

**THERMORESPONSIVE POLYMER INTEGRATED
METASURFACE SENSOR FOR CAPTURE AND
RELEASE OF EXTRACELLULAR VESICLES IN
REAL-TIME**

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By
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**THERMORESPONSIVE POLYMER INTEGRATED METASURFACE
SENSOR FOR CAPTURE AND RELEASE OF EXTRACELLULAR
VESICLES IN REAL-TIME**

By Beyza Nur Küçük

February 2025

We certify that we have read this thesis and that in our opinion it is fully adequate,
in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

THERMORESPONSIVE POLYMER INTEGRATED METASURFACE SENSOR FOR CAPTURE AND RELEASE OF EXTRACELLULAR VESICLES IN REAL-TIME

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M.S. in Materials Science and Nanotechnology

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Extracellular vesicles (EVs) play pivotal tasks in intracellular communication, carrying biomolecules such as proteins, lipids, and nucleic acids that reflect the physiological state of their originating cells. Isolating EVs purely from complex and protein-rich matrixes is crucial for understanding cellular processes and disease progression. The gold standard method for isolating EVs is ultracentrifugation, yet it has severe obstacles in terms of requiring expensive equipment, non-vesicular impurities, and possible damage on the EV surface. Owing to these drawbacks, their further analyses, isolation and detection through their surface markers are challenging. This study aims to integrate a smart thermoresponsive polymer with a metasurface plasmonic sensor, which is further decorated with anti-CD63 antibodies to capture EVs derived from MCF-7 breast cancer cells as a model system. We later isolate (release) these EVs by simply altering the local temperature above the lower critical temperature (LCST) of the polymer. Basically, the thermoresponsive polymer exhibits hydrophilic characteristics below its LCST and becomes hydrophobic when the temperature is increased above the LCST. The optic plasmonic sensor has a well-defined nanoperiodic array, presenting surface plasmons while exciting

the surface with a normal angle of incident light. Therefore, the metamaterial sensor denotes real-time and label-free detection of EV binding and release events. This enables the quantitative analysis of EVs. In a nutshell, leveraging the distinctive thermoresponsive characteristics of the polymer, we presented a novel methodology designed to selectively immobilize EVs through antibody interactions and subsequently release them by elevating the temperature. This process enables the isolation of EVs with a high degree of purity, free from non-specific molecular contaminants. Consequently, our pioneering approach opens a promising new way for studying EVs and their surface markers in great detail, unrestricted by the limitations of conventional separation techniques, and contributes to the understanding of complex cellular processes and the subtle development of illnesses.

Keywords : Microfluidic Chips, Cancer, Extracellular Vesicles (EVs), Plasmonic Metasurface, EV Isolation

ÖZET

HÜCRE DIŐI VEZİKÜLLERİN GERÇEK ZAMANLI OLARAK YAKALANMASI VE SALINMASI İÇİN ISIYA CEVAPLI POLİMER ENTEGRE METAYÜZEY SENSÖR

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Hücre dışı veziküller (EV), protein, lipit ve nükleik asit benzeri biyomolekülleri taşıyarak, salgılandıkları kaynak hücrelerinin fizyolojik durumunu yansıtan ve hücreler arası iletişimde kritik roller üstlenen yapılardır. Hücresel süreçlerin ve hastalık ilerleyişinin anlaşılması için, EV'lerin proteince zengin matrislerden saf bir şekilde izole edilebilmesi büyük önem taşımaktadır. EV izolasyonu için altın standart yöntem ultrasantrifüj olmakla birlikte; pahalı ekipman gerektirmesi, safsızlıklar içerebilmesi ve EV yüzeyinde olası zararlar oluşturabilmesi gibi kısıtlamalara da sahiptir. Bu dezavantajlar, EV'lerin moleküler düzeyde analizlerini, izolasyonunu ve tespitini zorlaştırmaktadır. Bu çalışmada, akıllı bir ısıya duyarlı polimer (PNIPAM) ve anti-CD63 antikorları ile fonksiyonelleştirilmiştir metayüzey plazmonik bir sensör kullanılarak, model sistem olarak belirlenen MCF-7 meme kanseri hücrelerinden türetilen EV'leri yakalamak amaçlanmıştır. Daha sonra, plazmonik metayüzey sensörün bölgesel sıcaklığı, polimerin alt kritik çözünürlük sıcaklığının (LCST) üzerine çıkarılarak EV'lerin izole edilmesi (salınması) sağlanmıştır. Isıya duyarlı polimer, LCST'nin altında hidrofilik özellik gösterirken, sıcaklık LCST'nin üzerine çıkarıldığında hidrofobik hale gelmektedir. Optik plazmonik sensör, nano-periyodik bir yapıya sahip olup, yüzeye belirli bir açıda gelen ışık ile uyarıldığında yüzey plazmonlarını ortaya çıkarmaktadır. Bu nedenle, metamateryal sensör, EV bağlanma ve salınım

olaylarının gerek zamanlı ve floresan iřaretleme gerektirmeyen bir řekilde tespit edilmesini saėlamaktadır. Bylece, EV'lerin kantitatif analizi mmkn hale gelmektedir. zetle bu alıřmada, polimerin ısıya duyarlı zelliklerinden yararlanarak, antikor etkileřimleri aracılıėıyla EV'lerin seici olarak yakalanmasını ve ardından sıcaklıėın artırılması yoluyla salınmasını saėlayan yeniliki bir metodoloji sunulmuřtur. Bu sre, EV'lerin zgn olmayan molekler kontaminantlardan arındırılarak, yksek saflıkta izolasyonunu saėlamaktadır. nerilen bu yeniliki yaklařım, geleneksel ayrıştırma tekniklerinin sınırlamalarından baėımsız olarak EV'lerin ve yzey belirtelerinin detaylı incelenmesine yeni bir perspektif kazandırmakta ve karmařık hcrel srelerin yanı sıra hastalıkların incelikli geliřiminin daha iyi anlařılmasına katkıda bulunmaktadır.

Anahtar szckler : Mikroakıřkan ip, Kanser, Ekstraseller Vezikller (EV), Plazmonik Metayzey, EV İzolasyonu



To my dear family...

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CHAPTER 1

1. INTRODUCTION

1.1 Cancer Diagnosis

In 2022, 9.7 million people died and 19.3 million people suffered from one of the distinguished 36 different types of cancer, which makes it the second leading source of death [1]. There is a variety of methods to diagnose cancer, such as physical examination, biomedical imaging methods (positron emission tomography (PET), computerized tomography (CT), magnetic resonance imaging (MRI), X-ray scans, etc.), and tissue biopsy each of which has unique advantages and limitations [2]. Physical examination is cost-effective and rapid, but has less sensitivity. Imaging methods are generally precise and highly sensitive. PET can provide 2D or 3D images of tumors, MRI has unique detailed imaging properties for soft tissue, and CT may offer spatial imaging; however, they have several disadvantages. They may require pre-treatment, such as high-contrast medicals. They are expensive techniques and require medical experts. Imaging a tissue requires prolonged analysis time and results in significant radiation exposure. Tissue biopsy, also called a surgical biopsy, is considered the gold standard cancer diagnosis method by providing tumor diagnosis by differentiating its subtypes; however, it is hard to collect tissue from patients [3]. Conventional solid tissue biopsy is mostly invasive methods since it is a surgical process, and not feasible to monitor disease progression. Moreover, unfortunately, tumors can grow significantly without causing symptoms, and it can be hard to find the diseased area at early stages.

Despite the latest developments in improved diagnostic techniques, a significant proportion of malignancies continue to be identified at an advanced stage, leading to a poor prognosis. Early detection of cancer prior to the development of symptoms is crucial to improve patient survival rates [4]. From this

perspective, liquid biopsy represents a potential concept for early detection of cancer that offers minimally or non-invasive analysis from biological fluids. In cancer diagnosis, resources define liquid biopsy as the detailed analysis of biological fluids such as blood, urine, cerebrospinal fluid, and saliva for the search of tumor-originated free cells, DNA, or other biomarkers [5], [6]. Liquid biopsy gained significant attention over the last decade with its superior features over conventional diagnostic techniques. First and foremost, since the analytes are fluids and not tissue, liquid biopsy is not as invasive as the conventional methods and causes less pain and anxiety for patients. Compared with tissue biopsy, liquid biopsy is rapid, cost effective, and requires less organizational needs [7] and can be obtained from fresh samples that are not contaminated by additional preservative chemicals utilized in tissue analysis in different steps such as freezing and fixation [6].

There are varieties of cellular and molecular indicators that can be targeted in liquid biopsy. In 1869, Thomas Ashworth reported tumor-like cells in the bloodstream, which are today named circulating tumor cells (CTCs), and this discovery paved the way for further research on unbound biomarkers navigating in the human body [3], [8]. Later, free nucleic acid molecules in plasma [9], circulating tumor DNAs [10] and EVs [11] were also discovered. If they can be detected at an earlier stage before the tumor metastasizes to different locations in the body, the solid tumor can be removed surgically. A recent paper demonstrated that a blood test may identify and locate eight different cancer tumors by evaluating the quantities of circulating proteins and mutations in ctDNA [12]. In another study, Buscail et al. showed that CTCs and EVs detached from non-metastatic pancreas tumors can be detected via liquid biopsy and tumor can be resected [13].

Even the liquid biopsy concept is quite promising considering the aforementioned advantages, it is not a standard method for diagnosis but an additional confirmation method after several imaging and biopsies. The technique suffers from several disadvantages, including the small concentration of targets in the vast amount of fluid that hinders their detection and their heterogeneous structure. Further clinical assessments are required to validate its usability. The scientific communities, notably the European Liquid Biopsy Society, are now focusing on demonstrating the effectiveness of these biomarkers to enable broader

clinical applications [14]. To encourage liquid biopsy utilization, more clinical studies and an algorithm are needed.

1.2 Extracellular Vesicles (EVs) in Cancer

EVs are lipid-bilayer enclosed cargo particles that may vary in nano-to-micron size, and they are not be able to replicate [15]. EVs were first thought to be the waste product bags released from the cells [16]; but, then, it was discovered that they may contain functional molecules that play roles in homeostasis and cell-to-cell communication, like speech bubbles. EVs transport a diverse spectrum of biological molecules [17]. Nucleic acids, specifically micro RNA (miRNA), messenger RNA (mRNA) and DNA, are the most studied cargo types in EVs [18]. EVs also include metabolites, lipids, and proteins such as transcription factors, enzymes, and surface proteins that may regulate cellular signaling. The myriad of cells, both eucaryotic and procaryotic, can secrete EVs such as bacteria, yeast, fungi and mammalian cells including stem cells, tumor-associated fibroblasts, immune cells and endothelial cells. Moreover, EVs can be isolated from a variety of resources, including blood, urine, saliva, cerebrospinal fluid, milk, and cell culture media [19].

Since EVs are quite heterogeneous in terms of their function, size, and biogenesis, they have several subtypes [20]. In the latest classification declared by the International Society for Extracellular Vesicles (ISEV) considering the biogenesis of the EVs, there are two main subtypes of EVs: (i) exosomes and (ii) ectosomes (Figure 1.1.). Exosomes are small vesicles varying in size between 50-150 nm. They are derived from inward budding, also called endosomal processes, released via the exocytosis of multivesicular bodies (MVBs) and amphisomes, which is described as a result of a combination of autophagosomes and MVBs [21]. They are highly rich in certain biomarkers, such as tetraspanin proteins (CD9 , CD63, CD81, etc.), biogenesis factors (ALIX, TSG101, etc.), intracellular trafficking proteins (Rab protein, etc.), and proteins that are specific to the cell type (MHC-1, FasL, etc.) [22]. Moreover, recent studies suggest that considering their nanoscale size and surface area to volume ratio, most of these markers are on the corona of the exosomes [18]. In contrast to the exosomes, ectosomes are larger with

size of 50-5000 nm and are produced through the outward budding and blebbing of the plasma membrane. Notably, certain ectosomes may also contain endosomal cargo components such as autophagosomes. Ectosomes may encompass small EVs, medium-sized microvesicles (MVs), and larger apoptotic bodies. MVs, also called shedding microvesicles (sMVs), vary in size from 50-1000 nm, and extracellular matrix components (integrin, etc.), cytoplasmic materials (Tau, GAPDH, etc.), and lipids can be found in MVs. As is the case in healthy cells, apoptotic cells can also secrete EVs, which are called apoptotic bodies. They are larger than the aforementioned exosomes and MVs, with a size of up to 5 μm [23]. Furthermore, they contain significantly higher amounts of RNA compared to the MVs and exosomes [24]. Even though the notion “EV” comprises each subtype, a variety of specialized terminology has been employed in literature to refer to EVs generated during particular cellular activities. EVs secreted during cell migration are named “migrasomes”, cancer cell-derived EVs are “oncosomes”, and “prostasomes” are specifically derived from the prostate [19].

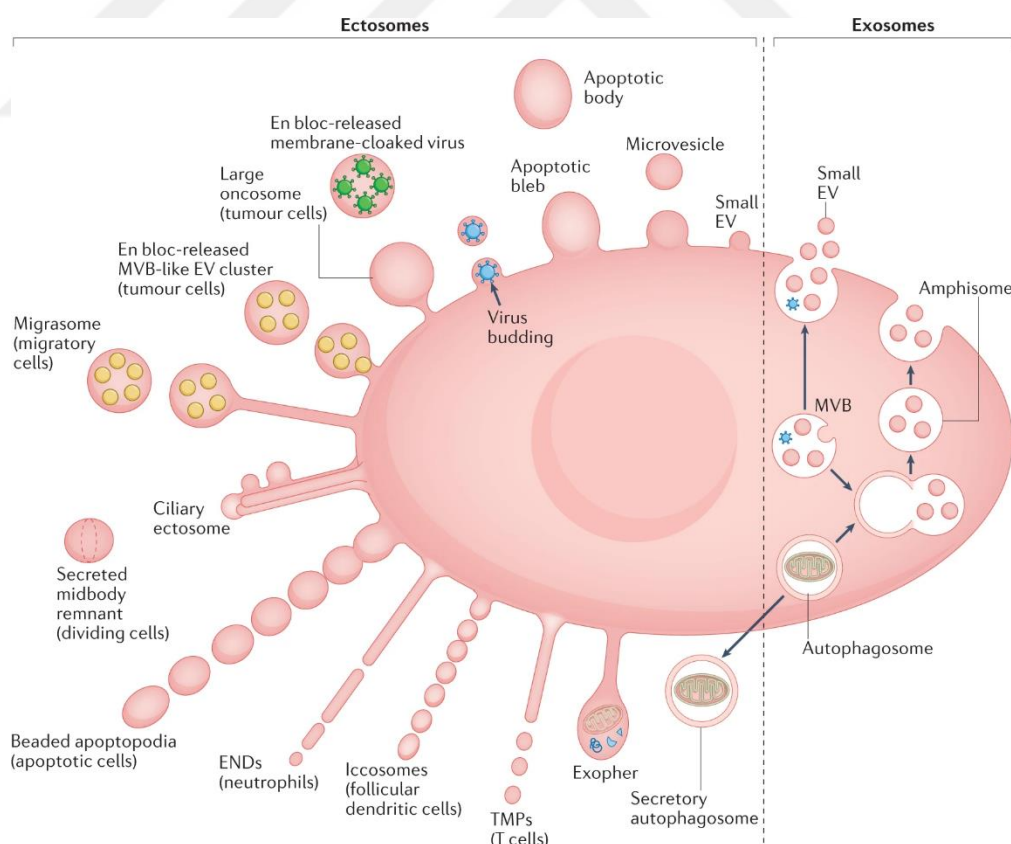


Figure 1.1 In this figure, different subtypes of EVs were illustrated. Reused from [21].

Scientists acknowledged that EVs released by cancer cells and tissues contribute to cancer progression and metastasis, considering their cargo and function in cellular communication. [25], [26]. EVs released from the tumor microenvironment (TME) can be attached to and uptaken by the healthy target cells through the direct fusion or binding process mediated by the membrane elements of EVs, such as glycans [27]. Upon internalization, EVs may trigger pivotal intracellular signaling pathways and affect the recipient cell's behavior and function with their cargo molecules (cancer-specific proteins, miRNA, etc.). Liu and coworkers demonstrated that the exosomal transfer of miR-93-5p enhances the growth of esophageal cancer cells through cellular interaction by targeting PTEN [28]. Moreover, EVs can create a susceptible and favorable environment in a secondary organ, which is called a pre-metastatic niche (PMN), for tumor metastasis [29]. For instance, Lin28B-overexpressing breast cancer cells facilitate suppression of the immune system in the lung pre-metastatic niche through exosomes containing low levels of let-7s and enhance cancer progression in the lung [30]. EVs have arisen as a promising strategy in cancer diagnosis in liquid biopsy owing to their aforementioned distinctive features and functionalities over other biomarkers. Compared with the CTCs and ctDNAs that occur in a very low frequency in biological samples, EVs can be obtained from multiple biological samples with high yield reflecting the origin of their released cells, and they can be secreted from various cell types, rendering them appropriate for the identification of multiple cancer categories. The lipid-bilayered and enclosed structure of EVs provides cargoes, identified as tumor indicators, to be protected from elimination and degradation [31]. Furthermore, EV detection strategies can be extended by combining with biosensor applications (optical or electrochemical sensors) or machine learning. Hinestrosa and their research team utilized data on varying EV protein levels to create a machine-learning system that identifies even a limited number of EV biomarkers, which enables the diagnosis of early-stage pancreatic, ovarian, and bladder malignancies [4]. Wei et. al. employed fluorescent aptamers for the efficient isolation and characterization of oncologic EVs from clinical samples with 91% of accuracy [32]. Even though EVs have superior properties as a diagnostic tool, their isolation and characterization methods need to be selected carefully depending on the application for a precise determination.

1.3 Extracellular Vesicles Isolation and Characterization

Regarding the highly heterogeneous subtypes of EVs, different separation approaches are distinguished by considering factors like density, size, and surface markers (Figure 1.2.) [33]. The traditional differential ultracentrifugation (dUC) method, noted as the gold standard for EV isolation, involves applying increasing centrifugal force and periods to specimens and separating particles according to size and density [34]. In theory, the expectation is to pellet larger/denser particles first and smaller/less dense particles in the following rounds. Despite dUC being acknowledged as the gold standard method due to its capacity to produce relatively pure populations with high sample volume, achieving complete isolation of EVs in reality remains challenging. Particles with similar density, such as high and low-density lipoproteins, may co-precipitate and mix with the EV solution. Additionally, small EV isolation is problematic in fluids that are rich in terms of proteins, such as blood or cell media [15]. Although elevated g-force does not facilitate the precipitation of small EVs, it may trigger their aggregation. Another limitation of dUC is the high cost of setup [35].

Similar to dUC, density gradient isolation (DG) provides to separate EVs according to their density, but in DG, a preconstructed media, such as sucrose, iodixanol, or iohexol, with a certain density is used [36]. When the EV-containing liquid is placed at the top or the bottom of the centrifuge tube with a high or low-density medium, the particles inside the sample traverse the gradient subsequent to the application of centrifugal force. Particles that generate fractions in the medium can be collected post-centrifugation; however, the gradients need to stay unaffected. Prior to the use of the EVs, the density medium needs to be eliminated from the environment by different methods. Although this method is suitable for differentiating EV subtypes from proteins, removing density media is problematic and the system has low recovery [37].

Size-exclusion chromatography (SEC) is an alternative method that sorts EVs depending on their size. There are two essential elements in SEC: (i) the stationary porous gel membrane located in the separation column and (ii) the biological sample with EVs. Once the biological specimen is applied, larger

particles, transported by gravity or a pressure pump, will pass the matrix rapidly without penetrating the pores and will be collected at the first fraction. Conversely, smaller particles compared to the pore size of the matrix, remain attached for an extended duration and mostly elute in subsequent fractions [38]. Different stationary phases can be used such as Sephadex, agarose or allyldextran [39] and the proper selection of the stationary phase can provide pure isolation with a reduced amount of lipoproteins and soluble proteins [40], [41]. Although SEC is a more accessible method compared to dUC, one main drawback of SEC is that the final product is a diluted version of the initial product. Moreover, SEC requires a long separation time and is not suitable for a high volume of samples.

Alternatively, affinity-based approaches are available to select and capture EVs with a determined charge or biomarker. The immunoaffinity capture method incorporates the isolation of EVs from non-EV components according to the surface expression of proteins, which leads to a more selective isolation. Tetraspanin markers (CD9, CD63, or CD81) are the commonly used markers to separate EVs [42] but other than tetraspanins, specific EV surface proteins also can be targeted [43], [44]. Affinity-based isolation results in highly pure EV isolation; however, it is challenging to separate the capture probe and EV. Another limitation is the system is generally not suitable for high sample volume [15].

Other than conventional isolation methods, recently conducted research studies presented alternative methods. One of them is the microfluidic-based EV separation, which can be incorporated with affinity-based or filtration methods [45], [46]. They are superior to the other methods when the sample volume is small. A notable microfluidic methodology employs a hybrid method that combines both dead-end and cross-flow operations on a single microfluidic chip [45]. This design integrates the benefits of both methods, potentially resulting in an elevated recovery rate and less pollution. As mentioned, each isolation method has advantages and limitations. In an EV-related study, the separation method needs to be selected depending on the biological source, sample volume, desired final concentration, and purity.

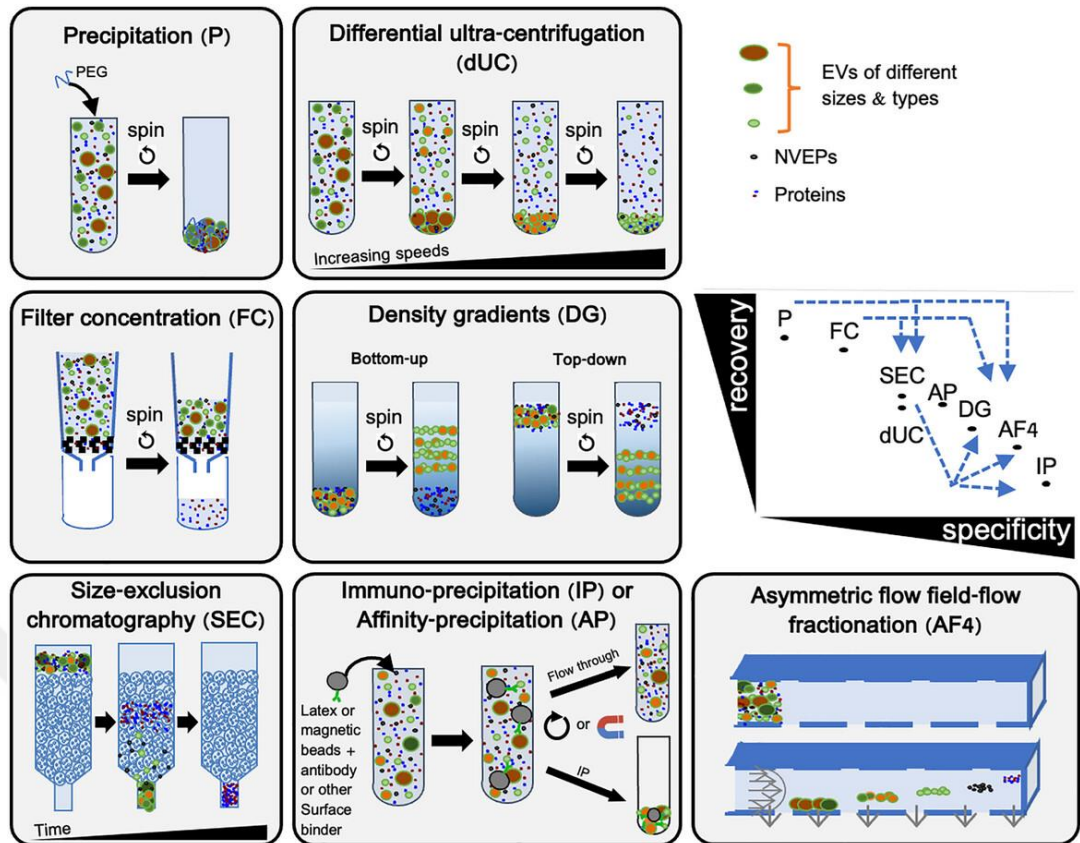


Figure 1.2. Different EV isolation methods were depicted. Reused with the permission of Creative Commons from ref. [15].

After the EVs were isolated, they need to be characterized depending on the application. One of the most standard methods for EV characterization is the nanoparticle tracking analysis (NTA). Similar to dynamic light scattering (DLS), NTA provides to achieve the size and concentration properties of the particles. It is an image-based method that comprises a laser module, a microscope, a sensitive charge-coupled device (CCD) camera, and a fluidic chamber [47]. The scattered signals from discrete particles inside the area of vision were monitored and captured on video. Another optical approach for multi-parametric screening and quantification of EVs is flow cytometry. A significant benefit of flow cytometry is its capacity to assess multiple tags on single particles and to distinguish different types and subsets. Despite the broad applicability of flow cytometry, a few limitations hinder its utilization, including the small size of EVs, which leads to a limited sensitivity in distinguishing them from background signals [48]. Thus, certain tagging components attached to the EVs for their identification may be needed for an accurate operation, such as fluorescent beads. Scanning electron

microscope (SEM) and transmission electron microscope (TEM) are the imaging methods to observe the morphological integrity of the EVs. Furthermore, the surface protein biomarkers and protein content of the EVs can be evaluated with diverse molecular biology techniques such as Western blot, bicinchoninic acid assay (BCA) analysis or, enzyme-linked immunosorbent assay (ELISA).

1.4 Optical Methods for EV Detection

Optical biosensor devices were proposed as an alternative for detecting cancer cell-derived EVs in the search for novel diagnostic instruments in cancer research (Figure 1.3.) [49]. Optical biosensor-based diagnostics, including plasmonic techniques such as surface plasmon resonance (SPR) and surface-enhanced Raman spectroscopy (SERS), colorimetric, and fluorescence techniques, demonstrate advantages over traditional methods, such as rapid assay time, easy assembly, instantaneous data collection, portability, and label-free detection [50], [51]. Furthermore, optic sensors exhibit enhanced sensitivity and specificity towards their targets via the modification of antibodies and binders [52]. Thus, these biosensors hold considerable potential in cancer diagnosis, presenting possible applications across many cancer types.

Plasmonic-based approaches are one of the most used optical sensors for biosensing applications. Briefly, plasmonic measurements investigate the physical interaction between light and metals, such as silver and gold, or nanoscale metallic structures, such as gold nanoholes or nanoarrays. In SPR, when polarized light is introduced to a plasmonic substance at a certain angle, surface electrons of the material oscillate coherently at the interface of two distinct phases [53], [54]. This phenomenon facilitates the observation of surface plasmons (SPs) and surface plasmon polaritons (SPPs) traveling along the surface, which are crucial for SPR and localized SPR (LSPR) applications and highly sensitive to refractive index alterations. When the analyte and immobilized capturing probe (antibody, aptamer, MIP, etc.) interact, the refractive index of the liquid phase changes, and it can be detected via SPR [49]. If the surface is designed with sub-wavelength nanostructures, the electron confinement is localized around these structures instead of propagating along the surface, and this is called LSPR. Briefly, SPR has a high

sensitivity with real-time detection without the need for a label, such as fluorescent probes. However, the setup is typically complex and necessitates trained technicians for sophisticated calibrations, making it impractical for point-of-care detection. Plasmonic sensing technologies can be improved by combining with additional techniques, such as fiber optics. Fiber optic SPR (FO-SPR) utilizes the total internal reflection (TIR) principle in the fiber and senses the environmental changes [55]. Researchers used portable FO-SPR to detect breast cancer-derived EVs with increased sensitivity [56]. SERS is another plasmonic technique for EV detection. Raman scattering depends on the energy gain (anti-Stokes) or loss (Stokes) of inelastically scattered photons resulting from molecular vibrational events. The SERS approach offers opportunities for on-site and real-time detection. However, conventional Raman spectroscopy is hindered by poor sensitivity due to low Raman scattering [57]. The SERS method, an improved form of Raman scattering in which plasmonic nanostructures were applied on the surface, may achieve an amplification factor of up to one million and significantly improve the sensitivity. As a result, the detection threshold can be reduced to the molecular level [58]. The SERS technique is now a widely utilized optical biosensor for the detection of biomolecules [59] and EVs [60]–[62] in cancer diagnosis.

Fluorescence and luminescence-based optical detection technologies constitute a further category of biosensing techniques. Photodiodes or photomultipliers can be used to collect emitted light from luminescent or fluorescent probes, allowing the detection of the spatial distribution and binding kinetics of the analyte being investigated. Recently, fluorescence-based instrumentations have achieved remarkable improvements since they facilitate sensitive and selective detection of microorganisms, as well as therapeutic and environmental chemicals [63]. A novel and sophisticated fluorometer can detect at the single photon level, and with advanced super-resolution technology, a fluorescence microscope can distinguish between two particles separated by less than 10 nanometers, which enables the detection of small concentrations of biotargets [64]. Förster Resonance Energy Transfer (FRET) is a widely utilized fluorescence sensing method that works to address the challenge of disorganized emission signals from complex biological matrixes. The essential components of a FRET system are the donor and the acceptor, in which the emission band of the former aligns with the absorption band of the latter when they are in close spatial

proximity [65]. Notably, FRET-used EV detection methods are available in the literature [66], [67]. Fluorescence-based diagnostic techniques are superior with their high sensitivity, allowing the detection and image of even a single molecule. Some of them, such as FRET, are also suitable for real-time analysis; however, the label requirement and photobleaching are certain drawbacks. Moreover, background noise is another issue that affects the sensitivity of the platform.

Colorimetric optical biosensors are a more user-friendly and achievable alternative for EV detection. They can utilize chemo-responsive compounds that exhibit a color shift upon interaction with the substrate molecule. The detection techniques of colorimetric biosensors may also employ nanoparticle aggregation [68], [69] and color alteration induced by catalytic enzymes [70]. Their features make them ideal choices for on-site testing systems, providing visually accessible, simple, and user-friendly operation in paper-based analytical devices (PADs) and lateral flow assays (LFAs) [71]. A research group developed an aptamer-based sensor that is modified with HRP-Streptavidin for the detection of cancer-derived exosomes. The system catalyzes dopamine to polydopamine, resulting in a brown coloration when hydrogen peroxide is available [72]. Although the colorimetric systems are feasible for POCT since they are cost-effective, user-friendly, rapid, versatile, and do not require any complex setup and training, they are limited in terms of sensitivity compared to other techniques. Their dynamic range is restricted, and further complementary techniques may be required to analyze a broad range of concentrations. Notably, they are relatively subjective when they are not assisted with a quantitative method, such as machine learning.

To sum up, choosing the optimal optical technique for EV detection in cancer diagnosis requires a thorough evaluation of the unique requirements and context of the assignment, which includes required sensitivity, specificity, cost, and practical factors. Every optical approach possesses distinct advantages and drawbacks, and their feasibility may differ based on the application.

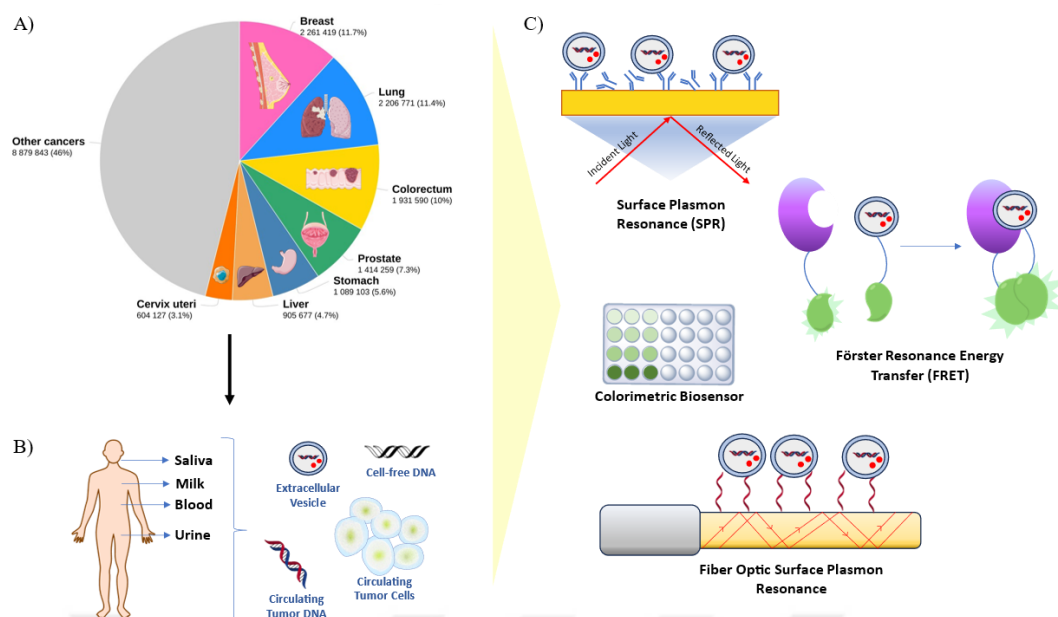


Figure 1.3. In this figure a) a pie chart that represents the anticipated global distribution of cancer patients across all genders and age groups, b) biological fluids employed in liquid biopsy and the cancer biomarkers contained with them and c) A schematic of optical biosensors, comprising SPR, FRET, FO-SPR, and colorimetric analysis for EV detection are illustrated. Reused from ref. [49] with the permission of Creative Commons Attribution (CC BY) license, 2024.

1.5 Thermoresponsive Smart Polymeric Biomaterials

Smart materials (SMs), also named advanced or responsive materials, refer to materials that can take environmental stimuli such as physical (light, temperature, pressure), biological (glucose, enzyme), or chemical (pH, ion, etc.) and adapt to that certain environment by creating a response [73]. This response of the material could vary, such as alterations of the morphology (shape-memory alloys) [74], color (photochromic films) [75], or physical properties (hydrophilic to hydrophobic transition) [76]. SMs typically turn to their initial state when the stimuli are removed, which makes them reversible. Tissue engineering, drug delivery systems, biosensor applications, robotic organs and artificial muscle structures are some of the examples of diverse SM utilization examples in medical applications [77], [78]. Moreover, SM applications can be supported with artificial intelligence (AI) and

AI-driven machine learning algorithms to clarify the action of these materials and their interactions with the environment [73], [79].

In the field of biomaterials, nanoparticles, micron-sized particles, hydrogels, and polymers are all frequently used tools and their integration with smart materials has recently garnered attention in localized and targeted drug delivery, biological substance detection and isolation (such as cells and EVs), and tissue engineering [80]. As previously stated, several types of stimuli exist, including temperature and among these, temperature is the most efficient response creator for biomedical approaches since the human body is set to 35-37° [81], [82]. In specific, polymeric thermoresponsive materials, which can be derived naturally or synthetically, are advantageous considering their biocompatibility and versatility. SMs that are responsive to temperature typically have a lower critical solution temperature (LCST) or an upper critical solution temperature (UCST) that indicates the phase separation points [83]. LCST subtypes are soluble in aqueous solutions at temperatures below the LCST of the polymer, and above the LCST, they become hydrophobic owing to increased molecular interactions. The reversible solubility alteration is extensively used in literature as on-off switches, cell sheet applications, and sensors. On the contrary, UCST subtypes are soluble at temperatures above the UCST and become insoluble at temperatures below the UCST. This phenomenon arises from certain interactions, including hydrogen bonding and electrostatic interactions. Additionally, dual-temperature-responsive characteristics may be engineered by the combination of segments of different LCST and UCST types. Poly(N-isopropylacrylamide) (PNIPAM) is one of the first reported and remains a widely utilized thermoresponsive polymer in biotechnology with its LCST around 33°C, which is close to the temperature of the human body and relatively easier to adjust [81]. Furthermore, PNIPAM shows low susceptibility of its phase transition to surrounding circumstances [84]. Consequently, little fluctuations in pH, concentration, or the chemical ambient result in minimal alterations to the transition temperature. Different polymerization techniques can be utilized to synthesize PNIPAM, including electron beam (EB)-induced polymerization [85], atomic transfer radical polymerization (ATRP) [86], or reversible addition-fragmentation chain transfer (RAFT) polymerization [87]. It is also suitable for copolymerization along with the different types of monomers to increase or decrease the LCST or provide dual-responsive properties for different stimuli, such as pH [88]. As stated

in Figure 1.4, they can be used in different areas, and biological substance separation and isolation is one of them. In 1996, Kanazawa and coworkers used the first PNIPAM-coated silica particle-modified high-performance liquid chromatography (HPLC) to achieve a temperature-sensitive chromatographic separation [89]. Since this system does not require harsh chemicals to separate the biomolecules (proteins, cells, etc.) and the temperatures are close to the body temperature, the biomolecules can be isolated without any biologically functional deterioration [90], [91]. Moreover, PNIPAM can be used at the bottom of the cell culture flasks for cell detachment. Canavan and their research group showed that conventional cell detachment methods typically involve an enzymatic activator (such as trypsin) or a mechanical force which can damage the surface proteins and extracellular matrix (ECM) of the cells and thermal detachment is preferable to preserve the cell environment [92]. The natural features of the thermoresponsive modified cell culture plate facilitate the creation of a "cell sheet", which is a monolayer of tissue from cells and a pivotal technique for tissue engineering. Despite the development of several cellular artificial tissue types, the cell sheet technique has remained as the most feasible among them due to its numerous benefits over alternative cellular tissues [93]. Firstly, it is possible to protect ECM since it functions as a cellular adhesive, allowing for the straightforward transplantation of cell sheets by basically positioning them at the affected spot without the need for sutures or further fixation techniques. Furthermore, the transplanted cell sheets are maintained at the site of transplantation, whereas cells introduced via cell suspension injection do not stay at that location. Polymeric thermoresponsive materials can also be used together with the nanoparticles. In literature, PNIPAM-coated magnetic nanoparticles were reported to separate DNA from complex biological mixtures [94]. Similarly, along with the silica beads, they are used for the charge-specific cell separation [95]. PNIPAM is also reported to be used for EV isolation in literature [96].

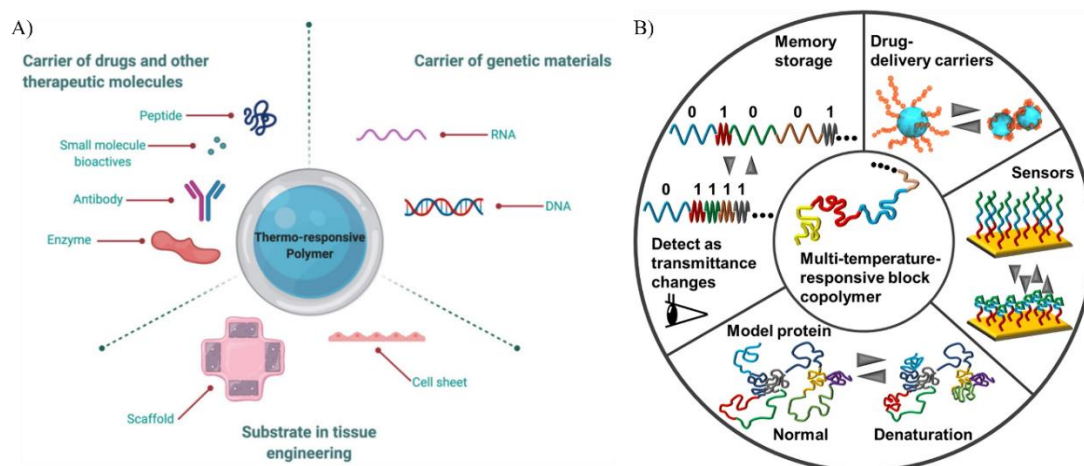


Figure 1.4 a) Illustration of thermoresponsive biopolymers as drug and therapeutic molecule carriers, genetic material carriers and tissue engineering tools and b) multi-temperature responsive polymers for biosensors, modeling, drug delivery, and memory storage agents were represented. Reused from ref [82] and [83] with permission from Springer Nature.

1.6 EV Capture, Detection, and Isolation via Plasmonic Measurements

EV, liquid biopsy, optic biosensor, and SPR notions were described and detailed in the previous sections. Cancer cell-derived EVs are suitable biological components to diagnose and understand cancer since they carry the cargo of the originated cells and represent the initiated cells. Plasmonic systems dominate the optical approaches for detecting EVs, with SPR being the preferred technique for the detection and separation of EVs in cancer diagnostics. It exhibits high sensitivity to specific target biomolecule binding events (such as proteins on the EV surface) within the wavelength range of approximately 200-1000 nm from the sensing surface, correlating closely with the dimensions of especially small EVs [97]. A planar metallic thin surface is utilized in conventional SPR, and it is highly sensitive to bulk refractive index alterations. Moreover, it can be combined with different sensing mechanisms such as angle or phase modulation [98] or SPR imaging technique (SPRi) as a refractive index visualization method [99]. Different SPR detection methods for various EV source are summarized in Table 1.

Table 1. Cancer cell-derived EV detection with surface plasmon resonance techniques.

Detection Method	Target Molecule	EV Source	Limit of Detection (LOD)	Ref.
AuNP enhanced SPR aptasensor	Oligonucleotide (CD63 Aptamers)	MCF-7 and MCF-10A breast cell lines	5.0×10^3 EVs/mL	[100]
	Human epidermal growth factor receptor 2 (HER2)	BT474 breast cancer cell line	8280 EVs/ μ L	[101]
SWCNTs integrated SPR	Programmed cell death-ligand 1 (PD-L1)	MDA-MB-231 breast cancer cells	31.9 particles/mL	[102]
Multifunctional (size, density, affinity) SPR	CD63	A549 lung cancer cell lines	-	[103]
SPRi	CD9, CD171, CD81 and GM1	Human Plasma	1 μ g/mL	[104]
SPR	Tetraspanin proteins (CD9, CD63, and CD81)	Human Lung Cancer Cells	$(0.6-1.8) \times 10^4$ particles/mL	[105]
SPR	CD81	Mouse	5.9×10^6	[106]
		Monocyte Cells	particles/mL	

Compared to the conventional techniques that require complex labeling processes (e.g. ELISA and Western Blot), SPR demonstrates considerable potential for label-free, rapid and real-time detection of EVs. The surface of the SPR system can be designed with various capture probes, including antibodies, aptamers, or MIPs and they can be used to target EV-specific markers such as tetraspanin proteins [107] or cancer-specific markers on EVs such as PD-L1 [102] and EGFR [108].

1.7. Aim of the Study

Despite the existence of several EV isolation methods documented in the literature, researchers were unable to identify a definitive technique that yields high purity from complicated biological samples in a practical configuration. Considering these, an EV isolation method that can specifically differentiate EVs in complex matrixes without harnessing the surface biomarkers is pivotal.

This study aimed to detect and isolate MCF-7 breast cancer cell-derived EVs from rich and complex cell medium by utilizing thermally responsive PNIPAM polymer. EVs were captured with tetraspanin protein-specific antibodies on a PNIPAM-coated plasmonic metasurface at room temperature and then released from the surface by increasing the local temperature. When the temperature increased, the PNIPAM switched from hydrophilic to hydrophobic, and with the effect of the flow in the microfluidic chip, EVs were released from the surface. The capture and release phenomena were observed in real-time with metasurface plasmonic measurements. Currently available EV isolation methods have certain advantages and disadvantages. In this study, a cost-effective and portable system was presented, which makes it suitable for on-site POC diagnosis and isolation.

CHAPTER 2

2. MATERIALS AND METHOD

2.1 Materials

Methanol ($\geq 99.9\%$), absolute ethanol ($\geq 99.5\%$), acetone ($\geq 99.5\%$), isopropanol, N-Isopropylacrylamide (NIPAM), 2-Bromoisobutanoic acid N-hydroxysuccinimide ester (NHS-BiB), copper(I) bromide (CuBr) and poly-L-lysine (PLL) were obtained from Sigma Aldrich (USA). Phosphate Buffered saline (PBS) tablets were purchased from Biomatik (Canada), and Tris 2-(dimethylamino)ethyl amine (Me6TREN) was purchased from Tokyo Chemical Industry (TCI, Japan). For metasurface fabrication, DVD-R discs from HP (USA) were used as the naturally grated structure. Metals (Au (999.9) and Ag (999.0)) were purchased from the Istanbul Gold Refinery (Turkey). For MCF-7 cell culture, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) with L-glutamine, sodium bicarbonate and HEPES, Fetal Bovine Serum (FBS), Trypsin-EDTA (0.25%) and 10,000 U/mL Penicillin- 10,000 $\mu\text{g/mL}$ streptomycin were purchased from Thermo Ficher Scientific, Gibco (USA). For microfluidic chip preparation, poly (methyl methacrylate) (PMMA) was taken from Sumitomo Chemical (Japan), and double-sided adhesive (DSA) was obtained from 3M (USA) and processed with a laser cutter. PTFE capillary tubings were obtained from Adtech (UK).

For the ATRP polymerization of the PNIPAM, we collaborated with the Olefin Metathesis Laboratory at Hacettepe University, Department of Chemistry, and their infrastructure was utilized. National Nanotechnology Research Center (UNAM) facility infrastructure was used for fabrication and characterization devices including thermal evaporator (MiDAS PVD 3T, VAKSIS, Turkey), X-ray photoelectron spectroscopy (XPS, K-Alpha, Thermo Scientific, USA), environmental scanning electron microscope (ESEM, Quanta 200 FEG, FEI, USA), contact angle instrument (OCA 30, DataPhysics Instruments, Germany),

nanoparticle tracking analysis (NTA, NS300, Malvern Instruments Ltd., UK) and laser cutter system (LazerFix, Turkey).

2.2 Metasurface Fabrication

Off-the-shelf optical discs (DVDs) were utilized for metasurface fabrication, as previously reported in the literature [110], [111]. Since the DVD surface has a grating pattern that is suitable for plasmonic detection, an additional method (such as electron beam lithography) is not required to fabricate micro/nanostructures. Additionally, DVDs are easily accessible materials with low cost. From top to bottom, there are several layers of DVDs, including (i) a protective top layer, (ii) a metal coating layer, (iii) a photoresist layer, (iv) surface grating and (v) a polycarbonate substrate. To fabricate the metasurface, first, the protective substrate was detached carefully with a knife. The resulting DVD was dipped in the ethanol and methanol mixture prepared as 1:1 (v:v) at room temperature. A chemical surface etching was then applied to adjust gratings with an acetone and isopropanol mixture with a 1:4 (v:v) ratio at room temperature for 60 seconds. The surface was rinsed with distilled water and dried with an air gun. To provide plasmonic properties, initially, the surface was coated with titanium (5 nm) that serves as an adhesion layer between the plastic substrate and the silver/gold layers. It ensures better mechanical stability and durability of the following metal coatings. Then, the surface was coated with silver (30 nm) and gold (15 nm) using a thermal evaporator under a vacuum environment. Herein, silver provides the main plasmonic property, and gold protects the silver layer from oxidation, increasing the storage shelf life of the metasurface. After the surface was prepared, it was cut with a scissor into 15 mm x 15 mm pieces to assemble with a microfluidic chip.

2.3 Microfluidic Metasurface Sensor Preparation

Poly (methyl methacrylate) (PMMA) and double-sided adhesive (DSA) layers were designed in RDWorks software and cut with a laser cutter (Laserfix, Turkey). They are used for microfluidic chip fabrication as an affordable and easy alternative compared to lithographic fabrications. The PMMA has a 2 μm thickness,

and it creates the upper layer of microfluidic with the inlets and outlets, as shown in Figure 2.1a. To cut it with the laser cutter, 40% of power and 40 m/s of speed was applied. The DSA is the middle layer with a 50 μm of thickness, and the channel structure is designed on it as in Figure 2.1b. DSA is also an adhesive layer between PMMA and plasmonic metasurface. In the laser cutter, 15% of power and 100 m/s of speed were utilized. Each layer was cut and prepared (Figure 2.1c) and prior to assembly, the PMMA layer was cleaned with 70% of ethanol and dried with an air gun. From bottom to top, metasurface, DSA and PMMA layers were integrated. Capillary tubings (length: 15-20 cm) were placed in the inlets and outlets and stabilized with epoxy and the microfluidic chip was completed. The flow in the channel was provided with syringe pumps (New Era Pump Systems Inc., USA). Details about the syringe pump system in plasmonic metasurface measurements were given in section 2.8.

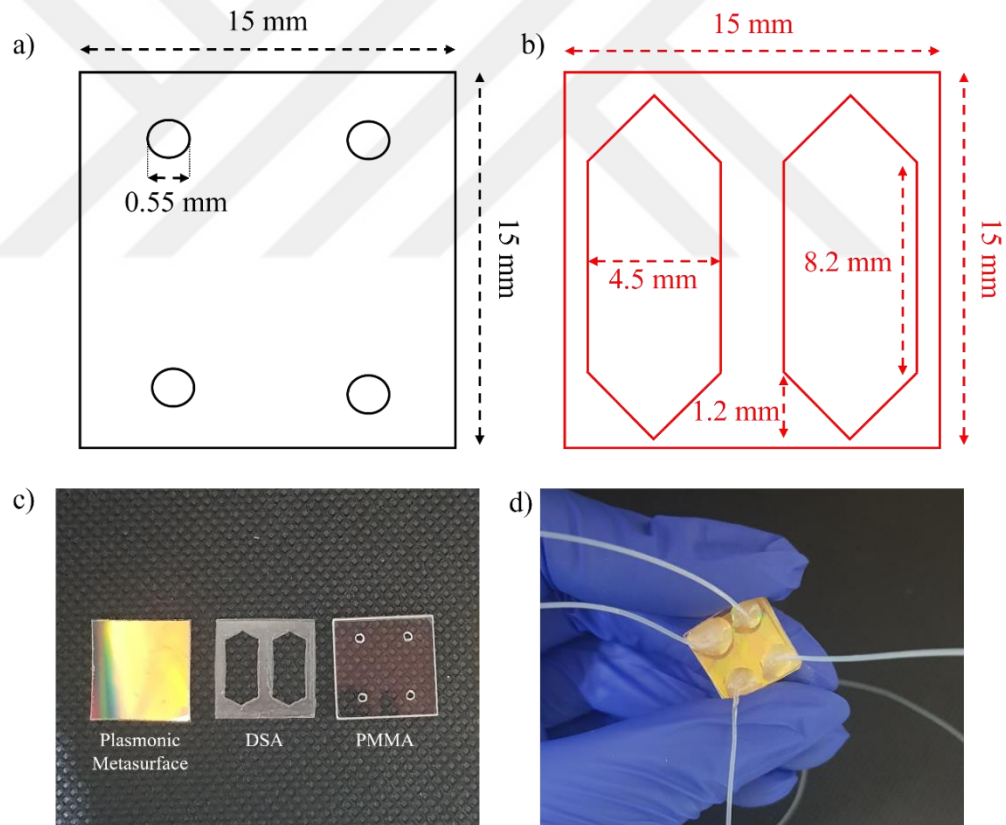


Figure 2.1 Metasurface integrated microfluidic chip preparation was shown. Illustration of the a) PMMA layer and dimensions, b) DSA layer and dimensions, c) plasmonic metasurface, DSA and PMMA layers separately and d) assembled microfluidic chip with capillary tubings were demonstrated.

2.4 PNIPAM Synthesis with ATRP

The synthesis was held at Hacettepe University, Department of Chemistry, Olefin Metathesis Laboratory. Before utilizing NIPAM monomer for ATRP, it was recrystallized in n-hexane and subsequently dried. Briefly, NIPAM (monomer), CuBr (catalyst), Me6TREN (initiator), and NHS-BiB (NHS ATRP initiator) were employed in a molar ratio of 100:1:1:1 for the ATRP polymerization. First, NIPAM (0.6 g) was dissolved in deionized water (10 mL) in a round-bottom Schlenk flask (100 mL) at 250 rpm for 10 minutes at 4°C. In a separate Schlenk flask (25 mL), Me6TREN (12 mg) and DI water (4 mL) were introduced. Both flasks were degassed under a nitrogen atmosphere while stirring at 250 rpm for 10 minutes at 4°C to completely cover the flasks with nitrogen. After that, CuBr (7.63 mg), NHS-BiB (14 mg), and Me6TREN were incorporated into the NIPAM flask, respectively, and the mixture was stirred during nitrogen degassing at 4°C for 30 minutes. The degassing process ended, and the reaction was allowed to stir at room temperature for a duration of two hours. When the reaction ended, the round flask was heated with a hot air gun, and hydrophobic PNIPAM aggregates were separated in a clean petri dish. Subsequently, the PNIPAM was dried for 24h at room temperature and preserved in a dark dry environment for further experiments.

2.5 Surface Modification and Characterization

To modify the metal-coated polycarbonate plasmonic metasurface with thermoresponsive PNIPAM and to use for EV capture and release, (i) poly-L-lysine (PLL), (ii) NHS-terminated PNIPAM, (iii) anti-CD-63 antibody, and (iv) bovine serum albumin (BSA) were applied to the surface respectively. Prior to use, the metasurface was cut with scissors as 15 mm x 15 mm and sterilized with 70% of ethanol. It was dipped in 0.1 mg/mL PLL prepared in PBS and incubated overnight at 4°C [111]. After incubation, the surface was washed with PBS three times and dried gently with an air gun. Subsequently, the modified surface was dipped in different concentrations of NHS- terminated PNIPAM (10 mg/mL, 5 mg/mL, and 2.5 mg/mL) prepared in distilled water and incubated overnight at 4°C. The surface was then rinsed with PBS and dried. The PNIPAM-modified surface was assembled

with a microfluidic chip, and 50 $\mu\text{g/mL}$ anti-CD-63 antibody (Santa Cruz Biotechnology, USA), which targets CD63 tetraspanin protein located on the EV surface, was introduced directly on the microfluidic channels (5 μL for each channel). It was incubated for 3 hours at 4°C and then rinsed with PBS three times to remove the excess amount of antibody that was not bound to the surface. 5 mg/mL BSA prepared in PBS was applied directly to the channels and incubated for 20 minutes at 4°C to prevent non-specific bindings on the surface. After rinsing with PBS, the surface was attached to the microfluidic chip and utilized for plasmonic measurement analysis in real time for EV capture and release experiments. Prior to use, contact angle, XPS, SEM and laser scanning microscope analyses were made to characterize the surface.

2.5.1 Contact Angle

Surface wettability plays an essential part in numerous processes in biology and chemistry and contact angle measurement is the standard methodology for its analysis [112]. The contact angle notion is defined as the angle at the interface where water, air, and solid connect. The contact angle value serves as an indicator of the tendency of the surface to be wetted by water. In other words, the contact angle analysis measures the hydrophilicity and hydrophobicity of the substrate. In this experiment, the hydrophilicity of the (i) bare plasmonic metasurface, (ii) PLL-modified surface, (iii) PLL and PNIPAM modified surface, and (iv) PLL, PNIPAM, and antibody-modified surfaces were analyzed via contact angle measurement. The sample that will be measured was placed on the sample tray on the device (Dataphysics, OCA 30, Filderstadt, Germany) and 5 μL ultra-pure water was dropped on the surface to use the sessile drop method. The image of the droplet was captured with the software immediately after it was dropped and the contact angle was measured. The measurement was repeated three times for each sample and the results were presented as mean $\pm$ standard deviation (SD).

2.5.2 X-Ray Photoelectron Spectroscopy (XPS)

XPS (K-Alpha, Thermo Scientific, USA), also called ESCA (Electron Spectroscopy for Chemical Analysis), is a surface characterization method that is

used in this study to validate surface modifications. It detects the elemental composition existing on material surfaces and characterizes their chemical states by sorting chemical bonds between them [113]. In XPS, typically the specimen is exposed to mild X-rays (energies below about 6 keV) under a vacuum environment, and the kinetic energy of the released electrons is examined. XPS approach is an efficient analytical and quantitative technique owing to its surface sensitivity and its capacity to disclose chemical state information from the sample's components. Varieties of materials, including metal, plastic, and fabric, can be analyzed, and almost every element can be quantified rather than helium (He) and hydrogen (H) [114]. In this study, first, the surfaces: (i) bare plasmonic metasurface, (ii) PLL-modified surface, (iii) PLL and PNIPAM modified surface, and (iv) PLL, PNIPAM, and antibody-modified surfaces were prepared as explained before. A complete XPS survey was performed, and separate carbon (C), nitrogen (N), and gold (Au) elemental spectrum peaks were analyzed. The resulting data was shifted according to the C1 peak and normalized using OriginPro.

2.5.3 Surface Morphology Analysis (Laser Scanning Microscope and Scanning Electron Microscope (SEM))

The surface topology of the plasmonic metasurface was observed after modifications to investigate whether the periodic grating structure on the surface is affected. First, a scanning electron microscope (SEM, Quanta 200 FEG, Field Electron and Ion Company, FEI, USA) was utilized to image (i) bare plasmonic metasurface, (ii) PLL-modified surface, and (iii) PLL and PNIPAM-modified surfaces. SEM mainly uses concentrated high-energy electron beams propagating on the specimen surface to stimulate the inelastic scattering of secondary electrons (SE), facilitating the production of high-resolution pictures of micro and nanostructures. For SEM, specimens were prepared with 0.5 mm x 0.5 mm size and stabilized on SEM stubs with double-sided carbon tape and imaged under a high vacuum environment. The working distance of the specimen to the electron gun was arranged as 10 mm. Afterward, the surface PLL-PNIPAM modified surface

was imaged with a laser scanning microscope (VKX-100, Keyence, USA) to observe the PNIPAM polymer coating on the surface.

2.6 MCF-7 Cell Culture

Maintenance of the mammalian breast cancer MCF-7 cell line was conducted in the sterile laminar flow cabinet. Prior to use, the safety cabinet was sterilized with UV light for 15 minutes and cleaned with 70% of ethanol. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12 with L-glutamine, sodium bicarbonate, and HEPES) was supplemented with 10% of fetal bovine serum (FBS) and 1% of penicillin (100 U/mL), and streptomycin (100 µg/mL) for MCF-7 cells [115]. The completed cell medium was placed in a water bath at 37°C before utilization.

The cell culture equipment that will be used, including cell culture flasks (75 cm², T75), centrifuge tubes, cell medium bottles and serologic pipettes, were disinfected with 70% of ethanol and placed in the cabinet. MCF-7 cell containing vial, preserved with DMSO at -80°C, was heated in the water bath. Immediately after thawing, cells and 5 mL of cell media were added in a 15 mL centrifuge tube and spun at 1500 x g for 5 minutes. The supernatant with DMSO, which is toxic for cells, was discarded, and the MCF-7 cell pellet was resuspended in 5 mL of fresh media. In T75 cell culture flasks, 6 mL fresh media and 2 mL cell suspension were added, and the flask was placed in a humidified cell culture incubator at 37°C with 5% of CO₂. In the first culturing, the medium was renewed after the cells were attached to the surface (after 24 hours), then it was changed bi-daily until they reached 80-90% of confluency for passaging. They were imaged with an inverted light microscope (Zeiss Inverted Fluorescence Microscope) in each medium change to assess their morphology and confluency. Before cell passage, the trypsin-EDTA (0.25) solution was heated in the water bath at 37°C to activate the enzyme and detach the cells from the surface. The medium in the flask was removed and the flask was rinsed with 2 mL DPBS. Then, 2 mL warm trypsin-EDTA was transferred to the flask and incubated for 5 minutes at 37°C with 5% of CO₂. The cells were observed with an inverted microscope to confirm if they were detached from the surface. Subsequently, 4 mL of fresh medium was added to inactivate trypsin-EDTA in the flask and cell suspension was centrifuged at 1500 x g for 5 minutes to

eliminate the enzyme from cell medium completely. The supernatant was discarded, and the cell pellet was resuspended in 2 mL of fresh medium. In a 200 μ L PCR Eppendorf, 10 μ L trypan blue, that stain dead cells, and 10 μ L cell suspension was added. The total cells were counted with a hemocytometer, and 300.000 cells were transferred to a new T75 flask with 7-8 mL of fresh medium. The cell culture flask was maintained at the incubator after the cell passage was completed.

2.7 Extracellular Vesicle Preparation and Characterization

The EV isolation was performed using a microfluidic chip, as reported in the literature previously [45]. Prior to introducing EV-containing cell media to the microfluidic chip, several pre-processing steps were applied. MCF-7 breast cancer cells were seeded in a T75 cell culture flask and cultured for 4 days without changing their medium. On the 4th day, the cell medium in the flask that contains secreted proteins, EVs, dead cells, etc., was collected and divided into 2 mL Eppendorf tubes. First, it was centrifuged at 14000 x rpm (20000 x g) for 15 minutes at room temperature (25 °C) to remove large molecules, dead cells, and waste products. The supernatant was collected in a 20 mL syringe carefully without disturbing the pellet and filtered with 0.22 μ m of syringe filters. After this step, the MCF-7 breast cancer cell-derived EV-containing media became suitable for a microfluidic ultrafiltration chip.

2.7.1 Microfluidic Ultrafiltration Chip Preparation

The microfluidic filtration chip was designed as reported in the literature by using RDWorks software [45]. It consists of five PMMA and five DSA layers (20 x 40 mm), two polycarbonate filters (200 and 50 μ m pore size), and capillary tubings, as depicted in Figure 2.2. The first PMMA and DSA layers (numbers 1 and 2) create the inlet and outlet of the microfluidic chip. The following layers until polycarbonate filters (numbers 3-6) provide a reservoir to collect the EV-containing media coming from the inlet and flowing through the outlet. Between two DSA

layers (numbers 6 and 7), polycarbonate filters are used to achieve size-based isolation. First, the EVs are isolated at the inlet side with a 200-sized filter and then isolated second times at the outlet side with a 50-sized filter. Numbers 8 and 9 provided a channel structure that transferred the liquid from the inlet to the outlet, and the bottom layer with no design (number 10) provided a base for the microfluidic chip. The tubings were then placed in the inlet and the outlets and stabilized with epoxy. A syringe needle was inserted at the inlet. The flow in the channel was provided with syringe pumps (New Era Pump Systems Inc., USA) at 40 $\mu\text{L}/\text{min}$. The outlet tubing was stabilized in a clean tube, and the EV solution was collected in it. For storage, it was divided into 500 μL Eppendorf tubes and stored at -20°C for single use to avoid a freeze-thaw cycle that may denature proteins [15].

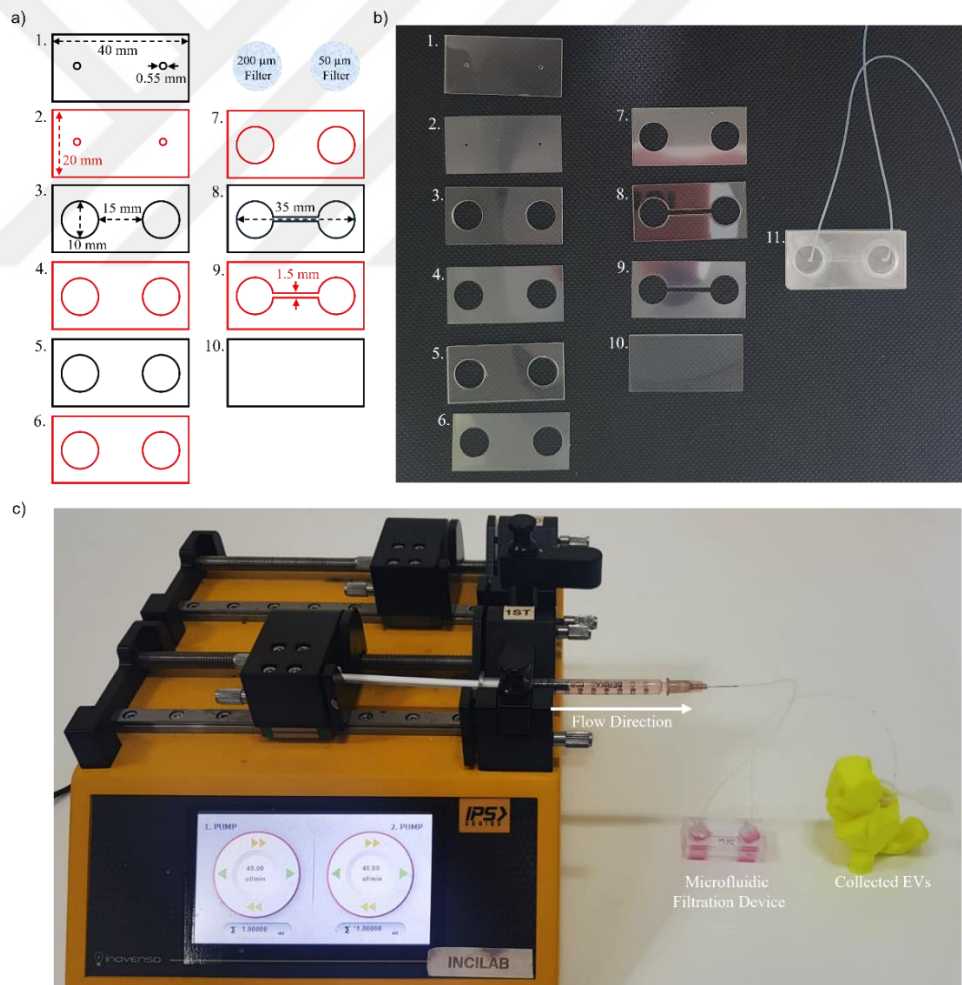


Figure 2.2 a) PMMA and DSA layers of microfluidic chip were demonstrated. Black colors are PMMA designs (thickness: 2 mm), and red colors are DSA layers

(thickness: 50 μm). Between two DSA (numbers 6 and 7), 200 and 50 μm pore-sized polycarbonate filters were placed. b) Laser-cut PMMA and DSA layers and completed filtration chip with tubings (number 11) and c) completed size-based EV isolation system with microfluidic chip and syringe pump were depicted.

2.7.2 Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA, NS300, Malvern Instruments Ltd., UK) is used to investigate the size and concentration of the nanoparticles (in our research, EVs) suspended in a liquid environment [116]. The EV solution was diluted 1:50 with DPBS filtered with a 0.22 μm syringe filter and introduced to the NTA sample chamber. This chamber is illuminated with a precisely configured laser beam (488 nm), and particles on the beam's trajectory scatter the laser. The scattered laser beam is captured by the 20x microscope lens and recorded with a digital camera (CCD) that helps understand the particles' Brownian motion. The NTA software conducts an individual and synchronized analysis of multiple particles and applies the Einstein-Stokes equation to estimate their hydrodynamic diameters [117]. In our experiment, viscosity was indicated as 0.92 in the software, and 5 captures were recorded for 60 seconds. After each capture, a 0.2 mL sample was introduced to the sample chamber.

2.7.3 Scanning Electron Microscope (SEM) for Biological Samples (EVs)

Similar to the metasurface characterization (section 2.5.3), the size distribution and morphology of the EVs were characterized with SEM. Silicon wafers were cut into 0.5 mm x 0.5 mm pieces and sterilized by sonicating in 70% of ethanol for 5 minutes. Then, it was dried with an air gun and stabilized on the SEM sample stubs via double-sided carbon tapes. Since EVs are biological and liquid samples, silicon wafers were used as a conductive substrate material to drop EV solution on them. The liquid EV solution was mixed with 4% of paraformaldehyde (PFA) with a 1:1 ratio (v:v) to fixate EVs and preserve their morphology under imaging. 5 μL of PFA and EV mix were dropped on the silicon wafer substrate with a micropipette and dried for 2 hours. To achieve a clear image,

the sample was rinsed three times with ethanol and settled to completely dry. Prior to imaging, the sample was coated with 10 nm of gold (Au) palladium (Pd) alloy for conductivity and then imaged.

2.7.4 Western Blot

Western Blot is a conventional gel electrophoresis technique to identify individual proteins in a mixed biological sample according to their molecular weights [118]. For better separation of the proteins, two types of polyacrylamide gels were used as resolving gel (10% of polyacrylamide) located on the bottom of the tank with larger pore sizes and stacking gel (4% of polyacrylamide) that includes sample wells with smaller pore diameters. Prior to starting, buffers and reagents were prepared as listed: 1.5 M Tris-HCl (pH 8.8) for resolving gel, 0.5 M Tris-HCl (pH 6.8) for the stacking gel, the 1X running buffer (25 mM of Tris, 192 mM of glycine, and 10% of SDS), the blocking buffer (5% of milk-TBST: non-fat milk powder, 10 mM Tris, 15.4 mM NaCl, and 0.1% of Tween 20).

The gel casting station was prepared by stabilizing the reusable glass cassettes to the front and the back. First, the resolving gel (10%) was prepared by adding 3.9 mL of ddH₂O, 2 mL of Tris-HCl (1.5 M, pH 8.8), 2 mL of 40% of Acrylamide mix, 80 μ L of APS, and 8 μ L of TEMED in a centrifuge tube (15 mL). Immediately after mixing, it was poured between two glass layers until the fill line and the remaining part was completed with isopropanol to remove bubbles in the gel to have a smooth structure. It was left for polymerization around 15 minutes. After polymerization, isopropanol was removed and dried carefully with Whatman paper without rupturing the resolving gel. The stacking gel was then prepared by adding 1.92 mL ddH₂O, 750 μ L Tris-HCl (0.5 M, pH 6.8), 300 μ L 40% of Acrylamide mix, 30 μ L APS and 3 μ L TEMED and poured on the stacking gel. The comb was placed in the stacking gel and waited for 15 minutes for polymerization. After drying, the comb was removed, and the gel was placed in the Western Blot tank filled with 1X running buffer. Loading dye and DTT mixture (20:1) were prepared before loading the samples in the wells. It was mixed with the EV sample with a 1:9 ratio (v:v) and boiled at 95°C for 5 minutes. 10 μ L of sample and dye mixture was loaded to each well, and proteins were run for 30 minutes at 90 V until they passed the stacking gel and reached the resolving gel. After 30 minutes, the

voltage increased to 120 V, and proteins were run for 90 minutes. Separated proteins were transferred from the gel to a PVDF membrane at 20 V for 105 minutes. Upon completing the transfer, the membrane was blocked with a 5% of milk-TBST blocking buffer for an hour at room temperature on a shaker. The blocked membrane was then washed three times, 10 minutes each with TBST. Anti-CD63 antibody (Recombinant Rabbit monoclonal antibody, 1 mg/mL), (Invitrogen MA5-30187, ThermoFisher) was prepared at a ratio of 1:500 in 5% of milk-TBST and applied to the membrane overnight at 4°C under medium shaking. The excess amount of primary antibody was washed three times with TBST to prevent non-specific bindings. Then, the secondary HRP conjugated goat-anti rabbit anti-CD63 antibody (1:5000 dilution) was prepared in 5% of milk-TBST and applied on the membrane at room temperature for one hour under gentle shaking. The membrane was washed three times with TBST from the secondary antibody and enhanced chemiluminescent (ECL) substrate (Luminol/Enhancer and Stable Peroxide Buffer (1:1 (v:v))) was applied on the membrane and visualized using ChemiDoc MP instrument (Bio-Rad, California, USA).

2.8 Plasmonic Measurements

A portable and user-friendly device that measures reflected light from the surface was designed and utilized for real-time EV capture and release plasmonic measurement monitoring experiments (Figure 2.3a), and for temperature alterations, an Arduino-assisted heating system was used (Figure 2.3b-c). The portable optic sensor consists of two main compartments: the spectrophotometer (Thorlabs, CCS175/M) and a fiber-coupled light source that operates in the visible spectrum. The generated light is focused on the sensor surface via diverse optical equipment, which was located in a 3D-printed outer protective layer. The light passed through the lenses, linear polarizer and beam splitter to illuminate the surface. The reflected light from the surface was then collected with a spectrophotometer and observed with its commercial software (Thorlabs OSA). Since the raw data provided by the Thorlabs OSA software is not feasible for real-time monitoring of biomarker binding events, incoming data was analyzed with a MATLAB GUI code. The final data was presented with time (s) at the x-axis and wavelength (nm) at the y-axis.

For the plasmonic measurements, the microfluidic metasurface chip was located under illuminated light and stabilized carefully to measure the exact same point during the experiment. The flow in the channel was provided with a syringe pump by withdrawing. One end of the tubings was placed in the sample, and the other was connected to the syringe pump to create a 10 $\mu\text{L}/\text{min}$ speed flow in the channel. As a typical SPR measurement, first, the PBS (running buffer) was introduced in the channel for 5 minutes, and a baseline was observed. The sample was then introduced for 45 minutes, which caused an upward wavelength shift (association phase). The unbound analytes in the environment were washed with PBS for 5 minutes (dissociation phase), and the wavelength change between the initial baseline and after washing was quantified to evaluate binding events on the surface.

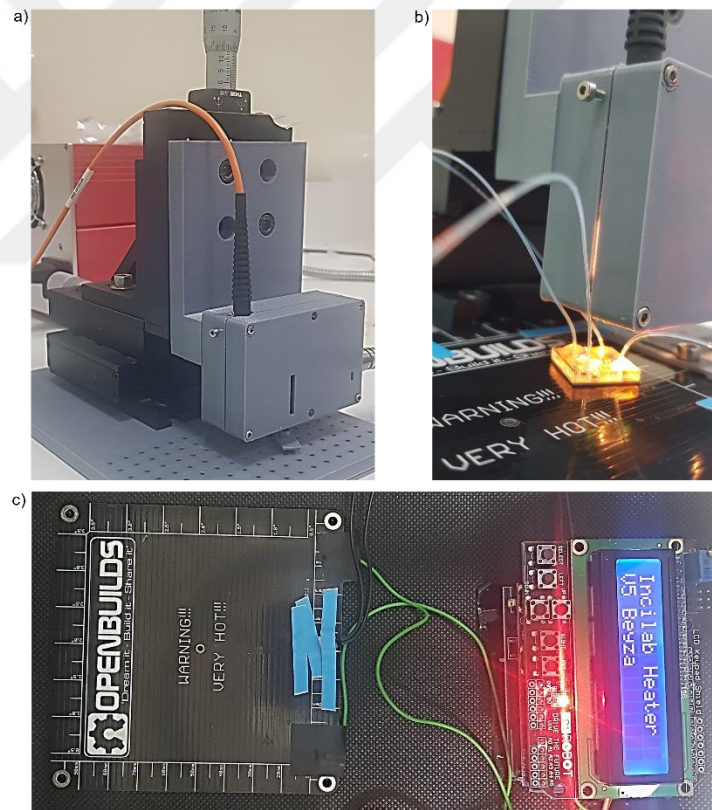


Figure 2.3 a) The portable surface polarizations measurement device, b) PCB heater and microfluidic plasmonic metasurface real-time data analysis, and c) Arduino-assisted PCB heater for PNIPAM phase transition were demonstrated.

In this experiment, a Printed Circuit Board (PCB) heating board was used for temperature control (Figure 2.3c, left). PCB is a thin panel built from composite materials and it includes conductive routes that are either etched or printed into its surface [119]. Temperature control was implemented with Arduino (Figure 2c, right), an electronic system that can implement the input and convert it to the output. In this scenario, input is a Python code that commands (i) to sense the available temperature, (ii) to heat it to the desired temperature, and (iii) to stabilize the temperature at a certain value.

The EV capture and release phenomenon in plasmonic measurement device via real-time monitoring is depicted in Figure 2.4. In capture-release studies, the PCB heater was placed under the microfluidic metasurface plasmonic sensor, and the distance of the light source was arranged accordingly. For capture, PBS for baseline (5 minutes), EV solution (45 minutes) and PBS washing (5 minutes) were conducted at room temperature (25 °C). The temperature was then increased to 35 °C and EVs were released for 45 minutes at 10 μ L/min. Following, the temperature was decreased to room temperature, and the final wavelength peak value was compared with the initial PBS baseline value. The portable real-time measurement system is illustrated in Figure 2.4b. Emitted unpolarized light from the light source passes through the lenses to collect and focus. Then, the light passes to an optical polarizer (LPVISC050, Thorlabs) that selects a specific polarization state of light. To excite plasmonic modes effectively, a specific polarization state (typically p-polarized for plasmonic measurements) is usually required, which strongly couples with surface plasmon waves. Since circularly polarized light consists of both s- and p-polarized components, linear polarization was applied in this study to maximize sensitivity. An optical aperture (SM1D25, Thorlabs) was utilized that limits the beam diameter to control the amount of light propagating in the system. Subsequently, the light passes through the beam splitter (50:50) that partially transmits and partially reflects the incoming light. The transmitted light is directed onto the plasmonic metasurface, and the modulated light, due to biomolecule bindings on the surface, reflects back toward the beam splitter, which can be detected via spectrometer.

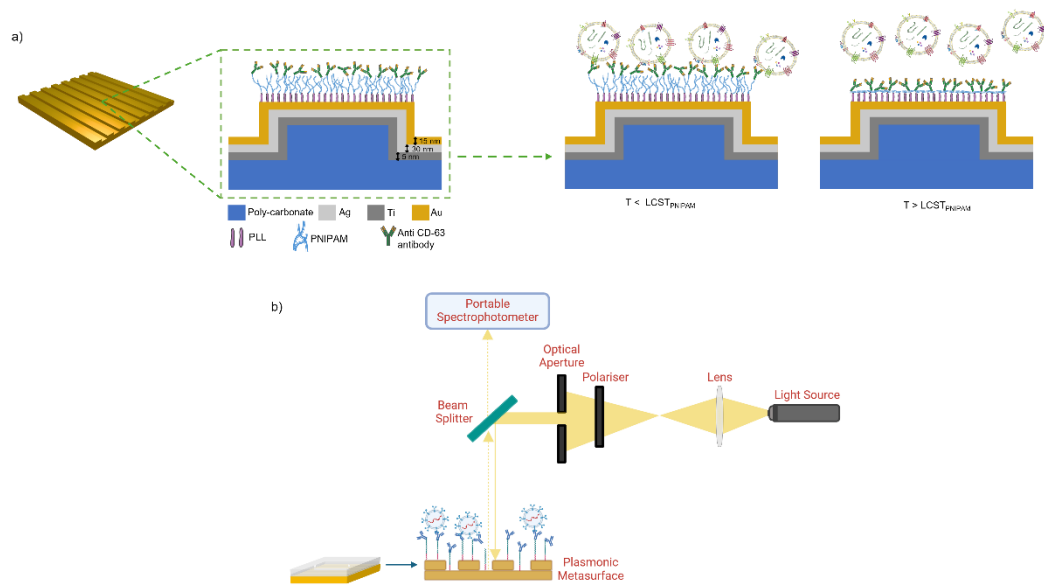


Figure 2.4 Overview schematics of a) capture and release of EVs on thermoresponsive polymer-modified plasmonic metasurface and b) portable plasmonic measurement system were depicted.

CHAPTER 3

3. RESULTS

3.1 Cell Studies

MCF-7 cells were cultured, as detailed in Chapter 2.6. Prior to collecting the cell media on the fourth day, which was reported as the optimum day for EV separation [45], the cells were imaged with an inverted fluorescence microscope (Zeiss). To observe their viability, calcein AM, which stains live cells and creates a green color, and ethidium homodimer, which stains dead cells and creates a red color, was utilized. Bright-field images and Hoechst 33342 cell nuclei staining were also utilized to clearly observe their confluency and morphology.

The imaging results are demonstrated in Figure 3.1. In the bright field image (Figure 3.1a), it can be seen that the cells adhered to the cell culture flask and created a cell sheet monolayer, which is essential for cell growth and proliferation. The morphology of the cultured MCF-7 cells was aligned with the results presented in the literature, which indicates the culture conditions are suitable for secreting healthy EVs [120], [121]. Live cells stained with calcein AM were observed with a GFP filter (Figure 3.1b), and dead cells stained with ethidium homodimer were observed with an RFP filter (Figure 3.1c). The merged image for both live and dead cells is presented in Figure 3.1d. The live-dead imaging results showed that the majority of the cells were alive on the 4th day without changing the cell culture medium, which is crucial for EV separation. Finally, Hoechst 33342 cell nuclei staining is shown in Figure 3.1e, which depicts the cell confluency on the cell culture flask with a higher constant compared to the bright field image.

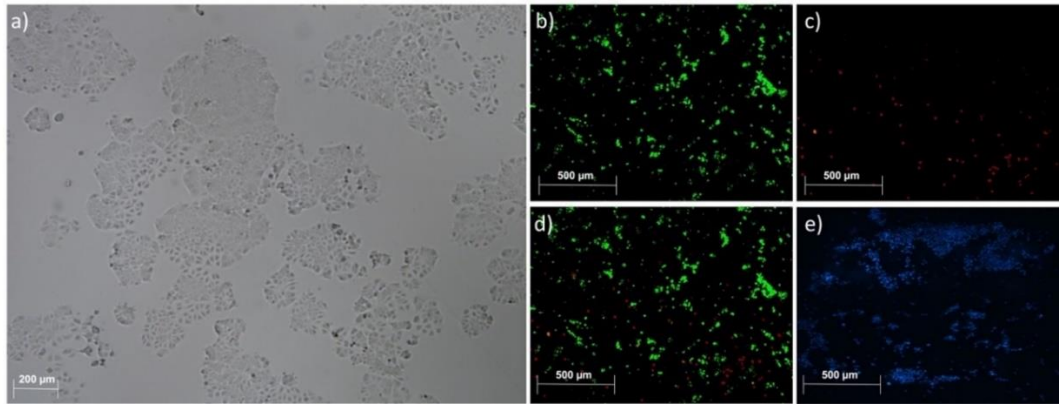


Figure 3.1 Cultured MCF-7 cells were imaged with the inverted light microscope: a) bright field, b) Calcein-AM-stained live cells (GFP filter), c) Ethidium homodimer stained dead cells (RFP) filter, d) merged image for live/dead staining and e) cell nuclei staining (Hoechst staining, DAPI filter).

The immunohistochemistry (IHC) staining of the MCF-7 cells was conducted to observe cancer-specific and cell type-specific markers on the cultured breast cancer cell line. The first marker investigated is cytokeratin-19 (CK-19), which is one of the principal structural proteins. It typically facilitates cytoskeletal structure; nevertheless, their abnormal levels of expression may induce oncogenesis in cells [122]. The second marker is the epithelial cell adhesion molecule (EpCAM, CD326) which commonly has high expression in carcinogenic cells [123]. For IHC staining, Hoechst 33342 (Figure 3.2a) was utilized to stain cell nuclei. It has excitation/emission (nm) peaks at 350/461, and the DAPI channel was suitable for observing blue-colored cell nuclei, which were smaller and centered compared to CK-19 and EpCAM located on the cell cytoskeleton and membrane. Alexa Fluor 647 tagged anti-EpCAM antibody (Figure 3.2b) has excitation/emission (nm) peaks at 650/671, and an RFP channel was used to investigate red-colored epithelial markers on the cell surface. FITC-tagged anti-CK-19 antibody (Figure 3.2c) has excitation/emission (nm) peaks at 495/519, and the GFP channel was utilized for cytokeratin observation. EVOS M5000 (Thermo Fisher Scientific, USA) inverted fluorescent microscope was used for imaging, and the presence of the cancer-specific biomarkers was validated.

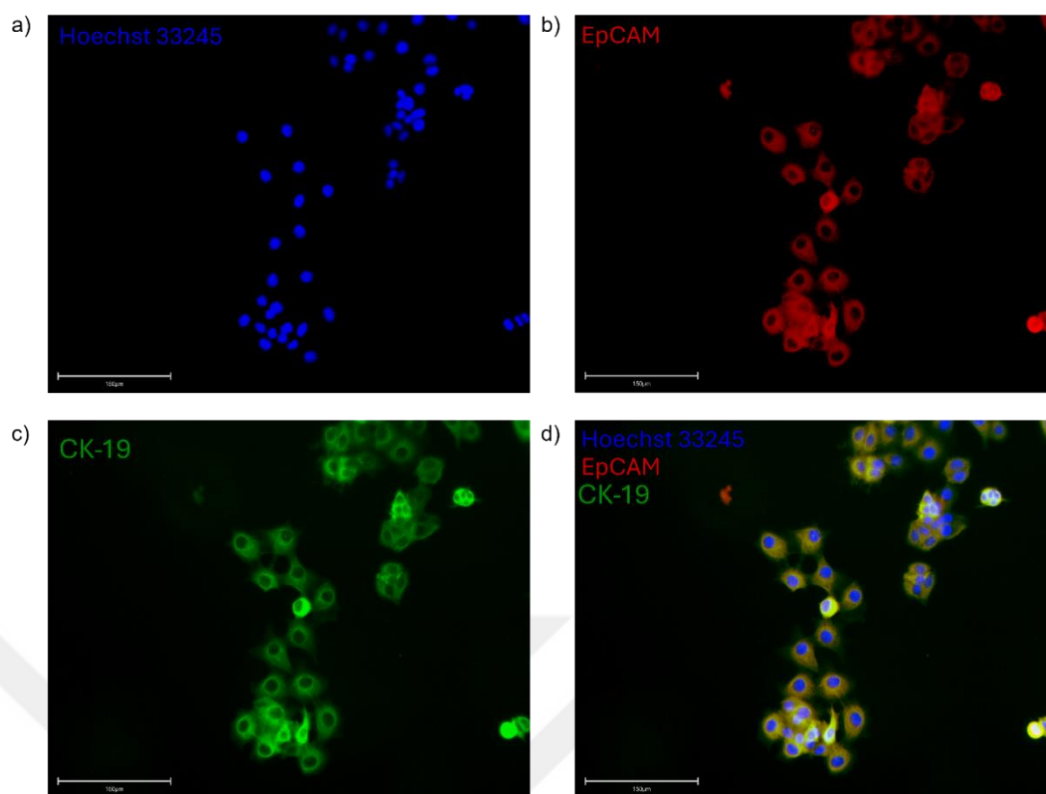


Figure 3.2 Immunohistochemistry (IHC) validation of MCF-7 breast cancer cells in the inverted fluorescence microscope (EVOS M5000) was demonstrated in a) Hoechst Staining (for nuclei) , b) Alexa Fluor 647-EpCAM (for epithelial cancer biomarkers), c) FITC- CK-19 (for epithelial cell cytokeratin biomarker), d) merged image for all channels

3.2 EV Isolation via Microfluidic Ultrafiltration Chip

EVs isolated with a microfluidic ultrafiltration chip were characterized with SEM and NTA to take as a reference point and compare after the second isolation via thermoresponsive polymer-modified plasmonic metasurface capture and release in real-time.

SEM is an electron microscope that assesses the morphology and arrangements of the micron and nano-sized structures. The SEM images of the EVs that were fixed with paraformaldehyde (1:1, v:v) and coated with 10 nm Au/Pd alloy for conductivity were demonstrated in Figure 3.3a-b at different magnifications. Since EVs have a lipid-bilayered close structure, it was expected to

observe spherical particles [124]. In Figure 3.3a, the spherical structures of the isolated EVs can be seen. Several aggregations during fixation were also observed. These aggregations caused a rough surface and non-uniform Au/Pd coating that ended up with electron accumulation at certain locations. These points were monitored brighter compared to more uniformly coated places. In Figure 3.3b a closure image of the isolated EVs at a higher magnification was depicted. They have a uniform and round-shaped morphology distribution without any contamination (bigger bioparticles, etc.), damage, or rupture.

The size and concentration of the isolated EVs via microfluidic chip were quantified with NTA. In Figure 3.3c, the NTA result of the EVs directly applied with 1/50 dilution in PBS can be seen. It was observed that the particles in the frames have a mean of 137.7 ± 3.6 nm size and a mode of 94.4 ± 4.7 nm. The result is consistent with the polycarbonate filter sizes (200 and 50 μ m). The concentration of the particles was recorded as $1.01 \times 10^{10} \pm 5.23 \times 10^8$ particles/mL. An immunostaining procedure was then applied specifically to EVs to evaluate the purity of the particles separated according to their size. Alexa Fluor 488 (excitation/emission (nm): 490/525) tagged CD63 antibody for EV selection and 488 nm blue laser in NTA (NS3000) for detection was utilized for immunostained EV detection. Prior to the measurement, the EV solution isolated with a filtration chip was diluted 1:50 with PBS and mixed with Alexa Fluor 488 anti-CD63 antibody. It was incubated in a dark chamber at room temperature for 30 minutes. The results are demonstrated in Figure 3.3d. The mean and the mode of the particles in the frame were decreased to 75.2 ± 13.6 nm and 54.1 ± 20.2 nm, respectively. Similarly, the concentration of the fluorescence-tagged EVs was found to be $1.98 \times 10^6 \pm 1.71 \times 10^5$ particles/mL, which is 10^4 times lower than the prior measurement.

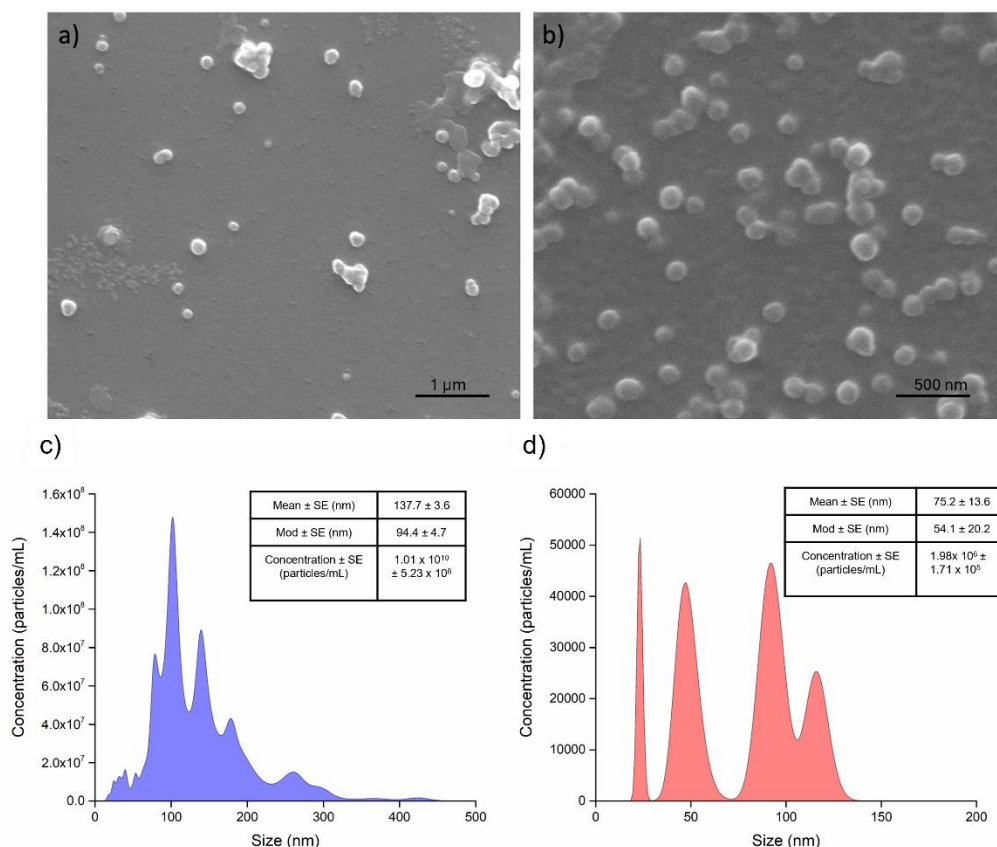


Figure 3.3 a-b) SEM images of EVs separated with microfluidic ultrafiltration chip, c) NTA of the EVs and d) Alexa Fluor 488 tagged CD63 immunostained NTA of EVs

3.3 PNIPAM synthesis

Thermoresponsive PNIPAM was synthesized through atomic transfer radical polymerization reactions. The ATRP was conducted in an aqueous solution. PNIPAM was purified from catalytic copper residues by precipitating the corresponding polymer through heating the aqueous solution to transform hydrophilic polymer into hydrophobic aggregates. The aggregates were transformed into a clean petri dish and dried. The solid PNIPAM was measured at the desired mass and utilized after being dissolved in distilled water. The LCST phase change of PNIPAM from hydrophilic to hydrophobic form was reported as 32-35 C in the literature [125]. To observe this phenomenon, 10 mg/mL PNIPAM solution in distilled water is prepared and placed on a hot plate (Figure 3.4). A temperature-sensing probe was dipped in the sample to measure the exact

temperature in real-time. The first image was taken at 30 °C which was lower than the LCST of the PNIPAM. At 30 °C, PNIPAM was observed as completely dissolved in water. The specimen exhibited no alteration in color and did not display any hydrophobic aggregates. At a temperature of 35 °C, the turbidity of the sample showed an increase, and it began to exhibit hydrophobic characteristics, particularly at the bottom of the vial, which is closer to the heating source. Lastly, at 40 °C, the specimen was completely heated. Its color switched to opaque white from transparent. The hydrophobic PNIPAM aggregations were formed and observed in the vial. This validation confirmed the thermoresponsive properties of synthesized PNIPAM. Since the plasmonic measurements are sensitive to refractive index and temperature changes, the exact transition point, which was 35 °C, was selected as the release temperature. It is also suitable for biological samples (such as surface proteins on the EVs) that are sensitive to higher temperatures.

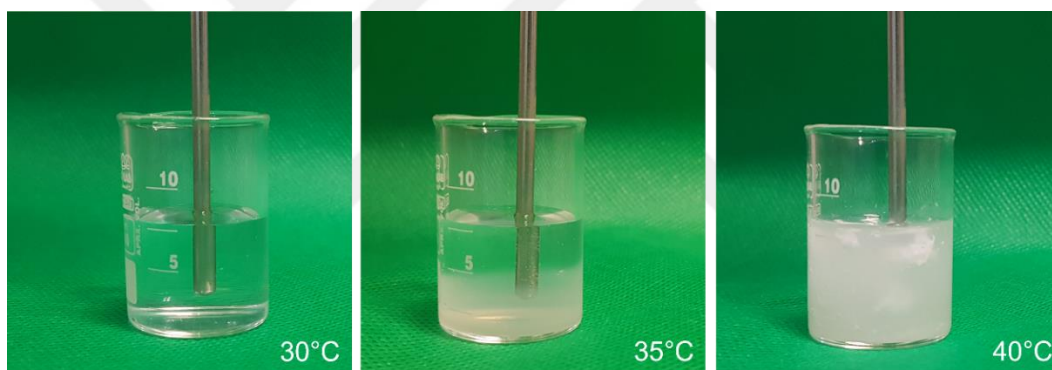


Figure 3.4 Hydrophilic to hydrophobic transition of PNIPAM at 30 °C (lower than the LCST), 35 °C (around the LCST), and 40 °C (higher than the LCST) were shown.

The PNIPAM was decorated with certain chemical groups for further bioconjugation studies. N-hydroxysuccinimide (NHS) ester groups have significant reactivity to amine nucleophiles, which are predominantly available in biological molecules such as proteins and antibodies [126]. In this context, NHS terminated ATRP initiator (NHS-BiB) was utilized for the polymerization to create NHS moieties on the PNIPAM chain. The NHS conjugation and PNIPAM polymerization were characterized by nuclear magnetic resonance (NMR). The spectrum was recorded in deuterated dimethyl sulfoxide (DMSO). The chemical

shifts are reported in parts per million (ppm) relative to the residual solvent peak of DMSO and demonstrated in Figure 3.5.

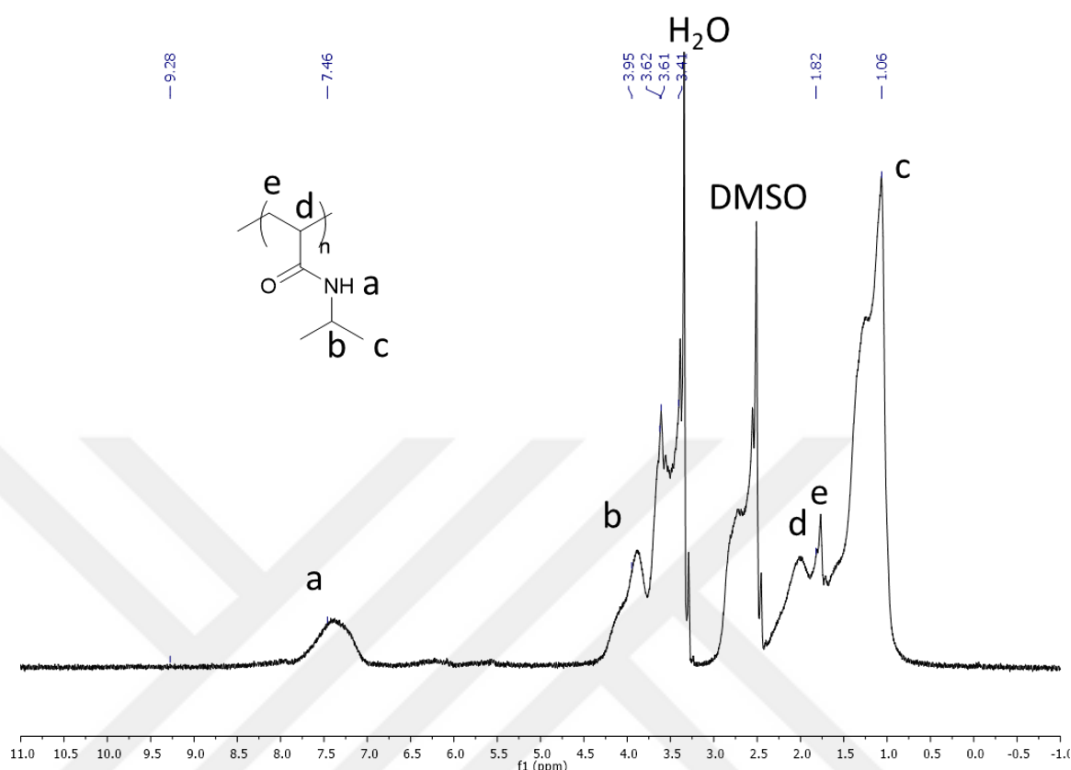


Figure 3.5 ^1H NMR spectrum of PNIPAM is depicted.

Table 2. ^1H NMR spectrum proton peaks

Peak Label	Peak Shift (ppm)	Origin
a	~7.46	Amide proton of PNIPAM (-NH)
b	3.95 – 3.44	Backbone methine proton (-CH)
c	1.82 – 1.06	Methyl groups (CH_3)
d	1.5 – 2.0	Methine proton (-CH)
e	0.9 – 1.2	Methylene groups ($-\text{CH}_2$) and succinimide terminal protons
-	3.3 – 3.9	Residual water
-	2.5	DMSO

The characteristic proton resonances in the PNIPAM backbone and side groups were investigated with NMR analysis and presented in Table 2. The peak at around 7.46 ppm (a) corresponds to the amide (-NH) proton of PNIPAM. This peak

confirms the presence of the amide functionality, which is essential for the polymer's thermoresponsive behavior [127]. Peaks around 3.95 – 3.44 ppm (b) are related to the methine (-CH) groups at the PNIPAM backbone (NH-CH-), confirming successful polymerization. The characteristic strong methyl protons of the isopropyl group of the PNIPAM (c) around 1.82 – 1.06 ppm and methine protons from the backbone (d) around 1.5 – 2.0 ppm are observed [128], [129]. Around 0.9 – 1.2 ppm (e), methylene groups from the polymer backbone were observed. Succinimide termination was observed around 2.8 – 3.00 ppm as a small shoulder peak [130]. The NMR analysis of the synthesized PNIPAM validated the successful polymerization and succinimide termination of the PNIPAM, which is suitable for further bioconjugation studies.

3.4 Surface Characterization

The modifications on the plasmonic metasurface were characterized with different methods, including contact angle, SEM, and XPS.

3.4.1 Contact Angle

Contact angle analysis was utilized to investigate the hydrophilic and hydrophobic characteristics of the plasmonic metasurface, which is strongly associated with surface energy. Here, (i) bare plasmonic metasurface, (ii) 0.1 mg/mL PLL modified metasurface, (iii) 0.1 mg/mL PLL and 5 mg/mL PNIPAM modified surface and (iv) 0.1 mg/mL PLL, 5 mg/mL PNIPAM and 50 µg/mL Anti-CD63 antibody modified metasurface were analyzed respectively and the result were presented in Figure 3.6. The angle between the specimen-water intersection and droplet-air intersection was taken as the contact angle. The surface is considered hydrophilic when the contact angle is between 0 – 90°. On the contrary, if the angle was higher than 90°, (90 - 180°) the surface is validated as hydrophobic.

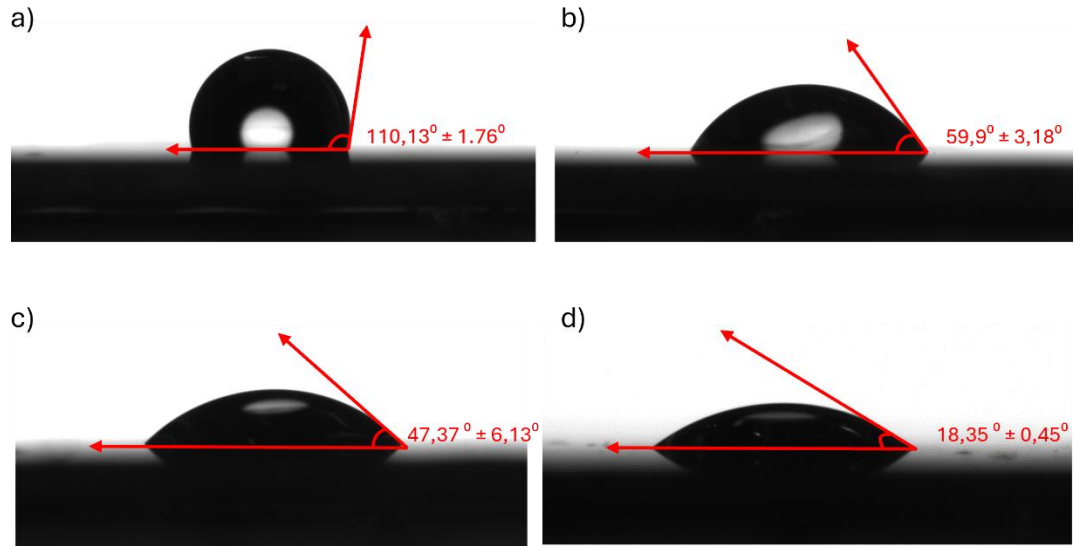


Figure 3.6 The contact angle measurement for a) bare plasmonic metasurface, b) PLL modified surface, c) PLL and PNIPAM modified surface and d) PLL, PNIPAM and anti-CD63 antibody modified surfaces were demonstrated.

The contact angle in the bare surface (Figure 3.6a) is measured as $110.13^\circ \pm 1.76^\circ$, which indicates a hydrophobic structure with low surface energy. It was decreased to $59.9^\circ \pm 3.18^\circ$ after PLL modification and the surface became hydrophilic. After PNIPAM and anti-CD63 antibody modifications, the contact angles were recorded as $47.37^\circ \pm 6.13^\circ$ and $18.35^\circ \pm 0.45^\circ$ respectively. These measurements validated that the surface was hydrophobic initially, and it became more hydrophilic after each modification.

3.4.2 Scanning Electron Microscope (SEM) and Laser Scanning Microscope Images of the Metasurface

The gratings on the DVD surface have pivotal importance in achieving SP coupling and, thus, plasmonic signals during real-time analyte attachment and detachment monitoring [131]. Considering this, preserving the periodic structure of the gratings is crucial during metal coating and surface modifications. To observe the surface topology after completed polymer modification (PLL and PNIPAM), a laser scanning microscope (Keyence, VKX-100) was utilized (Figure 3.7a). SEM

images of bare surface (Figure 3.7b), PLL modified surface (Figure 3.7c) and PLL and PNIPAM modified surfaces (Figure 3.7d) were investigated. The result of the bare surface showed that the gratings on the surface were protected during metal coating via a thermal evaporator. Moreover, the applied surface chemistry did not affect the surface topology and was suitable for EV capture and release monitoring in real-time plasmonic measurements.

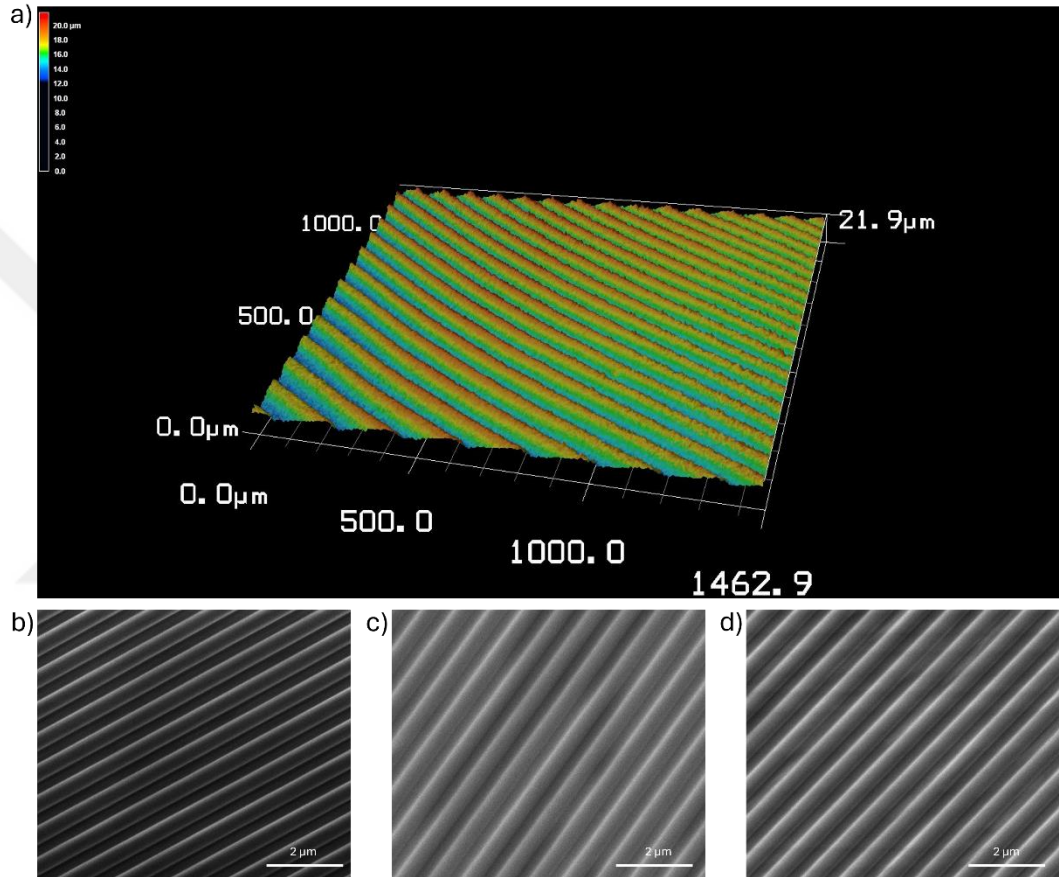


Figure 3.7 a) Laser scanning microscope of PLL and PNIPAM modified surface and SEM images of b) bare DVD metasurface, c) PLL coated and d) PLL and PNIPAM coated surfaces were demonstrated.

3.4.3 X-Ray Photoelectron Spectroscopy (XPS)

Analysis

XPS analysis was performed to investigate the surface chemistry of the modified surfaces. Gold (Au4f), carbon (C1s), and nitrogen (N1s) spectrums of

bare, PLL-modified, PLL-PNIPAM-modified, and PLL-PNIPAM-Anti CD63 antibody-modified surfaces were analyzed and presented in Figure 3.8.

In the Au4f, peaks at 87.88 eV and 84.2 eV were observed for all surfaces, which indicates the gold coating presence in all samples. Significant shifts in gold peaks were not observed; only the peak strength diminished with each alteration, which satisfies the fact that surface modifications do not have a negative impact on the underlying gold layer.

In the C1s peaks, in the bare surface analysis (Figure 3.8e), there is one peak from air contamination, and there are no functional groups on the surface. After PLL modification (Figure 3.8f), there is a strong peak around 284.32 eV, representing C-C, C-H, and C=C bonds from the PLL backbone. There are moderate peaks around 285.81 eV and 287.48 eV, representing C-O and C=O bonds in the PLL structure. Following the PNIPAM coating (Figure 3.8g), the intensity of the peak at 284.25 eV increased, indicating the PNIPAM backbone. Similarly, the intensity of the peaks at 280.99 eV and 287.43 eV increased compared to PLL results. When the anti-CD63 antibody was applied (Figure 3.8h), the peak intensity at 287.7 eV (C=O) slightly decreased, which shows amide formation with the NHS group in the PNIPAM.

N1s spectrum is an important indicator of the presence of organic and biological compounds. Initially, there was not a significant peak in the N1s spectrum of bare surface (Figure 3.8i). Along with the PLL modification, peaks at 399.26 eV for amine (-NH₂) groups and 401.08 eV for amide bonds (HN-C=O-) in the PLL were observed (Figure 3.8j). In PNIPAM modification, an additional peak at 398.9 eV was observed, indicating secondary amines available in the isopropyl group of PNIPAM. At 400.53 eV a peak that might be amide (N-(C=O)-) or imide (-(C=O)-N-(C=O)-) bonds noted (Figure 3.8k). Lastly, at 399.58 eV, amine bonds from the PNIPAM backbone were observed. After antibody conjugation (Figure 3.8l), the intensity of the peak at 399.5 eV was increased since NHS in the PNIPAM was incorporated into an amide bond. Overall, XPS data validated that the PLL, PNIPAM, and antibody modifications were completed successfully.

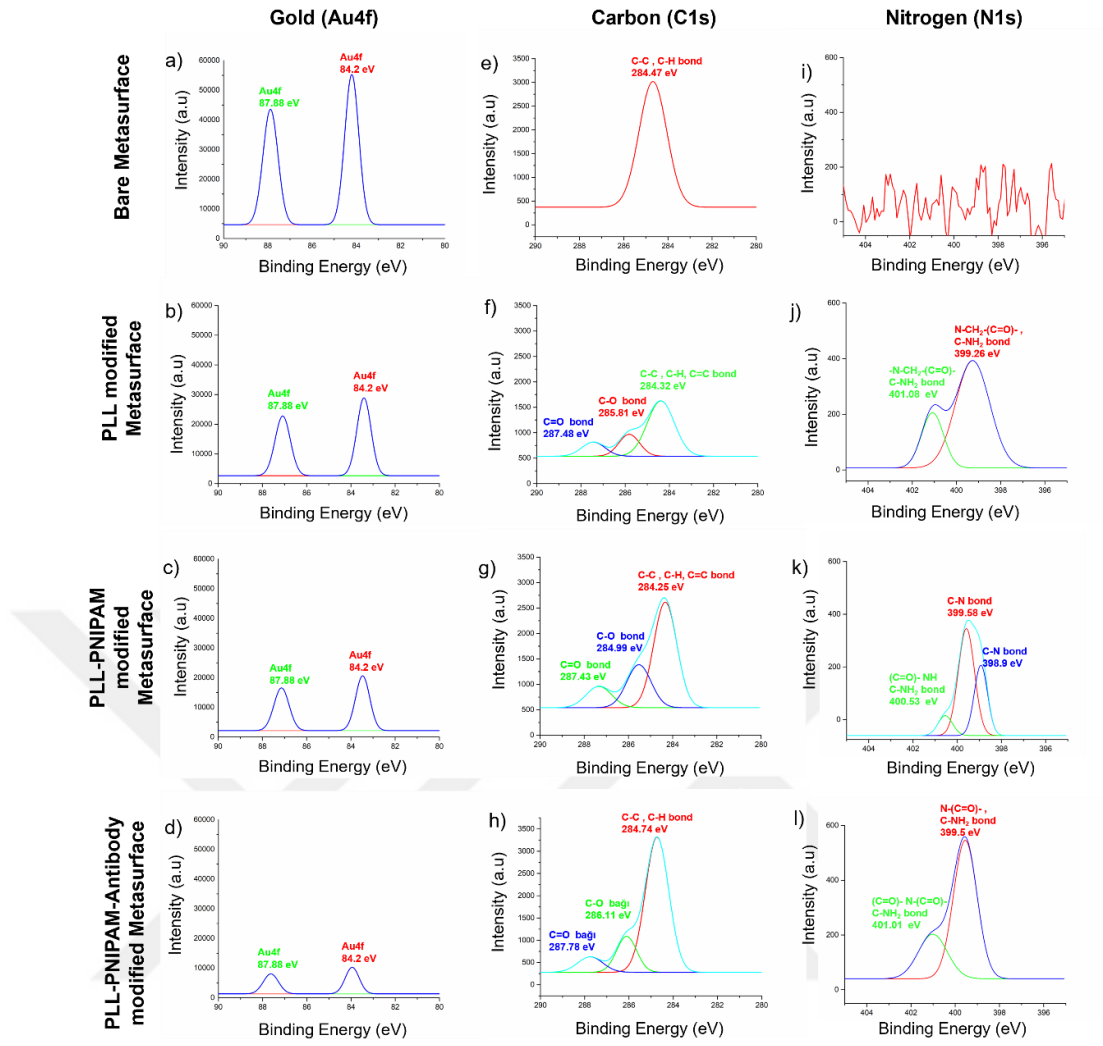


Figure 3.8 XPS spectra for the modified surfaces were demonstrated. Au4f scans are presented for a) bare metasurface, b) PLL-modified surface, c) PLL-PNIPAM modified surface, and d) PLL-PNIPAM-Anti CD63 antibody modified surfaces. C1s scans are shown for e) bare metasurface, f) PLL-modified surface, g) PLL-PNIPAM modified surface, and h) PLL-PNIPAM-Anti CD63 antibody modified surfaces. N1s scans are presented for i) bare metasurface, j) PLL-modified surface, k) PLL-PNIPAM modified surface, and l) PLL-PNIPAM-Anti CD63 antibody modified surfaces.

3.4.4 Thermal Camera Evaluation of the PCB Metasurface Heater

The PCB heater was placed under the plasmonic metasurface microfluidic chip to control the capture and release temperature precisely. The thickness of the

PCB heater was measured and the light source moved upward accordingly to focus the light on the chip from optimum distance. Since the temperature of the room where the experiment was conducted may change, the PCB heater is arranged at 27 °C (<LCST of the PNIPAM) for capture experiments, and the exact temperature of the microfluidic chip was measured with a thermal camera (Figure 3.9a). Previous PNIPAM characterizations satisfied that (Section 3.3) 27 °C is in the hydrophilic temperature range of PNIPAM and is suitable for EV capture. The temperature then increased to 35 °C, hydrophilic to the hydrophobic transition temperature of the PNIPAM. The stability of temperature was validated with a thermal camera (Figure 3.9b). Lastly, the PNIPAM formation in the chip was observed at higher temperatures. When the chip was heated up to 45 °C, a solid PNIPAM formation in the channel was seen (Figure 3.9c). This observation also confirmed the successful PNIPAM modification, but the hydrophobic aggregation would disturb the laminar flow in the channel and create a chaotic flow. Moreover, since it would affect the plasmonic signal and deteriorate the real-time analysis, the release temperature was selected around the transition point (35-37 °C).



Figure 3.9 Thermal camera images during a) capture at room temperature (~27 °C < LCST of PNIPAM) and b) at higher temperature (~35 °C > LCST of PNIPAM) were validated. c) PNIPAM formation in the channel at 45 °C was also observed.

3.5 Thermoresponsive Polymer Modified SPR Optimization

For the real-time analysis, first the concentration of the PNIPAM was arranged on the surface. Different concentrations of PNIPAM were utilized for diverse applications and surfaces in the literature [132], [133]. In this study, 10

mg/mL, 5 mg/mL and 2.5 mg/mL PNIPAM dissolved in distilled water homogenously were applied in different metasurface specimens and their plasmonic signals were evaluated with SPR (Figure 3.10). In the 10 mg/mL PNIPAM applied surface, the wavelength peak was observed around 529 nm. The standard deviation of the signal data point was 1.3 nm, which can be considered as a strong noise. To properly identify the collected EVs on the surface, the background noise needs to be minimized. If the fluctuations caused by the surface chemistry increase, the binding events between the immobilized capture element (anti-CD63 antibody) and target (EV) cannot be differentiated. In this scenario, 10 mg/mL PNIPAM caused a strong noise and fluctuation in the signal and was not used for further experiments. When the concentration decreased to 5 mg/mL, the peak was around 525 nm and the standard deviation decreased to 0.3 nm, a tolerable noise compared to the former. At 2.5 mg/mL, PNIPAM formation was not observed in the channel at higher temperatures due to low concentration. Considering the results of the experiments, the optimum concentration of the PNIPAM was decided as 5 mg/mL for further capture and release experiments.

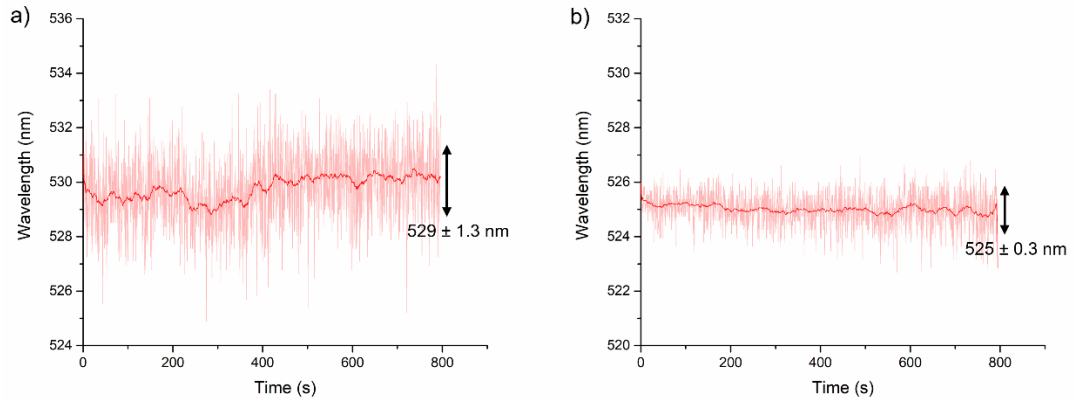


Figure 3.10 PNIPAM concentration optimization with SPR for a) 10 mg/mL PNIPAM and b) 5 mg/mL PNIPAM after PLL modification.

Since different temperatures are required for the capture and release of the EVs (27 °C and 35 °C), the temperature effect on the plasmonic signal was investigated (Figure 3.11). The plasmonic metasurface was modified with PLL-PNIPAM and anti-CD63 antibody. While PBS was applied with a 10 μ L/min flow rate, the temperature of the PCB heater increased from 30 °C to 40 °C with a 1°C step size increment. At each temperature, the real-time plasmonic metasurface

measurement data was taken for 10 minutes (Figure 3.11a). A decreasing pattern was observed when the temperature increased, which was previously reported in the literature [134]. To observe the wavelength values in detail at each temperature, the temperature ($^{\circ}\text{C}$) versus wavelength (nm) graph was investigated (Figure 3.11b). The spectrophotometer records two data points (wavelength values) at each second, which makes 1200 data points for one temperature value for 10 minutes. The mean and the standard deviation of the last 500 data points for each temperature were observed separately in Figure 3.11b). Following, the temperature change ($^{\circ}\text{C}$) versus wavelength shift (nm) was derived from the former graph. It was observed that the temperature change-dependent wavelength shift can increase up to ~ 0.58 nm in 10°C alteration. To observe if this change affected the reflection peak of the plasmonic metasurface reversibly or irreversibly, the wavelength (nm) versus reflection (a.u.) graph was investigated. It was noted that there were no significant alterations and deteriorations in the reflection peaks at three critical temperatures: 30°C ($<\text{LCST}$), 35°C (transition point), and 40°C ($>\text{LCST}$).

Considering the results, it was decided to add an additional step after the release at higher temperatures. Since the wavelength shift caused by temperature change and EV release may interfere with the real-time data, after release, the temperature increased back to the temperature during the capture (27°C). The initial PBS value was compared with the final capture temperature values to quantify the capture-release efficiency.

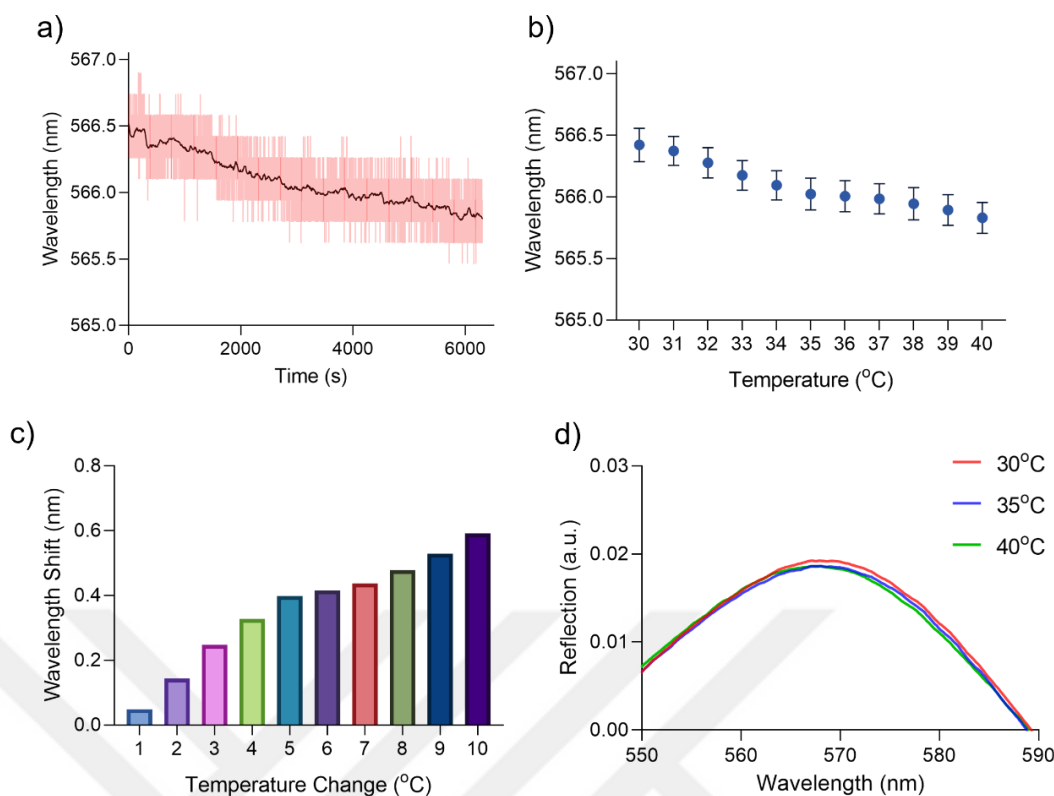


Figure 3.11 Refractive index changes with temperature alteration were evaluated, and a) real-time metasurface plasmonic measurement data, b) temperature-dependent wavelength shift, c) wavelength shift values at increased temperatures, and d) plasmonic reflection signal measurements taken from portable spectrophotometer at different temperatures (30, 35, and 40 $^{\circ}\text{C}$) were demonstrated.

Prior to EV capture with PLL-PNIPAM-antibody surface chemistry, different modifications on the metasurface were validated to assess the optimum procedure. First, 3-Mercaptopropionyl-N-hydroxysuccinimide ester (3-MNHS) was applied directly on the gold surface. 3-MNHS is a heterobifunctional crosslinker that has a thiol (-SH) group at one end and a succinimide (-NHS) group at another end. In theoretical knowledge, the thiol groups can bind directly on the gold surface and create self-assembled monolayers (SAM), and the metasurface can present succinimide groups that are reactive towards primary and secondary amines to bind PNIPAM. The second alternative surface chemistry is to apply 3-aminopropyl triethoxysilane (APTES) to the surface. APTES has a Si that is bound to three ethoxy groups ($\text{O}-\text{CH}_2\text{CH}_3$) at one end and an amine ($-\text{NH}_2$) group at another end. In theory, it can bind a surface that is rich in terms of hydroxyl ($-\text{OH}$) groups from the Si side and can present free amines for further NHS-PNIPAM and antibody

conjugations. These alternative EV capture strategies were evaluated and compared with PLL-PNIPAM chemistry. For both, two replicates of EV capture experiments were conducted, and the results are presented in Figure 3.12. The EV stock achieved after the microfluidic filtration chip and measured with NTA as 10^{10} EVs/mL concentration was diluted to 10^9 EVs/mL with PBS and used for each experiment. During the experiment, (I) PBS for 5 minutes at a 10 μ L/min flow rate (to observe the background signal), (II) EV solution for 45 minutes at a 10 μ L/min flow rate, and (III) washing with PBS for 5 minutes at 10 μ L/min flow rate were applied. The initial and final signal data points were compared to record the total wavelength shift after EV binding.

In Figures 3.12a and 3.12b, the 3-MNHS applications were depicted. The wavelength shifts after the washing steps were 0.18 nm and 0.2 nm, respectively (0.19 ± 0.1 nm). Following, APTES-applied surfaces were validated in Figures 3.12c and 3.12d. Similarly, the wavelength shifts after the washing steps were 0.01 nm and 0.32 nm, respectively (0.165 ± 0.155 nm). With regard to the high concentration of EVs, the consequent wavelength changes were fairly slight and even lower than the potential noise of the microfluidic chip. Consequently, APTES and 3-MNHS modifications were not utilized for EV capture and release investigations owing to their low sensitivity.

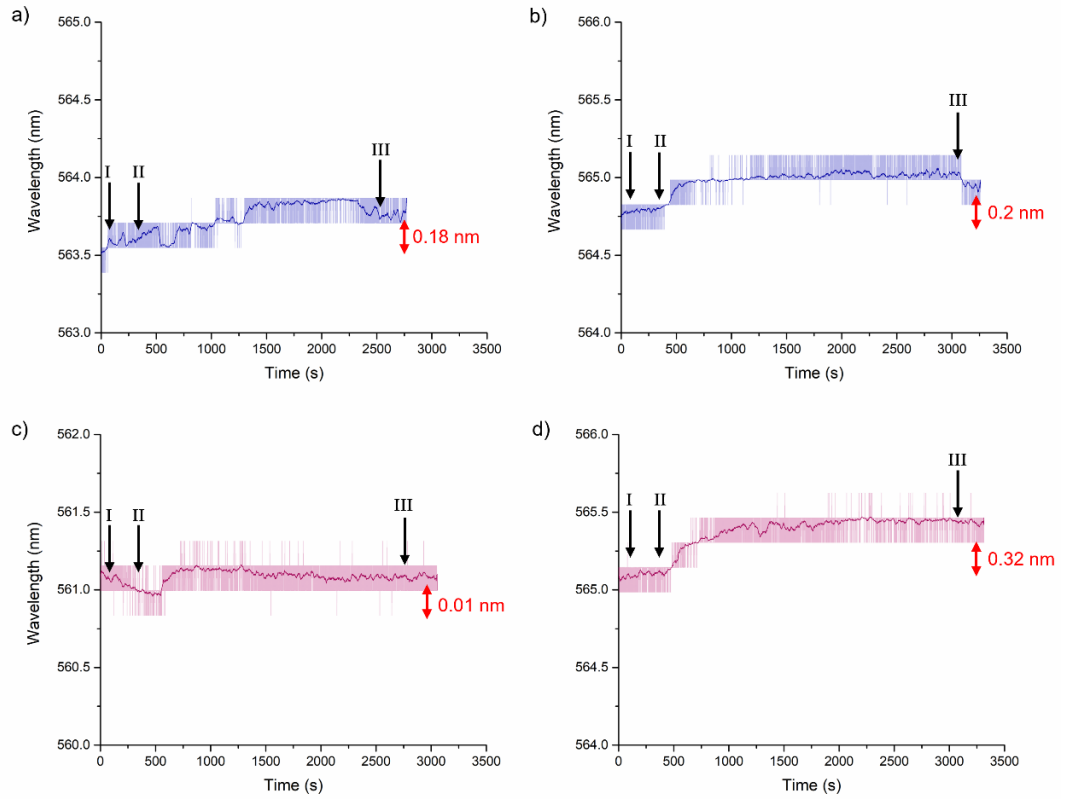


Figure 3.12 Plasmonic metasurface chemistry optimization studies for 10^9 EVs/mL particle detect and capture with a-b) 3-MNHS, PNIPAM, and anti-CD63 antibody applied and c-d) APTES, PNIPAM, and anti-CD63 antibody applied surfaces were demonstrated.

3.6 EV Capture and Release

Under the light of the optimization studies, the plasmonic metasurface was modified with 0.1 mg/mL PLL, 5 mg/mL PNIPAM (dissolved in distilled water), and 50 μ g/mL anti-CD63 antibody. The capture temperature was arranged at 27 $^{\circ}$ C. The maximum EV concentration achieved with a microfluidic filtration chip was 10^{10} particles/mL. Starting from the maximum concentration, 10^9 particles/mL, 10^8 particles/mL, and 10^7 particles/mL EV solutions were introduced to the microfluidic channel for EV detection and capture. The experiments were repeated three times, and the results are presented in Figure 3.13. Similar to the previous capture study, (I) PBS for 5 minutes for baseline, (II) desired concentration of EVs for 45 minutes and (III) PBS for 5 minutes for washing were applied respectively

and the initiation point for each alteration was marked on the graph. The difference between the final value and the initial baseline was recorded as the wavelength shift.

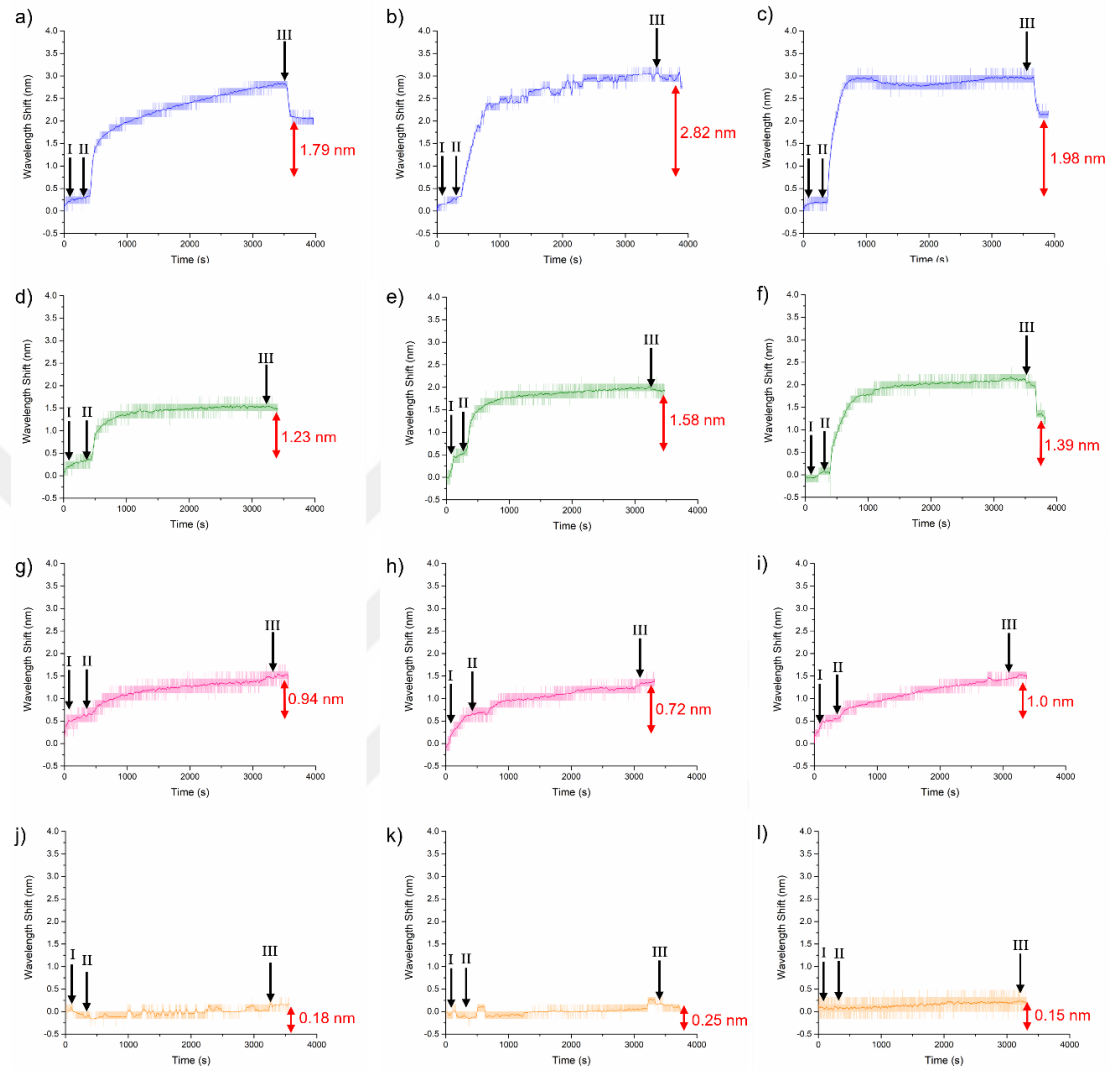


Figure 3.13 Real-time EV capture monitoring for three replicates of a-c) 10^{10} particles/mL, d-f) 10^9 particles/mL, g-i) 10^8 particles/mL, and j-cl) 10^7 particles/mL were demonstrated.

When 10^{10} particles/mL EVs were applied (Figure 3.13a-c), 1.79 nm, 2.82 nm, and 1.96 nm increases (2.20 ± 0.45 nm) were recorded. After, 10^9 particles/mL EVs were introduced to the different channels (Figure 3.13d-f), and 1.23 nm, 1.58 nm, and 1.39 nm increments (1.40 ± 0.14 nm) were observed. Upon decreasing the sample concentration to 10^8 particles/mL, 0.094 nm, 0.72 nm, and 1.0 nm, a total upward shift (0.89 ± 0.12 nm) was seen. Lastly, for 10^7 particles/mL, 0.18 nm, 0.25 nm and 0.15 nm increments (0.2 ± 0.04 nm) were recorded. These wavelength shift

measurements were significantly higher than the previously conducted 3-MNHS and APTES-modified surface increments.

When the results were presented as the mean $\pm$ standard deviation of the three replicates and fitted to Pearson's correlation, the graph in Figure 3.14 was achieved. It was observed that the dynamic range of the EV detection and capture capacity of the system was around 3-4 logarithmic decades (from 10^{10} particles/mL to 10^7 particles/mL), and the limit of detection (LOD) for this sensor system was around 10^7 particles/mL.

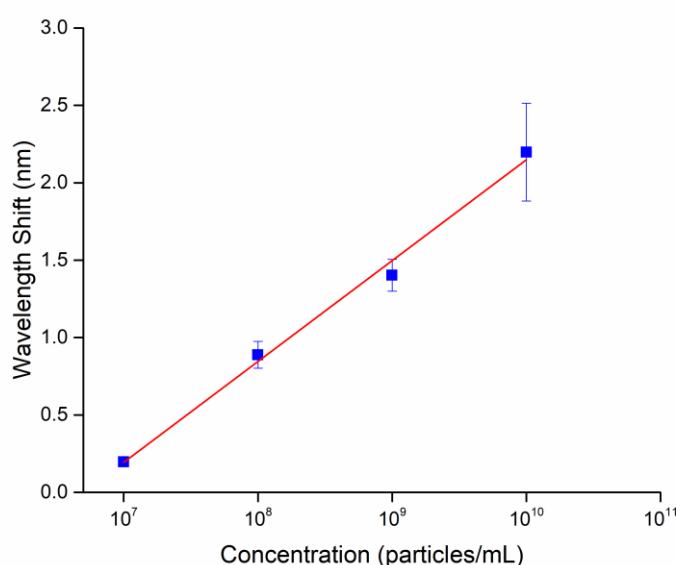


Figure 3.14 Different concentrations of EV detection and capture were depicted. The error bars are the standard deviation of triplicate experiments (Pearson Coefficient (r-value) = 0.94, $R^2 = 0.89$).

Release experiments were conducted with 10^9 particles/mL EV sample concentrations. Real-time plasmonic polaritons monitoring of five repeats of the capture and release experiments is presented in Figure 3.15. Subsequent to the capture and washing, the temperature was increased to 35 °C, and release was initiated for 45 minutes at under 10 μ L/min flow rate. Considering the simultaneous wavelength shift effect of the temperature elevation, as detailed in section 3.5, the temperature was decreased to the capture temperature (27 °C) right after release. At this temperature, PBS was introduced to the chip for 10 minutes to stabilize the temperature. These alterations were marked on the graphs as: (I) PBS baseline for

5 minutes at 10 $\mu\text{L}/\text{min}$ flow rate, (II) 10^9 particles/mL EV sample for 45 minutes (10 $\mu\text{L}/\text{min}$), (III) PBS for washing for 5 minutes (10 $\mu\text{L}/\text{min}$), (IV) temperature increase to 35 $^{\circ}\text{C}$ and release for 45 minutes (10 $\mu\text{L}/\text{min}$) and (V) temperature decrease to 27 $^{\circ}\text{C}$ for 10 minutes (10 $\mu\text{L}/\text{min}$). The wavelength shifts after capture/release and release efficiency values are given in Table 3. The release efficiency rates were fluctuated between 18 – 49 % (36.35 ± 16.06 %). A significant pattern that affects the release rates was not observed.

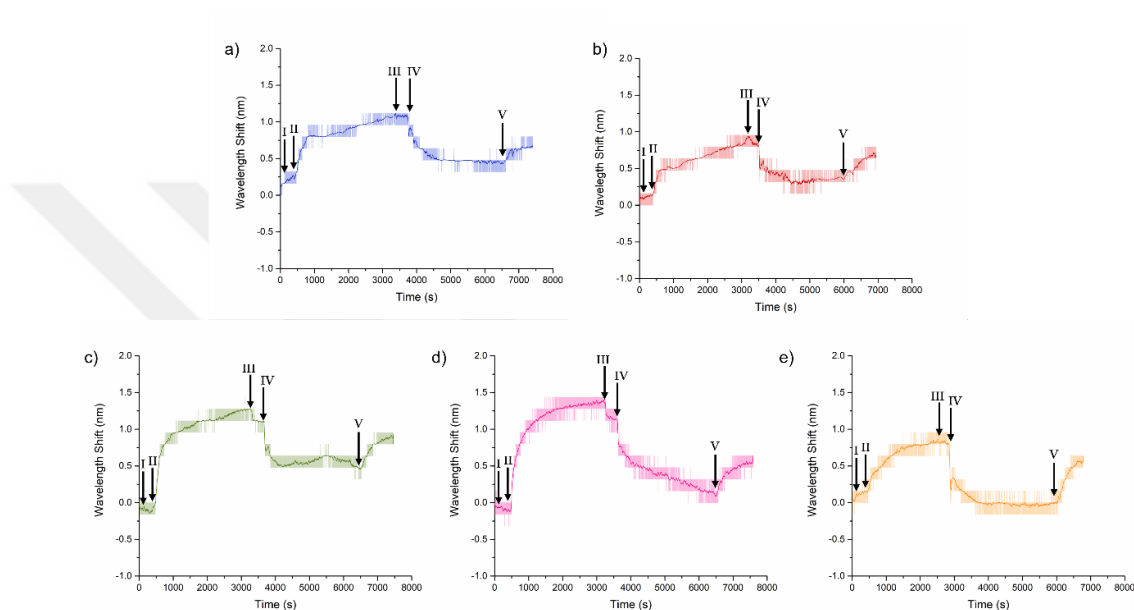


Figure 3.15 Real-time plasmonic measurement data of the five replicates of EV capture and release experiments for PLL-PNIPAM-Anti-CD63 antibody applied surfaces (without any surface blocking) were depicted.

Tablo 3. Wavelength increase values at capture and wavelength decrease values at release, and release efficiency percentages were given.

Experiment	Wavelength Increase (Capture) (nm)	Wavelength Decrease (Release) (nm)	Release Efficiency (%)
a	0.85	0.42	49.05
b	0.73	0.2	27.62
c	1.23	0.22	18.14
d	1.22	0.6	48.81
e	0.70	0.27	38.15

Subsequently, the same experimental setup was utilized for the blocked plasmonic metasurfaces for EV capture and release. The common drawback of label-free approaches is, they may suffer from poor specificity [135]. Surface blocking is a pivotal strategy for biosensing approaches that reduce non-specific bindings on the surface and provide enhanced selectivity towards the selected target molecule, especially in the complex fluids that are rich in terms of proteins and biomolecules (e.g., blood, urine, cell medium, etc.). There are varieties of surface-blocking strategies, including milk powder application, PEGylation, surfactants (Tween 20), or certain sugar molecules that create a hydration layer on the surface [135]–[137]. One of the most common blocking approaches, also used in conventional molecular biology techniques such as Western Blot, is the bovine serum albumin (BSA) utilization [138]. To observe the surface-blocking effect on the release efficiency, after the PLL-PNIPAM-Anti-CD63 antibody surface modifications, 5 mg/mL BSA was introduced to the channels and incubated at 4 °C for 20 minutes. After incubation, the channels were rinsed with PBS three times, and an excess amount of BSA was removed. Subsequently, the same procedure was applied to the previous capture and release group (without BSA). The results are presented in Figure 3.16. In the graphs: **(I)** PBS baseline (5 min, 10 μ L/min), **(II)** 10^9 particles/mL EV sample (45 min, 10 μ L/min), **(III)** PBS washing (5 min, 10 μ L/min), **(IV)** temperature increase to 35 °C and release (45 min, 10 μ L/min) and **(V)** temperature decrease to 27 °C for (10 min, 10 μ L/min).

Compared with the non-blocked surface, the release rates of the BSA-applied capture and release experiments were higher. For five different experiments, the release percentage of the captured EVs varied between 50.18 – 118.8 % (87.03 ± 23.5 %). This can be caused by reduced unspecific protein binding events on the surface. In the previous experiment, non-targeted biomolecules may be trapped on the surface and remained unreleased. The wavelength shift after EV detection and capture phenomena were also lower than the non-BSA applied experiments, which confirms the successful blocking strategy. For one specific experiment, the release rate was higher than 100%. The reason for this event could be the detachment of the antibodies from the surface, which is a limitation of the immunoassay-based separation techniques [15].

To sum up, the PLL-PNIPAM-tetraspanin protein-specific antibody and BSA applied surface can successfully detect and capture EVs specifically from

complex fluids in low sample volume (microchannel volume: $\sim 5 \mu\text{L}$). The captured EVs can then be isolated via release from the surface by changing the hydrophilicity of the thermoresponsive PNIPAM brush-like polymer.

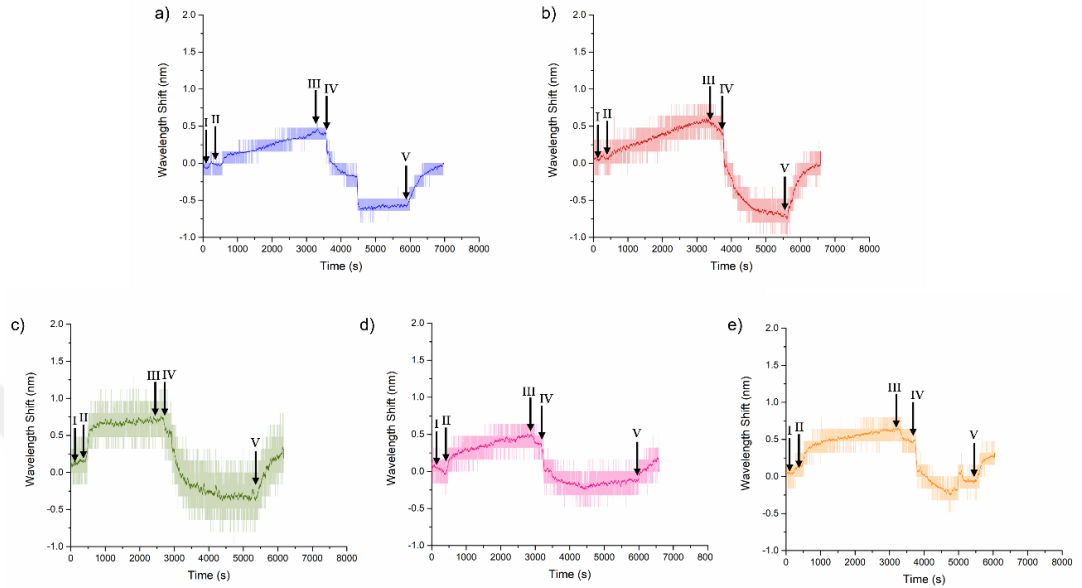


Figure 3.16 Real-time metasurface plasmonic measurement data of the five replicates of EV capture and release experiments for PLL-PNIPAM-Anti-CD63 antibody and BSA blocking-applied surfaces were demonstrated.

Tablo 4. Wavelength increase values at capture and wavelength decrease values at release, and release efficiency percentages were given

Experiment	Wavelength Increase (Capture) (nm)	Wavelength Decrease (Release) (nm)	Release Efficiency (%)
a	0.44	0.43	98.8372
b	0.44	0.53	118.8046
c	0.45	0.426	94.44445
d	0.40	0.29	72.93167
e	0.40	0.20	50.18039

3.7 Characterization of Released EVs

The primary objective was to separate EVs with excellent purity, avoiding the use of harsh enzymes and chemicals. To validate this, isolated EVs from plasmonic metasurface via capturing and releasing were characterized with SEM, NTA and Western Blot.

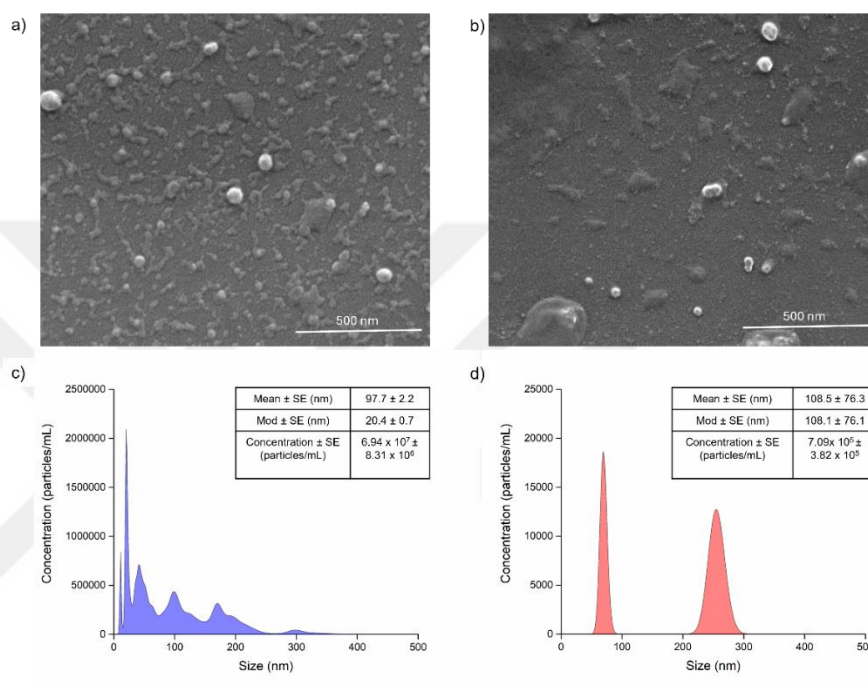


Figure 3.17 a-b) SEM images of EVs isolated with capture and release from PLL-PNIPAM-anti-CD63 antibody and BSA applied surface, c) NTA of the isolated EVs, and d) Alexa Fluor 488 tagged CD63 immunostained NTA of EVs were demonstrated.

The morphology and spherical shape of the EVs were observed with SEM (Figure 3.17a-b). Compared with the SEM images of the EVs isolated with the microfluidic filtration chip (Figure 3.3a-b), there were fewer particles. However, their morphology and size were consistent. NTA measurements were utilized for the size and concentration investigation. First, the sample achieved from the plasmonic metasurface was directly measured via NTA. The mean and mode of the particle size were 97.3 ± 2.2 nm and 20.4 ± 0.7 nm, respectively. There were small peaks at bigger sizes as well (299, 507 and 579 nm). The concentration of the particles was recorded as $6.94 \times 10^7 \pm 6.31 \times 10^6$ particles/mL. Following, the Alexa

Fluor 488 tagged CD63 immunostaining was conducted and NTA analysis was made with a blue laser source. The mean and mode values of the EVs on the frame shifted to 108.5 ± 76.3 and 108.1 ± 76.1 , respectively. The higher peaks around 500 nm were diminished which could be the contaminations from liquids (dust, salt). The size peak shifted to between 200 and 300 nm. This change may be due to the detachment of the antibody immobilized on the metaplasmonic surface, emerging simultaneously with the EV [15].

Moreover, the concentration was noted as $7.09 \times 10^5 \pm 3.82 \times 10^5$ particles/mL. In the previous measurement (Figure 3.3c-d), the concentration difference between bare and immunostained EVs was $\sim 10^4$, which indicates high impurities. Here, this difference decreased to $\sim 10^2$ and it was observed that the sample was purified 100 times successfully.



Figure 3.18 Western blot validation of the CD63 tetraspanin protein presence on the EVs released from plasmonic metasurface was depicted.

Western blot analysis was performed to validate the released EVs and the functionality of the CD63 tetraspanin proteins on their surface. It is the gold standard for identifying proteins and deciding their presence in a mixture of proteins. Five samples captured and released from blocked (BSA applied) surfaces were collected and analyzed. CD63 is recognized for its impact on cancer malignancy and post-translational modifications can be seen on CD63, specifically N-glycosylation [139], [140]. Due to that, the CD63 was observed as a smeared band between 35-80 kDa rather than a thin band at a certain value. The western blot results confirmed the presence of the CD63 tetraspanin protein in the collected PBS sample. Consequently, it was achieved that the EVs captured on the PNIPAM-coated plasmonic surface were released and isolated successfully.

CHAPTER 4

4.CONCLUSION AND FUTURE PROSPECTS

In this thesis, a microfluidic chip integrated plasmonic metasurface was designed. This surface was modified with thermoresponsive PNIPAM polymer for label-free detection, capture, and isolation via the release of the captured EVs from the surface.

Initially, the MCF-7 breast cancer cells were cultured to isolate EVs secreted from these cells and their culture conditions were evaluated. It was known that the physiological condition of a cell, covering its culture conditions (cell culture medium, pH, electrolytes, etc.) and stress levels, substantially influences the types and quantities of molecules encapsulated in EVs [22], [141]. Stress and pathological circumstances also have significant impacts on their function in cellular communication and the development of diseases (including spread and growth) [142]. The breast cancer cells were cultured in the optimum conditions as stated in the literature [143] and detailed in the methodology section. Since the cells created a monolayer and grew without any contamination and stress factor, their morphology and physical states were evaluated as suitable for further EV isolation. The cell specific EpCAM and CK19 markers of MCF-7 breast cancer cells were also evaluated with IHC staining. EpCAM is also known to play a role in the secretion and function of EVs derived from epithelial tissues [144]. The fluorescent immunolabeling of the EpCAM and CK19 biomarkers of the cultured MCF-7 cells validated their presence, confirming the healthy culturing of the MCF-7 cells.

Following the cell culture, a microfluidic double filtration chip was utilized for the first EV isolation based on their sizes. This chip is a cost-effective and rapid alternative to commercially available isolation techniques such as SEC [145]. EVs are highly heterogeneous in terms of their size, which has a significant impact on their content and density. Moreover, even though it is not a direct indicator, the size of the EV can be a preliminary sign of its biogenesis [21]. After the first isolation of EVs from cell culture media, the heterogeneity of the EVs was reduced. 200 and

50 μm pore-sized filters were utilized for double filtration. The SEM results confirmed that the isolated particles have similar size and spherical morphology. The NTA results also confirmed that the size distribution is focused between 15 – 300 nm. Even the small pore sizes and relatively bigger EVs were observed in NTA results. There are three main reasons for this: (i) the non-uniform distribution of the pores on the polycarbonate filters, (ii) the elastic structure of the EVs, and (iii) aggregations. In the first scenario, if the pores on the polycarbonate membrane were not uniformly constituted, they may overlap and emerge bigger pores. Additionally, the EVs are not rigid particles. They have a lipid-bilayered outer structure, and even though their Young modulus (stress/strain) may vary depending on the cell type, they typically display an elastic structure with a relatively high elastic modulus [146]. Consequently, they may squish and pass through the comparatively smaller pores without any morphological alteration or damage. EVs also may aggregate in the liquid sample and create bigger particles.

The NTA measurement was then conducted with immunostaining that target CD63 proteins on the EVs specifically. A blue laser (488 nm) was utilized in the NTA setup for this measurement. The aim of this measurement was to observe the EV purity of the size-based isolation from the cell culture medium. The results in Figure 3.3d showed that the concentration given as particles/mL decreased across the four logarithmic ranges. In brief, only 1 particle in 10.000 particles is labeled and verified as an EV that has a CD63 surface marker. Owing to this, the size-based isolation does not contain only EVs but also includes different biomolecules that remain in the same size interval. An additional step is required for certain applications that require extremely pure EV.

Among different controlled radical polymerization methods (such as nitroxide-mediated polymerization (NMP) and reversible addition–fragmentation chain-transfer polymerization (RAFT)), ATRP was selected for the synthesis of PNIPAM for this study. Controlled radical polymerization offers advantages for the synthesis of highly branched polymers, including dendrimers and hyperbranched polymers. They are entities characterized by a compact structure, extensive branching linkers, a three-dimensional spherical form, and numerous terminal groups [147]. Here, the NHS conjugated ATRP initiator was utilized to terminate the PNIPAM chain with NHS (succinimide) groups that have high reactivity towards amine groups in biological molecules, including antibodies. The

hydrophilic to hydrophobic transition of the synthesized PNIPAM was evaluated by controlled heating on the hot plate and hydrophobic aggregate formation was observed. The LCST transition point was also measured as ~ 35 °C. Following the NMR analysis of the PNIPAM was performed. The obtained peaks were aligned with the literature, confirming the effective synthesis of PNIPAM by the ATRP technique [148]. Since the succinimide functional groups are located at the end of the polymer chain, they were not observed as a strong peak [149].

To prepare the surface for EV capture and release, surface modifications were applied. During this step, the pivotal issue was to prevent the plasmonic properties of the metasurface and not damage the periodic nanostructures and metal coating on the surface with harsh chemicals or intense concentrations. Prior to PNIPAM, PLL was applied on the surface, which is a commonly used positively charged linker to bind the gold surface electrostatically [150], [151]. PNIPAM was then applied, and lastly, the anti-CD63 antibody was conjugated through the NHS groups in the PNIPAM. The contact angle results validated that the metasurface was initially hydrophobic and became hydrophilic after each modification. Following the PNIPAM density on the surface and its effects on the surface topology were observed. The SEM and laser microscope results validated the surface gratings were not affected by the modifications and are suitable for plasmonic biomarker detection in real-time. The XPS analysis was then conducted, and PLL-PNIPAM and antibody modifications were confirmed.

The optical measurement setup with PCB heater was optimized for release studies. It was observed that the temperature alteration can be controlled easily and precisely via a keyboard on the Arduino heater. The temperature of the microfluidic chip at capture and release temperatures was measured with a thermal camera. The primary concern at this stage was that the light source should not produce a thermal effect. The photothermal effect is also driven by surface plasmons, and the fiber-coupled light source may have a heating impact on a certain location if the light intensity is higher [152]. The thermal camera measurement was crucial for the light intensity optimization for precise temperature control, along with the plasmonic signal. Subsequently, how the PNIPAM concentration affects the plasmonic detection signal was investigated, and 5 mg/mL was decided as the optimum concentration that creates reduced background noise.

The plasmonic signal alteration over temperature increase was then investigated. In a previously conducted study, the refractive index of the acetone from 20 °C to 45 °C changed [134]. Both experimental and theoretical results of acetone showed that the wavelength decreased when temperature increased. A similar result was obtained for PBS on the PLL-PNIPAM-antibody-modified plasmonic surface. The temperature increased from 30 to 40 °C, and the wavelength decreased sequentially. This temperature-dependent signal change was considered in release experiments.

Surface chemistry has an inevitable impact on biosensing applications, and a proper design on the sensing surface provides enhanced sensitivity and specificity. Besides PLL, different linker molecules and surface modifications were applied on the surface to select the optimum detection and capture method for EVs. First, 3-MNHS was applied, which is a single-stepped alternative of MUA and EDC/NHS chemistry [153]. This is an effective method for directly binding a molecule to the gold surface from the amine functional group however, it was not suitable for PNIPAM after antibody [154]. Secondly, APTES was applied on the surface to create amine groups on the sensor surface. In literature, mostly hydrolyzation (-OH) on the gold surface prior to APTES application was made with acidic solutions in special conditions (such as 30 minutes at 60 °C) [155], [156]. The metasurface used in this study was made from cost-effective, off-the-shelf materials, and it has a plastic polycarbonate base. Oxygen plasma was utilized for hydroxylation at mild conditions (at 200 kW for 2 minutes) to not harm the base material, gratings and metal coating. This might result in insufficient hydroxylation on the surface. In both alternative approaches, the capture efficiency was extremely poor, and they were not selected for surface chemistry.

In Figure 3.13 and 3.14, the detection and capture capacity of the surface was validated by applying different concentrations (particles/mL). Considering the maximum concentration achieved with the microfluidic double filtration chip, 10^{10} particles/mL, 10^9 particles/mL, 10^8 particles/mL, and 10^7 particles/mL were applied. A typical limit of detection (LOD) linear regression plot was observed at a 3-4 log dynamic range when the x-axis was presented as a log range [157]. Moving forward, the release studies were conducted. For release, (i) non-blocked (no BSA applied) and (ii) blocked BSA-applied surfaces were evaluated. Respectively, 36.35 ± 16.06 % and 87.03 ± 23.5 % of release efficiencies were achieved for the former

and the latter. The capture efficiency in the BSA-blocked surface was also lower compared to the non-blocked surfaces. BSA blocking is a conventional strategy to provide selective bindings on the surface. Especially for single molecule or single EV detection systems, BSA can be applied to reduce background scattering signals coming from non-specific protein adsorption [158][159]. In this study, the wavelength decrease values were similar for blocked and non-blocked surfaces, but the release efficiency was lower in non-blocked surfaces compared to the overall increase due to the capture (such as a 0.42 nm decrease in total 0.85 nm increase). this rate increases in the blocked surface (such as 0.43 nm decrease in total 0.44 nm increase). This may occur because non-specifically adsorbed proteins can be kept on the surface without desorbing.

Lastly, the released EVs were validated with SEM, NTA and Western blot analyses. The SEM results showed that their morphology was preserved without any changes. In NTA results, the non-tagged concentration was $6.94 \times 10^7 \pm 6.31 \times 10^6$ particles/mL and it decreased to $7.09 \times 10^5 \pm 3.82 \times 10^5$ particles/mL. Compared with the microfluidic filtration chip, the achieved EV solution has enhanced purity 100 times. The presence of the CD63 protein on the released EVs was validated and confirmed with Western Blot analysis. The overall results were satisfying in detecting EVs and achieving pure EV isolation while monitoring in real-time.

This system is a proof-of-concept platform presenting some pros over the existing literature, and at the same time, requiring some improvements. The microfluidic chip utilized in this study has an extremely low cost compared to commercial lithographic chips and commercial SPR surfaces. They can be practically used without the requirements for additional training, and the detection can be repeated multiple times. Moreover, unlike commercial SPR devices with advanced setup and prism, the utilized plasmonic measurement system is a portable alternative. It is suitable for POC diagnosis and isolation. The sample taken from a patient can be analyzed directly on-site without additional device requirements. The detection, capture, and isolation analysis can be made with a low-volume sample (300-450 μ L). However, the system needs to be improved prior to clinical applications. Initially, the blocking step is essential for more selective capture and release of the EVs. BSA, PEG, or diverse blocking agents can be utilized on the surface. One critical point here is the electrical field on the surface dominated by surface plasmons decreases when the distance from the surface increases [160]. The

optimum surface chemistry needs to be assessed with the optimum distribution of the molecules on the surface. Secondly, even though the system provides enhanced purity, it decreased the concentration of the particles in the mL of sample. When the non-tagged NTA results of the microfluidic filtration chip and metasurface release were compared, the total particle concentration was decreased from $1.01 \times 10^{10} \pm 5.23 \times 10^8$ particles/mL to $6.94 \times 10^7 \pm 6.31 \times 10^6$ particles/mL. This issue can be solved by enhancing the chip's surface area by designing a channel with a higher length and width. Since the surface will saturate at a certain point during capture, the concentration will be limited with the surface area of the microfluidic chip.

As previously discussed, EVs represent compelling candidates for the early detection of various diseases, including cancer, through liquid biopsy. However, their inherent heterogeneity and structural diversity pose significant challenges in classification and isolation. Furthermore, their precise biogenesis and molecular architecture remain subjects of ongoing investigation, with novel insights continuously emerging [161]. In this study, we present an alternative method for the detection and isolation of EVs selectively. This approach holds great promise for advancing diagnostic applications and separation methodologies.

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Appendix A

Appendix

A.1 Declaration of Copyright Clearance Agreements

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