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Light and Electron Microscopy Analysis of Gold Nanoparticle Penetration in Stiffness varying 3D Tumor Spheroids

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Light and Electron Microscopy Analysis of Gold Nanoparticle Penetration in Stiffness varying 3D Tumor Spheroids

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University of Connecticut
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ABSTRACT

The prime purpose of this thesis is to study and analyze the penetration of gold nanoparticles in different stiffness and same sized 3D tumor spheroids of varying time-points day 5 and day 20 using light and electron microscopy techniques. Culturing cells in 3D (spheroids) is a smart way of mimicking the in vivo environment than the conventional 2D cultures (monolayer cell cultures). Being a 2D model, it does not represent the detailed the morphological details of the cells. The substantial difference has been observed in the effect of chemotherapy drugs when they are tested as 2D cancer cell monolayers versus 3D (Fig. 1.4 A & B) whereas, in reality, cancer cells exist as clusters of cells called spheroids(Mehta, Hsiao, Ingram, Luker, & Takayama, 2012). Therefore, recently a lot of attention is being given to the culture of cancer spheroids(Zanoni et al., 2016) for applications such as drug delivery, nanoparticles interaction, and penetration in spheroids(Goodman, Chen, Matveev, & Pun, 2008). Such analysis requires high-quality imaging modalities like transmission electron microscopy (TEM). In this study, the penetration of the gold nanoparticles in size-matched day 5 and day 20 spheroids was analyzed using two different modalities- Light microscopy and Electron Microscopy. The size-matched spheroids were cryosectioned to study the entire morphology and penetration of the particles using light microscopy and transmission electron microscopy was used to analyze the distribution of gold nanoparticles qualitatively and determine the changes in sub-micron cell features such as cell-cell adhesion, Golgi bodies, nucleus size, (Mehta et al., 2012). The analysis after TEM imaging showed the accumulation of gold nanoparticles were likely found to be more at the periphery for day 20 as compared to day 5. As per initial statistical analysis it was found that at the center, the particles were found to be less for day 20 as compared to day 5 spheroids. The area of nucleus remained higher for day 20 as compared to day 5 irrespective of the peripheral region or the central region.
Qualitative observations with respect to cell-cell junctions showed that junctions were intact for the day 20 when compared to day 5. In conclusion, the two microscopy techniques were used to analyze the gold nanoparticle penetration and it was found that electron microscopy was better to analyze the particles and morphological changes in the size matched spheroids in a more quantitative way and light microscopy worked well to study the entire morphology and accumulation of particles qualitatively.
Chapter 1

Introduction

1.1 Background on Breast Cancer

Breast cancer is one of the most common cancers that has been occurring in women, with about 200,000 new cases diagnosed per year in the United States alone, and it is also the second leading cause of cancer-related deaths in women. Breast cancer and benign breast tumors can also occur in men, adolescents, and children rarely. Breast cancer is of various subtypes of both invasive and non-invasive cancers composed of a heterogeneous group of diseases, including those that have varying treatment options. During the process of drug development process, it is a very crucial factor to know that the experimental model of the disease used in the testing is highly comparable to the real-life scenario (Duval et al., 2017). This piece of information forms the base in a laboratory setting where the goal is to mimic as best as possible the in vivo environment during the pre-clinical studies. For a qualitative breast cancer research, it is very important that the cancer cell models represent the disease in terms of expression of target receptors, drug transporters and proteins essential for cell survival and growth, as well as the activity of enzymes responsible for drug metabolism. Recent advances in the field of nanotechnology have given a pathway to nanoparticles formulations that can provide significant improvements in the diagnosis and treatment of cancer. By controlling the nanoparticles shape, surface chemistry and size, the penetration, uptake and retention of the same by the tumor can be investigated and this could form a basis to develop the ideal drug formulation that can potentially be personalized to achieve optimal diagnostic and therapeutic outcomes as per the person’s disease state (Sykes et al., 2016).
1.2 Cancer Models: Monolayer and 3D

Monolayer model is the platform for cell cultures grown in dishes and on coverslips where the static 2D culture systems used as reference models. The three-dimensional cultures comprise of pseudo-vascularized cultures, fiber and bead scaffold cultures, and spheroid cultures (Breslin, O’Driscoll, Breslin, & O’Driscoll, 2016).

1.2.1 Monolayer

![T47D cancer cell line grown in a petri dish](image)

Fig 1.1 T47D cancer cell line grown in a petri dish

This technique is known as two-dimensional (2D) cell culture and was first developed by Wilhelm Roux who, in 1885, removed a portion of the medullary plate of an embryonic chicken and maintained it in warm saline for several days on a flat glass plate. From the advance of polymer technology arose today’s standard plastic dish for 2D cell culture, commonly known as the Petri dish (Fig 1.1). Julius Richard Petri, a German bacteriologist, is generally credited with this invention while working as an assistant to Robert Koch. Various researchers today also utilize culturing laboratory flasks, conical, and even disposable bags like those used in single-use bioreactors. 2D cell culture platforms remain as the gold standard approach for the in vitro testing
of new therapeutic agents. These types of cultures are simple, cheap, and compatible with the different equipment used for high-throughput screening (HTS).

Extensive research has confirmed that 2D experiments are subject to various limitations, such as dissimilarities in cell adhesion and migration or in cytoskeletal organization, along with a poor analysis of complex cell-substrate interactions. Consequently, 2D in vitro models are often associated with contradictory results, typical of transposing new medical and anticancer compounds from the bench to the bedside. In particular, the lack of reliability seems to be associated with the following main aspects: cell source (e.g. phenotype selection), model dimensionality and microenvironment complexity.

1.2.2 3D or Spheroids

![Fig 1.2 T47D breast cancer spheroid formed by liquid overlay technique](image)

In the human body, the cells exist in their own 3D microenvironments with complex cell-cell and cell-extracellular matrix interactions. This multicellular arrangement of cells allows the cells to interact with one another and with the extracellular matrix (ECM), thus providing a much clearer
understanding of cellular complexities like dynamics of nutrients transport, cell polarization which are particularly crucial in cancer and stem cell research – an area where spheroids have huge potential. They can serve a wide range of applications starting from studying the complexities of the malignant tissues up to therapeutics research. Three-dimensional (3D) cell culture systems have gained increasing interest in drug discovery and tissue engineering due to their evident advantages in providing more physiologically relevant information and more predictive data for in vivo tests. There are different zones in the spheroid as compared to the morphology of a single cell(Duval et al., 2017). The outer layer is the proliferation zone which mainly contributes to the cell multiplication, the middle layer is the quiescent zone and the central layer is the core that is particularly the necrotic zone which is a region devoid of oxygen and other nutrients and leads to the accumulation of dead cells (Fig 1.3).

![Diagram of spheroid zones](image)

**Fig 1.3 Zones of the cancer spheroid showing the three zones namely proliferation, quiescent and necrotic zone**

As the spheroid increases in age, the necrotic core starts to develop gradually and this can be easily visualized using an optical microscope.
1.2.3 Monolayer versus 3D

Fig 1.4 A) Monolayer T47D cancer cell line grown in 2D on a petri dish (left) and B) T47D cancer cell line grown in 3D(Spheroid)

It has been observed after multiple research and experiments that culturing cells in three-dimensions mimics the *in vivo* environment in a more qualitative way than traditional two-dimensional cultures. In this review, we discuss the characteristics of 3D cell culture systems in comparison to the two-dimensional (2D) monolayer culture (Fig 1.4 A), focusing on cell growth conditions, cell proliferation, population, and gene and protein expression profiles. The research and the development in 3D culture techniques in the field of drug discovery over the past 5 years have been improved, where the emphasis has been mainly to retrieve the cellular response in response to the different classes of anti-cancer drugs, focusing mainly on similarities and distinctions between 3D and 2D cancer cell culture models. To understand the cell behavior and other related interactions the cell-based assays play a very crucial role as an important aspect of the drug discovery process to provide an accurate, quick and cost-effective tool to cut down on the animal testing. The key element—cultured cells—is the most critical part of such technique, since
results are based on the cellular responses to drugs or compounds or external stimuli. As part of the initial experimental model, the majority of cell-based assays use traditional two-dimensional (2D) monolayer cells cultured on flat and rigid substrates like Petri dishes. The traditional 2D cell cultures were initially used for quantifying the cell-based assays but still failed to mimic the 3D tumor microenvironments which involve the cell-cell, cell-ECM interactions, varying gene expression profiles, and cell signaling pathways. All the cells in the three-dimensional in vivo environment is surrounded by other cells wound in their own extracellular matrix (ECM). As a result, 2D cell culture tests sometimes provide misleading and nonpredictive data for in vivo responses. Currently, in drug discovery, the standard procedure of screening compounds starts with the 2D cell culture-based tests, followed by animal model tests, to clinical trials. Only about 10% of the compounds progress successfully through clinical development. Many of the drugs fail during clinical trials, especially during phase III, which is the most expensive phase of clinical development largely due to the lack of clinical efficacy and/or unacceptable toxicity. A portion of these failures is attributed to data collected from the 2D monolayer culture tests in which the cellular response to the drug(s) is altered due to their unnatural microenvironment. To lower the cost of failed compounds/molecules, the dismissal of ineffective and/or unacceptable toxic compounds should happen as early as possible, ideally before animal tests. Therefore, it is imperative to develop or establish in vitro cell-based systems that can more realistically mimic the in vivo cell behaviors and provide more predictable results to in vivo tests.

Recently, a growing body of evidence has suggested that 3D cell culture systems (Fig 1.4 B), in contrast to the 2D culture system, represent more accurately the actual microenvironment where cells reside in tissues. Thus, the behavior of 3D-cultured cells is more reflective of in vivo cellular responses. In fact, research has found that cells in the 3D culture environment differ
morphologically and physiologically from cells in the 2D culture environment. It is the additional dimensionality of 3D cultures that is the crucial feature leading to the differences in cellular responses because not only does it influence the spatial organization of the cell surface receptors engaged in interactions with surrounding cells, but it also induces physical constraints to cells. It has been demonstrated that cell responses in 3D cultures are more similar to \textit{in vivo} behavior compared to 2D culture. In the past several years, tremendous effort has been put into the development of a variety of 3D culture systems, as well as the adoption of 3D cell culture systems in drug discovery, cancer cell biology, stem cell study, engineered functional tissues for implantation, and other cell-based analysis. Such 3D culture systems provide excellent \textit{in vitro} models, allowing the study of cellular responses in a setting that resembles \textit{in vivo} environments(Edmondson, Broglie, Adcock, & Yang, 2014). One of most important aspect that differentiates the ages of the spheroids is hypoxia which is a condition in the central region of the spheroid (necrotic core). Research proves that the hypoxia was only seen in the dense 3D multicellular spheroids, which failed in the 2D monolayer cell cultures(Shi et al., 2016). Hence 3D cell cultures mimic the hypoxic condition in a better way than the standard 2D cell cultures. Furthermore, 3D \textit{in vitro} models have an edge over the 2D models in the aspect of animal models for cancer researchers including immune-deficient mice which have multiple ethical issues(Zheng et al., 2012).
Chapter 2

Gold Nanoparticle penetration and Stiffness study in 3D tumor models

2.1 3D spheroid culture via Liquid Overlay Technique (LOT)

![Diagram of 2D to 3D transition of cells](image)

Fig 2.1 The transition of single cells from 2D monolayer form to aggregation in the form of spheroid using a liquid overlay technique

This is one of the widely used techniques for culturing spheroid. First introduced by Costachel et al. and Yuhas et al. This technique helps form aggregate of the cells on a nonadhesive substrate (Fig 2.1). They are superhydrophilic and promote the cell-cell and cell-ECM interactions. Some of the frequently used non-adhesive coatings are poly-Hema, agarose. LOT is preferred over other techniques like hanging drop method because it is cheap, aids long-term culture and is least affected by shear stresses which can change the morphology of the spheroids. This helps mimic the actual tumor microenvironment in an efficient manner as compared to the standard 2D cultures.

The spheroids were grown on 96 well plates coated with a fixed concentration of agarose which is 50 microliters/well. Agarose is preferred since it can be prepared easily in a serum-free media. It is quick, cost-effective and the sterilization can be done by autoclave or UV radiation. The formation of spheroids for most of the cell lines usually takes place within 1-3 days post
incubation. One of the studies where this technique was proved successful was when Mayer and co-workers demonstrated that out of 17 gastric cancer cell lines, 12 of them successfully mimicked the features of actual parental gastric carcinoma (Costa, de Melo-Diogo, Moreira, Carvalho, & Correia, 2018).

2.1.1 Spheroid Culture Protocol

In this study, the human mammalian epithelial breast cancer cell line (T47D) was used for the growth of spheroids (American Type Culture Collection, Manassas, VA). The prime components used for the culturing of the spheroids were Dulbecco’s Modified Eagle’s Medium(DMEM), 10% of Fetal Bovine Serum(FBS), and 1% Penicillin-Streptomycin (Gibco, Fisher Scientific, USA). The spheroid formation was initiated by the liquid overlay technique where they were grown on the surface treated 96-well plate (Corning, NY, USA) with 50 μl of agarose in each well. The monolayer cancer cells in 60 mm petri dish were washed with 1ml of 0.025% of trypsin to remove the excess media and finally trypsinated with 500 μl of 0.25% trypsin. The cells were kept in incubation for 2-3 minutes. Trypsin helps in the detachment of the cells from the surface of the plate. The cell suspension was prepared by diluting the trypsin with media (1:4). Next 10 μl of the cell suspension was loaded into the Hemocytometer (Hausser Scientific, Horsham, PA, USA) and finally the cells were counted under an inverted microscope. The number of cells was calculated per ml. In this study, two differently aged, size-matched, stiffness varying spheroids were cultured, day 5 (Fig 2.2 B) and day 20(Fig 2.2 A) respectively. The day 5 spheroids were seeded on a 96 U-bottom well plate with a density of 4300 cells/well and the day 20 spheroids were seeded at a density of 900 cells/ well. The average size of the spheroids was from 350μm to 425μm (Fig. 2.2 A & B). The spheroids were then incubated under standard conditions of 37°C temperature and
5% CO2 in humidified incubators (Jaiswal et al., 2017). The size characterization of the spheroids was evaluated with the help of ImageJ software.

![Fig 2.2](image)

Fig 2.2 (A) T47D day 20 spheroid with necrotic center size matched with (B) T47 D day 5 spheroids. The scale indicates the size of the spheroid

### 2.2 Stiffness analysis in a single cell/tissue

A lot of research in the field of cancer have revealed that molecular, cellular and genetic changes influence the cancer progression. However, very few details are known about the cellular mechanical properties associated with it. The mechanical properties associated with the extracellular matrix and the cells play a very crucial role in terms of differentiation of cells, cancer metastasis, and wound healing (Thomas et al., 2013). One of the parameters among the mechanical properties is the stiffness of the cancer cells and tissues. Stiffness plays a major role in the transmigration of cancer cells via their basal membrane (Barnes et al., 2014). The stiffness of the cancer cells and normal cells vary. Various techniques have been used to characterize the mechanical stiffness of cancer cells like atomic force microscopy (AFM) where the principle was
to cause indentation to the selected cell using the atomic force microscopic tip of defined dimensions (Fig 2.3). The force inflicted due to the deflection of the cantilever was fitted with the indentation curve using the Hertz model to characterize the stiffness of the cell.

Fig 2.3 Depicts the cantilever deflection to characterize stiffness by Hertz models(Thomas et al., 2013)

2.2.1 Stiffness study in 3D spheroids

Stiffness is one of the prime characteristics to differentiate between the healthy cells and the tumor cells. Research study on stiffness characterization of spheroids using micromechanical tweezers have shown that the breast tumor spheroids (T47 D in this case) is softer by a scale of 3-6 times as compared to healthy epithelial breast cancer spheroids like MCF10A(Table 2.1) (JAI SwAL, DEVINA, "Analysis of 3D Tumor Model with respect to Diffusion and Morphology"(2017). Doctoral Dissertations.1376.

https://opencommons.uconn.edu/dissertations/1376.
Table 2.1) Stiffness data for different cancer cell lines compared to the noncancer cell line (MCF10A) JAISWAL, DEVINA, "Analysis of 3D Tumor Model with respect to Diffusion and Morphology"(2017), DoctoralDissertations.1376. https://opencommons.uconn.edu/dissertations/1376.

2.3 Previous diffusion studies in Monolayer cancer cells

After identifying the markers like stiffness that differentiate the normal cells from the tumors, the challenge still remains to target the drug diffusion at the cancer site to inhibit the cancer progression which leads to the cancer metastasis. For this, first, it is important to study the diffusion pattern in the cancer cells and how the mechanical characteristics affect this diffusion pattern. In this study, there will be a study of Nanoparticle diffusion and how the stiffness affects the diffusion pattern. A lot of research has been successfully done in case of delivering the drug to the tumor sites. One of the most important drug delivery vehicles is the nanoparticle drug delivery systems such as gold, lipid-based, polymeric, pH-sensitive that have played a significant role in cancer treatments. The characteristics of the tumor microenvironment are very complex and the cancer drugs show very feeble effects and are almost immune to the drugs(Shi et al., 2016). Hence, it is important first to analyze the diffusion effect of such anti-cancer drugs. A specific type of a pH-sensitive nanoparticle drug delivery system called the acid-labile chemical bond-based
nanoparticle system was used that would inhibit the release of the drug in the blood vessel which is at pH 7.4. On the contrary, the release of the anti-cancer drug takes place at acidic pH 5.7, since the chemical bonds break at that particular pH causing the drug to release. Other nanoparticle drug delivery systems include lipid-based (E.g. Liposomes, micelles), polymeric based (polyethylene glycol (PEG) FDA approved.

![Diagram](image.png)

Fig 2.4 Acid-labile chemical based nanoparticle delivery system (Shi et al., 2016)

### 2.4 Diffusion studies in 3D spheroids

Diffusion is one of the key parameters to evaluate the efficacy of targeted site-specific drug delivery, controlled delivery, enhanced permeation and retention effect (EPR). The three-dimensional (3D) cell aggregates called the spheroids are being widely considered since they replicate the in vivo 3D tumor microenvironment with the actual cell-cell and cell-ECM interactions, gene expressions, and cell signaling pathways very well. The cells grow in a culture medium which supplies nutrients like proteins, enzymes, and aids the diffusion of oxygen. As the contact of the cells increase with the culture medium, there is an increase in the oxygen diffusion
which is directly proportional to the cell viability. Cell viability is a direct measurement of the cell proliferation rate. The morphology and the vasculature of the 3D tumor spheroid are so complex that some cells within the tissue of the spheroids do not get sufficient oxygen and nutrients from the media (Dernowsek et al., 2016). This condition is called hypoxia. Research has proved that this condition is directly related to the age of spheroids. As the spheroid increases in age, there is the development of necrosis core which is cell death. There is a low proliferation in these zones due to the lack of oxygen and nutrients. The present study mainly focuses on such 3D cell cultures and drug delivery systems that are applied in cancer research and treatments.

In terms of evaluating the efficacy of drug delivery systems, the three-dimensional (3D) cell culture models which mimic the native complex tumor microenvironment in vitro have been studied for a long time.

### 2.4.1 Nano Particle diffusion in 3D spheroids

**Introduction:**

Nanoparticles (NPs) range in the size from 1 to 100nm. They have a very wide application in the field of cancer diagnosis and therapeutics for almost two decades widely applied in the field of cancer therapy since two decades as part of pharmacological approach (Sykes et al., 2016). The nanoparticle morphological features like the size, shape and surface characteristics can affect the penetration ability, its retention in the cells, pattern of accumulation, and uptake by the cells. Moreover, sizes of NPs can affect toxicities causing them to accumulate in liver, kidney and even leading them to cross the blood-brain barrier. This can be mitigated by changing the surface characteristics of the NPs by masking them. The nanoparticle drug delivery system includes Gold, polymeric NPs, lipid-based carriers, quantum dots, carbon nanotubes, metal-based NPs which have an edge in drug delivery and distribution over the other conventional drug delivery systems (Mc
Some of the advantages of the nanoparticle systems over the conventional drug delivery system is minimizing the premature degradation of drug, increasing the efficiency of its local concentration and control the releasing kinetics with respect to time. Furthermore, this system reduces immunogenicity, increases permeability and retention effect (EPR), improves the solubility, half-life, and bioavailability of drugs. Cancer drug delivery systems require precision targeting ability so that the drug can be targeted specifically to the tumor tissue, thereby minimizing cytotoxicity and improving therapeutic efficacy.

Fig 2.5: Depicts the different kinds of nanoparticles used for the drug delivery systems (Mc Carthy, Malhotra, O’Mahony, Cryan, & O’Driscoll, 2015).
2.4.2 Gold nanoparticle diffusion on day 5 and day 20 size-matched 3D tumor spheroids

Fig 2.6: Schematic diagram of the Citrate-coated Gold Nanoparticle structure showing the Au core and the Citrate particles bound on the surface of the core

In this study, we will discuss the diffusion of gold nanoparticles in 3D breast cancer epithelial cell line T47D. Two spheroids of similar sizes, varying ages day 5 and day 20 (average size ~ 400µm) (JAISWAL, DEVINA, "Analysis of 3D Tumor Model with respect to Diffusion and Morphology" (2017). Doctoral Dissertations. 1376. https://opencommons.uconn.edu/dissertations/1376) were being considered to study if the stiffness of the spheroids affects the diffusion of the gold nanoparticle. The day 5 spheroids were seeded with a density of 4300 cells/ well and the day 20 ones were seeded with 900 cells/well for the T47D breast cancer cell line. As per the research studies, the day 5 T47D spheroids were found to be softer than the day 20 T47D spheroids. Diffusion with liposome studies proved that fluorescence for day 20 spheroids ~ 1.6 times lesser in intensity than the day 5 spheroids because of the necrosis formation at the central core due to hypoxia condition which leads the older spheroids to deposit more and more extracellular matrix(ECM) and restricting the entry of
nanoparticle. In this study spherical gold nanoparticles (AuNPs) coated with citrate (Fig 2.6) were considered. These were obtained from Luna Nanotech (Toronto, ON, Canada). The citrate-coated gold nanoparticles were used to offer a high degree of control over core diameter of 45nm ±0.8 nm. The AuNPs were supplied as liquid suspended in water with 0.01 % (w/v) and administered to each tumor spheroid at concentrations of 3 μL in 12 μL Phosphate-buffered saline PBS. The AuNPs were chosen over other more clinically appropriate polymeric materials. The below is Transmission electron microscopy image of Citrate coated AuNps of size 45nm (Fig 2.7).

![TEM Image of Citrate Coated AuNPs](image)

**Fig 2.7** Transmission Electron Microscopy (TEM) ultrathin section image of a T47D Day20 spheroid with each of the 45nm AuNPs localized at the intracellular junction within the cytoplasm. The scale bar is 500nm. Magnification is 49000X

The breast cancer tumors usually have leaky vascular structures. The passive mechanism called the enhanced permeability and retention (EPR) has an edge during this situation where this triggers the AuNPs in the size range 10–400 nm to preferentially extravasate from the leaky vasculature
into the interstitial space between the breast cancer tumors. Furthermore, the other factors that contribute to the cytotoxicity and pharmacodynamics of the AuNps are the size, shape, surface charge and overall morphology. These properties of the nanoparticles are efficiently tailored to design systems that can improve the uptake by the tumor cells (England et al., 2013). The therapeutic efficacy can be improved and toxicity can be reduced by using the nanoparticle delivery systems. To be more specific, gold nanoparticles have shown great promise in cancer treatment.
Chapter 3

Gold nanoparticle penetration analysis using Light Microscopy in day 5 and day 20 size-matched 3D tumor spheroids

This chapter discusses the analysis of the gold nanoparticle diffusion in two different aged spheroids day 5 seeded with a density of 4300 cells/well and day 20 seeded with the density of 900 cells/well. The spheroids are sectioned using frozen section procedure which will be discussed in detail in this chapter. This is done to better understand the diffusion in both the size matched day 5 and day 20 spheroids. Post sectioning, they are stained with H&E staining to distinguish the cell morphology and the gold nanoparticles.

3.1 Frozen Section procedure

3.1.1 Importance:

The frozen section procedure is a pathological technique in order to perform a quick analysis of a specimen in this case cancer spheroid using microscopy. This procedure is well known as the cryosection procedure. In a clinical aspect, this is widely used in the diagnosis of cancer by determining the malignancy/benignancy of the suspected tissue or cell section to further confirm the metastasis condition. In case of the cell, it is very helpful to identify the subcellular structures like mitochondria, ribosomes, peroxisomes, cell-cell junctions, nuclear areas, nucleus to cytoplasm ratios etc.(Nakazawa, Rosen, Lane, & Lattes, 1968).

3.1.2 Frozen section protocol:

Each of the sizes matched T47D spheroids at day 5 (4300 cells/well) and day 20 (900 cells/well) was diffused with the AuNPs which were supplied as liquid suspended in water with 0.01 % (w/v) at concentrations of 3 μL in 12 μL Phosphate-buffered saline PBS. The spheroids were incubated
in the humidified incubators at the temperature of 37º C and 5% CO₂ for 24hrs. After 24 hrs, the day 5 and day 20 spheroids were observed under the inverted microscope with 10X. To begin with the protocol, the individual spheroids for each condition (day 5 with AuNPs, day 5 control, day 20 with AuNPs and day 20 control) were transferred to a 15 ml tube respectively with the P1000 pipetteman. The tip was cut off to easily lift the spheroids. Next, the spheroids were allowed to settle down for 5 mins. The media was carefully pipetted out without disturbing the settled spheroids. Around 300 µL of media was left behind. Next, 3ml room temperature 1X PBS was added to the tubes with cap and inverted 2X, to wash out the media. The spheroids were allowed to settle down for 3-5 mins. This PBS washing protocol was repeated for a total of 3x. As a next step, 3ml Buffered Formalin was added to each of the tubes. Formalin is mainly added to fix the cells. The tube was inverted every 60 secs for 5 mins. Finally, the formalin was removed and again 3ml of PBS was added to wash out the formalin since this might destroy the cell morphology if left for a long time. The washing with PBS step was repeated 4x. After the 4th time, the PBS was removed as much as possible and replaced with 3ml of 30%w/vol sucrose in 1X PBS and was left to settle for 5 mins. The sucrose solution is added to maintain the cell morphology. Next, the optimal cutting temperature(OCT) compound was prepared by adding 1/10th volume of 0.5% Methylene Blue dye and inverted to mix well. OCT is added to embed the cell samples before the frozen sectioning on a cryostat. To efficiently cut the sections, the sucrose solution was removed and all the spheroids were transferred to bullet foil mold prepared for each sample(Fig 3.1). The molds prepared were placed in Dry Ice: Isopropanol bath as a slurry to freeze molds. The outside of the foil was labeled for each condition with a sharpie marker. Additional sucrose was removed and a drop of OCT was dropped into the bullet foil mold. See mold diagram below. Next, enough methylene blue dyed OCT was added to cover spheroids halfway. The molds were allowed to
freeze completely. The foils were folded and stored in zip-lock bags at -80 °C.

Fig 3.1 A) The bullet foil mold for transferring the spheroids and coating with OCT compound to form the frozen section B) The interior view of the mold C) The full view of the bullet foil mold.

3.1.3 Cryosectioning:

The Frozen section procedure is a very quick and rapid way to fix histology sections and mount them on a refrigeration device called a cryostat (Fig 3.2 A &B). This is used to cut thin sections of the sample. In clinical application, it is used post the surgical removal of tumors to allow rapid quantification and determination of margin and check for any remaining cells post the surgery. These cryosections also have a cutting edge to detect the presence of substances that are lost in the conventional histology technique such as the lipids. This is also necessary to define organelles and cell membrane structures using light microscopy. They have been widely used to detect some antigens that are being masked by formalin(Peters, 2010). The below flow diagram describes the steps for the cryosectioning until the staining of the samples by H&E staining.
Fig 3.2 A) The frontal view of the cryostat used for the sectioning of the spheroid. B) Internal view of the cryostat.

Flow diagram showing the steps for cryosectioning:

1. Molds were transferred on dry ice and placed in cryostat for at least 20 mins to reduce temperature down to -23°C.
2. Cryo sections of (6-10um) were cut and placed onto charged Plus slides and allowed to air dry.
3. They were dipped in 50% ETOH for 2-3 mins to elute excess OCT.
4. Next, they were transferred to 100% ETOH for 3 mins and then cold methanol: acetone (1:1) for 10 mins.
5. Dried at RT for 30 mins and stored in slide boxes at -20°C.
6. Next, the slides were removed from freezer and allowed to dry at RT for 20 mins.
7. Re-hydrated in H2O for 15 mins.
8. Next, they were taken for H&E staining.
3.2 Hematoxylin and eosin (H&E) Stain

This staining is one of the most common histological stains and it is highly essential to recognize various morphological changes which includes the changes in the cytoplasm, nucleus, and extracellular matrix features. Hematoxylin dye has a deep blue-purple color and this specifically stains the nuclei. The Eosin dye is pink in color and stains nucleoli, cytoplasm and all extracellular matrix.

3.2.2 Protocol

All of the slides after cryosectioning and post wash with 50% ethanol, 100% ethanol and fixation in cold methanol: acetone were allowed to come to RT for around 20 minutes. Place the sample slides in H2O for 10 mins at room temperature and allowed to hydrate. The slides were first dipped in 95% EtOH for 1 min and then placed under running water. Next, they were dipped in Hematoxylin stain solution for 2 mins and then placed under the running water till the color clears out. Next, they were placed in Bluing solution for 1 min and again dipped in running water till the water went clear. Later the slides were dipped in 90% EtOH for 30 secs and then in Eosin Stain for 15 secs. Then they were dipped 10 times in 3 different absolute ethanol bottles and later dipped 15 times in 3 different toluene solutions to dehydrate the samples. The slides were then mounted with the mounting media and coverslip.

3.3 Results and Discussion

3.3.1 Analysis of the cryosection of day 5 control spheroid

Each of the sizes matched T47D spheroids day 5(4300 cells/well) and day 20 (900 cells/well) was diffused with the citrate-coated AuNPs which were supplied as liquid suspended in water with 0.01% (w/v) at concentrations of 3 μL in 12 μL Phosphate-buffered saline PBS. The spheroids were
incubated in the humidified incubators at the temperature of 37°C and 5% CO₂ for 24hrs. Similar researches with AuNPs have proved that penetration and uptake of the AuNPs by the cells improve with the incubation time (Huang et al., 2012). After the sectioning, they were stained with H&E staining and observed under the light microscope to observe the penetration in the day 5 and day 20 size matched spheroids. The below table summarizes the conditions that were considered for this study.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Spheroid Age</th>
<th>Seeding density</th>
<th>Nano-Particle Size or Control</th>
<th>Concentration of NP</th>
<th>Time points for penetration study</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>Day 5</td>
<td>4300</td>
<td>45 nm</td>
<td>3 μL Np/ 12 μL (PBS)</td>
<td>24hrs</td>
</tr>
<tr>
<td>T47D</td>
<td>Day 5</td>
<td>4300</td>
<td>Control</td>
<td></td>
<td>24hrs</td>
</tr>
<tr>
<td>T47D</td>
<td>Day 20</td>
<td>900</td>
<td>45nm</td>
<td>3 μL Np/ 12 μL (PBS)</td>
<td>24hrs</td>
</tr>
<tr>
<td>T47D</td>
<td>Day 20</td>
<td>900</td>
<td>Control</td>
<td></td>
<td>24hrs</td>
</tr>
</tbody>
</table>

Table 3.1) Detailed plan indicating the conditions for the day 5 and day 20 size matched T47D breast cancer spheroids for the analysis of AuNPs penetration using light microscopy.

The day 5 and day 20 spheroids of different stiffness and this study we tried to see if the penetration of the AuNPs is affected by the stiffness, age and necrosis condition of the spheroids. The below table indicates the stiffness data for the size matched spheroids (Table 3.2).
Table 3.2) Average Size, stiffness data and seeding density of the size matched T47D spheroids
day 5 (4300 cells/well) and day 20 (900 cells/well) JAISWAL, DEVINA, "Analysis of 3D Tumor
Model with respect to Diffusion and Morphology" (2017). DoctoralDissertations.1376.
https://opencommons.uconn.edu/dissertations/1376.

The figure (Fig 3.3 A) indicates the complete sectioned image of day 5 control spheroids after the
frozen OCT and cryosectioning procedure. The figure on the right (Fig 3.3 B) indicated the dark
field image of the section. This section is unstained and the size analysis of the section was
computed using ImageJ software. As per the study, there were no changes observed in the size of
the spheroid section and it was found to be around ~ 390μm which is close to the size of the
spheroid that was computed using ImageJ prior to the sectioning.
Fig 3.3 A) Cryosection image of T47D Day5 Control spheroid using light microscope of magnification 20X. The scale bar indicates the radius of the section which is 184.54µm quantified with the help of ImageJ. B) Darkfield image of T47D Day5 Control spheroid cryosection at a magnification of 20X.

Fig 3.4 A) Analysis of the original size of Day 5 T47D control spheroid observed under a light microscope of magnification 10X using ImageJ software. The scale bar indicates the complete
diameter of the section which is 386.60µm. B) Analysis of the original size of another Day 5 T47D control spheroid observed under a light microscope of magnification 10X using ImageJ software. The scale bar indicates the complete diameter of the section which is 373.68µm.

3.3.2 Analysis of day 20 spheroids treated with 45nm AuNPs post sectioning and H&E staining

Fig 3.5 A) Cryosection image of a T47D Day20 spheroid with 45nm AuNPs and stained with H&E staining observed using light microscope of magnification 40X. The scale bar indicates the radius of the section which is 217.31µm quantified with the help of ImageJ. B) Unstained section of a T47D Day20 spheroid with 45nm AuNPs observed using light microscope of magnification 40X. Scale bar 213.63 µm

The figure (Fig 3.5 A) shows the cryosectioned image of day 20 spheroids diffused with 45nm AuNPs and stained with H&E staining observed under a light microscope of 20X magnification. The nucleus is stained with purple and the cytoplasm is stained in pink. The red spots on the image may indicate the accumulation of the AuNps, however, this is not clear and will be analyzed using dark field and bright field microscopy to confirm the presence of the accumulated particles. The
figure (Fig 3.5 B) shows the unstained section of day 20 spheroid with 45nm AuNPs. The sizes of both the sections were being computed using ImageJ software.

Fig 3.6 A) Cryosection image of a T47D Day20 spheroid with 45nm AuNPs and stained with H&E staining analyzed using bright field microscope of magnification 40X. The scale bar is 75µm. B) Analysis of 45nm AuNPs observed using light microscope with phase contrast of magnification 40X. Scale bar 75 µm. The arrows indicate the accumulation of AuNPs.

This image (Fig 3.6) was observed under a light microscope at 40X magnification to see the localization of the AuNPs, however, the black spots could have been the accumulation of particles at those sites but this was not clear. Hence it was very difficult to quantify the particles without considering high resolution microscopes like Transmission electron microscope. The next chapter will discuss about why electron microscopes are used for quantification of the gold nanoparticles and to observe the subcellular, cellular features in a more qualitative way.
3.3.3 Analysis of day 20 control spheroids post sectioning and H&E staining

This image (3.7 A) indicates the stained section of day 20 control spheroid with H&E staining, however, the image on the right (Fig 3.7 B) is unstained. The sections have been observed under the light microscope at 20 X magnification. Again, the cytoplasm is stained in pink and nuclei in purple.

Fig 3.7 A) Cryosection image of T47D Day20 control spheroid stained with H&E staining observed using light microscope of magnification 60X. The scale is 150 µm. B) Cryosection image of T47D Day20 control spheroid unstained observed using light microscope of magnification 60X. The scale is 150 µm.
Chapter 4

Gold nanoparticle penetration analysis using Electron Microscopy in 3D tumor models

4.1 Transmission Electron Microscopy (TEM): An Introduction

The electron microscope is a special type of microscope that utilizes a beam of electrons to project an image of the specimen. It provides very much higher magnifications and has a very great resolving power as compared to a conventional light microscope, which allows it to see much tinier objects in fine resolution. Conventional microscopy is not able to achieve such high resolution. In our study, the focus is to identify the gold nanoparticles in the various sections of the spheroids which have a thickness of 70nm and to further quantify the cell-cell junctions, subcellular features, nucleus to cytoplasm ratio etc. Hence such analysis requires high-quality imaging modalities including scanning electron microscopy (SEM) and transmission electron microscopy (TEM). We will study the spheroid sections to be imaged using TEM based on sub-micron features such as cell-cell adhesion, Golgi bodies and nucleus size, nucleus to cytoplasm ratio. TEM provides Nano-scale resolution to give detailed comparative results with respect to cell morphology change as well as mechanical characterization. High-quality imaging techniques, such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are required for these studies. An optical microscopy is a useful tool for observing cells but it has limited resolution and needs expensive fluorescent stains for observation of nanoparticle-cell interaction. TEM is able to provide nanoscale resolution to precisely locate a diffused particle in a tumor, and also give detailed comparative results with respect to cell morphology changes.

4.1.1 Sample preparation for TEM

The cultured T47D breast cancer epithelial spheroids were processed for TEM at the Biosciences Electron Microscopy Laboratory (UConn). The sample preparation included fixation, agarose
embedding, oxidation, dehydration, and embedment of the spheroid samples as well as ultramicrotomy. Few of the steps with respect to the sample preparation was handled in the lab before the processing by the TEM team. This included –

1) Primary Fixation

The spheroids with four conditions day 5 (4300 Cells/ well) with 45nm AuNPs, day 5 control, day 20 (900cells/well) and day 20 control were transferred from the 96-well plate to individual Eppendorf tubes designated for each condition with pipette P1000 tip cut off. The cells were rinsed with PIPES buffer and later the buffer was removed and immediately replaced with 1,500 µl EM fixative (2.5% glutaraldehyde + 4.0 % paraformaldehyde in 0.1M PIPES + 2 mM CaCl₂ + 4 mM MgCl₂, pH 6.8). **The cells were not allowed to dry between solution changes.** Later the samples were fixed overnight at 4°C.

2) Agarose Embedding

3% low gelling temperature agarose was melted and held at ~42°C in water bath. The fixatives from the Eppendorf tubes were removed carefully using micropipettes. A Layer of 500 µl of liquefied 3% agarose was added on top of the cell suspension for each condition and was allowed to solidify for 1 hour in Eppendorf tube in a bucket with ice in the refrigerator.

TEM (FEI Tecnai 12 G2 Spirit BioTWIN) was used for 2D imaging. For image analysis, ImageJ (NIH) was used to measure nuclei area, cell junctions, and other inter-cellular dimensions. The below table indicates the plan for the Electron Microscopy Imaging.
Table 4.1) Detailed plan indicating the conditions for the day 5 and day 20 size matched T47D breast cancer spheroids for the analysis of AuNPs penetration using Transmission Electron Microscopy (TEM). For image analysis, ImageJ (NIH, Bethesda, MD, USA) [10] was used to measure nuclei area, cells junctions, and other intercellular dimensions.

4.2 Analysis and discussion

4.2.1 Analysis of penetration of 45nm AuNPs using TEM for day 20 spheroids

This section focusses on the study of penetration of 45 nm AuNPs on day 20 spheroids that of size ~400μm. Each spheroid was seeded with 900 cells/well. The figure (Fig 4.1 A) indicates the depth of penetration of the AuNPs from the periphery of the spheroid ultrathin section. At a depth of 70 μm the accumulation of AuNPs was observed. The figure (Fig 4.1 B) describes the better-magnified image with a scale of 2 μm. The particles were up taken at the intracellular spaces and junctions.

The next image (Fig 4.2 A) shows the nanoparticle uptake at a depth of 20 μm. The AuNPs seems to have been accumulated at the intracellular spaces in the cytoplasm. The image (Fig 4.2 B) is an enlarged region of the image that shows much clearer accumulation of the particles at a higher
The accumulations of the AuNPs were observed to be maximum at the periphery of the section. To actually quantify the number of AuNPs, the section (Fig 4.3 A) gives a clearer count and this was being used to estimate the number of particles in the adjoining areas (Fig 4.3 B) using Image J. However, it was observed that, after the penetration of the AuNPs there were disruptions in the cell membrane observed but there wasn’t any change in the nuclei (Fig 4.5 A).

Fig 4.1 A) Transmission Electron Microscopy (TEM) ultrathin full section image of a T47D Day 20 spheroid with the 45nm AuNPs penetration localized at the depth of 70 μm from the periphery in the intracellular junction within the cytoplasm. The scale bar is 10μm. Magnification is 440X. B) Higher magnified image of the 4.1 (A). The scale bar is 2μm. Magnification is 6800X.
Fig 4.2 A) Transmission Electron Microscopy (TEM) ultrathin section image of another T47D Day20 spheroid with the 45nm AuNPs penetration localized at the depth of 20 µm from the periphery in the intracellular junction within the cytoplasm. The scale bar is 10µm. Magnification is 1200X. B) Higher magnified image of the 4.2 (A). The scale bar is 2µm. Magnification is 9300X.
Fig 4.3 A) Transmission Electron Microscopy (TEM) ultrathin section image of T47D Day20 (same section as Fig 4.2 B) spheroid with the 45nm AuNPs penetration localized at the depth of 20 µm from the periphery in the intracellular junction within the cytoplasm. The scale bar is 500nm. Magnification is 4900X. B) ImageJ analysis of the depth of AuNPs from periphery found to be 20 µm. The scale bar is 10µm. Magnification is 1200X.

Fig 4.4 A) Transmission Electron Microscopy (TEM) ultrathin section image of a T47D Day20 spheroid with the 45nm AuNPs penetration localized very clearly in the intracellular junction within the cytoplasm. The scale bar is 500nm. Magnification is 49000X. B) Surrounding regions localized with AuNPs clearly visible. The scale bar is 500nm. Magnification is 14500X.

4.2.2 Analysis of cell-cell junctions after penetration of 45nm AuNPs using TEM for day 20 spheroids

There was disruption of the cell membrane (Fig 4.5 A) as a result of penetration of the AuNps into the intracellular regions but there were no changes observed in the nuclei membrane which confirmed that there wasn’t any penetration into the nucleus. The cell-cell junctions were again
observed to be disrupted whereas the normal day 20 control sections had tight cell-cell junctions (Fig 4.5 B).

Fig 4.5 A) Transmission Electron Microscopy (TEM) ultrathin section image of a T47D Day20 spheroid with the 45nm AuNPs showing the disruption of the cell membrane and weak cell-cell junctions. The scale bar is 2µm. Magnification is 6400X. B) Day 20 Control spheroid section indicated tight cell-cell junctions. The scale bar is 10µm. Magnification is 1400X.

4.2.3 Analysis of penetration of 45nm AuNPs using TEM for day 5 spheroids

The penetration of AuNPs in case of Day 5 spheroids showed that the more penetration was observed at the periphery. At a depth of 50 microns (Fig 4.6A), the particles were accumulated in the adjoining regions as well. The disruption of the cell membrane (Fig 4.6 B) was observed because of the penetration into the cytoplasm but the nucleus was unaffected indicating no particles. The cell-cell junctions were observed to be weak because of the disruption. The figure (Fig 4.6 C) showed that there were accumulations of the NPs at the center of the spheroid and the
quantification was possible with the help of (Fig 4.6 D) to estimate the count of the NPs at the central region.

Fig 4.6 A) Transmission Electron Microscopy (TEM) ultrathin section image of T47D Day5 spheroid with the 45nm AuNPs penetration localized at the depth of 50 µm from the periphery in the intracellular junction within the cytoplasm. The scale bar is 10µm. Magnification is 890X. B) Higher magnified image of
Fig 4.6 B) Magnified image of T47D Day5 spheroid region highlighted in 4.6 A better localization of particles in the adjoining areas. The scale bar is 2µm. Magnification is 6800X. C) Localization of particles at the center (250µm). The scale bar is 2µm and magnification 4800X. D) Localization at the center to count the particles and quantify the ones in the regions around. The scale is 500nm and Magnification 23000X

4.2.4 Analysis of day 20 control and day 5 control size matched spheroids characteristics using TEM

The Control spheroids for both days 20 and day 5 were size-matched using the ImageJ software and the sizes were found to be the same (Fig 4.7) of around 420 microns. In terms of quantitative analysis, the area of nucleus was computed for both day 5 and day 20 spheroids at the periphery using ImageJ where 5 to 6 cells were considered at the periphery for both the cases (Fig 4.8 B & C) and it was found that Day 20 spheroids had larger nuclear cross-sectional area compared to the day 5 spheroids using bar graph statistics (Fig 4.8 A). The computation was done again for both day 5 and day 20 size matched spheroids but now at the center using ImageJ where 5 to 6 cells
were considered at the center for both the cases (Fig 4.9 B & C) and it was found that Day 20 spheroids had larger nuclear cross-sectional area compared to the day 5 spheroids using bar graph statistics (Fig 4.9 A). Thus, it was inferred that irrespective of the consideration of the locations the statistics continued to be the same. The results could be corroborated with continued analysis of more sections of the spheroids.

Fig 4.7 A) TEM full section view of Day 5 control T47D spheroid. The scale bar is 10µm. Magnification is 460X. The size was quantified using ImageJ and found to be 204 µm B) TEM full section view of Day 20 control T47D spheroid size-matched with day 5. The scale bar is 10µm. Magnification is 200X. The size was quantified using ImageJ and found to be 205.7 µm
Fig 4.8 A) Bar graph statistics of cross-sectional area of the nucleus computed for regions at the periphery for Day 5 and day 20 control size matched T47D spheroids.

B) Areas of interest marked using ImageJ at the periphery for day 20 control spheroid size-matched with day 5. C) Areas of interest marked using ImageJ at the periphery for day 5 control spheroid size-matched with day 20.
Fig 4.9 A) Bar graph statistics of cross-sectional area of the nucleus computed for regions at the center for Day 5 and day 20 control size matched T47D spheroids. B) Areas of interest marked using ImageJ at the center for day 5 control spheroid size-matched with day 20. C) Areas of interest marked using ImageJ at the center for day 20 control spheroid size-matched with day 5.
4.2.5 Analysis of day 20 control and day 5 size matched spheroids with 45nm AuNPs using TEM

Fig 4.10 A) Bar graph statistics of Day 5 and day 20 size matched T47D spheroid with 45nm AuNPs distribution at the periphery B) Bar graph statistics of Day 5 and day 20 size matched T47D spheroid with 45nm AuNPs distribution at the center

For this analysis, the quantification of the AuNPs distribution was done at both the periphery and the center of the size matched spheroids. For day 5 and day 20, 5 to 6 cells were considered at the periphery and at the center, the regions were mapped using ImageJ and the individual AuNps were counted. The statistics showed that the number of AuNps per cell were more at the periphery for day 20 and less as compared to day 5. This could have been due to the nonuniform distribution of the particle and accumulations at certain junctions in the intracellular regions. However, at the center for the day 20 spheroids, there was an almost null value of the nanoparticles and the count was better in case of the day 5 spheroids. The reason is that the day 20 spheroids were older in age that leads to the formation of the necrotic core. Excess deposition of ECM matrix inhibits the
penetration of the particles into the central region. On the contrary, the day 5 spheroids are younger in age and the necrosis does not develop leading to better penetration of nanoparticles.

4.2.6 Analysis of cell-cell junctions using TEM for day 20 and day 5 spheroids at the center

![Day 20](image1)
![Day 5](image2)

Fig 4.11 A) Cell-cell junction observed for Day 20 spheroid section at the center. Scale is 10µm and magnification is 2900X B) Cell-cell junction observed for Day 5 spheroid section at the center. Scale is 10µm and magnification is 1900X.

The cell-cell junction is seen to be tightly packed for day 20 spheroid section which is as shown in Fig 4.11 A. As this is the necrotic core region where there is excess deposition of ECM as compared to day 5 spheroid shown in 4.11 B. The excess deposition tightens the region causing no fluid spaces between cells. This further restricts the entry of nanoparticles in the center.
4.2.7 Statistical Significance: T test using GraphPad

Finally, the statistical significance T test was performed on a sample set of 5 for both day 5 and day 20 spheroids. The area of nucleus was quantified for both cases at the periphery and at the center and the quantitative distribution of the nanoparticles both at the periphery and center showed statistically significant data.

Table 4.2: Data for Day 20 spheroids. Mean cross-sectional area of nucleus nuclei and No. of AuNPs distribution per cell for Day 20 spheroids at necrotic region (center) and proliferating zone (side). (Mean ± SD, Sample size = 5 each)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Necrotic Zone (center)</th>
<th>Proliferating Zone (side)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cross-sectional area of nucleus</td>
<td>89±6</td>
<td>112±28</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>No of AuNPs distribution per cell</td>
<td>6.2±1(P&lt;0.01)</td>
<td>1250±290(P&lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3: Data for Day 5 spheroids. TABLE 2. Mean cross-sectional area of nucleus nuclei and No of AuNPs distribution per cell for Day 5 spheroids at necrotic region (center) and proliferating zone (side). (Mean ± SD, Sample size = 5 each)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Center</th>
<th>Proliferating Zone (side)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cross-sectional area of nucleus</td>
<td>32±6</td>
<td>37±8</td>
<td>P&lt;0.01</td>
</tr>
<tr>
<td>No of AuNPs distribution per cell</td>
<td>87±2P&lt;0.01</td>
<td>700±188 (P&lt;0.05)</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSION

In this thesis, two 3D breast cancer spheroids of the T47D epithelial breast cancer line were developed by using the liquid overlay technique. The spheroids were of two different ages day 5 and day 20 but where developed with same sizes and different stiffness. The citrate coated gold nanoparticle penetration study was conducted for both day 5 and day 20 spheroids. Two modalities were considered for this analysis, the light microscopy and the electron microscopy. Light microscopy was used to understand and study the complete morphology of the spheroids. For this, the spheroids were first diffused with gold nanoparticle concentration of 3 µl per 12 µl of phosphate buffered saline. They were left for incubation for 24 hrs. as incubation time improves the particle penetration. Later they were sectioned using the cryosectioning technique where they were sliced at a thickness of 10 microns and stained with H& E staining. The results showed accumulation of the gold nanoparticles at the periphery for day 20 section but no penetration at the center due to the necrotic core leading to excess deposition of ECM and inhibition of the entry of the particles. For better qualification of individual gold nanoparticles and better analysis of sub cellular structures like cell-cell junctions, nucleus, cytoplasm, Golgi bodies, mitochondria, vesicles etc. a higher resolution, higher magnification modality was considered. The ultrathin sections with a thickness of 70nm was cut using an ultramicrotome for both day 5 and day 20 spheroids. The gold nanoparticle penetration study was done using statistical analysis. Based on the initial trials, it was found that the gold nanoparticles were engulfed into vesicles inside the cytoplasm by endocytosis process for both day spheroids. The quantification of individual nanoparticles was done, and it was inferred that the number of particles at the periphery were more for day 20 compared to day 5 spheroid. At the center, however due to excess ECM deposition, it was observed that the day 20 spheroids had very few particles compared to day 5. As part of the morphological analysis, the area of nucleus was computed both at the periphery and at the center of both the spheroids. The
area of nucleus was observed to be large for day 20 compared to day 5 both at the center and at the
periphery. T statistical analysis was done to prove the initial inferences. The cell-cell junctions
were found to be tighter at the center for day 20 compared to day 5 spheroids. The importance of
the mechanical characteristics and cellular uptake process play a major role in the gold nanoparticle
penetration and uptake the cells and through this thesis we were able to study these effects.
REFERENCES


