Cancer is a complex and dynamic disease which changes quickly. One of every four deaths in the United States and Canada is due to cancer. Thus, the early diagnosis of cancer is of much significance because the chances of survival of patients would increase. This is visible only if proper methods and devices of identification of biomarkers or targets are developed. In general, the cancer diagnosis is carried out, through imaging, endoscopy and tissue biopsy. Currently, focus of the researchers is toward the liquid biopsy for clinical trials.

Liquid biopsy is a minimally invasive technique in which sampling of a biological fluid is carried out in order to detect the cell derived material, indicating the presence of an underlying pathology such as cancer or other diseases. The interesting key feature of liquid biopsy is that it enables the monitoring of the diseases over the entire time line, from healthy to advanced stages. During the last few years, the role of exosomes in cancer diagnosis is becoming predominant and promising due to their usefulness as valuable sources of material for research. It is well-known that the structure and the plasmonic properties of noble metal nanoparticles can be customized for specific applications such as biosensing, diagnosis, imaging, etc. by tuning the size and shape of the nanoparticles. Thus, by using the plasmonic property of noble metals, the detection at the nano-scale is possible by monitoring the shift of Localized Surface Plasmon Resonance (LSPR) band with respect to the changes in the dielectric constant of the surrounding medium. The present study is aimed to the evaluation of the quality and performance of two nano-plasmonic platforms for detection and capture of exosomes.

Over the past few decades, fabrication of optimal nanostructures for detecting specific bio-entities remained an active area of research. There is no specific methodology to isolate and detect them. Currently, the exact science behind the major standard techniques of isolation or targets are developed. In general the cancer diagnosis is carried out, through imaging, endoscopy and tissue biopsy. Currently, focus of the researchers is toward the liquid biopsy for clinical trials.

Exosomes are nanoscale heterogeneous vesicles in the size range of 30–100 nm, which are released by cells as shown in Figure 1. These vesicles play a significant role in intercellular communications, and transport of proteins, RNA, and other molecular information. In the last decade, researchers have shown substantial interest in this field as there is no specific methodology to isolate and detect them. Currently, the exact science behind the major standard techniques of isolation of exosomes is not clearly understood. Thus, the limitations due to low yield and poor quality of exosomes may compromise further the molecular analysis for diagnosis. The ultracentrifugation method of isolation of exosomes is time consuming, laborious, infrastructure intensive and it may lack specificity. Therefore, a lot of challenges exist in this field in order to develop next generation affinity-based technologies to capture the exosomes selectively and use them for further diagnosis at the clinical level.

In the present work, two different sensing platforms, developed in our laboratory for the detection of exosomes, are compared. The two platforms are the ex-situ gold (Au) nano-islands on glass substrates and the in-situ prepared silver (Ag) - polydimethylsiloxane (PDMS) nano-composite. The first platform is fabricated by depositing colloidal Au nanoparticles on a glass substrate by the thermal convection method, followed by morphological tuning of the formed nanoparticles to nano-islands by annealing at 560 °C. The second is the nano-composite platform prepared by the in-situ reduction of Ag ions in the silver nitrate solution by the curing agent of the PDMS polymer. This study is carried out in order to choose the most sensitive platforms to detect and capture the nano-sized exosomes. The exosomes are rich sources of associated biomarkers, containing specific proteins, mRNA, miRNA, tRNA, DNA and thus having the potential for early cancer detection along with the information on the tissue and cell of origin.

Given the growing evidence that EVs/exosomes may be a clinically-relevant biomarker source, there is a great demand for simple and efficient EV isolation from bio fluids. Currently, the gold standard, the time-consuming method of isolation by ultracentrifugation is not a clinically-viable method. Furthermore, polymer-based EV isolation methods are not suitable for therapeutic applications because these polymers are toxic. Most affinity-based EV-isolation methods rely on antibodies directed against EV surface marker(s).

In both the platforms developed in our laboratory, the detection and capture of exosomes is based on the strong affinity of heat shock proteins contained in exosomes to a polypeptide called Venceremir or Vn96 (27 amino acids), specifically developed and validated. The Vn96 peptide targets the canonical heat shock proteins (HSPs) present on the surface of exosomes. The Vn96 based exosome detection and capture method is further validated for downstream analyses, clinical compatibility, and liquid biopsy assays (biomarker and mutation detection) and platform versatility, using cell-culture conditioned media and human body fluids as sources of exosomes. Overall, Vn96 provides multiple advantages over currently available affinity-capture methods for EVs/exosomes isolation: the scalability, quality, platform versatility, and cost-effectiveness.

By using the Au-nano-islands platform, a biotinylated Vn96 peptide is bounded onto the streptavidin coated Au nano-islands, and the subsequent steps of binding of nano-sized vesicles (exosomes) are monitored through the Localized Surface Plasmon Resonance (LSPR) band of Au nanoparticles. The Ag-PDMS platform is used in a similar way and the shift of the Ag LSPR band is monitored after each sensing step. Generally, the magnitude of the LSPR shift depends on the size and shape of the nanoparticles and on the dielectric constant (refractive index) of the surrounding environment. Following a binding event, the refractive index will change and the change will trigger a shift in the position of the LSPR band.
the shift reflects the strength of the interaction and it is proportional to the concentration of the analyte, in this case the exosomes content in the cell culture. The platform based on gold islands is suitable for sensing of various peptides and proteins by using the corresponding antibodies.5–7

It is found that the results of the sensing process depend on the two major things: the molar ratios of streptavidin to biotin-PEG-Vn96 and the final step, the capture of exosomes by the biotin-PEG-Vn96 complex. The morphology of Au nano-islands and Ag-PDMS nanocomposite were further investigated by SEM and the LSPR techniques.

Experimental

Materials.—Gold (III) chloride trihydrate (HAuCl₃·3H₂O), Sodium citrate, Silver nitrate, 11-mercaptoundecanoic acid in ethanol (Nano Thinks Acid 11), phosphate buffered saline (PBS), N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-hydroxy succinimide (NHS) were obtained from Sigma-Aldrich, Canada. Sylgard 184 elastomer kit and curing agent for the PDMS fabrication purchased from Dow Corning. De-ionized (DI) water with a resistivity of 18MΩ is used obtained from the NANO pure ultrapure water system (Barnstead). Streptavidin is from IBA GmBH, Biotin-PEG-Vn96 is from New England Peptide and the MCF7 exosomes is from the Atlantic Cancer Research Institute (ACRI), Moncton, Canada.

Fabrication of the platform by ex-situ synthesis.—In the ex-situ synthesis, Au nanoparticles were deposited on a glass substrate by the convective assembly of Au nanoparticles from an Au colloidal solution. The colloidal Au solution was prepared by using the Turkevich method.17 During the evaporation process, the Au nanoparticles present in the colloidal suspension are deposited at the interface of the substrate and solution resulting in multi-layers of Au structures. The thickness of the layer depends on the concentration of Au particles, their diameter, and the interaction forces between the particles and the glass surface. The synthesis, as well as the convective assembly is shown in Figure 2a. By varying the size of nanoparticles and their morphology, the peak resonance wavelength of the Au LSPR band

Figure 1. Biogenesis and Release of Extracellular Vesicles (EVs).

Figure 2. a. Schematic of the convective assembly for the Au nanoparticle synthesis and the multi-layers formation (ex-situ synthesis); b. Schematic of the fabrication of Ag nanocomposite (in-situ synthesis). c. Schematic of nano-islands on glass substrate d. Schematic of Ag nano particles on the polymer (PDMS) surface.
can be shifted to other wavelengths when the refractive index of the surrounding environment is changed.

During each step of the sensing protocol, the local refractive index changes due to the binding of various bio molecules to the immobilized nanoparticles and result in a shift toward longer wavelengths. The Au nano-island structures were fabricated by annealing the deposited Au multilayers at 560°C for an hour.

Fabrication of the platform by in-situ synthesis.—Nanocomposites seem to be alternative and promising substrates for label-free biosensing.7,8 In this process Ag ions from the silver nitrate aqueous solution are reduced by the curing agent present in the polydimethylsiloxane (PDMS) matrix, a good reducing agent of Ag ions in order to form the Ag-PDMS nanocomposite as shown in the Equation 1.

As shown in Figure 2b the first step is the fabrication of the PDMS substrate. The substrate is prepared by mixing the PDMS base (prepolymer) and curing agent in a ratio of 3:1 by weight and then spin coated on a glass substrate of the 25mm × 25mm × 1mm, using the LAUREL spin coating machine at a speed of 2000 rpm, to obtain a thickness of around 10 μm. After the spin-coating, the polymer is cured at 70°C, for about 2 hours. The cured substrates were then immersed in the Ag precursor solution, prepared by adding 1g of silver nitrate into 100 ml of DI water. The immersed substrates were kept in an oven at 50–60°C, for about 2 days in order to increase the rate of the reduction reaction. The synthesized platforms were annealed at 250°C, for 10 minutes in order to tune the morphology of Ag aggregates to nanoparticles.

\[ R−Si−H+AgNO_3 \rightarrow Si−O−Si±Ag+N_2O+H_2O \]  

**Results and Discussion**

Morphological tuning and SEM characterization.—To tune the morphology of the ex-situ synthesized Au nanoparticles, the platform was annealed at 560°C for an hour. The scanning electron microscopy (SEM) images of the Au structures, before and after annealing are shown in Figure 4. It is visible that before annealing, the nanoparticles appear as aggregates, with several layers of Au one above the other, due to the strong attractive van der Waals forces between Au and 0.05M of N-hydroxy succinimide (NHS), an additive used for the improved amidasations and peptide/protein couplings. The substrate is incubated for about 3 hours. The streptavidin-biotin complex is the strongest known noncovalent complex (Kd = 10^-15 mol/L). The bonding is very rapid and the bond is unaffected by extreme values of pH, temperature, organic solvents and other denaturing agents. Streptavidin (0.01mg/ml) is immobilized on to the activated linker layer and incubated for an hour. Further, the Biotin-PEG-Vn96 (0.003 mg/ml) is immobilized on the streptavidin layer and then incubated again for about 4 hours. In the last step of the bio sensing protocol, the MCF7 (breast cancer cell line) cell culture conditioned media, containing EVs/exosomes is immobilized onto the Vn96 layer, a synthetic peptide designed to capture them.

The biosensing protocol for the case Ag-PDMS nanocomposite platform is similar to that of Au nano-islands platform until the streptavidin stage, the only difference is in the concentration of Biotin-PEG-Vn96 (0.015 mg/ml).

**Biosensing protocol.—**The various steps involved in the biosensing protocol for both platforms are shown in Figures 3a and 3b. In the case of the Au nano-islands platform, initially the Au nano-islands were functionalized by immersing the substrate into the linker solution, which is Nanothink (11-mercaptooundecanoic acid in ethanol) in order to form a self-assembled monolayer on the top of Au nano-islands. In the next step, the structure is activated by adding a few microliters of the cross-linker which is a 1:1 mixture of 0.1M N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) - a carboxyl activating agent for amide bonding with primary amines.

Figure 4. SEM images of Au nano structure a) before annealing; b) after annealing (560°C for 1 hour).
Figure 5. SEM image of Ag-PDMS a) before annealing; b) after annealing (250°C for 10 minute).

Figure 6. Absorption spectra corresponding to each stage of the sensing protocol (ex-situ synthesis).

Figure 7. Absorption Spectra corresponding to each stage of the sensing protocol (in-situ synthesis).

Detection of exosomes.— The Au-LSPR absorbance spectrum for the ex-situ platform is recorded at each stage of the biosensing protocol: after the functionalization of the nano-islands with the linker, followed by the cross linker, the streptavidin, and biotin-PEG-Vn96 using a lambda 650 UV-visible spectrophotometer. The spectra show a shift of the Au-LSPR band of around 6 nm upon the interaction of biotin-PEG-Vn96 and exosomes (MCF7) as shown in Figure 6. Similarly, for the in-situ synthesized platform the Ag-PDMS LSPR band is recorded at every stage of the biosensing protocol. In this case a shift of 4 nm is noticed, between the Biotin-PEG-Vn96 and exosomes (MCF7) stage as shown in Figure 7.

Substrates characterization using confocal microscopy.— In order to determine whether the roughness of substrates play any role in the sensitivity of the platforms, measurements were carried out by using an Olympus LEXT OLS 4100 laser scanning digital microscope. Further, it can be seen in Table I that, in the case of ex-situ synthesized Au nano-island substrates, the roughness value (Ra & Sa) increases considerably after the immobilization of the chemicals and biomolecules, whereas, in the case of the in-situ synthesized Ag platform, the roughness (Ra & Sa) does not change significantly. This indicates that, in the case of ex-situ synthesized Au platform, the number of Au nano-islands present on the substrate is considerably larger as shown in Figures 8a–8b, than in the case of the in-situ synthesized Ag platform where the number of Ag nanoparticles is low as shown in Figures 8e–8h. Thus, the average LSPR shift in the case of ex-situ Au platform varies considerably with the concentration of exosome nanoparticles and the glass surface, whereas the annealed samples show well separated nano-island structures with an average diameter of islands of around 110 nm. Because of the size of the exosomes, this size is adequate for sensing taking into account the depth of penetration of the plasmonic field.

Table I. Average line roughness for both the sensing platforms, before and after the immobilization of analytes.

| Sample Description                | Horizontal Ra [μm] | Vertical Ra [μm] | Roughness Sa [μm] |
|-----------------------------------|--------------------|-----------------|-------------------|
| Au nano-islands substrate         | 0.443              | 0.324           | 0.38              |
| Au nano-islands substrate +       | 2.671              | 0.965           | 3.68              |
| + Biosensing analytes             |                    |                 |                   |
| Ag-PDMS substrate                 | 0.248              | 0.206           | 0.291             |
| Ag-PDMS substrate +               | 0.290              | 0.192           | 0.294             |
| + Biosensing analytes             |                    |                 |                   |

nanoparticles and the glass surface, whereas the annealed samples show well separated nano-island structures with an average diameter of islands of around 110 nm. Because of the size of the exosomes, this size is adequate for sensing taking into account the depth of penetration of the plasmonic field.
but the in-situ synthesized Ag platform is not that sensitive. Most of the Ag NPs are completely embedded in the PDMS surface.

**Dependency of the LSPR shift on the concentration of exosomes.**—In order to quantify the exosomes (MCF7) by using the two platforms, five experiments were carried out for each dilution factor (D) (1x, 5x, 10x). The concentration of exosomes/particles present in the cell culture is of the order of 1.33 \times 10^{10} \text{ per ml}. In the present study ‘1x’ denotes the undiluted solution. It is clear from Figure 9, that the sensitivity of the Au platform is higher than that of the Ag one because of the availability of Au nano-islands (ex-situ) on the surface of substrate, (Figure 2c). As noted before, in the case of the in-situ synthesis platform, Ag is embedded in the sub-surface layer, and thus the nanoparticles are not exposed to the surrounding medium as shown in Figure 2d. Further it can be seen that the slope of the graph in the case of Au nano-island platform is larger, because this platform ‘feels’ much more the change of the refractive index with the concentration of exosomes than the embedded Ag.

**Conclusions**

It is found that the refractive index sensitivity of the ex-situ synthesized Au nano platform is considerably higher than that of the in-situ synthesized Ag-PDMS nanocomposite and consequently, this platform is much more performant for sensing of exosomes. Two principal reasons were identified in order to account for this difference. It is thought that, because of the low temperature annealing of Ag-PDMS, contrary to the Au nano-islands, a non-suitable morphology is formed. It has been demonstrated that nano-island structures have a higher sensitivity due to their morphological characteristics. On the other hand, due to the in-situ formation mechanism, a large proportion of the surface Ag particles will diffuse inside the polymer, sub-surface and, hence they will not be available anymore for sensing. The studies carried out on various platforms so far show that a label-free LSPR sensing method can be used for detection of EVs (Exosomes) by using the Vn96 peptide for capturing them through their interaction with the proteins on their surface.

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**References**

1. Canadian Cancer Society’s Advisory Committee on Cancer Statistics. Canadian Cancer Statistics 2017. Toronto, ON: Canadian Cancer Society; 2017. Available at: cancer.ca/Canadian-Cancer-Statistics-2017-EN.pdf.
2. S. EL. Andaloussi, I. Mager, X. O. Breakefield, and M. J. Wood, Nat. Rev. Drug Discov., 12, 347 (2013).
3. E. Van der Pol, A. N. Boing, P. Harrison, A. Sturk, and R. Nieuwland, *Pharmacol. Rev.*, **64**, 676 (2012).
4. C. Thery, S. Amigorena, G. Raposo, and A. Clayton, *Curr Protoc Cell Biol.*, Chapter 3: Unit 3, 22 (2006).
5. J. Ozhikandathil, S. Badilescu, and M. Packirisamy, *Journal of Biomedical Optics.*, **17**(7) (2012).
6. J. Ozhikandathil and M. Packirisamy, *Sensors.*, **14**(6), 10497 (2014).
7. H. SadAbadi, S. Badilescu, M. Packirisamy, and R. Wuthrich, * Biosensors and Bioelectronics.*, **44**, 77 (2013).
8. P. Devi, A.Y. Mahmoud, S. Badilescu, M. Packirisamy, P. Jeevanandam, and V.V. Truong, pp. 1, *The First International Conference on Biosciences* (2010).
9. A. Ghosh, M. Davey, I.C. Chute et al. *PLoS ONE* **9**(10), e110443 (2014)
10. N. Nath and A. Chilkoti, *Anal. Chem.*, **76**(18), 5370 (2004).
11. A. L. Weikel, S. D. Conklin, and J. N. Richardson, *Sens. Actuators B: Chem.*, **110**(1), 112 (2005).
12. F. Frederix et al, *Anal. Chem.*, **75**(24), 6894 (2003).
13. K. Fujiwara et al. *Anal. Bioanal. Chem.*, **386**(3), 639 (2006).
14. M. D. Malinsky et al. *J. Am. Chem. Soc.*, **123**(7), 1471 (2001).
15. L. Guo, G. Chen, and D. H. Kim, *Anal. Chem.*, **82**(12), 5147 (2010).
16. F. Toderas et al. *Nanotechnology.*, **18**(25), 255702 (2007).
17. J. Turkevich, P. C. Stevenson, and J. Hillier, *Discuss. Faraday Soc.*, **11**(0), 55 (1951).
18. J. J. Diao et al. *J. Phys. D.*, **36**(3), L25 (2003).
19. S. Bathini et al., *Research*, **2018**, 1–10 (2018).