

The Effects of Gold and Silver Nanoparticles on an Enzymatic Reaction Between Horseradish Peroxidase and 3,3',5,5'-Tetramethylbenzidine

Barry Morris and Farhad Behzad*

British Institute of Technology and E-commerce, London, UK

Abstract

The purpose of this investigation was to study the effects of gold (Au) and silver (Ag) nanoparticles on a commonly used enzymatic reaction between horseradish peroxidase (HRP) and the substrate, 3,3',5,5'-tetramethylbenzidine (TMB).

Two different methodologies were used in this study. The first was the addition of small quantities of nanoparticles, directly onto a solution of streptavidin peroxidase (streptavidin bound to HRP), prior to its reaction with TMB. The second, was the addition of nanoparticles to immobilised streptavidin peroxidase, covalently bounded to wells of a microtitre plate. In both cases, reactions with TMB were measured by visible absorbance spectroscopy, using a microtitre plate-reader.

The results indicate that both gold and silver nanoparticles have a significant affect; with gold suppressing the reaction and silver enhancing it.

Keywords: Nanoparticles; Horseradish peroxidase; Tetramethylbenzidine

Introduction

The reaction between the enzyme, horseradish peroxidase (HRP), and the substrate, 3,3',5,5'-tetramethylbenzidine (TMB), is well established and frequently used in Enzyme-Linked Immunosorbent Assays (ELISAs) for detecting and measuring analytes, such as proteins and antibodies. HRP has a metal ion cofactor, known as haem, which is a prosthetic group consisting of an iron ion. When haem, in the peroxidase, is combined with TMB, it produces a one-electron oxidation product, which is a free radical cation that is responsible for the change in colour from an almost colourless solution to dark blue as explained [1]. The degree of this colour change is directly reflective of the amount and activity of the enzyme; hence its use in ELISAs as the signal generating stage. When an acid is applied to the enzyme-substrate complex, a further reaction takes place, producing a two-electron diimine product, which changes the colour from dark blue to yellow.

The purpose of the following experiments was to investigate the effects of gold and silver nanoparticles on the HRP activity, using TMB as the substrate. Visible absorption spectroscopy was used to obtain readings of the amount of light transmitted through the wells of a microtitre plate. These readings, known as transmittance data, I_o , were then converted into relative absorbance, A_r , using Equation 1:

$$A_r = \frac{A_w}{A_p} \times 100\% \quad \dots \text{Equation 1}$$

—Where,

A_w = Absorbance of the contents of a microtitre well

A_p = Absorbance of the contents of a microtitre well with a positive control reference

The absorbance, A_w or A_p , was calculated using the generic equation for absorbance, A , shown in Equation 2:

$$A = \text{Log}_{10} \left[\frac{I_i}{I_o} \right] \quad \dots \text{Equation 2}$$

Where,

I_i = Intensity of light entering a microtitre well

I_o = Intensity of light leaving a microtiter well (transmittance)

Materials and Apparatus

Materials

A Hyaluronidase Assay Kit, from Razie Biotech Ltd., was purchased from AMS Biotech.com.

The following materials were purchased from Sigma-Aldrich UK: 3,3',5,5'-tetramethylbenzidine; streptavidin peroxidase (streptavidin bound to HRP; 1 mg of lyophilised powder was dissolved in 1 ml of phosphate buffered saline (PBS) solution, a 100 times (v/v) in PBS was used in the procedure); gold colloid solution, 20 nm; gold colloid solution, 10 nm; gold colloid solution, 5 nm; silver nanoparticles dispersed in water, citrate stabilised, 157 nm.

Apparatus

1. Two, 96-well microtitre plates; one of them coated with biotin
2. Shaker/incubator; set at 390 r.p.m. and 37 °C
3. Microtitre plate-reader

Experimental procedure

The experimental procedure was carried out using two methodologies, a direct method and a method using a biotin bounded

*Corresponding author: Farhad Behzad, British Institute of Technology & E-commerce, London, United Kingdom; E-mail: farhad.behzad@talk21.com

Received: July 22, 2014; Accepted: September 11, 2014; Published September 15, 2014

Citation: Morris B, Behzad F (2014) The Effects of Gold and Silver Nanoparticles on an Enzymatic Reaction Between Horseradish Peroxidase and 3,3',5,5'-Tetramethylbenzidine. *Biochem Pharmacol* 3: 146. doi:10.4172/2167-0501.1000146

Copyright: © 2014 Morris B. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

plate. Both methods required streptavidin, dilute 100 times (v/v) in PBS, which was the first task to be performed.

The direct method used six wells of a non-coated, 96-well microtitre plate. Transmittance readings, through the empty wells, were recorded using the plate-reader. A 2 μ l drop of streptavidin peroxidase:PBS solution was placed in the centre of one of the wells, which was used as a positive control reference well, i.e. without the addition of any nanoparticles. A 5 μ l drop of 20 nm gold nanoparticles was placed in the centre of the second well, which was used as a negative control reference well, i.e. without the addition of any streptavidin:PBS solution. A 2 μ l drop of streptavidin peroxidase:PBS solution was placed in the centre of each of the four remaining wells and 5 μ l of nanoparticles were added to each of these drops, using the following materials: 20 nm Au nanoparticles were added to the first drop, 10 nm Au nanoparticles were added to the second drop, 5 nm Au nanoparticles were added to the third drop and 157 nm Ag nanoparticles were added to the fourth. The plate was placed in the shaker/incubator for a period of 10 minutes. It was then removed from the shaker/incubator and each of the six wells were filled with 50 μ l of TMB, prior to the plate being returned to the shaker/incubator. Another set of transmittance readings were recorded after 40 minutes incubation.

The bounded method, used four biotin coated wells of a 96-well, microtitre plate. Each well was filled with 50 μ l of streptavidin peroxidase:PBS solution and placed in the shaker/incubator for a period of 30 minutes. The wells were emptied, washed in distilled water and dried, prior to the addition of nanoparticles. One well was left void of nanoparticles and used as a positive control reference well. The remaining three wells were filled with 50 μ l of nanoparticles. The first with 5 nm Au nanoparticles, the second with 20 nm Au nanoparticles and the third with 157 nm Ag nanoparticles. The microtitre plate was returned to the shaker/incubator for a period of 10 minutes; then, emptied, washed with distilled water and dried again. Transmittance readings, of the empty wells, were recorded using the plate reader, prior to filling each well with 50 μ l of TMB. The plate was returned to the shaker/incubator for a further 30 minutes, and then another set of transmittance readings were recorded.

Both sets of transmittance readings, obtained from each of the above procedures, were used to calculate the absorbance of the contents of each well, relative to the absorbance of the positive control reference wells, using Equations 1 and 2.

The procedures were repeated a number of times to obtain mean values.

For the direct method, no colour change was detected in the negative control well, where 20 nm gold nanoparticles were added to TMB on its own.

The results, expressed for each well, are the mean values of six wells, in three sets of experiments.

Results

When the wells were observed by eye, both experiments revealed that only the positive control reference wells and those containing silver nanoparticles appeared to be dark blue in colour; the remaining wells appeared to be either transparent or a very pale blue.

Discussion

The results, obtained from the two different methodologies, indicate that gold and silver nanoparticles have a significant effect on

the enzymatic reaction between HRP and TMB.

The positive control reference wells contained the enzyme-substrate complexes only, without any nanoparticles. These were expected to produce normal reactions and the relative absorbance of these complexes was used as reference points of 100%.

In the direct method, a 5 μ l drop of 20 nm gold nanoparticles was placed in the centre of the negative control reference well, without the addition of any streptavidin:PBS solution. This was expected to produce no reaction and the relative absorbance of the nanoparticle/TMB solution was used as a baseline reference (i.e. the minimum amount of relative absorbance). The results clearly show that the nanoparticles did not react with the TMB and played no part on the conversion of TMB into colour products. The addition of streptavidin:PBS solution, on the other hand, together with the same size gold nanoparticles, provides evidence that colour products were produced by the reaction between streptavidin:PBS and TMB alone, while the gold nanoparticles inhibited this reaction by almost 40% relative absorbance (Figure 1).

The direct method also provides evidence that biotin, which was coated on the microtitre plate for the bounded method, had no part to play in the reactions. The role of biotin was only to bind HRP to the walls of the microtitre wells.

In both experiments, the relative absorbance of the enzyme-substrate complex, containing silver nanoparticles, exceeded that of the complex contained in the positive control reference well. Thus, indicating that the silver nanoparticles enhanced the reaction between HRP and TMB [2]. Who demonstrated that silver nanoparticles not only enhances the activity of HRP, but also alters its structure. However, their results also indicate that this only occurred within a specific concentration range of silver nanoparticles (Figure 2).

On the other hand, the relative absorbance of the enzyme-substrate complex, containing gold nanoparticles, was less than that of the complex contained in the positive control reference well. Thus, indicating that the gold nanoparticles inhibited the reaction between HRP and TMB. Furthermore, the results also show that these reactions appear to be influenced by the relative size of the nanoparticles, with 20 nm gold nanoparticles having the least effect and the smaller nanoparticles exhibiting greater inhibition.

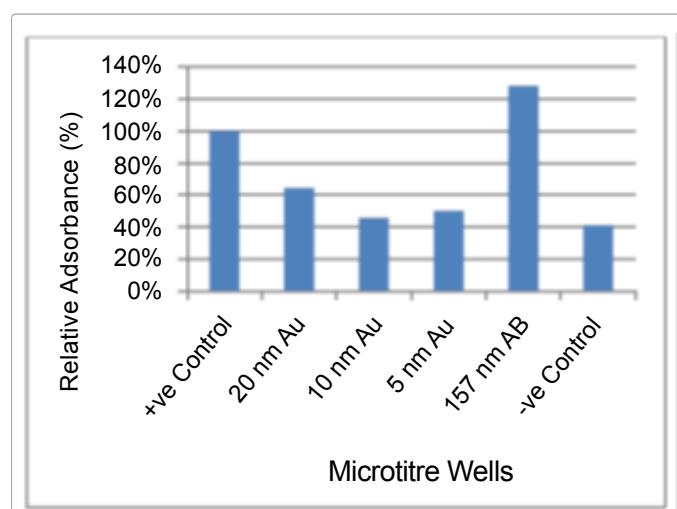


Figure 1: Results from the Direct Method- Relative Absorbance of the Wells, containing Gold (Au) and Silver (Ag) Nanoparticles using, the Direct Method

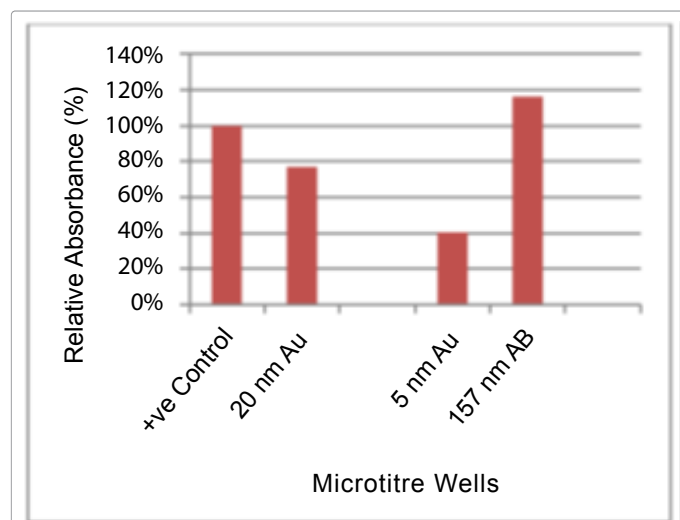


Figure 2: Results from the ELISA Method- Relative Absorbance of the Wells, containing Gold (Au) and Silver (Ag) Nanoparticles, using the Bounded Method

Considering these results, it is not unreasonable to suggest that other enzymes may also be affected by nanoparticles, and that different types of nanoparticles, of different shapes and sizes, may affect enzymatic reactions, in different ways [3], has shown that the properties of nanoparticles, such as size, shape, surface chemistry and charge, can alter the structure and function of enzymes [4], has also demonstrated that nanoparticles are able to induce protein modifications.

A great deal of research work is being carried out on the effects of nanoparticles on biological systems. It comes as no surprise that these systems are influenced by nanoparticles, since the common ground between nanoparticles and biology is the nanoscale [5], has demonstrated that gold and silver nanoparticles could enhance the radiation sensitivity of hepatocellular carcinoma cells (HCC), a common type of liver cancer. The mechanism for this is thought to be

due to programmed cell death (apoptosis) or elevated DNA damage.

Veeraapandian demonstrated that protein capped gold nanoparticles have been shown to exhibit good antioxidant activity, which reduces cell damage or death, while protein capped silver nanoparticles are able to inhibit the growth of several Gram-positive and Gram-negative microorganisms [6].

Conclusion

Enzymatic reactions are influenced by nanoparticles. The mechanism of these nanoparticle-enzyme interactions are currently under investigation. This is an important area of research, since the ability to regulate enzymatic activity could potentially lead to new processes for enzyme control and modification, with possible use in new drug developments and industrial applications. It also signifies probable toxicity of nanoparticles as they become more widely present in everyday life via off-the-counter available cosmetics and household goods.

References

1. Tatzber F, Griebenow S, Wonisch W, Winkler R (2003) Dual method for the determination of peroxidase activity and total peroxides-iodide leads to a significant increase of peroxidase activity in human sera. *Anal Biochem* 316: 147-53.
2. Karim Z, Adnan R, Ansari MS (2012) Low Concentration of Silver Nanoparticles Not Only Enhances the Activity of Horseradish Peroxidase but Alter the Structure. *PLoS ONE* 7: e41422.
3. Wu ZC, Zhang B, Yan B (2009) Regulation of enzyme activity through interactions with nanoparticles. *Int J Mol Sci* 10: 4198-4209.
4. Sanfins E, Dairou J, Rodrigues-Lima F, Dupret JM (2011) Nanoparticle-protein interactions: from crucial plasma proteins to key enzymes. *Journal of Physics: Conference Series* 304: 012039.
5. Zheng Q, Yang H, Wei J, Tong JL, Shu YQ (2013) The role and mechanisms of nanoparticles to enhance radiosensitivity in hepatocellular cell. *Biomed Pharmacother* 67: 569-575
6. Veeraapandian S, Sawant SN, Doble M (2012) Antibacterial and antioxidant activity of protein capped silver and gold nanoparticles synthesized with *Escherichiacoli*. *J Biomed Nanotechnol* 8: 140-148.