

High-Q whispering-gallery mode sensor in liquids

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ABSTRACT

Optical sensing of biomolecules on microfabricated glass surfaces requires surface coatings that minimize nonspecific binding while preserving the optical properties of the sensor. Microspheres with whispering-gallery (WG) modes can achieve quality factor (Q) levels many orders of magnitude greater than those of other WG-based microsensors: greater than 10^{10} in air, and greater than 10^9 in a variety of solvents, including methanol, H₂O and phosphate buffered saline (PBS). The presence of dyes that absorb in the wavelength of the WG excitation in the evanescent zone can cause this Q value to drop by almost 3 orders of magnitude. Silanization of the surface with mercapto-terminal silanes is compatible with high Q ($>10^9$), but chemical cross-linking of streptavidin reduces the Q to 10^5 - 10^6 due to build-up of a thick, irregular layer of protein. However, linkage of biotin to the silane terminus preserves the Q at a $\sim 2 \times 10^7$ and yields a reactive surface sensitive to avidin-containing ligands in a concentration-dependent manner. Improvements in the reliability of the surface chemistry show promise for construction of an ultrasensitive biosensor.

Keywords: whispering-gallery mode; microresonator; streptavidin-biotin; Q factor; evanescent wave; biosensor

1. INTRODUCTION

1.1 Background

Optical biosensors are typically transducers that detect the presence of molecules at a surface. They have several desirable features, particularly for the detection of biological molecules: (1) They can be extremely sensitive (nanomoles or less); (2) they are non-destructive to the sample; (3) the transduction processes in optical biosensors generally take place on a surface and can be tailored to sense almost any kind of molecule, chemical and pre-biotic as well as biological.

Among the most sensitive class of biosensors are the evanescent wave sensors [Lukosz, 1995 #21] [Schult, 1999 #16], in which an evanescent wave produced by the total reflection of light within the waveguide interacts with analytes on the waveguide surface. The evanescent wave protrudes above the waveguide surface by ~ 100 nm (the actual distance depends on the relative index of refraction of the waveguide and the sample medium) and samples only analyte on the surface. Surface treatments such as antibodies or oligonucleotide strands can provide specificity for the analyte; the sensor then detects only that bound to the surface. Transduction mechanisms for bound analyte include fluorescence, mass change in the evanescent region [Foster, 1995 #30], and change in index of refraction [Weisser, 1999 #20]. Typical sensitivity of evanescent wave biosensors based on fiber optic sensors or planar waveguide sensors is in the range of nM to pM.

1.2 Very high Q microspheres

Whispering gallery modes of microspherical and other micro-resonators are known to have extremely high quality factors (Q) in the optical domain [Gorodetsky, 1996 #24]. Microspherical (50-500 μ m diameter) glass resonators with Q's in excess of 10^{10} have been demonstrated, and Q's as high as 10^{11} are, in principle, possible [Vernooy, 1998 #23]. The very high quality factors Q of microspheres may be attributed to several factors. One is that the dielectric materials for microspheres, fiber grade fused silica, have ultra-low optical loss at the frequencies of the supported whispering gallery modes. Another is that the surface of the sphere is specially fabricated to minimize the size of any surface inhomogeneities, e.g., on the order of a few \AA by processes such as fire polishing; these can scatter photons out of the WG mode and decrease the Q [Vernooy, 1998 #23]. Perhaps mostly important, microspheres have curved

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circumferential edges above and below their equators to provide a two-dimensional confinement to the optical energy in a WGM for grazing reflection of all wave vector components. This grazing incidence in a sphere is essential for minimizing surface scattering that would otherwise limit the Q far below that imposed by attenuation in the material. For example, there are other WG mode planar waveguide microcavity geometries, optical micro-ring and micro-disk, formed from cylinders with straight sidewalls. As a result, the light is confined only by a curved geometry along one dimension and effectively bounces from flat surfaces under *finite* angles. Hence, typical Q-factor of such cavities is limited to about $10^4 \sim 10^5$ [Kuwatagonokami, 1995 #28] [Blair, 2001 #25].

The basic detection scheme is that binding of molecules to the micro-resonator surface induces an optical change proportional to the amount of bound molecules. The paradigm for this process is a change in the cavity Q as the surface bound molecules affect the photon storage time either through increased scattering or increased absorption. In effect, the analyte spoils the Q and we measure that change.

However, any protein will adhere to glass, and hence fire-polished spheres are entirely nonspecific. Two conditions must be met for chemical modification of the microsphere surface: first, the glass must be coated with a compound that will minimize nonspecific binding. Second, an antibody or other protein with sensitivity to a particular ligand must be linked to the sphere in such a way that both the protein's functionality and the sphere's Q are preserved.

A thin film of a material with thickness smaller than the WG mode's evanescent field will not significantly alter the Q of the micro-resonator, thus, a thickness of ~ 10 -100 nm can be applied to the microsphere while retaining its high Q. Silane +biotin can result in layers < 3 nm thick [Weisser, 1999 #20]. For larger proteins, such as streptavidin and antibodies, the shape of the protein and its distribution on the microsphere surface may be as important as its molecular weight [Pearson, 1998 #29], and empirical measurements of coating density vs. Q are required to optimize the sensor surface.

We can increase the sensitivity of the sensor by using a detection scheme in which the molecule to be detected absorbs at the laser wavelength used to excite the WG mode. If the wavelength of the WG mode overlaps the absorption spectrum of the probe, the cavity photon losses increase even more as the probe fluoresces and commensurately increases the sensor sensitivity. With a WG of 670 nm, the fluorophores Cy5.5 or Alexa 680 are ideal.

In preliminary experiments, we were able to obtain 8-10% surface coverage of streptavidin (SA) and biotin onto fire-polished and silanized spheres. Even this small amount of SA reduced Q levels to 10^6 - 10^5 , rendering the sensor essentially useless for monitoring changes in Q. However, the same amount of biotin allowed for $Q > 10^7$, and binding of SA-Alexa 680 resulted in a concentration-dependent reduction in Q. Further improvements in the experimental setup and the surface chemistry will allow this sensor design to be optimized.

2. THEORY

2.1 Detection limits and kinetics

The effect of Q attenuation by the absorption in an immersion liquid can be described as follows:

$$Q^{-1} = Q_0^{-1} + k_{WG} \frac{\alpha \lambda}{2\pi n} = Q_0^{-1} + k_{WG} \frac{\lambda}{2\pi n} \epsilon C \quad , \quad (1)$$

where Q_0 is the intrinsic Q factor of the resonator immersed in pure solvent; α is the linear attenuation for a plane wave propagating in the solution; ϵ is the molar extinction coefficient of the analyte at measurement wavelength λ ; C is the molar concentration of the solution, and k_{WG} is a dimensionless coefficient that accounts for the fraction of the luminous energy flux of the whispering-gallery mode circulating outside the solid dielectric in the form of an evanescent field.

The value of k_{WG} depends significantly on both the dimension of the resonator and on the difference between the refraction index of the resonator, n_{res} , and of the immersion liquid, n_{sol} . As the refraction contrast decreases, the evanescent field extends further into the fluid, and k_{WG} changes from roughly 0.001 in air for typical few hundred micron diameter resonators, to 0.01 and more when resonator is immersed in solvents. Fig. 1 presents the results of calculations of k_{WG} for several diameters of silica resonators immersed in fluid of different refraction indices.

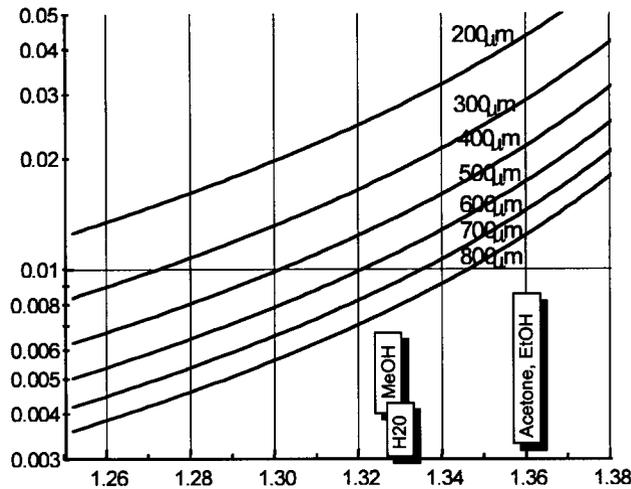


Figure 1. Dimensionless inclusion coefficient k_{WG} vs. index of refraction n for microspheres of different diameters (200 – 800 μm) immersed in solvent. Based on analytical estimates published in [Savchenkov, 2000 #31].

An upper sensitivity limit can be estimated by calculating the point at which sensor's Q has fallen to 1/2 of its initial value. For typical conditions, this occurs at a local concentration of $\sim 10 \mu\text{M}$ for initial $Q_0 = 10^7$, and $0.1 \mu\text{M}$ for $Q_0 = 10^9$ (Fig. 2).

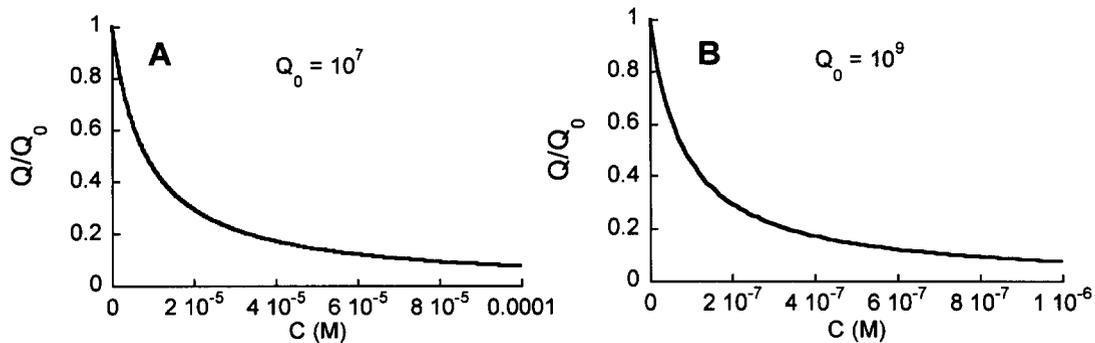


Figure 2. Estimates of Q attenuation with analyte concentration for (A) $Q_0 = 10^7$, and (B) $Q_0 = 10^9$. Parameters assumed were $k_{WG} = 0.01$ and $n = 1.32$ for aqueous solution, $\lambda = 670 \text{ nm}$, and $\epsilon = 150,000 \text{ M}^{-1}\text{cm}^{-1}$ (Alexa 680).

The effective local concentration at the sphere will depend upon diffusion or mass transport rates in the solution as well as on binding probability. The binding constant is very high for strong chemical reactions such as streptavidin-biotin, and thus the concentration on the sphere will essentially be a function of the number of active molecules present at the surface.

The surface area of an average sphere is $\sim 10^{13} \text{ \AA}^2$. Close-packed SA will result in approximately one molecule/3000 \AA^2 , or 3×10^9 SAs per sphere. AFM studies showed approximately 10% surface coverage, implying a total of at most 3×10^8 molecules available for binding.

It is unlikely that a large biotinylated molecule will bind at more than a 1:1 ratio with SA; thus 3×10^8 molecules will saturate the sensor. The time and concentration required to achieve this depends on whether the flow is diffusion-limited or mass transport limited (see next section).

Conversely, biotin packs densely at 1 molecule/40 \AA^2 , although this is too high to achieve maximum SA binding. The

ideal density in other studies has been shown to be ~ 1 biotin/ 140 \AA^2 ; our preliminary work has yielded again $\sim 10\%$ coverage, so that a biotin-bound sensor will be saturated at between 10^9 and 10^{10} molecules of bound ligand.

2.2 Finite volume stirred cell

If the sphere is immersed in a finite volume at low concentration, depletion of the ligand reservoir becomes significant. Thus, the simplest model for the dimensionless fraction of the surface bound, θ , is given by the differential equation

$$\frac{d\theta}{dt} = k_a C(t)(1 - \theta) \quad , \quad (2)$$

with

$$C(t) = C_{bulk} - C_{sphere} \frac{A}{V} \theta \quad , \quad (3)$$

where C_{sphere} is the concentration of binding sites available on the coated sphere (mol/m^2), adjusted for the ratio of the sphere area to solution volume A/V , and k_a is the binding constant. This quadratic differential equation has a solution of the form

$$\tau = \frac{-1}{\left|1 - \frac{C_{sphere}A}{C_{bulk}V}\right|} \ln \left(\frac{1 - \theta}{1 - \frac{C_{sphere}A}{C_{bulk}V} \theta} \right) \rightarrow -\ln(1 - \theta) \text{ as } \frac{C_{sphere}A}{C_{bulk}V} \rightarrow 0 \quad , \quad (4)$$

where τ is a dimensionless parameter $\tau = k_a C(0)t$.

Experimentally, depletion will be negligible unless $C_{sphere}A > C_{bulk}V$. For example, 2 mL of a 10 fM solution contains $\sim 10^7$ molecules, and 2 mL of a 10 pM solution contains $\sim 10^{10}$ molecules. If there are 10^{10} binding sites available on the sphere, $C_{sphere}A/C_{bulk}V = 10^3$ for the fM solution and 1 for the pM solution.

This leads to two important differences between stirred-cell data and flow-cell data, especially at longer times. First, the binding will rapidly saturate in a finite cell, so that the number of total sites bound does not exceed the number of particles in the bulk. Second, binding sites will remain on the sphere, and may be filled by exposure to fresh solutions.

2.3 Unstirred cell

In a diffusion limited system, with depletion, the diffusion rate $d_0 = (D/\pi t)^{1/2}$ replaces k_a in Eq. (2) above. This leads to an identical equation (4), except in this case the dimensionless parameter τ is proportional to the square root of time:

$$\tau = \sqrt{\frac{4D\tau}{\pi} \frac{V}{A}} \quad , \quad (5)$$

where the diffusion constant D has the units m^2/s . For solutions more dilute than ~ 1 nM, no appreciable binding will be seen after any practical experimental time (Fig. 3). For the short time periods we consider (~ 10 min), the unstirred solution binds a significant amount more quickly than the stirred solution (Fig. 3), but this binding represents only $\sim 4\%$ of the sphere surface.

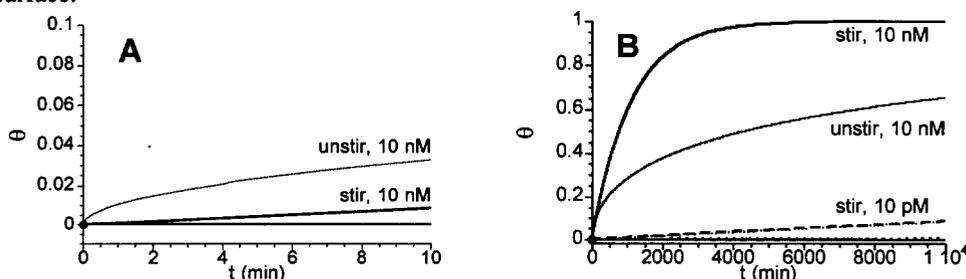


Figure 3. Coverage vs. time for biotin-coated spheres immersed in SA solutions of given concentration. Parameters assumed: surface binding sites available, 10^9 ; diffusion coefficient $D = 6 \times 10^{-7} \text{ cm}^2/\text{s}$; volume of cell = 2 mL; size of sphere = 500 μm ; reaction

coefficient $k = 7 \times 10^4 \text{ m}^3/\text{mol}\cdot\text{s}$ [Balgi, 1995 #32]. (A) Short time scale. A 10 nM solution will bind only ~4% of surface area in 10 min. In these very short times, the diffusion-limited case is faster because it assumes immediate depletion of the diffusion radius. (B) Longer time scale. The differences between the stirred and unstirred kinetics begin to be noticeable only after hundreds of hours.

The effective concentration within the evanescent field (Eq. 1) may be related simply to the fraction of surface coverage fraction of surface coverage by:

$$c [\text{M}] = \theta N_{total}/N_A V_{mode}, \quad (6)$$

where N_{total} is the number of binding sites, N_A is Avogadro's number, and the volume of the evanescent field V_{mode} is approximately 10^{-10} L for a $600 \mu\text{m}$ sphere and 10^{-12} L for a $100 \mu\text{m}$ sphere. In Fig. 4, it is shown that a 1 nM analyte solution can be quickly resolved by the smaller sphere, whereas with the larger sphere, hundreds of minutes are required to obtain a significant decay in Q.

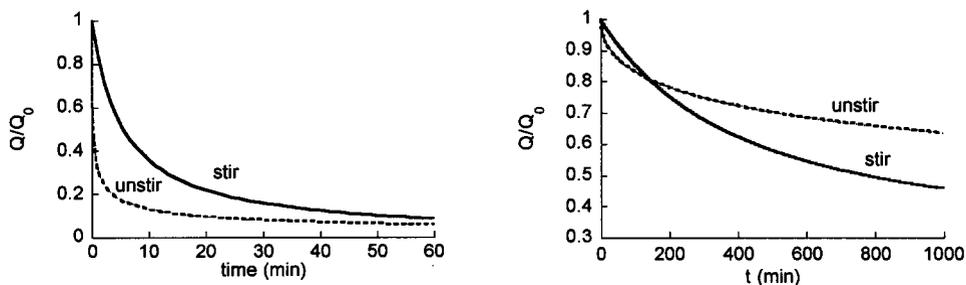


Figure 4. Time course of Q change for (A) a $100 \mu\text{m}$ diameter sphere and (B) a $600 \mu\text{m}$ diameter sphere in a 1 nM solution of Alexa 680. Both stirred and unstirred cells are shown, as in Fig. 3.

3. METHODOLOGY

3.1 Silanization

Spheres were fire-polished prior to coating. Polished spheres were immersed in 3 % mercaptopropyltrimethoxy silane (MPTS) in methanol for 30-40 min under a N_2 atmosphere. They were then removed into room air and washed 3-4 times with methanol. Cross-linking was carried out immediately with 20 mM GMBS in ethanol for 60 ± 5 min. After 3-4 washes in filtered phosphate-buffered saline (PBS, pH 7.5), the spheres were dipped in a solution of the protein to be conjugated, 10 mg/mL in PBS, for 1-1.5 hr. A final PBS rinse and overnight soak in large volumes of clean PBS preceded all experiments.

3.2 Fluorescent labeling

Fire-polished, uncoated spheres and streptavidin-coated spheres were immersed in $1 \mu\text{M}$ fluorescein-biotin (Molecular Probes) for 10 min, then rinsed thoroughly with PBS. Fluorescent imaging of the spheres was performed using a Nikon $10\times$ Plan Fluor (N.A. = 0.3).

3.3 AFM studies

A PTrak3 atomic force microscope by Pacific Scanning (Pasadena, CA) was used to study the surfaces of blank and coated microspheres and planar slides.

3.4 Laser

4. RESULTS

4.1 Nonspecific sensing

Our first experiment to demonstrate a microsphere sensor was in a liquid sample. The approach was to sense an absorbing analyte that would spoil the microsphere cavity Q over and above that caused by any surface effects. We chose a Molecular Probes dye, TO-PRO-3, which absorbs between $\sim 500\text{-}700$ nm, as shown in Fig. 5. The excitation source was a tunable 670 nm diode laser, which is just at the tail end of the absorption.

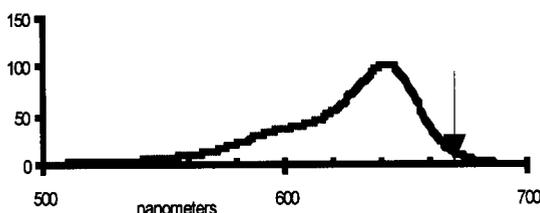


Figure 5 Absorption spectrum of dye used in microsphere sensor experiments. The arrow shows the location of the laser wavelength used for measuring Q.

After fabrication, the microsphere Q was measured in air to be $\sim 3 \times 10^9$. After immersion in methanol, a solvent for TO-PRO-3, the Q dropped to $\sim 1.5 \times 10^9$. The cavity Q when immersed in pure methanol is the sensitivity floor for the sensor, as we cannot measure changes in Q below that limit. Cavity Q was measured at a variety of dye concentrations, as shown in Fig. 6. Two techniques were used to measure the Q, depending on the Q value. For $Q > \sim 10^8$, the cavity ringdown time was measured to obtain Q, while for values less than this, the tunable laser swept through the microsphere line width.

As Fig. 6 indicates, there were two separate experiments with two spheres. The data overlap demonstrates the reproducibility of the measurements. In this case, we are relying on absorption at the laser wavelength to affect the cavity Q. Note that, according to Fig. 5, the absorption at 670 nm is only $\sim 12\%$ of the peak absorption; changing to a shorter wavelength could increase the sensitivity by about an order of magnitude.

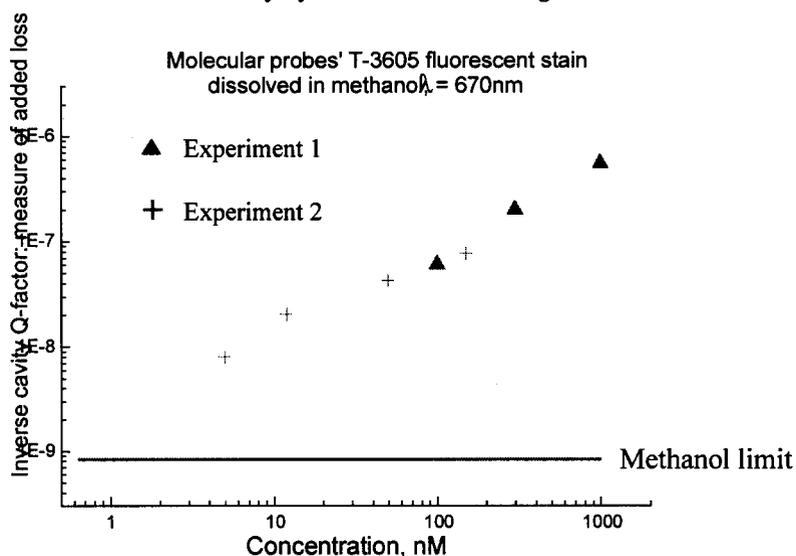


Figure 6. Plot of microsphere Q against analyte concentration.

4.2 Visualization of resonant modes

A fresh uncoated sphere was immersed in a micromolar solution of Cy-5. A fiber coupled laser diode emitting at 635 nm was used for WG excitation via a fiber coupler. The sphere was observed through a microscope equipped with a CCD camera. An interference filter centered at 670 nm was used to block the excitation light. Fluorescence is excited in the evanescent zone around the resonator where the light of the resonant mode interacts with environment. Several modes are excited simultaneously as the laser source has broad emission bandwidth. Interference of these modes on the resonator surface creates a Lissajous pattern visible in Fig. 7.



Figure 7. Photo of a 400 μm silica sphere with several whispering gallery modes excited around its equator.

4.3 Q with streptavidin coating

SA coating introduces surface structure which degrades the quality factor of the resonator. In our experiments the best Q of the SA coated spheres was 6.6×10^6 . However, on average only one of three streptavidin-coated spheres exhibited $Q > 10^6$ (Fig. 8 A), with results varying from batch to batch and within a batch. This forced us to reverse our chemistry by linking biotin to spheres and exposing them to dilution series of streptavidin.

4.4 Q with biotin coating

Coating of spheres with biotin yields more consistent sensors. Initial Q factors (in PBS, prior to exposure to SA) are in low 10^7 range with 90% repeatability. Examination of most spheres reveals surface coverage similar to that in the SA case, $\sim 8\text{-}10\%$ (Fig 8 B). However, in 20-30% of cases, the reaction is very inefficient and essentially no biotin is present on the sphere's surface. This is easily determined by any of several experiments (see below).

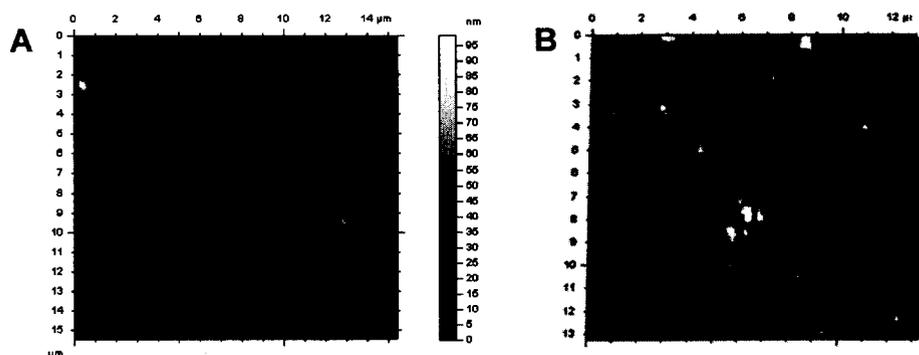


Figure 8. (A) AFM image of the highest Q SA-coated sphere ($\sim 6 \times 10^6$). Surface coverage $\sim 9\%$. Scale is given in μm . Most SA-coated spheres have large aggregates which degrade resonator performance through scattering ($Q \sim 10^5$).

(B) AFM image of a typical “successful” biotin-coated sphere (success rate $\sim 60\%$). Coverage is $\sim 8\%$, $Q \sim 10^7$. Unsuccessful spheres show no appreciable coverage and $Q \sim 10^5$.

4.5 Specific binding

Fig. 9 shows Q change with changing concentration of SA-Alexa680 for two spheres. Initial values of Q in pure PBS are indicated on the vertical axis. Sphere #1 shows Q drop progressing with increasing concentration of analyte. After this experiment the sphere was observed under a fluorescent microscope and fluorescence from bound analyte was detected. Sphere #2 shows less of a Q drop. No fluorescence from bound analyte was detected during later inspection. This indicates that specific binding did not occur efficiently, and may be attributed to failure of the silane-biotin reaction at the sensor surface. However, it bodes well for the silane-biotin combination's ability to block nonspecific binding.

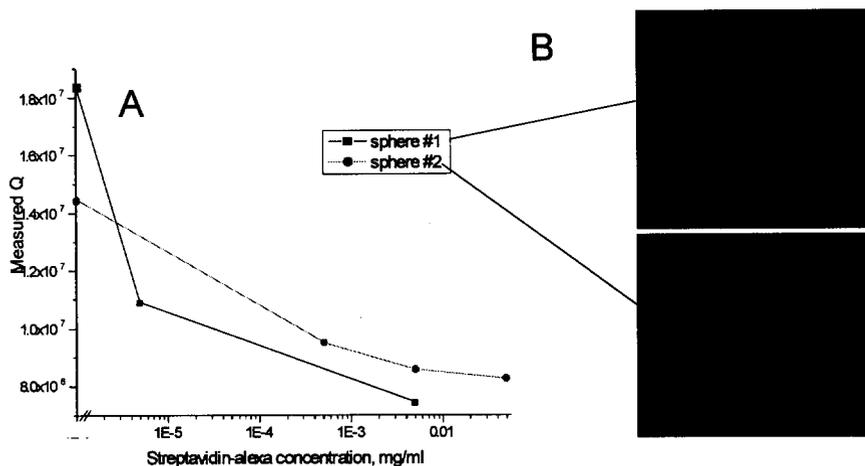


Figure 9. (A) Biotin-coated spheres of different surface coverage. (B) Fluorescent images of each (Filter: Ex. 510-560; Em. 590 LP).

5. CONCLUSIONS AND FUTURE WORK

The microsphere system shows initial potential as a biosensing system. However, four main issues must be addressed before they are practical. First, the surface chemistry must be made more reliable, and the ideal percentage of coverage determined. As in photolithography, we need to find the correct recipe; in our case the goal is to immobilize molecules to the surface without destroying the cavity Q. Use of different silanes, different solvents for the silane, and different times for silanization are the most obvious angles of approach for this issue.

Second, we need to measure binding kinetics and compare data with theory. Addition of a flow cell system will simplify the model as it eliminates local analyte depletion, and may allow for detection of more dilute samples. Additionally, a flow cell makes the experiments more repeatable as the microsphere has a stable aqueous environment.

Third, an experimentally tested model for Q change with binding needs to be worked out, and the difference calculated between binding of streptavidin alone vs. streptavidin-Alexa 680.

Finally, model systems with clinical or research applicability need to be selected and implemented. We need to demonstrate sensitivity and detectivity for a useful system. Any system which involves immobilizing a large, bulky molecule to the sensor surface will probably prove lethal to the Q; hence, smaller target molecules with large ligands are the best candidates.

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Yes, Karina still has the Purcell paper. Jennifer has the Adami paper. and we never got the Nadeau paper.
That's the status.

Carla