at 20% and it fell even more with bigger concentrations. This observation is consistent with previous studies that suggest selenium as an anticancer agent **[89].**

Furthermore, Katifelis et al examined the cytotoxic effects of AgAu bimetallic nanoparticles on several cell lines. Toxicity in cancerous cell lines reaches 50% from a concentration of 20μg/ml and above, while at the same time toxicity in HEK293 is 20%. Interestingly enough, at 50μg/ml HEK293 toxicity remains still at 20%, while the toxicity in cancerous cell lines reaches 90%. More specifically, AgAu(3:1) nanoparticles seem to be the most suitable ratio of metals, since they are highly cytotoxic against cancer cells and not in HEK293. AgAu(1:1) bimetallic nanoparticles are also cytotoxic, but to a lesser extent and the least cytotoxic are AgAu(1:3) ones. The same study suggests that tryptophan, which was used in nanoparticle synthesis, has a protective role for HEK293 cell lines, while it increases toxicity against cancer cells **[78].** The same observation for tryptophan was made by Shmarakov et al, who noticed that the utilization of tryptophan as a reducing factor during bimetallic nanoparticle synthesis, reduces the nanoparticle toxicity in the liver and kidneys **[90].**

AgAu nanoparticles can also be used in ***photothermal therapy***. According to Nasrabadi, when oral cancer cells are incubated with such nanoparticles and are exposed to laser radiation, significant cytotoxic effect can be observed after 1 minute. On the contrary, cells do not exhibit cell death, when they are irradiated without incubation with the nanoparticles **[4].**

Several bimetallic nanoparticles have also been reported as successful agents for ***magnetic hyperthermia***. For example, CuNi and FeNi nanoparticles display a significant self-heating effect over 40 ◦C. Last but not least, gold-based bimetallic nanostructures can as drug carriers, because they have small size, they are biocompatible, and their surface is easily modifiable **[79].**

Apart from therapeutic applications, bimetallic nanoparticles can have future utilization in diagnostic applications, which include sensing and imaging, thanks to their optical

and magnetic properties. Plasmonic nanoparticles can be employed as ***plasmonic biosensors,*** in order to detect specific biomolecules. For example, nanoclusters of copper, gold and silver have already been established as imaging agents in several in vivo or in vitro systems. Magnetic nanoparticles cause low background signal in most samples, which is an important advantage. Apart from sensing based on magnetic separation, magnetic nanoparticles can act as ***contrast agents*** in imaging applications. In fact, FeNi, CuNi, FeCo and Pt3Co nanoparticles have been reported as T2 signal contrast agents in MRI. Moreover, bimetallic nanoparticles can enable dual modality imaging after their surface is modified, which offers a more comprehensive diagnosis of several diseases, including cancer. For example, FePt bimetallic nanoparticles can be conjugated with anti-HER2 antibodies or cysteamine to allow dual CT/MRI molecular imaging **[79].**

2.3.3. Bimetallic nanoparticle conjugates with polymers

The entrapment, encapsulation and conjugation of bimetallic nanoparticles on polymer nanomolecules is a field of nanoscience that has yet to be explored. Until now there are only a few studies in literature and in most of them the polymer is used as a scaffold for the manufacturing of bimetallic nanoparticles or as a platform to create biosensors. There are very few studies regarding the utilization of bimetallic nanoparticle-polymer conjugates in cancer treatment. Maney et al created PtAu bimetallic nanoparticles, bound them with chitosan (which is a biopolymer) and loaded them with doxorubicin (anticancer agent). Then, the nanocarrier was studied in terms of provoking cytotoxicity against several cell lines. It became obvious that PtAu-chitosan nanocomposites can encapsulate large quantities of doxorubicin and exploit tumor acidic conditions to release their cargo (pH-dependent release) in a prolonged way **[5].**

In a similar study, which whoever included monometallic gold nanoparticles, Liebig and his colleagues focused on the toxicity of coated gold nanostars. These nanoparticles were coated with poly(ethyleneimine) (PEI), maltose-modified poly(ethyleneimine) (PEI-Mal) and heparin. Then their cytotoxicity was evaluated on HEK293 and YTS cell lines. Interestingly, coating the nanoparticles with heparin reduced toxicity and improved biocompatibility of the Au nanostars, as compared with plain ones. On the

contrary PEI-coated and PEI-Mal-coated gold nanostars were highly toxic for both cell lines **[91].**

2.4. Mechanisms implicated in nanocarrier-based drug delivery to cancer tissues

2.4.1. Passive targeting - Enhanced Permeation and Retention (EPR) phenomenon

When a tumor starts to develop, its cells proliferate rapidly, in an unsustainable way, so that the offered nutritional supply from blood circulation (mainly via diffusion) is limited and not enough to meet the metabolic needs of the total tumor volume **[92].** For that reason, after some point the tumor size reaches a plateau, which is called 'diffusion-limited size' and has a diameter of around 2 mm **[92, 93].** In order for the tumor to satisfy its constantly growing needs for nutrients, cancer cells enhance ***neoangiogenesis***, the creation of new blood vessels **[92].** In this phase excessive secretion of angiogenic factors is observed, such as vascular endothelial growth factor (VEGF), in addition to other factors (e.g., cytokines, matrix metalloproteinases (MMP) etc.). These factors enhance neoangiogenesis and increase vascular permeability **[93-95].** The newly created blood vessels are characterized by an abnormal and leaky structure. The basal membrane exhibits pores and pericytes and smooth muscle cells are almost absent **[93, 96].** This creates fenestrations in the blood vessel architecture. Their size is heterogeneous and can even reach 200 nm or more. This abnormal structure potentiates the extravasation of circulating molecules and nanocarriers into the tumors (an effect describes as ***enhanced permeation***), which normally is not observed in healthy organs with intact vessels **[93].** Another aspect that characterizes ***EPR*** is the ***enhanced retention*** and long-lasting accumulation of extravasated molecules and nanoplatforms within tumor tissues. This phenomenon is partly due to the poor lymphatic drainage which is displayed by tumor tissues. The abnormal vessel structure is also responsible for the access of cancer cells to the circulation, a fact that causes metastasis **[93].** The leaky vasculature phenomenon is a research target for many human tumors (such as pancreatic cancer) as far as both chemotherapeutic and imaging applications are concerned **[95].**

Although EPR effect is very promising in terms of antitumor clinical applications, many challenges still need to be assessed. One of them is tumor heterogeneity. Among different cancer types, many differences in vasculature formation and architecture can be observed. Moreover, we need to consider the effect of the cancer stage on the EPR, the delivering of nanomedicines to the primary or metastatic lesions, the tumor stroma characteristics, as well as patient-to-patient variability and the necessity for an individualized nanomedical approach. Tumor heterogeneity should also be taken into consideration in the preclinical context. Animal tumor model selection can significantly affect decisions regarding subsequent clinical trials. It is rather notable that subcutaneous xenografts are tumors that form very fast and usually exhibit an exaggerated EPR effect, which may mislead clinical translation **[97].** Genetically engineered mouse models (GEMM) are of greater value when EPR effect is being studied **[97 –100].**

Scientific efforts are oriented towards augmenting EPR effect, in order to increase drug accumulation in tumors. Maeda et al proposed that, since tumor vasculature lacks integrity, when a vasoconstrictor agent (e.g. angiotensin II) is administered, the tumor blood vessels will not exhibit vasoconstriction, in contrast to other healthy blood vessels in the body. Thus, the tumor blood flow is expected to peak, which in turn will increase the concentration of nanomedicines to the desired tumors via EPR effect, an assumption which was proven scientifically **[96, 101].**

Although the abnormally formed tumor vasculature is regarded as a feature that should be exploited in nanomedicine delivery, there are interestingly other researchers, who consider it an obstacle that must be 'normalized' in order achieve the delivery of chemotherapeutics to tumors. Jain et al introduced the idea of ***"tumor vasculature normalization"*** **[102].** They suggested that the defective tumor vasculature may have a negative influence, as far as the delivery of nanomedicines and generally chemotherapy to tumors is concerned **[103].** According to their assumption, tumor vascular "normalization" shall alleviate tumor hypoxia, which increases radiation toxicity on tumors, reduces metastatic potential and tumor resistance, and ultimately improve chemotherapy effectiveness **[103–104] (Figure 14).** In tumors, the balance between antiangiogenic and proangiogenic activity is shifted towards the second, in

order to promote tumor neoangiogenesis **[105].** Using antiangiogenic drugs (e.g., monoclonal antibodies for VEGF and VEGF receptor tyrosine kinase inhibitors) is the most common way to reestablish the normal balance between pro- and anti-angiogenic factors **[103, 105].** Unfortunately, the vascular ***"normalization"*** effect lasts for a small period of time (about 7–10 days), and during this window period chemotherapy or radiotherapy should start **[105, 106].** Jain et al found that tumor vasculature "normalization" improved tumor uptake of nanoparticles with a size between 10-12 nm (i.e., albumin-bound paclitaxel), but not larger ones (for example doxorubicin) **[18, 107].**

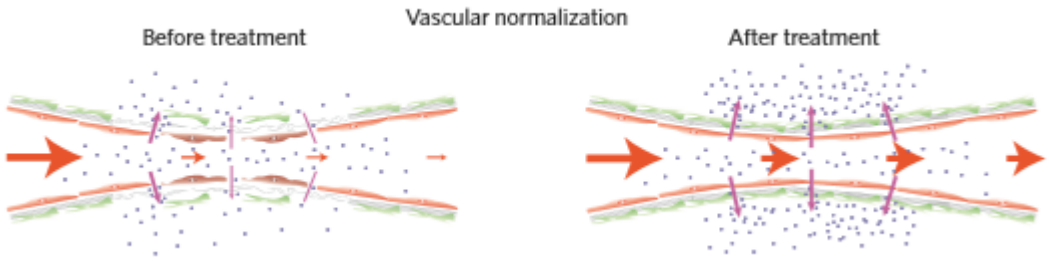


Figure 14: Vascular normalization repairs vasculature, making it more mature, homogenous and less leaky. This results in lower interstitial fluid pressure. Transvascular fluid pressure difference is restored, which improves blood flow and nanoparticle penetration in tumors **[108].**

2.4.2. Active Targeting

The term ***active targeting*** describes the exploitation of proteins or any other molecular targets, which are expressed or overexpressed exclusively on tumor cells or vasculature **(Figure 15).** The exploitation takes place via the attachment of a specific ligand, which is complementary to the tumor molecular target, on the nanocarrier surface. These ligands exhibit high affinity to the tumor targets. The receptor bearing nanocarriers are then internalized via endocytosis inside tumor cells **[109].** While tumor accumulation is facilitated via EPR, active targeting can be thought of as method to promote cellular internalization of nanocarriers. Various possible targets for active targeting have been identified in tumors, such as vascular endothelial growth factor receptors (VEGFR), several integrins, epidermal growth factor receptor (EGFR), human epidermal growth

factor receptor 2 (HER-2), transferrin and folic acid receptors among others **[110, 111].** Two main EGFR tyrosine kinases are well- studied and characterized, namely, EGFR and HER-2. Sandoval et al. manufactured an EGFR-targeted SLNs, which contained lipophilic gemcitabine in order to target human breast cancer cells, which are known to overexpress EGFR. Compared to non-targeted gemcitabine SLNs, the EGFR-targeted once displayed enhanced antitumor activity and accumulation in mice breast cancer tumors. Other nanocarrier surface modifications include the conjugation with transferrin and folic acid **[112–116].**

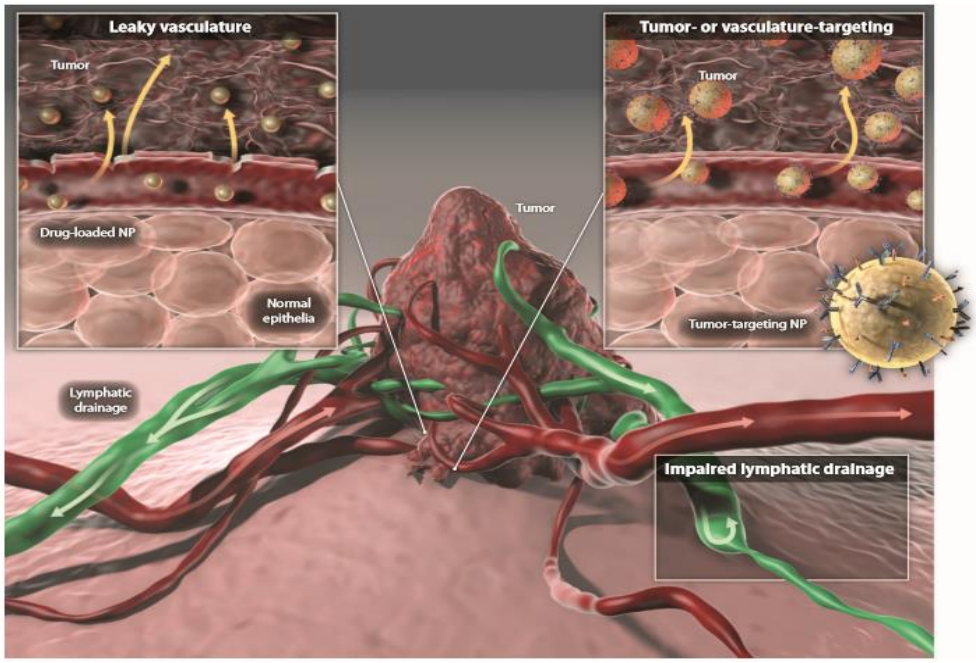


Figure 15. Passive targeting (EPR effect) and active targeting **[117].**

2.5. Body barriers to the delivery of nanomedicines to the tumors

This section displays the major barriers that prevent the delivery of nanomedicines to tumors. Once we administer a nanomedicine, it has to pass several physiological barriers before it reaches blood circulation, unless it is administered intravenously. Once injected, it travels long before it reaches its target, which is the cancer cell. The body reacts, tending to clear the blood from the newly injected nanomedicine. Even the small fraction of the nanomedicine that overcome these clearance mechanisms, encounter a totally different environment (the tumor) that should also be tackled. In order to tackle the tumor microenvironment, two approaches are applied. One includes

the modification of the tumor microenvironment physical barriers. The other approach is to take advantage of the unique features of the tumor microenvironment in order to deliver more drugs to the tumor cells. Unfortunately, reaching these cells is not the end of the journey, since the cells themselves have their own defense mechanisms **[18].**

2.5.1. Nanomedicine circulation time and drug release

Once the nanomedicines enter the circulation, they adsorb plasma proteins, like albumin, complement proteins and immunoglobulins. This process is called ***opsonization.*** After opsonization, the nanocarriers are recognized and phagocytosed, a process delivered by the ***mononuclear phagocyte system (MPS)*** (e.g., monocytes and macrophages) and are carried to the MPS organs (e.g., liver, spleen, lymph nodes). This phenomenon significantly shortens the circulation half-life of the nanomedicines. Opsonization is observed in large nanoparticles, with a diameter >100 nm. Small nanoparticles below 10 nm (such as some quantum dots and carbon nanotubes) are excreted through the kidneys after glomerular filtration. Nanocarriers are opsonized based on several factors, such as surface characteristics (hydrophobicity vs. hydrophilicity), zeta potential, size, and shape. It has been observed that opsonization happens more efficiently and faster to hydrophobic particles. This is the reason why the surface modification of nanoparticles with hydrophilic molecules is the most widely applied strategy in order to prolong the nanomedicine circulation time **[118-122].**

PEG is the most commonly used molecule to achieve this goal, in a process called PEGylation **(Figure 16).** The protruding PEG chains are believed to sterically prevent the adsorption of plasma proteins on the nanocarrier surface. Also, its hydrophilic nature is believed to minimize non-specific interactions of the nanocarrier surface with MPS cells **[123]**. The term ***"stealth"*** is used to describe PEGylated, long circulating nanocarriers, since PEGylation has become the cornerstone in the design of chemotherapeutic nanocarriers.

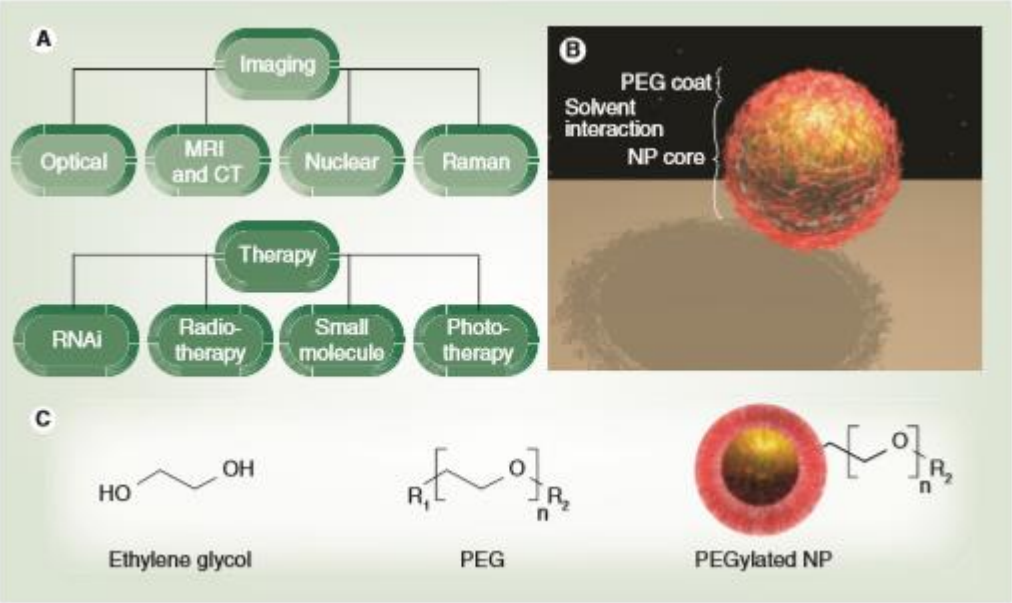


Figure 16. Nanoparticle applications of PEG (polyethylene glycol). a) The use of nanoparticles for imaging and therapy. b) A PEGylated nanoparticle, the surrounding cloud of PEG chains (red). c) Monomers of ethylene glycol are polymerized and create PEG for nanoparticle coating **[124].**

Drug release from the nanocarrier is also a factor that needs to be carefully considered when we design a nanoparticle. A very fast drug release can lead to the total loss of the drug in the circulation before it reaches their target. As a result, prolonged circulation time should happen simultaneously with slow drug release to avoid drug leakage **[125].**

Biomimetic particles, which are nanoparticles that mimic biological entities, such as bacteria, viruses, and several blood cells have been extensively studied as well, for drug delivery purposes. These biological entities exhibit tremendous capabilities when it comes to evading biological and cellular barriers. The camouflaged nanoparticles can be expected to have the same properties **[110].** This strategy and the control of shape and size of the nanoparticles is commonly adopted to increase the blood circulation time and improve drug delivery to tumors **[122].** Recently, Taciotti et al synthesized leukocyte-mimicking non-porous silicone nanoparticles, which coated with a leukocyte cell membrane in order for the nanocarrier to exhibit white blood cell properties **[126].** These particles have in fact exhibited significantly less opsonization rates and cellular uptake by human macrophages and may be promising in the delivery of drugs to

tumors, since they display delayed liver clearance and they exploit EPR effect, while they can also be actively targeted to bind on the tumor endothelium **[126].**

2.5.2. Other Biological Barriers

Another biological barrier regarding the delivery of nanomedicines to cancer tumors is the ***blood brain barrier (BBB)*** **(Figure 17).** Its presence and the existence of tight junctions between its endothelial cells, leads to low permeability of nanodrugs into the tumor parenchyma. Its vascular pore size is around only 12 nm, so the EPR effect is probably not useful in terms of increasing the delivery of nanomedicines in brain tumors **[118].** Using targeting ligands to enable the transportation of the nanocarriers across the endothelial boundary of the tumor vasculature is considered a very promising approach **[127-129].**

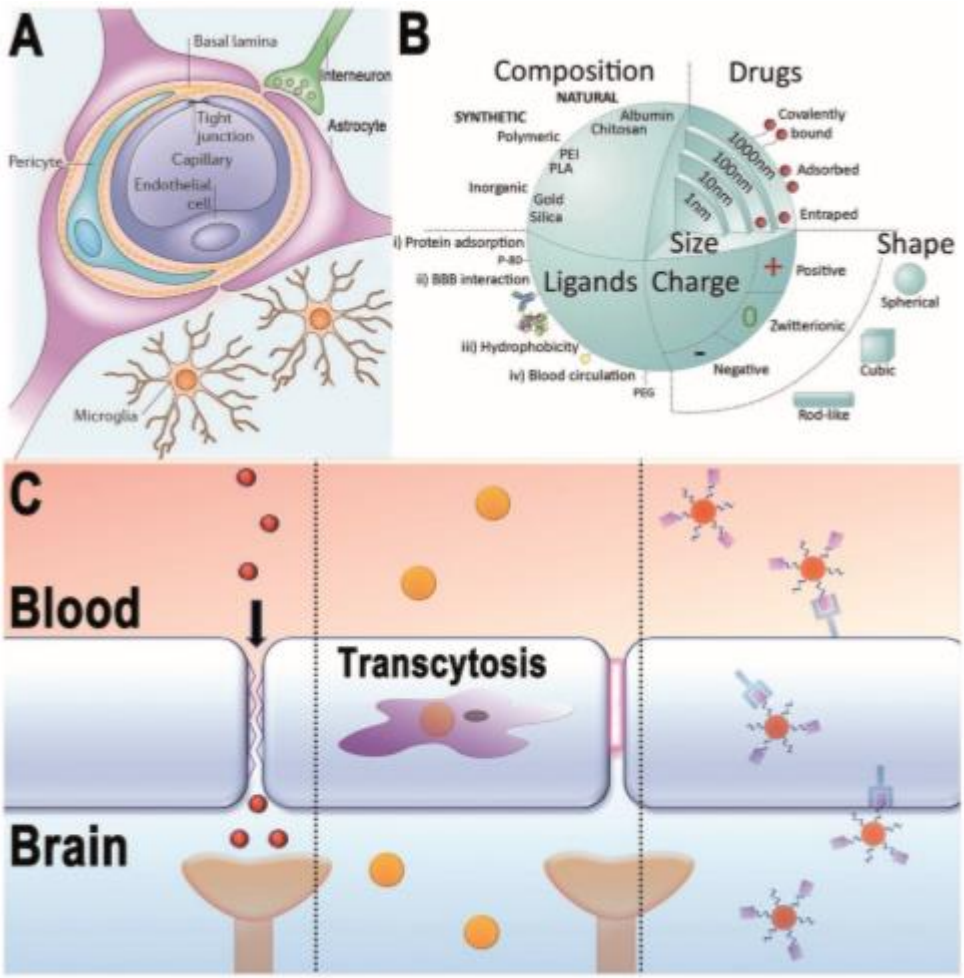


Figure 17. a) The figure shows the BBB structure. b) The properties of nanocarriers which affect the BBB penetration and targeting. c) The several methods of transport of nanomaterials through the BBB for brain drug delivery **[130].**

2.6. Nanomedicine and tumor microenvironment

A tumor shall not be described as a bunch of cancer cells, all packed together in a specific region of the body. It shall be conceived as an *"organ",* with well- and ill-formed structures. Tumors include various cell types, such as tumor cells, fibroblasts, immune cells (e.g. macrophages), blood and lymphatic vessels, all held together with the help of ***extracellular matrix (ECM)*** **[131, 132].**

The ECM consists of various proteins (e.g., collagen), glycosaminoglycan, proteoglycans, the glycoprotein SPARC and polysaccharides **[133–135].** A major factor that determines the heterogeneity and rigidity of tumor matrix is the presence of ***matrix metalloproteinases (MMPs),*** which are overexpressed in many tumors. These proteins are responsible for the collagen proteolysis within the tumor matrix **[133, 136, 137].** Their overexpression is associated with minimized matrix rigidity, increased metastatic potential low or non-existent apoptosis **[138].** MMPs overexpression is directly correlated with poor cancer patient prognosis **[137, 138].** The variability between ECM components stresses out the importance of tumor heterogeneity and plays a significant role in determining the effectiveness of chemotherapy. Tumor cells are encapsulated within this matrix and are connected to the blood circulation by aberrant vasculature **[18].**

Three important features that characterize the tumor matrix are hypoxia, extracellular tumor acidic environment and increased interstitial fluid pressure (IFP). ***Hypoxia*** is observed in the core part of many solid tumors and is due to the abnormal vasculature, which is unable to deliver oxygen and nutrients deeply inside the tumor tissues, when they lie more than 100 μm from the neighboring blood vessel. These cells no longer depend on aerobic metabolism to produce ATP and turn to anaerobic glycolysis, which leads to the accumulation of lactate deeply in tumor tissues, thus ***lowering the pH*** of the extracellular matrix. These hypoxic cells are viable and are usually accompanied by

necrotic tissues. Unfortunately, this process creates a group of cancer cells which is outstandingly resistant, (via selection of cells which have lost p53 function), and prone to give metastases. Both hypoxia and the acidic tumor environment lead to cancer resistance to chemotherapeutics. Furthermore, hypoxic conditions reduce the efficacy of radiation therapy as well **[18].**

Another feature of many solid tumors is the ***increased interstitial fluid pressure (IFP),*** which can hinder the efficient delivery of drugs to the tumor site **[18].** The poor tumor lymphatic drainage, the accumulation of metabolic products and the abnormal vasculature structure significantly contribute to this phenomenon **[133].** The rapidly dividing cancer cells and the dense ECM structure compress the blood and lymph vessels and do not exhibit edema, because they do not expand freely. As a result, the increased IFP cannot be alleviated. In such cases EPR effect might not be enough **[18].**

Tumor-associated macrophages (TAMs) are inflammatory cells in the tumor microenvironment. Although they may be expected to fight against the aberrantly proliferating cancer cells, interestingly enough, they are not only blocked, but also "enslaved" by tumor cells and they help them proliferate, progress and metastasize. TAMs improve neoangiogenesis, immune system suppression and metastasis and they have been correlated with poor prognosis **[18].**

After this brief overview regarding tumor microenvironment, it is clear that exploiting the intrinsic anticancer activity of nanomedicines is not sufficient. In the literature, the evaluation of the cytotoxic effect on cell cultures is generally accepted as a means to determine the activity of the nanomedical formulation. This approach is the cornerstone of this research thesis, as well. In the following section, we describe some basic principles on overcoming the tumor microenvironment barriers. The following image **(Figure 18)** depicts all important aspects of tumor microenvironment.

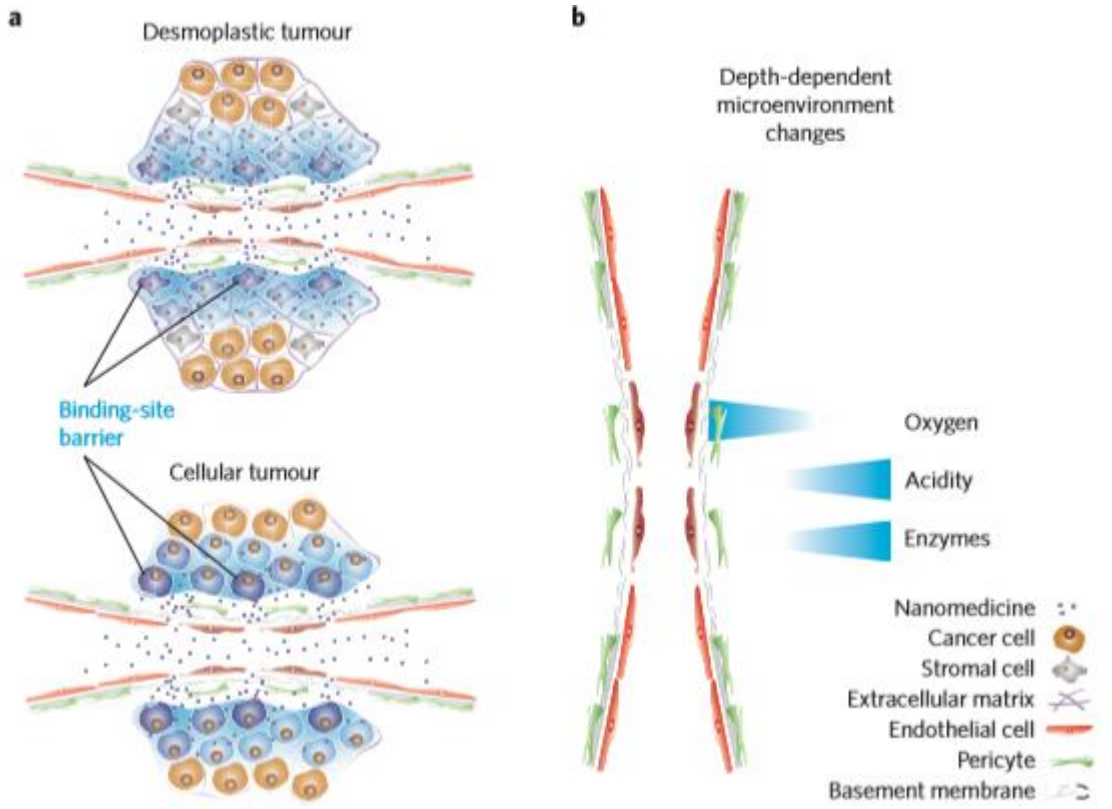


Figure 18. a) Tumors can be "desmoplastic", i.e. they have a rich ECM, or they can be "cellular", i.e. they are largely composed of cancer cells. Targeted nanomedicines can bind on both ECM and cancer cells, exploiting surface and matrix moieties. This binding can sometimes delay the nanoparticle movement in the tumor. b) Microenvironment properties change, as we move deeper inside the tumor. Oxygen decreases, tumor density and acidity increase. Different levels of various enzymes can be measured **[108].**

2.6.1. Stimulus-responsive nanocarriers

This approach exploits some tumor environment in order to improve the anticancer activity of the chemotherapeutics. Various tumor stimuli are involved in this approach, such as low extracellular pH, hypoxia, and MMP overexpression **(Figure 19).**

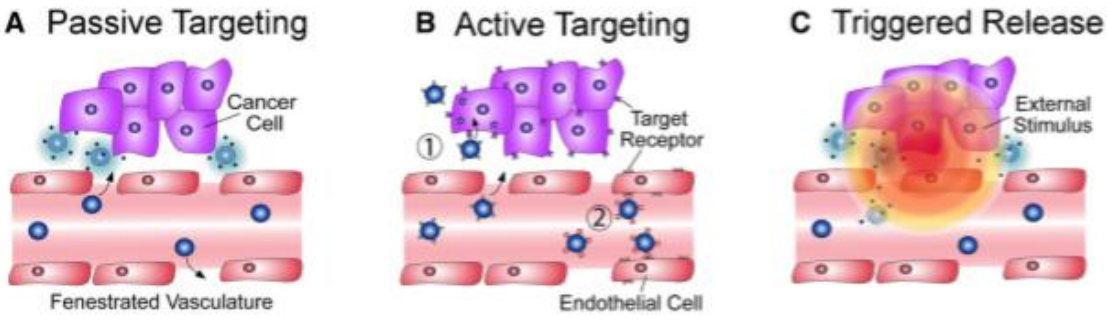


Figure 19. Comparison of passive target, active target and triggered release of nanoparticles **[139].**

Several ***pH-sensitive polymers*** have been utilized in order to deliver drugs in the acidic extracellular tumor environment, while at the same time drug leakage is minimized. The idea of pH-dependent loss of PEG chains is also an interesting approach that has been described in literature. Using PEG shedding in order to expose another hidden surface moiety is a mechanism that can be exploited. Torchilin et al manufactured an MMP2-sensitive micellar formulation, which loses its PEG chains and exposes on its surface a cell penetrating Tat-peptide, only when the micelles reach the MMP2-rich tumor matrix. The PEG chains are connected to the MMP sensitive peptide and provide a long plasma circulation time of the micelle formulation and the Tat-peptide conjugated PEG chains keep the Tat shielded and protected until the micelles reach the tumor site. The following picture **(Figure 20)** depicts polymers that can be used for triggered release of nanomedicines **[18]:**

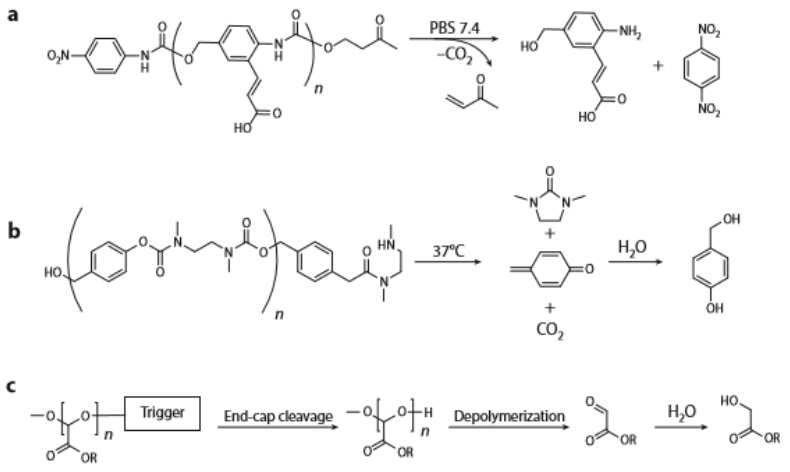


Figure 20. Different polymers that can be used for triggered release of nanomedicines **[140].**

2.7. Cellular barriers

2.7.1. Nanocarrier internalization and endosomal escape

After the nanocarrier passes all the above-mentioned barriers, it contacts the tumor cells and interacts with tumor cell membranes in order to get internalized ***(endocytosis).*** The process of endocytosis forms a vesicle (called ***endosome*** or ***phagosome***), where the nanocarriers are entrapped. Five major endocytic pathways have been identified, which include phagocytosis, macropinocytosis, clathrin- mediated and caveolin-mediated endocytosis, and clathrin and caveolin-independent endocytosis **(Figure 21) [18].**

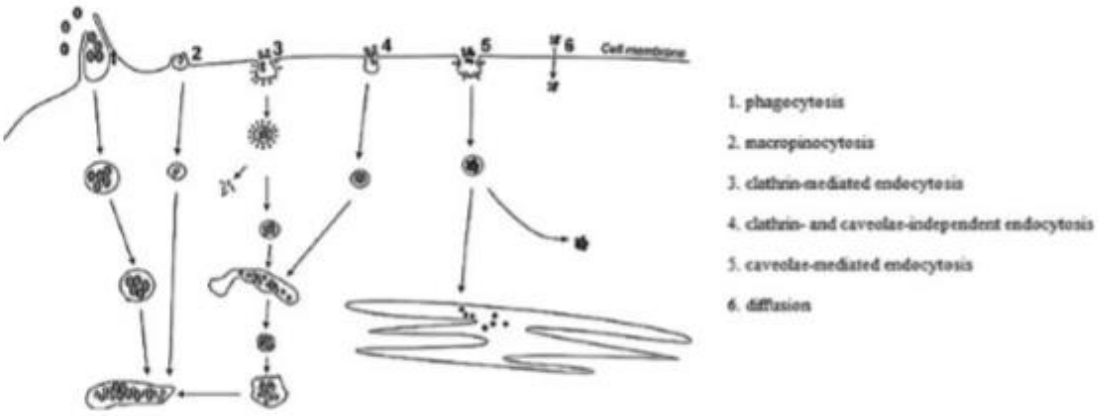


Figure 21. All possible pathways with which a nanoparticle can enter into a cell **[7].**

The type of endocytosis depends on several factors, like the size, surface chemistry and ligands of the nanocarrier, and the tumor cells. The pH inside these vesicles is usually acidic. Clathrin-mediated and caveolin-mediated endocytosis lead to the formation of early endosomes (pH 6.5–6.8), which later become late endosomes (pH 5.2–6.2). Phagocytosis and macropinocytosis lead to the formation of phagosomes and macropinosomes, respectively. All these vesicles fuse with lysosomes (pH 4.5–5.2). The lysosomal acidic conditions and the existence of hydrolytic enzymes facilitate the digestion of the nanocarriers and destroy them. Thus, it is very important for the nanocarriers to find a way to escape the endosomes before fusion with lysosomes. A method that to enables the endosomal escape of nanocarriers is the ***"proton-sponge effect"*** **(Figure 22).** In this method, nanocarriers are protonated by the low pH inside the endosome, which leads to an extensive flow water and ions and water inside them,

with subsequent osmotic swelling and rupture. The nanocarriers can then escape into the cytosol **[18].**

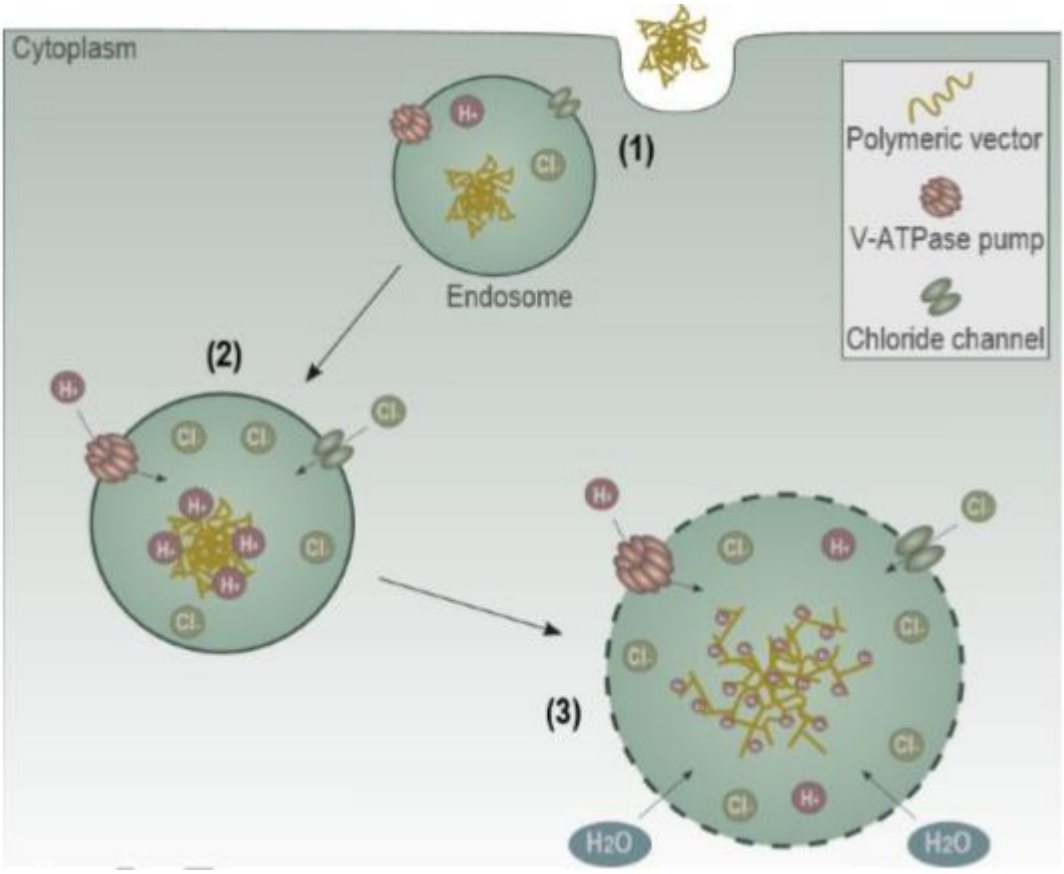


Figure 22. The proton sponge effect **[141].**

3. Aim of the study

The great scientific interest regarding metallic and bimetallic nanoparticles in cancer therapy led to the conception and organization of this research protocol. The aim of the study is to evaluate the cytotoxicity of polymer encapsulated AgAu 3:1 bimetallic and Au monometallic nanoparticles on non-cancerous embryonic kidney HEK293 and colorectal HTC116 cell lines. Furthermore, this study aims to compare the obtained results with the toxicity induced by plain Au and AgAu bimetallic nanoparticles, as well as the polymer itself on the same cell lines. The co-polymer that will be used is poly[2-(dimethylamino)ethyl methacrylate-*b*-(oligo ethylene glycol)methacrylate] (PDMAEMA-*b*-POEGMA).

4. Materials and methods

4.1. Materials

Tetrachlorauratic acid, silver nitrate, tryptophan aminoacid, 2-(Dimethylamino)ethyl methacrylate (DMAEMA), Poly-(oligo-ethylene-glycol) methacrylate (OEGMA) from Aldrich, 1,4-dioxane (99,8% pure), from Aldrich, tetrahydrofuran (THF, 99,9%), 4-cyano-4-87-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid (CDTP), 2,2'-Azobis(isobutyronitrile) (AIBN), high purity nitrogen gas, n-hexane, NAOH 1M solution, DMEM High Glucose culture medium (BioSera), FBS, trypsin EDTA, HEK193 and HCT116 cell lines, DMSO, MTS assay.

4.2. Methods

4.2.1. Construction of AuNPs and AgAu bimetallic nanoparticles

Colloidal solutions of monometallic gold nanoparticles and bimetallic silver and gold NPs were created via chemical reduction of tetrachlorauratic acid and silver nitrate (HAuCl4, AgNO3, Merck, Germany) using tryptophan aminoacid (Trp, SC12–20120713, China). The obtained bimetallic nanoparticles were of alloy type and were manufactured by simultaneous metal ion reduction. The metal molar ratio was Ag:Au = 3:1. For all colloidal solutions the TRP solution was adjusted to pH = 10 using 1N NaOH and was later heated until boiling. The following step was the injection of AgNO3/HAuCl4 solutions. The absorption spectra of the manufactured colloidal solutions were measured in the UV-visible region using a Lambda 35 (Perkin-Elmer) spectrophotometer and 1cm quartz cells. A transmission electron microscope JEOL JEM-1230 was used to determine the size and morphology of the nanoparticles. The nanoparticle diameter was measured by dynamic light scattering (Zeta Sizer Nano S spectrometer, Malvern, UK) **[78].**

4.2.2. Synthesis of PDMAEMA homopolymer

In order to prepare the PDMAEMA block, DMAEMA was polymerized in 1,4-dioxane solution to produce PDMAEMA macro-CTA chains. The initiator used was AIBN and 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl] pentanoic acid (CDTP) acted as the CTA (moles CDTP: moles AIBN = 10:1). The AIBN monomer and CDTP were dissolved in dioxane and magnetically stirred in a 25 mL round bottom flask with a rubber septum. The obtained solution was degassed using high purity nitrogen gas and was subsequently placed in an oil bath at 65^{o}C for 18 h. Following the polymerization

reaction, the solution was cooled at -20^{o}C and exposed to air. The PDMAEMA macro-CTA was isolated in excess of n-hexane by precipitation, after being redissolved in THF. The product was then dried at room temperature for 2 days. The above procedure enabled the preparation of PDMAEMA homopolymers as the first blocks of diblock copolymers **[142].**

4.2.3. Synthesis of PDMAEMA-b-POEGMA diblock copolymer

The procedure for the preparation of the PDMAEMA-b-POEGMA diblocks is described as follows: the PDMAEMA block was used as the macro-CTA, 1,4-dioxane as the solvent and AIBN as the radical initiator. In order to achieve the synthesis of PDMAEMA-b-POEGMA, PDMAEMA, OEGMA, AIBN of 1,4-dioxane were added in a round flask. The flask included a magnetic stirrer and was sealed using a rubber septum. The mixture was then degassed with nitrogen and was incubated in an oil bath at 70^{o}C for 1 day. After the polymerization reaction took place, the solution was cooled at -20^{o}C and exposed to air. Last but not least the product was isolated in an excess of n-hexane by precipitation and was later dried at room temperature under vacuum for 2 days **[142].**

4.2.4. Cell culture and exposure to NPs

Human embryonic kidney 293 (HEK293) and human colorectal carcinoma 116 (HCT116) cell lines were grown using DMEM High Glucose culture medium (BioSera), which contained 10% FBS, 100U/ml penicillin, 100g/ml streptomycin and 2 mmol/L glutamine. The culture took place at 37^{o}C. The culture medium was changed every 2 days and cells were passaged once every week using standard concentrations of trypsin EDTA. Cells were then frozen using a freezing medium which contained FBS and 5% DMSO. HCT116 cancer cell lines are adherent and Ras-mutant. HEK293 is a non-cancerous cell line and is often used as a control group. The ***HEK293*** cell line was incubated at 37^{o}C for 24h with plain PDMAEMA-b-POEGMA polymer, Au nanoparticles encapsulated in the same polymer, AgAu 3:1 bimetallic nanoparticles encapsulated in the polymer, plain Au nanoparticles and plain bimetallic AgAu nanoparticles. Various concentrations were applied (1, 2.5, 5, 10, 15, 20, 25, 30, 40 and 50 μg/mL). The ***HCT116*** was incubated with plain PDMAEMA-b-POEGMA polymer, Au nanoparticles encapsulated in the same polymer, Ag/Au 3:1 bimetallic nanoparticles encapsulated in the polymer using concentrations of 1, 2.5, 5, 10 and 20 μg/mL, as well as plain Au nanoparticles and plain

AgAu 3:1 bimetallic nanoparticles using concentrations of 5, 10, 15, 20, 25, 30, 40 and 50μg/mL. After the incubation, an MTS viability assay was performed **[78].**

4.2.5. Viability (MTS) assay

An ***MTS assay*** was used to determine and quantify the viability of the cells that were exposed to the aforementioned nanoparticles in different concentrations. In MTS assays, the tetrazolium (3-(4,5 dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide), which is yellow in color, is reduced to formazan, which is purple. Only living cells can potentiate the formation of formazan, since the reaction is mediated by mitochondrial enzymes. Formazan levels can be later quantified using spectrophotometry. MTS assay can also be performed in order to evaluate and quantify cellular death, since in that case formazan formation is decreased. For this MTS assay, a 96-well plate (corning-Costar, Corning, NY) was used. Each well contained approximately 5000 cells. A control included increasing numbers of cells in consecutive wells. These cells were unexposed to nanoparticles, only in culture medium. The cells were incubated with nanoparticles for approximately 24h and were rinsed once before the addition of 100μL of serum free medium, which contained contain 0.5mg/mL MTS. They were then incubated for approximately 4h at 37^{o}C. The optical densities were measured by using a Microplate Spectrophotometer (SPECTROstarNano, BMG LABTECH) and were read at 570nm (reference filter was set at 690nm). Spectrometry was followed by the normalization of absorbance measurements with respect to control cultures in order to reliably calculate changes in cell viability **[78].**

5. Results

5.1. Characterization of the particles

Colloidal solutions of noble monometallic Au and bimetallic Ag and Au nanoparticles were synthesized, while tryptophan aminoacid was used in order to achieve metal reduction and as a particle stabilizer. The formation of stable bimetallic silver-gold colloids is promoted by an alkaline medium with anionic TRP. These colloidal solutions maintain their stability for more than one year because the negative charge accumulates around nanoparticles. Absorption spectra of obtained colloids included the expected typical bands of ***localized surface plasmon resonance (LSPR),*** which can

be observed in metals (**Figure 23**). The maximum of LSPR band of nanosized gold was at 527nm. The band maximum of colloid AgAu(3:1) bimetallic nanoparticles was located at 435nm. The data obtained by ***dynamic light scattering (DLS)*** suggests that the average diameter of gold nanoparticles was 10 nm and the average diameter of AgAu nanoparticles was around 10nm, as well **[78].** (**Figure 24**)

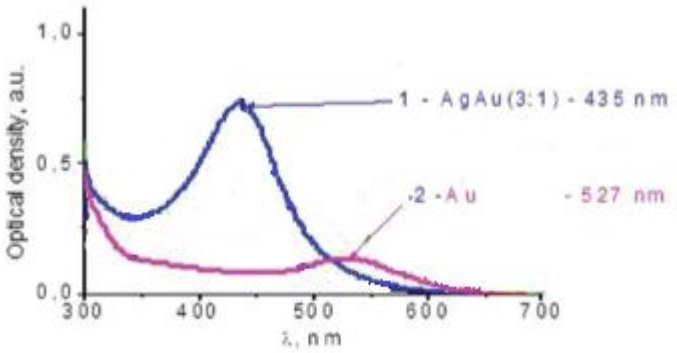


Figure 23. LSPR spectra of gold and bimetallic AgAu nanoparticles **[78].**

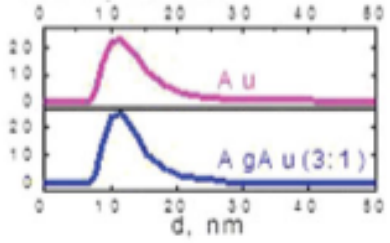


Figure 24. Average diameter of gold and bimetallic AgAu nanoparticles **[78].**

The molecular weight and polydispersity index of the PDMAEMA-b-POEGMA polymer were calculated by size exclusion chromatography. The molecular weight was 12600 $g.mol^{-1}$ and the **polydispersity index** was 1.26**.** Finally, the **hydrodynamic radius (R_h)** of Au nanoparticles encapsulated in the polymer was ca. 25nm and the R of encapsulated bimetallic nanoparticles was near 70nm, as measured by dynamic light scattering **(Figure 25, above).** The maximum LSPR spectra obtained by UV-Vis was 529 nm for Au-polymer conjugates and 443 nm for AgAu(3:1)-polymer conjugates, as depicted below **(Figure 25, below).**

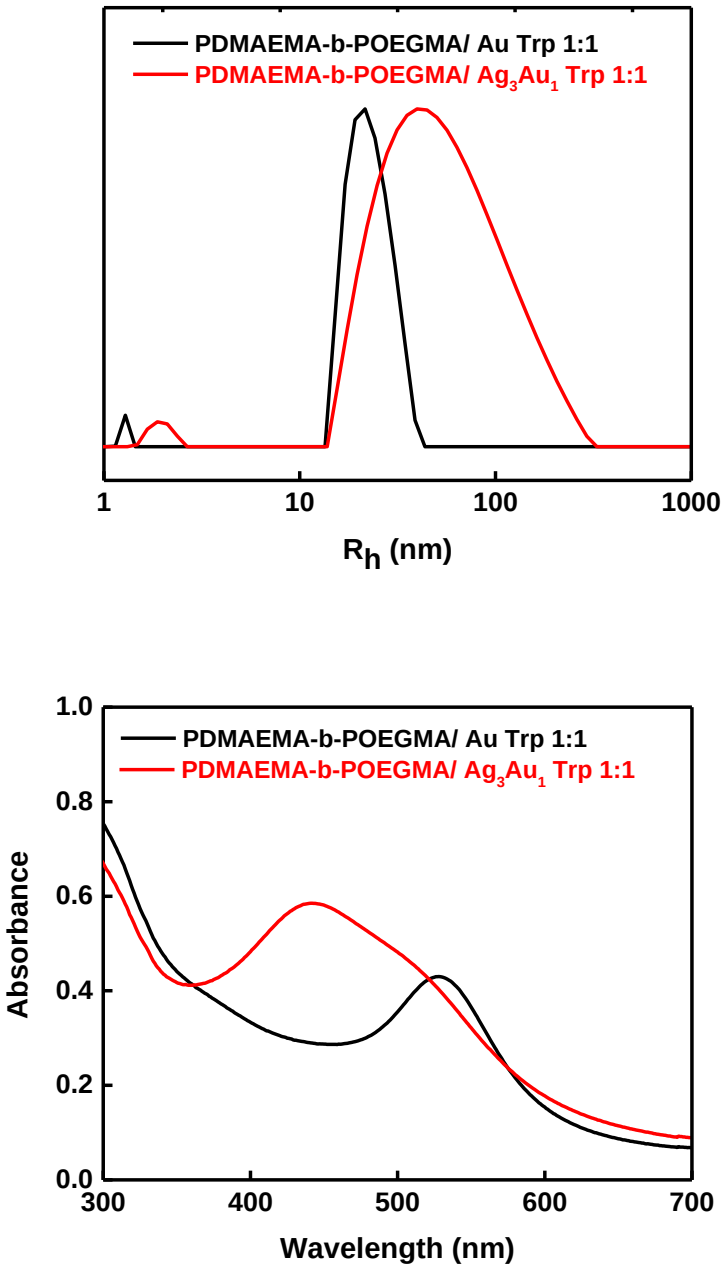


Figure 25. Hydrodynamic radius of the polymer conjugated nanoparticles (above) and their LSPR spectra (below) **[Reproduction from A. Skandalis].**

5.2. Toxicity studies

Two cell lines were used for the assessment of the nanoparticle toxicity, namely HEK193 and HCT116 cell lines. The aforementioned cell lines were incubated with nanoparticles

and an MTS assay was performed. The results regarding the HEK293 are as follows **(Table 5):**

	AuNP	AgAuNP 3:1	Polymer	Au-polymer	AgAu-polymer
% toxicity HEK293					
1 μg/ml			94,58	100	74,1
2,5 μg/ml			81,3	97,5	61,4
5 μg/ml	83,09	82,73	98	96,4	52,6
10 μg/ml	82,67	79,82	61	64	56
15 μg/ml	71,98	70,72			
20 μg/ml	70,05	68,9	26	43	31
25 μg/ml	67,31	54,34			
30 μg/ml	62,85	57,82			
40 μg/ml	60,58	48,88			
50 μg/ml	31,49	39,18			
% toxicity HCT116					
1 μg/ml			70	100	67
2,5 μg/ml			90	81	46
5 μg/ml	82,69	87,34	98	100	23
10 μg/ml	76,76	68,28	86	100	53
15 μg/ml	75,92	66,6			
20 μg/ml	86,29	75,07	94	63	48
25 μg/ml	96,44	77,397			
30 μg/ml	64,07	48,89			
40 μg/ml	40,15	37,61			
50 μg/ml	11,37	11,16			

Table 5: Percentage (%) toxicity of AuNPS, AgAuNPs, plain polymer, Au-polymer and AgAu-polymer conjugates in HEK293 and HCT116 cell lines.

5.2.1. Toxicity studies on the HEK293 cell line:

Incubation of the HEK293 cell line with plain bimetallic AgAu nanoparticles in a concentration of 5μg/ml resulted in 83% viability, while 80% viability was observed for a concentration of 10 μg/ml and 69% after incubation with 20 μg/ml. Cell death was greater with higher concentrations between 25 and 40μg/ml, with viability fluctuating between 39 and 58%. Incubation of the HEK293 cell line with the bimetallic nanoparticle, which was encapsulated in the polymer showed the following results: cell viability was measured at 74% with a concentration of 1 μg/ml of the encapsulated nanoparticle and at 61% when the concentration was 2,5μg/ml. Viability percentages were also 53% (5μg/mL), 56% (10μg/mL) και 31% (20μg/mL).

Incubation of the HEK293 cell line with plain Au nanoparticles showed the following viabilities, with respect to the applied nanoparticle concentrations: viability was standing at 83% when the Au nanoparticle concentration stood at 5μg/mL and 10 μg/mL, 72%, 70%, 67%, 63%, 61% and 31% with respective nanoparticle concentrations at 15, 20, 25, 30, 40 and 50 μg/mL. When incubating with the Au-Polymer conjugate, viabilities were the following: 100%, 97% and 96% when small concentrations of 1, 2.5, and 5 μg/mL were used, while viability decreased with larger concentrations, namely 64% (10 μg/mL) and 43% (20 μg/mL).

Last but not least, incubation with plain polymer depicted the following results: viability remained high with polymer concentrations of 1, 2.5 and 5 μg/mL (95%, 81% and 98% respectively) and showed a rapid declining tendency when polymer concentrations were increased. Viability stood at 61% and 26% with polymer concentrations of 10 and 20 μg/mL respectively. The above results are shown in the following tables (**Figure 26, Figure 27**):

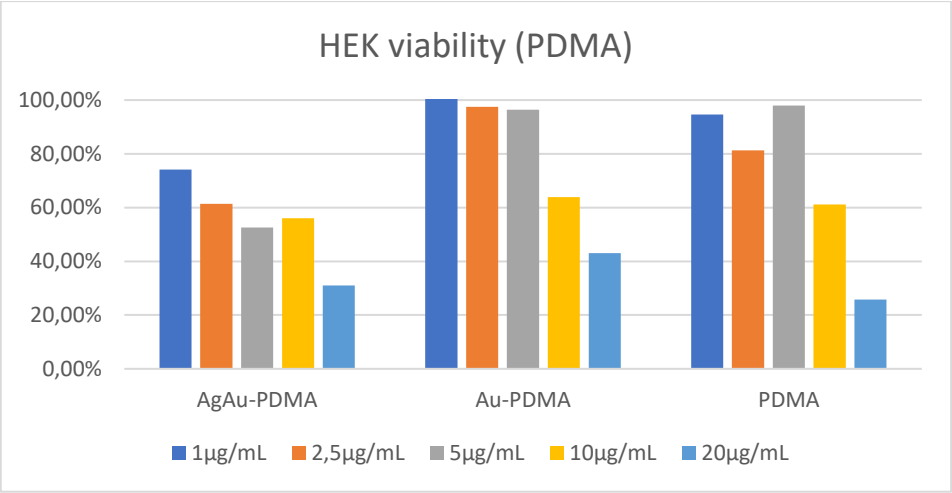


Figure 26: HEK293 viability assay. Incubation with bimetallic NP and polymer conjugate, gold and polymer conjugate, and plain polymer respectively in different concentrations.

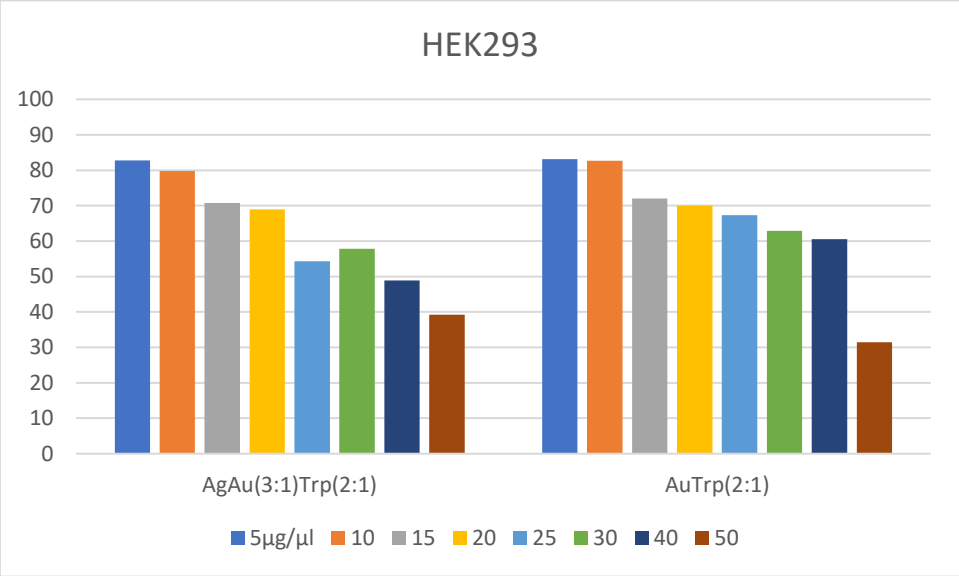


Figure 27: HEK293 viability assay. Incubation with plain bimetallic NP and plain gold NP respectively in different concentrations.

5.2.2. Toxicity studies on the HCT116 cell line

Incubation of the HCT116 cell line with the encapsulated bimetallic nanoparticles showed the following results: at a concentration of 1 µg/mL viability stood at 67%. The increase in concentration showed a decrease in viability until the concentration of 5 µg/mL (46% and 23% with concentrations of 2,5 and 5 µg/mL). Surprisingly further

increase in the conjugate concentration led to an increase in cell viability. It stood at 53% and 48% when concentrations were 10 and 20 µg/mL respectively. Incubation with monometallic gold-polymer conjugated nanoparticles showed much higher viability rates. Viability stood at 100% at concentrations of 1, 5 and 10 µg/mL and it fell at 81% and 63% when the concentrations were 2,5 µg/mL and 20 µg/mL respectively.

Furthermore, the HCT116 cell line was incubated with plain bimetallic nanoparticles and the following results were obtained: increasing the concentration of the bimetallic nanoparticles provoked a decreasing pattern in cell viability (68%, 68%, 66%, 49%, 38% and 11% with concentrations of 5, 10, 15, 30, 40 and 50 µg/mL respectively). Interestingly the concentrations of 20 and 25 µg/mL showed higher viability rates of 75% and 77% as compared with smaller concentrations. The same pattern was observed when the cells were incubated with plain Au nanoparticles. Viabilities were as following: 83%, 77%, 76%, 86%, 96%, 64%, 40% and 11% with concentrations at 5, 10, 15, 20, 25, 30, 40 and 50 µg/mL respectively.

Lat but not least the HCT116 cell was incubated with plain polymers at concentrations of 1, 2.5, 5, 10 and 20 µg/mL. The viability observed was 70%, 90%, 98%, 86% and 94% respectively. The results are depicted in the following tables (**Figure 28, Figure 29**):

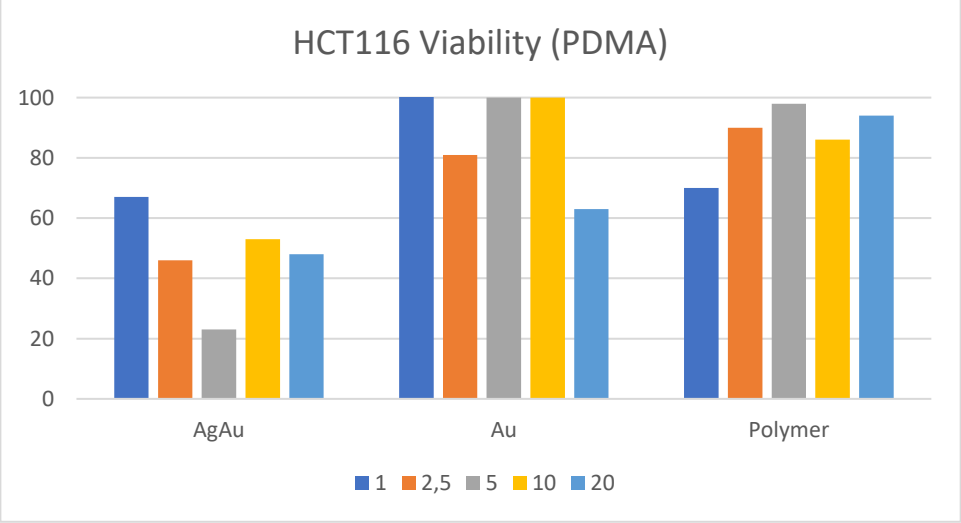


Figure 28. HCT116 viability assay. Incubation with bimetallic NP and polymer conjugate, gold and polymer conjugate, and plain polymer respectively in different concentrations.

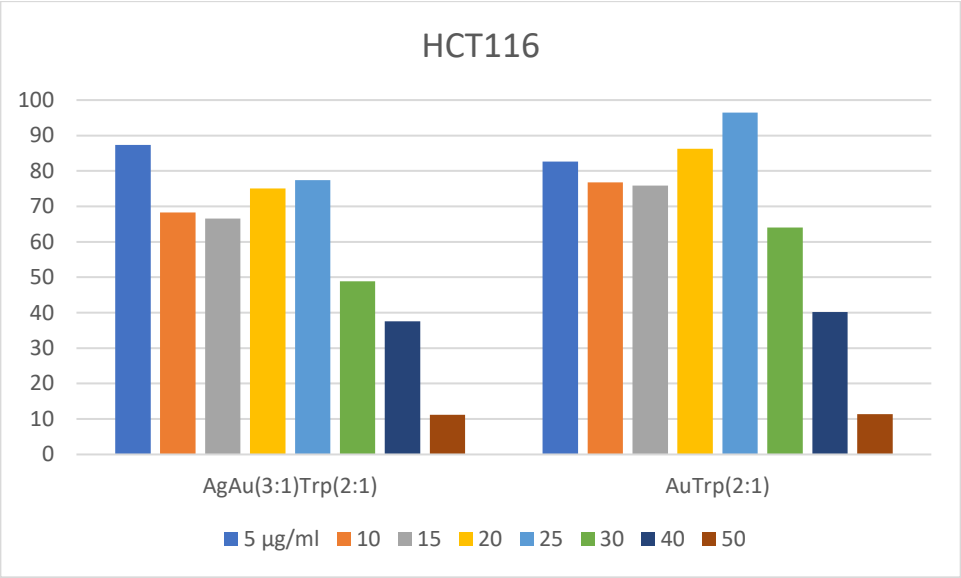


Figure 29. HCT116 viability assay. Incubation with plain bimetallic NP and plain gold NP respectively in different concentrations.

The following table (**table 6**) summarizes the viability rates of both cell lines when incubated with plain Ag/Au bimetallic nanoparticles and Ag/Au bimetallic nanoparticles encapsulated in the PDMAEMA-b-POEGMA polymer:

	5µg/mL	10µg/mL	20µg/mL
HEK	83%	80%	69%
HEK-PDMA	53%	56%	31%
HCT	87%	68%	75%
HCT-PDMA	35%	53%	48%

Table 6: Summary of the viability rates of HEK293 and HCT116 cell lines. The first and third line of the table depict the observed viability when the cell lines were treated with plain bimetallic Ag/Au nanoparticles. The second and fourth line depict the effect of the polymer on viability, when it encapsulates the aforementioned nanoparticles.

6. Discussion

AuAg bimetallic nanoparticles have attracted scientific interest because they exhibit several exploitable properties on par with the monometallic nanostructures of the same metals. In this work, we decided to manufacture alloy type AgAu bimetallic nanoparticles with Ag:Au ratio of 3:1, since the properties of the monometallic Ag and

Au nanoparticles are very promising. In fact, AgNPs share antibacterial, antiviral, antifungal, anti-inflammatory, anticancer and antiangiogenic effects **[91].** Furthermore, AuNPs have been proposed as successful diagnostic and therapeutic agents. They exhibit low toxicity as far as in vivo systems are concerned and they are easily functionalized, having a high area to volume ratio and being able to incorporate several different functional moieties on and in their structure (e.g. antibodies, drugs etc.) **[78].** The element ratio of Ag to Au was chosen to be 3:1, since according to a scientific project conducted by Katifelis et al., AgAu nanoparticles which have a metal ratio of 3:1, exhibit the maximal antitumor effect in cancer cell lines, as compared with metal ratios of 1:1 and 1:3, while at the same time the toxicity regarding normal HEK293 cells was found significantly decreased **[78].** As discussed above, metallic nanoparticles can be easily functionalized and conjugated with many other molecules. For that reason, the obtained nanoparticles were functionalized using the PDMAEMA-b-POEGMA diblock copolymer.

The aim of the study was to compare and assess the cytotoxicity effects of plain AuNPs, AgAuNPs and the polymer nanoparticles on one side and on the other side to study the change in cytotoxicity induced by the conjugation of the above-mentioned polymer on the AuNPs and AgAuNPs. While monometallic and bimetallic nanoparticles have started to attract scientific interest for their novel properties, research regarding their combination with other molecules and in this case, biopolymers, is still in its infancy. Maney at al manufactured PtAu bimetallic nanocomposites, which were conjugated with chitosan biopolymer and assessed their cytotoxicity on several cell lines with and without the encapsulation of doxorubicin. Interestingly the cytotoxic effect did not have a significant correlation with the nanocomposite, rather than doxorubicin itself. Cell viability was maximum when plain PtAu and PtAu-chitosan nanocomposites were used it was even observed that the addition of chitosan on the structure had a protective and cell-growth effect **[5].** In another study conducted by Liebig et al, gold nanotriangles were manufactured and were entrapped in hyperbranched polyethyleneimine (PEI), maltose modified polyethyleneimine (PEI-Mal) and heparin polymers. The cytotoxicity of the polymer-trapped and naked gold nanotriangles was evaluated and compared in non-cancerous HEK293 and NK-cell leukemia cell lines (YTS).

Results showed that heparin coating reduces cytotoxicity of Au nanotriangles in both cell lines and can be used as a coating molecule in order to increase biocompatibility. Coating gold nanotriangles with PEI increases cytotoxicity drastically, while PEI-Mal coating also confers high cytotoxicity, but to a lesser extent and is by far less toxic to HEK293 than YTS cells **[91].**

In this study we examined cell viability in HEK293 and HCT116 cell lines. HEK293 is a non-cancerous cell line, while on the contrary HCT116 are colorectal cancer cells. Incubation of HEK293 cells with bimetallic AgAu nanoparticle showed a toxicity which increased proportionally with increasing nanoparticle concentration. The same effect was observed when Au nanoparticles were used. The addition of Ag and creation of bimetallic nanoparticles did not show any significant change in cell toxicity, as compared with plain Au nanoparticles. On the contrary, the addition of the polymer on the bimetallic nanoparticle exhibits a significant cytotoxic effect. HEK293 viability was reduced from 83% (AgAu 5 μg/ml) to 53% (AgAu-polymer 5 μg/ml) and the same effect was observed with other concentrations as well. In fact, viability was reduced from 80% to 56% when using concentrations of 10 μg/ml and from 69% to 31% with concentrations standing at 20 μg/ml. The plain polymer showed a significant cytotoxicity at concentrations above 5 μg/ml. HEK293 cell viability stood at 61% with a concentration of 10 μg/ml and only 26% with a concentration of 20 μg/ml.

Incubation of HCT116 cells with bimetallic AgAu nanoparticles showed an increasingly cytotoxic effect, which was proportionate with increasing nanoparticle concentrations. The same pattern was observed when the cells were incubated with plain Au nanoparticles. Interestingly enough, viability seemed to increase when both AgAu and Au nanoparticle concentrations stood at 20 μg/ml and 25 μg/ml, as compared with lower concentrations of 10 μg/ml and 15 μg/ml. In fact, viability stood at 75% and 77% with AgAu nanoparticle concentrations of 20 μg/ml and 25 μg/ml respectively, while it was decreased at 66% and 68% when the concentrations stood at 10 μg/ml and 15 μg/ml respectively. Similarly, when the HCT116 cells were incubated with plain Au nanoparticles at concentrations of 10 μg/ml and 15 μg/ml, viability stood at 76% and increased to 86% and 96% with increasing concentrations standing at 20 μg/ml and 25 μg/ml, respectively. This observation needs further explanation, since higher

concentrations are expected to provoke higher cytotoxicity. Thus, further verification is needed.

The incorporation of the polymer on the bimetallic nanoparticle structure exhibited a significant cytotoxic effect in the case of HCT116 cell line. The highest cytotoxicity was observed when the cells were incubated with a concentration of AgAu-polymer conjugate standing at 5 μg/ml and the observed viability was 35%. In contrast, cell viability when the cells were treated with plain AgAu nanoparticles stood at 87%. Similar results were observed when other concentrations were used as well. It seems that the addition of the polymer increases the cytotoxicity of the nanocomposite. As a result, the observed viabilities were 68% versus 53% (10 μg/ml) and 75% versus 48% (20 μg/ml).

The encapsulation of the AgAu nanoparticles in the PDMAEMA-b-POEGMA polymer increases toxicity significantly in both cell lines, even more than twice when we use a concentration of 20 μg/ml in normal HEK293 cells. Using plain AgAu nanoparticles at a concentration of 10 μg/ml without polymer seems the most effective strategy, since HCT116 cell viability decreases to 68%, while HEK293 is maintained simultaneously at 80%. This is important, since the target in cancer therapy is to achieve on one hand high cytotoxic effect on cancer cells, but on the other hand to protect and preserve normal cells. On the contrary, the same concentration of 10 μg/ml of the AgAu-polymer conjugate exhibits a similar viability of 56% and 53% for both cell lines respectively, a cytotoxicity rate which is particularly high and unacceptable for normal HEK293 cells. Toxicity was even greater at a concentration of 20 μg/ml, as viability stood at a mere 31% for HEK293 and 48% for HCT116 cells. This concentration even seems to induce higher toxicity to normal rather than cancerous cells.

AgAu bimetallic nanoparticles are investigated mainly because they are easily manufactured and they show enhanced photoluminescent and catalytic properties **[78].** Their therapeutic potential, especially in combination with other molecules and moieties has not yet been adequately researched.

7. Conclusion

In the next 10–20 years, nanotechnology is expected to fundamentally transform technology, science and society. This offers a significant opportunity to promote human health and well-being in novel ways, mostly by enabling early disease detection, as well as new, precise and effective therapeutic strategies, which are specifically tailored for each patient (personalized medicine) **[7].**

Cancer nanomedicine, a major sector of nanotechnology, is a rapidly growing field of medicine. Effective diagnostics and therapeutics for cancer require delivery of drugs to tumors with appropriate spatiotemporal resolution to achieve favorable pharmacokinetics. Various methods of tumor targeting can address this need. The development of new nanocarriers will be crucial in inducing progress in this field **[140].**

Especially bimetallic nanoparticles and their combinations with other nanomolecules have a lot to offer. They offer advances in several of biological applications, ranging from diagnostics, such as sensing and imaging to therapeutics, for example tumor hyperthermal ablation and drug delivery. Since most imaging techniques require the utilization of a contrast agent, bimetallic nanoparticles can act as such in MRI, CT and dual modal imaging. Additionally, magneto-plasmonic nanomaterials, such as Au-Fe nanoparticles, enable imaging techniques based on optical properties, as well. Bimetallic nanoparticles have also been used for therapeutic reasons, including hyperthermia and drug delivery. By taking advantage of their tunable characteristics we can achieve specific targeting and minimize systemic and tissue toxicity. Furthermore, Au-based nanocarriers are excellent candidates for drug delivery strategies due to their biocompatibility. Moreover, Fe-based nanomaterials can be modified with pH- or temperature-sensitive coatings. The inherent optical and magnetic properties of bimetallic nanoparticles render them promising candidates theranostic approaches. Unfortunately, there are still few examples in the literature as far as their applications, especially against cancer, are concerned. The use of multi-component nanoparticles, which comprise of three or more metals have also been proposed as materials that will enhance the magneto-optical properties discussed herein **[79].**

For sure, apart from appropriate nanoparticle manofacturing research, we also need to increase our understanding regarding the fundamental processes involved, in order to overcome major obstacles in cancer nanomedicine, such as nanoparticle circulation, biodistribution, targeting and penetration of tumors. Further knowledge of oncology and cancer biology will strongly enhance the rational design of nanodrugs for specific cancer types. More research is needed in order to be able in the future to treat metastatic tumors, which cause the majority of deaths related to cancer. Also, the utilization of nanotechnology for the early detection of tumors is very useful for diagnosing cancer at an early stage. We shall also not forget that biocompatibility, toxicity, and the appropriate drug formulation are of great importance for therapeutic success **[140].**

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8. References

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