First steps towards simultaneous isolation and detection of exosomes with carbon nanotube-based SMRs

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Abstract—Exosomes are small (20-150nm) intercellular vesicles carrying valuable information regarding disease presence or evolution. Their isolation from body fluids is one of the most challenging targets. Although many techniques already exist, these are still inefficient, expensive and time-consuming. One of these new methods is nanofiltration. Within this technique, carbon nanotube (CNT) forests have been proposed as good candidates owing to their easily controllable tube separation and growth. Previous works have proven the isolation of different particles; however, optical read-out methods are still needed to confirm the success of the process. Here we propose and start studying the integration of CNT forests in solidly mounted resonators to simultaneously isolate exosomes and gravimetrically estimate the trapped amount. As a first step we prove the ability of our devices to trap and detect CD63 antibodies, due to their high affinity to exosomes. We demonstrate a frequency shift of around 280 ± 8 kHz with an antibody concentration of 8 nM, four times lower than previously reported concentrations used for the detection of antibodies with thin film acoustic resonators, for similar frequency shifts.

Keywords—carbon nanotubes, solidly mounted resonator, biosensor, antibody.

I. INTRODUCTION

Exosomes are extracellular vesicles (20-150 nm) carrying valuable information within cells. They have become important in disease diagnosis or disease evolution tracking [1]. The biggest bottleneck in exosome research are the inefficient and time-consuming standard purification methods from body fluids (e.g. ultracentrifugation [2]). The newest technologies for exosomes isolation include nanofiltration [3], or affinity capture methods [4]. Although efficient, these technologies still present disadvantages, like the use of expensive nanolithography, isolation without size discrimination below 150 nm, or the need of optical read-out means in all cases. Microfluidic devices including carbon nanotube (CNT) forests have proven to be an alternative filtering method overcoming the nanolithography need by controlling the tubes distance for specific size isolation [5]. Nevertheless, optical read-out methods are still needed and the success of the process can only be verified at the end.

Film bulk acoustic wave resonators (FBARs) have been widely investigated as highly sensitive gravimetric sensors proving mass detection in the ng or even pg ranges [6]. More

particularly, they can also be used as gravimetric biosensors by properly designing them to operate in liquid media [7], [8]. FBARs are based on piezoelectric materials, like ZnO or AlN, which are excited through a pair of electrodes giving rise to a resonant frequency directly dependent on the thickness of the piezoelectric stack [9]. FBARs can be found either on their suspended form or as solidly mounted resonators (SMR), where the piezoelectric stack lays on a Si substrate acoustically isolated from it with an acoustic reflector.

In this work we propose the combination of CNT forests with SMR biosensors for the simultaneous isolation and in-situ gravimetric detection of exosomes. While exosomes of specific sizes can be trapped by controlling the tube separation in CNT or designing appropriate nanoporous structures, the gravimetric ability of SMRs can give an accurate estimation of the mass (exosomes amount) attached to them. To enhance the trapping ability of CNT forests, the tubes should be functionalized with antibodies possessing high affinity to the targeted exosomes. As a first step towards the simultaneous isolation and gravimetric detection of exosomes we present here our initial progresses by monitoring the step where CD63 antibodies [10], specific to exosomes, are attached to the CNTs directly grown on our SMRs. Such mass attachment is translated to shifts in the SMR resonant frequency. By comparing these results with previously reported ones regarding antibodies detection with FBARs, we demonstrate the improvement of the limit of detection of the sensors explained by the surface area enhancement with CNT forest, hence being able to get similar frequency shifts with even four times less concentration of antibodies.

II. EXPERIMENTAL

A. The device

We used SMRs specifically designed for biosensing applications. These are composed of fully-insulating reflectors made of seven alternating layers of SiO_2/AlN , on which we deposited a bottom electrode/AlN with tilted grains/top electrode sandwich (Fig. 1a). In this case we used Ir and Mo as bottom and top electrodes, respectively. The piezoelectric AlN film with tilted grains was deposited following a previously described two-steps process [11] and is crucial for the excitation of the shear mode, suitable for in-liquid operation since it does not radiate acoustic energy to the liquid [12]. All the materials deposition processes were

carefully tuned so that the final structures can sustain the high temperatures needed for the ulterior integration of the CNT forests (600°C), which are directly grown on the top electrode. These SMRs have proved to sustain temperatures up to 1000°C without delamination [13].

The final devices were tested in liquid media, and for that a fluidic system was used; it consists in sealing the active area of the devices containing the CNTs, leaving the pads outside for RF testing. To ease contacting the devices outside the fluidic system the top electrodes were extended to 1.7 mm (Fig. 1b). To avoid the presence of parasitic capacitances and inductances, the bottom electrode located below the extensions of the top electrode was patterned. Therefore the fully-insulating reflector was pivotal to avoid the appearance of parasites due to the capacitive coupling of the top electrode with the last layers of the reflector [14].

Fig. 1. Device structure: (a) cross sectional sketch of the SMR, (b) optical top view of an actual device with electrical extension and integrated CNT forest. Red lines indicate the mark of the sealing gasket used in the fluidic system.

B. CNT forest integration and functionalization

The CNT forests were grown using low pressure chemical vapor deposition (CVD) [15] and a catalytic system composed of a blocking SiO_2 layer underneath a metallic catalyst (Fe). The blocking layer was crucial in this case to avoid bulk diffusion of the catalyst into the metallic top electrode substrate. The thicknesses of the catalytic system were 50 nm for the SiO_2 film and 6 nm for the Fe. The catalytic bilayers were selectively deposited over the Mo top electrodes by a photolithographic lift-off process. For the CVD process, NH_3 and C_2H_2 were used as reducing and carbon source gases, respectively. We used the same temperature, namely 600°C, for the nanoparticle formation and the CNT growth.

After their integration on the SMRs top electrode we functionalized the CNT forest using a covalent method based

on an Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N hydroxysuccinimide (NHS) chemistry to attach streptavidin to their walls, which has a strong affinity with our target exosome antibodies since they are biotinylated. All the functionalization process was monitored through the response of our resonators towards the attachment of the different bioparticles to the CNT walls. As mentioned, this mass attachment was directly translated to shifts in the resonant frequency of the SMRs.

CNT forests are inherently hydrophobic, hence the first step was to expose the samples to a low damage oxygen radical plasma treatment using a reactive-ion etching system in order to introduce chemically active carboxyl groups ($\text{COOH}-$) on their surface. For the liquid functionalization we used an activation buffer (0.2 mM EDC with 0.8 mM of NHS in a 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) solution at pH 5), a coupling buffer consisting in the previous activation buffer at pH 7.4 with the streptavidin at a 248 nM concentration, and a washing buffer consisting in a 1X phosphate buffered saline (PBS) solution with a Tween 20, a polysorbate-type nonionic surfactant at 0.05%. Here, the Tween 20 detergent acts as a blocking agent avoiding non-specific bindings. At this point the devices were ready for the CD63 antibody detection.

C. Measurement set-up

The CNT-based sensors were characterized by measuring their electrical impedance with a network analyzer using calibrated RF probes for on-wafer contacting. For the in-liquid measurements, we used a fluidic system made of PMMA that includes a PDMS chamber with a 30 μl cavity sealed around the active area of the device (Fig. 2). A peristaltic pump was used to feed the solutions into the cavity at a rate of 100 $\mu\text{l}/\text{min}$. To track the resonant frequency during the biodetection process, the real part of the admittance was fitted to a rational function in a narrow frequency interval around its maximum at the resonant frequency.

Fig. 2. Experimental set-up for biodetection.

This process was controlled with a LabView® application that acquires the data from the network analyzer, and allows assessing the frequency with less than 1 kHz accuracy with an interval of around 3 s.

III. RESULTS

Prior to the sensing test we characterized the response of the devices after the direct growth of the CNT forests on their top electrodes. In previous works we concluded the optimal forest height to be around 5 μm [16]. We found out this is the optimal tradeoff between surface area enhancement for binding sites and device performance degradation in terms of quality factor. The height was controlled by the time left for the C_2H_2 to flow inside the CVD chamber, which in this case was 3 min (Fig. 3a). By using a 6 nm-thick Fe catalyst we grew multi-walled forests, which we characterized by Raman spectroscopy (Fig. 3b), with an average tube separation of around 10-25 nm (see inset in Fig. 3a).

The electrical response of the SMR after the CNT integration is shown in Fig. 4, where the spectrum of the real part of the admittance is plotted. This peak is the one fitted and monitored during the CD63 detection. The quality factor of the device (Q) is reduced from 320 to 203, as expected with the 5 μm forest height, but the electromechanical coupling coefficient (k_{eff}^2) is maintained. When immersed in liquid the Q decreases to 150 and the frequency shifts around 50 MHz due to the losses and changes in density and viscosity of the surrounding medium.

Fig. 3. (a) SEM image of the carbon nanotubes grown on the electrode (in the inset the black bar corresponds to 30 nm); (b) Raman spectrum of the forests obtained with the SiO_2/Fe catalyst.

Fig. 4. Spectrum of the real part of the admittance. The maximum is located the resonant frequency.

To begin the functionalization process, we applied the described oxygen plasma to the CNT-based devices and subsequently mounted them inside the fluidic system. The response of the device to each step of the in-liquid process is shown in Fig. 5, where the shifts in resonant frequency are plotted against time. The sequence started with the activation buffer, which we left flowing and pre-incubating until the response of the device was stabilized, or for at least 30 min. At approximately 3000s in the graph we introduced the coupling buffer containing the previous activation buffer with diluted streptavidin at a concentration of 248 nM. Streptavidin is used in this case owing to its high affinity to biotin since the CD63 antibodies are biotinylated. The coupling buffer was left for incubation for around 2h to guarantee streptavidin was bound to all possible sites. Owing to this mass attachment, we observed frequency shifts of 100 ± 15 kHz. After the incubation, the device was thoroughly cleaned in the washing buffer with PBS and finally the CD63 antibody was introduced to the system diluted in PBS in a concentration of 8 nM. The flow was left until the shift in frequency saturated. This gave rise to a total shift in frequency due to the antibodies attachment of 280 ± 8 kHz. This result can be, for example compared to previously reported ones where they targeted human prostate-specific antigens using suspended-FBARs [17]. In that work, the authors presented shifts due to IgG antibodies (150kDa similar to the CD63 used here) of around 230 kHz with a concentration of 33 nM, four times higher than the concentration used here. This demonstrates the ability of our CNT-based SMRs to not only be able to trap and detect CD63 antibodies needed for the future specific detection of exosomes, but also to improve the sensing ability of already existing gravimetric biosensors by reducing the solution concentration needed to provide the same frequency variations.

Fig. 5. Resonant frequency evolution with time during the functionalization process of the CNT-based SMR up to the CD63 antibody detection.

CONCLUSIONS

As a first step towards the simultaneous isolation and detection of exosomes with specific size, we have proven the ability of AlN-based SMRs with integrated CNT forests to trap and detect CD63 antibodies. These antibodies will act as the subsequent binding sites of exosomes. Moreover, we have proven the enhancement in terms of limits of detection of our devices compared to FBARs without CNTs. This was demonstrated by measuring the same frequency shift for an antibody concentration of four times lower than in previous works.

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