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# Microarray-Based Kinase Inhibition Assay by Gold Nanoparticle Probes

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**We report on the development of a new class of kinase microarray for the detection of kinase inhibition based on marking peptide phosphorylation/biotinylation events by attachment of gold nanoparticles followed by silver deposition for signal enhancement. The α-catalytic subunit of cyclic adenosine 5′-monophosphate-dependent protein kinase (PKA), and its well-known substrate, kemptide, were used for the purpose of monitoring phosphorylation and inhibition. As expected, highly selective inhibition of PKA is demonstrated with the four inhibitors: H89, HA1077, mallotoxin, and KN62. Furthermore, an inhibition assay demonstrates the ability to detect kinase inhibition as well as derive $IC_{50}$ (half-maximal inhibitory concentration) plots.**

The phosphorylation of proteins by kinases plays various vital regulatory roles in metabolic pathways and cell communication.[1−4] The family of mammalian protein kinases consists of over 500 members of which only a fraction has been characterized. Therefore, the identification of kinases, their substrates, and, in particular, potential inhibitors is necessary for the understanding of many fundamental biochemical processes. This knowledge is also necessary in drug discovery.[5−7] Radiolabeling of the substrate using $\gamma$-$^{32}$P-ATP as cosubstrate is the standard method to monitor kinase activity.[1−8] High-throughput, nonradioactive alternative methods based on microarray or other techniques, e.g., fluorescent/luminescent labels and mass spectrometry, have been used to monitor kinase activity on the surface of peptide chips.[8−14] These techniques have enormous benefits to biomedical and kinase-based research in particular.

We have recently shown that kinase substrates can be identified by the specific binding of gold nanoparticles to the phosphorylated product immobilized on a microarray.[15] This is achieved by replacing the radiolabeled cosubstrate with $\gamma$-biotin-ATP which would bind specficially to the gold nanoparticles modified with avidin.[15,16] These binding events are readily detected by resonance light scattering (RLS) of nanoparticles. The detection of RLS by metal particles is the key step toward higher sensitivity as the eventual goal would be to detect single biomolecular binding events.[17−25] Mirkin and co-workers have developed a highly sensitive DNA-based, bio bar code for the specific detection of proteins on microarrays using a similar principle.[24,26−29] They have used DNA hybridization as the specific surface recognition process for detecting genomic DNA and proteins from patient serum.

Here, we report a highly sensitive, selective, and simple microarray method, which can be employed for kinase inhibition study. As in our previous report, this method is based on labeling peptide phosphorylation events on a microarray with gold nano-

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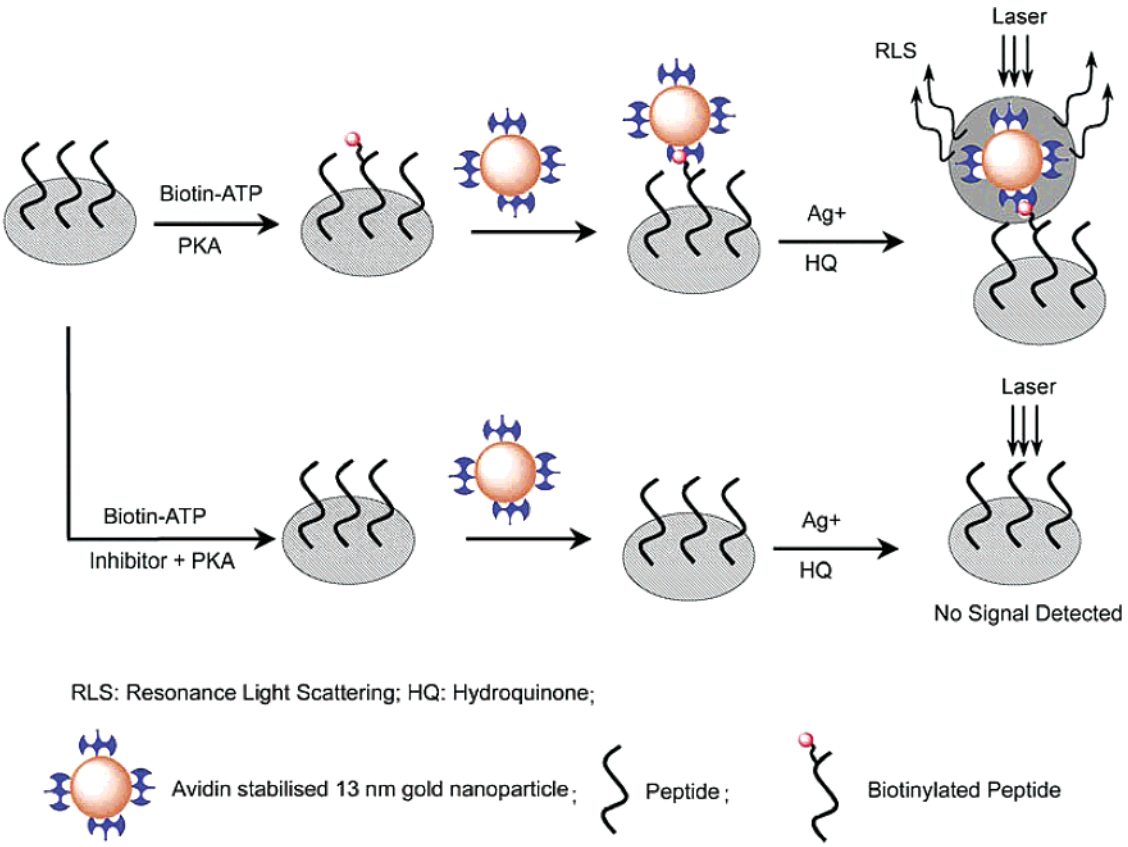


**Figure 1.** Schematic representation of phosphorylation/biotinylation and inhibition of the spotted peptide. This starts with the attachment of avidin-stabilized gold nanoparticles followed by silver enhancement, and, finally, detection by resonance light scattering.

particles using avidin−biotin chemistry followed by silver enhancement and RLS detection.[15] We demonstrate that it is possible to screen kinases with single or multiple inhibitors simultaneously on the same microarray format, thereby increasing assay throughput in this proof-of-concept study. Compared with the previously reported microwell approach,[16] this new method is directly amenable to very high throughput in terms of both the number of substrates and of inhibitors. Processing and reading the array is quick and used significantly reduced reagent volume, e.g., single−spot inhibition assays required only ~1 nL of reagent, while determination of a $IC_{50}$ value required less than 100-nL samples per replicate, thus providing significant cost and time savings over plate-based assays.

## EXPERIMENTAL SECTION

**Materials and Reagents.** Tetrachloroaurate ($HAuCl_4$), bovine serum albumin (BSA), and avidin were purchased from Sigma-Aldrich Co. α-Catalytic subunit cyclic adenosine 5′-monophosphate (cAMP)-dependent protein kinase (PKA) was purchased from New England Biolabs (Beverly, MA). Kemptide (LRRASLG) and control peptide (LRRAGLG) were purchased from Shenggong Ltd. (Shanghai, China). Biotin-ATP (adenosine 5′-triphosphate [γ]-biotinyl-3,6,9-trioxaundecanediamine (ATP [γ]biotin-LC-PEO-amine)) was purchased from Alt. Inc. (Lexington, KY). Inhibitors (H89, HA1077, mallotoxin, and KN62) were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA. USA). NHS-ester Hydrogel-coated glass microscope slides (Nexterion Slide MPX 16) were purchased from Schott AG. The MPX 16 slide consists of a coated glass slide partitioned into 16 individual wells by an ultrahydrophobic patterned layer. All other chemical reagents were purchased from Sigma-Aldrich Co. Milli-Q water (18.2 MΩ) was used throughout the experiments.

**Preparation of Gold Nanoparticle Probes.** The citrate-stabilized, 13-nm gold nanoparticles were synthesized by the Frens−Turkevich method.[30] Avidin-stabilized gold nanoparticles were prepared by stirring an aqueous mixture of 2 mL of succinylated avidin (1 mg/mL in 50 mM PBS, 0.15 M NaCl, pH 7.5) and 20 mL of citrate-stabilized, 13-nm gold nanoparticles ($3.8 \times 10^{-9}$ M) for 30 min at room temperature. Excess protein was removed by repeated centrifugation at 13 000 rpm, (~16100*g*, 3×) using an Eppendorf centrifuge . The gold nanoparticles were resuspended in PBS and stored at 4 °C.

**Peptide Microarray Fabrication and Phosphorylation.** Peptide microarrays were manufactured by the standard procedure using a SmartArrayer 48 system (Capitalbio Ltd.).[31−33] To obtain 16 independent 4 × 4 subarrays, kemptide or a control peptide was spotted in different wells of NHS-ester-functionalized MPX 16 slide in 0.3 M PBS (pH 8.5, 0.2 M NaCl) with 20 μg/mL BSA. After printing, the slides were allowed to age at 15 °C with a relative humidity of 75% for 3 h. Each slide was then quickly rinsed with 20 mL of phosphate buffer (pH 7.5, 50 mM) containing 1% (w/v) BSA. The slide was next immersed in blocking buffer (pH 7.5, 50 mM PB, 0.15 M NaCl containing 1% w/v BSA and 0.1

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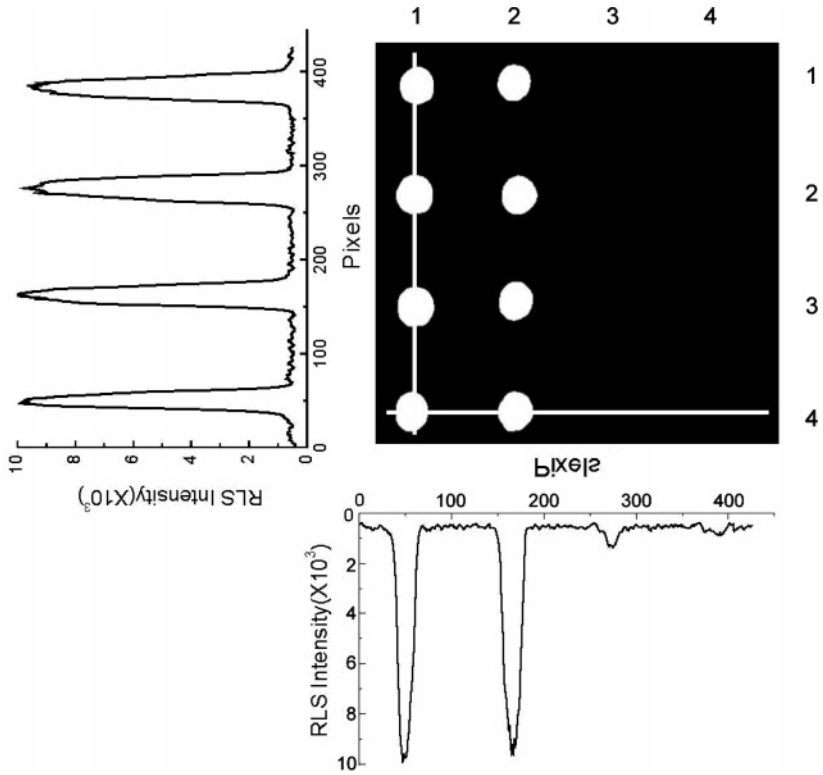


**Figure 2.** Light scattering image of peptide microarray after phosphorylation, labeling positives with gold nanoparticles, enhancement by silver deposition, and corresponding RLS intensity curves of column 1 and row 4 of the array (marked by white lines). Kemptide was spotted on columns 1 and 2, while control peptide was spotted at columns 3 and 4. The concentrations of spotted peptides were 1 $\mu$g/mL.

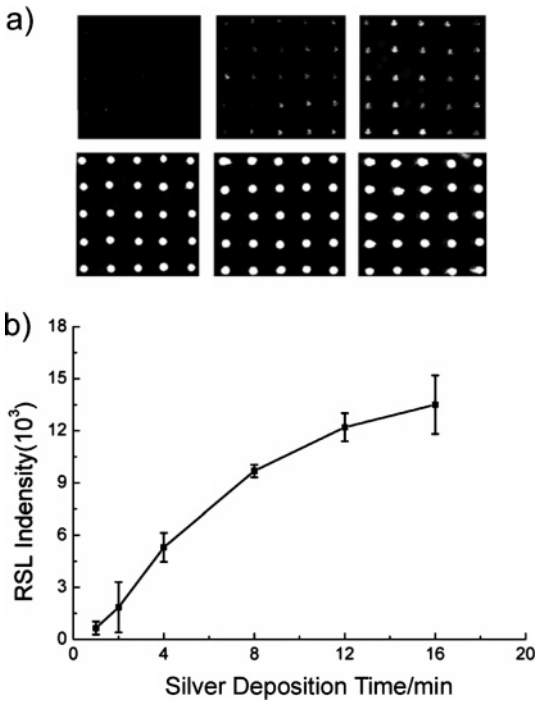


**Figure 3.** (a) Light scattering images of peptide microarrays showing the effect of different time for silver deposition; (b) corresponding curve of average RLS intensity change of (a). The concentration of spotted kemptide was 1 $\mu$g/mL.

M ethanolamine) for 1 h to inactivate any free NHS-ester groups. The slides were then washed with washing buffer (pH 7.5, 20 mM tris, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA with 0.1% Triton X-100) for 10 min (3×) before washing with kinase buffer (pH 7.5, 50 mM tris, 10 mM $MgCl_2$, 1 mM DTT) for another 10 min. The cleaned slide was then incubated with kinase buffer containing 100 $\mu$M ATP for 10 min. The slide was next washed for an additional 10 min with kinase buffer and incubated for 1 h at 30 °C with 200 $\mu$L of kinase solution. The kinase solution is PKA buffer (supplied together with PKA from New England Biolabs) with an addition of 50 $\mu$M biotin-ATP and 100 units of PKA. This solution was applied to the slide under a removable frame-seal incubation chamber that was supplied together with the MPX 16 slide. Following the 1-h incubation period, the slide was rinsed with washing buffer for 5 min (3×), washing buffer without Triton X-100 for 5 min (3×), and water for 3 min (3×), respectively. The slide was then centrifuged at 200*g* for 1 min to remove excess water. The microarray slide was then treated with 200 $\mu$L of gold nanoparticle probes ($6.1 \times 10^{-9}$ M) in PBS (pH 7.5, 50 mM PB, 0.15 M NaCl) for 2 h at room temperature. The slide was next subjected to a series of rinsing steps: (1) PBS buffer with 0.1% Tween-20 for 3 min (3×), (2) PBS buffer for 3 min (3×), and (3) water for 3 min (3×), respectively. The rinsed slides were dried by centrifugation as before.

**PKA Inhibition and $IC_{50}$ Assays.** The inhibition efficiency test was performed using the inhibition solution (kinase solution with each inhibitor at 10 $\mu$M) on different subarrays. The arrays were then incubated, washed, spun-dry, and probed by gold nanoparticles as described in the phosphorylation process. To measure the $IC_{50}$ values of inhibitors (H89, HA1077, mallotoxin, KN62), different concentrations of the inhibitor (0.0001−50 $\mu$M) in the kinase solution were applied to individual subarrays, then incubated, washed, spun-dry, and probed by gold nanoparticles as described previously.

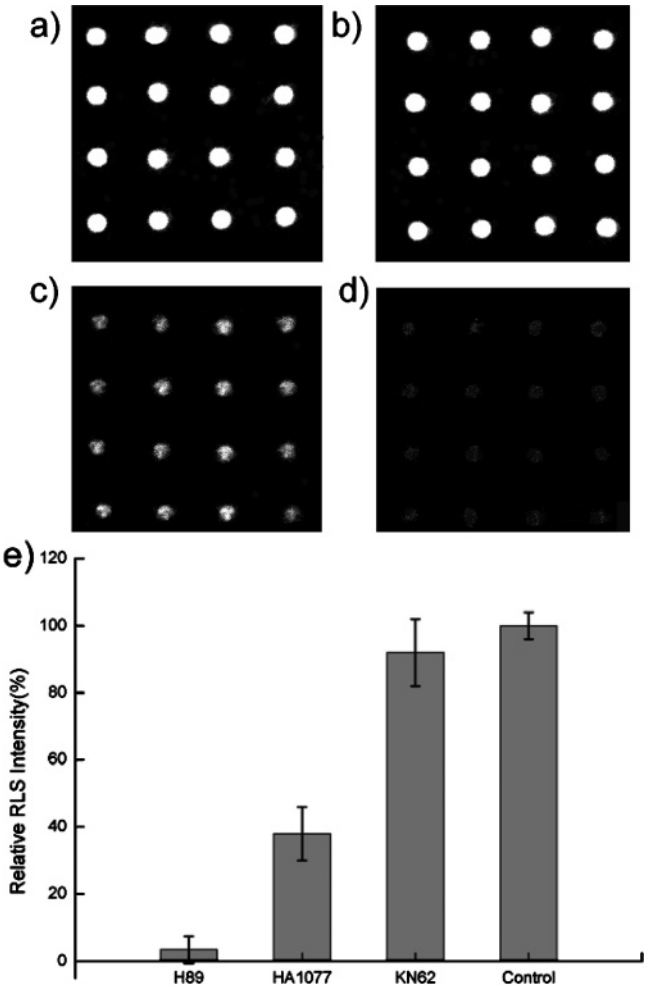


**Figure 4.** Light scattering images of peptide microarrays with (a) in the absence of inhibitor (control experiment) and in the presence of 10 $\mu$M inhibitors: KN62(b), HA1077(c), and H89(d). (e) Shows the preliminary quantitative analysis of RLS signals from arrays a−d. The signals have been corrected for background noise and normalized to the average RLS intensity obtained in the absence of inhibitors. The concentration of spotted kemtide was 1 $\mu$g/mL.

**Silver Enhancement and Detection.** After labeling with gold nanoparticles, a 1-mL silver enhancer solution consisting of equal parts of solutions A ($AgNO_3$) and B (hydroquinone) was applied to each microarray slide and washed with 10 mL of water (3×). Upon signal amplification by silver deposition, the slides were imaged with a highlight scanner using white light (Qiagen Instruments) and analyzed with the Array Analyser software. In each case, the average signal intensity of three replicate assays (total of 24 or 48 spots in three independent 2 × 4 or 4 × 4 subarrays) is reported.

## RESULTS AND DISCUSSION

**PKA Phosphorylation Arrays and Signal Amplification.** Multiple copies of two different peptides, kemptide (LRRASLG, substrate of PKA) and control peptide (LRRAGLG which is not a substrate of PKA), were spotted and immobilized on commercial NHS-ester MPX 16 glass microscope slides by a standard robotic procedure.[31−33] The reaction scheme for the determination of kinase functionality/inhibitation is shown in Figure 1. The substrate peptide (kemptide) is phosphorylated on the microarray spot by the kinase, PKA, using biotinylated ATP (Biotin-ATP) as a cosubstrate. Through this reaction, together with the phosphate, a biotin site is transferred to the kemptide. In a subsequent step, the biotinylated peptide is specifically labeled by attachment of avidin-stabilized gold nanoparticles. Subsequently, a silver enhancement step was applied to the microarrays for signal amplification since the light scattering properties of gold nanoparticles by themselves are relatively poor, if the particles are smaller than ~40 nm.[17] The microarray image is shown in Figure 2, as previously reported, which shows the selective biotinylation of the spots that contain the kemptide, while no significant reaction occurs on the control spots.[15] Integration of experimental parameters, i.e., signal intensity, signal-to-noise ratio, and samples cost, 1 $\mu$g/mL kemptide in the spotting solution was used for the inhibition study.

The effect of silver deposition with regard to the RLS intensity was investigated since silver enhancement is a vital step for good signal detection. Figure 3 shows the RLS array images with different deposition times. This clearly indicates that the average RLS intensity increases with an increase of silver deposition on the gold seeds. We have found that the detection sensitivity and dynamic range were critically related to the amount of silver deposited. No spots can be detected when exposure time is less than 1 min. For short exposure times (2−4 min), there was poor sensitivity while long exposure times (>15 min) had significant background noise and saturated signal output, which also reduced sensitivity and dynamic range. With the optimum exposure time of ~8 min, good features of the spots and a relatively higher signal-to- background noise ratio can be achieved (Figure 3a, left bottom). In this study, all results shown were obtained under these conditions.

**PKA Inhibition Assays.** Inhibition of PKA phosphorylation activity was tested by comparing signals from samples incubated with three established kinase inhibitors, H89, HA1077, and KN62, against those obtained from a sample without any inhibitor. In this case, for PKA, the inhibited efficiency of H89 is stronger than that of HA1077, while KN62 is a noninhibitor. Different RLS images of 4 × 4 arrays of the inhibition assays are shown in Figure 4a−d. As expected, in the presence of inhibitors, the RLS intensity decreases with increasing efficiency of inhibitor (see Figure 4e). This suggests that our method not only have the potential to screen for inhibitor activity qualitatively but can also be developed to yield quantitative data on the efficiency of different inhibitors. More efficient inhibitors lead to lower levels of biotinylation, which in turn gave a decreased RLS intensity.

**$IC_{50}$ Value Determination.** We determine the $IC_{50}$ values for the PKA inhibitors (H89, HA1077, mallotoxin, KN62) to further demonstrate the utility of this gold nanoparticle-based microarray format for a quantitative inhibition assay. Figure 5 shows the resultant array images for H89. In this case, the RLS intensity decreases with increasing the concentration of H89, indicating the relative levels of substrate phosphorylation and inhibition. This result allowed for determination of the $IC_{50}$ based on the relative RLS intensity. The corresponding $IC_{50}$ curve for H89 is shown in Figure 6, and the $IC_{50}$ values of all inhibitors are shown in Table 1. The $IC_{50}$ values determined using the assay was comparable with the literature vales. More interestingly, mallotoxin (Rottlerin),

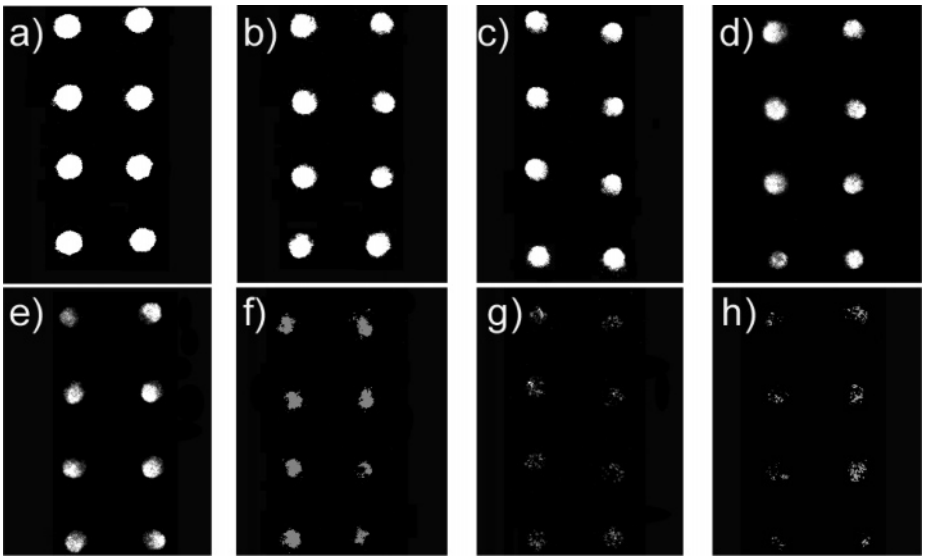


**Figure 5.** Effect of various concentrations of inhibitor H89 in the kinase solution on the light scattering images of the peptide microarrays. The concentration of spotted kemtide was 1 $\mu$g/mL.

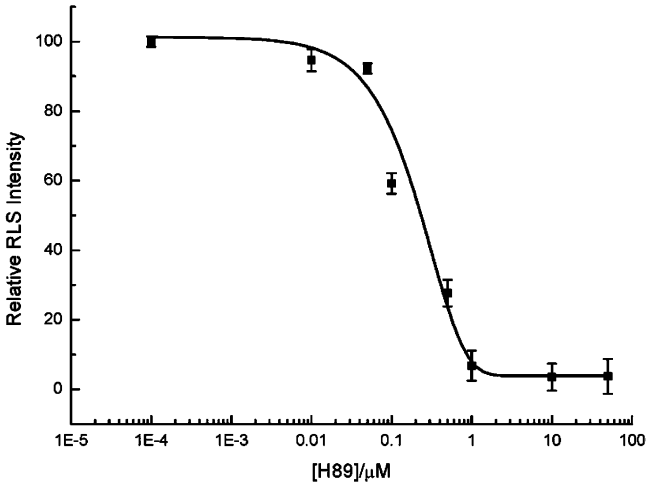


**Figure 6.** $IC_{50}$ curve for H89. The signals have been corrected for background noise and normalized to the average RLS intensity obtained in the absence of inhibitor.

**Table 1. IC50 Comparison**

| inhibitors | $IC_{50}$, found | $IC_{50}$, reported |
|---|---|---|
| H89 | 200 nM | 135 nM[7] |
| HA1077 | 1.9 $\mu$M | 1.6 $\mu$M[35] |
| mallotoxin | 2.7 $\mu$M | none |
| KN62 | no inhibition | no inhibition[7] |

the well-known inhibitor of PKC isoforms,[34] also inhibited PKA potently in the inhibition assay. These results confirm that the gold nanoparticle-labeled microarray format has the potential to screen for PKA inhibition as well as determine the $IC_{50}$ values.

Overall, this microarray assay holds great promise as a tool for screening kinase inhibitors. There are several advantages of this technique over others as the assay: (1) does not pose any radioactive hazards, (2) is highly selective, and (3) is simple and low cost since RLS can be easily detected by even an office flat bed scanner.[24,26] As a proof-of-concept, only one inhibitor was employed per subarray in the present study. However, it is possible to develop a single-spot inhibition assay using a noncontact overprinting mode during the microarray fabrication for multiple inhibitors screening.[14,36]

## CONCLUSIONS

A versatile, gold nanoparticle-based microarray format with high selectivity and sensitivity for kinase functionality and inhibition has been developed. Well-known kinase inhibitors were used to establish this new microarray format method. This technique can be applied to other analytical problems such as the quantitative determination of kinase functionality and inhibitor activity. This would open up possibilities for the future use of nanoparticle-based, high-throughput technologies in drug discovery. Future work will develop such high-throughput assay formats based on increasing the number of kinases and inhibitors per array.

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