

A New Cost-Effective Method to Evaluate Collagenase Activity Using Nano-Graphene Oxide

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Abstract

Graphene oxide (nGO) is considered as a good nano carrier for enzymes to protect them in biological media against proteolytic activity and extend their activity and stability. In this paper, we reported a new inexpensive collagenase assay using graphene oxide nanosheets (nGO) for Coomassie brilliant blue (CBB) staining. Collagenase enzymes were attached to nGO and added to microwells containing dried collagen that covered the bottom of wells. The optical absorption (590 nm) was linearly increased parallel to increasing the concentration of collagen. The concentration of collagenase enzyme can be measured by this assay after staining with CBB after overnight incubation with collagen. This technique is a simple, safe, inexpensive and relatively fast method for evaluation of collagenase enzyme and could be applicable for pharmaceutical and industrial purposes.

Keywords: Collagenase assay, Graphene oxide nanosheets, Spectrophotometric assay, Protein stability, Graphene oxide functionalization.

1. Introduction

collagenolytic enzymes have an essential role in regulation of extracellular matrix remodeling in several tissues, including skin, blood vessels, the heart, cartilage, and synovial fluid (1). This metalloprotease, as its name implies, is capable of specifically degrading native triple helical or denatured collagen that constitutes 30% of the total structural proteins in many tissues. Nowadays, collagenases have been applied in pharmaceuticals, medical, food, cosmetic, textile and leather industries. From a medical point of view, it has been widely investigated for enzymatic treatment

of ulcers and burns, elimination of scars, for treatment of Dupuytren's disease and various types of fibrosis such as liver cirrhosis (2). One of the most prospective collagenase medical applications is in the transplant surgery success of some specific organs and in preparing samples for diagnosis purposes. Also, collagenase is considered as the standard of care for chronic, nonhealing wounds in some patients (3) as well as biomarkers for osteoarthritis and rheumatoid arthritis (4) and a new therapeutic approach for treating such diseases is preventing collagenase production and/or their activity. Therefore, it is important to find a feasible and cost-effective way for measurement of collagenase amount and activity. Some relatively intricate and expensive methods were currently em-

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ployed including: sandwich enzyme-linked immunosorbent assay, enzyme-specific substrates, zymography, and film in situ zymography (5). However, these methods cannot be applicable in the industry due to their abundant restrictions. Spectrophotometric collagenase assay was suggested as a simple alternative method that could minimize manipulation and avoid specific reagent as well as radiolabeling (6). But this method also has some limitations including relatively complexity and taking a lot of time (7). In addition, this type of protein was rapidly denatured specially in the biological fluids which limits their biological uses. It's obviously clear that biopharmaceutical science have to find a simple and effective way for evaluating the activity of a protein preparation. Recently, we report a novel method for protecting collagenases using graphene oxide nanosheets (nGO) (8).

nGO is a type of carbon nanomaterials that forms a monolayer carbon nanosheet with oxygen functionalities. Because of its sp² structure and favorable hydrophobicity, nGO can attach to aromatic compounds via van der Waals forces and π - π interactions and offers excellent prospect and innovative solution in medical application for diagnostic purposes and drug delivery (9, 10).

Here, we describe a relatively simple colorimetric assay using UV spectrophotometer using nGO to assess the activity of collagenase. To the best of our knowledge, there are no document which is shown using graphene oxide for assessing the activity of enzymes.

2. Materials and methods

2.1. Synthesis of nGO

According to Hummer's preparation method, nGO was synthesized in several steps. Briefly, the graphites (Merck, Germany) was dispersed and stirred gently in absolute sulfuric acid for 72 h and then potassium permanganate was slowly added to make the solution color dark green. The magnetic stirring was done using a magnetic stirrer (Heidolph, Germany) at 40 °C for 30 min, and at 70 °C for 45 min to make the solution color dark brown. After adding deionized water for the first time, the heating was continued at 105 °C for 10 min and adding water for second time the temperature was

reduced to 100 °C and remained for 15 min. For the last time, the addition of water and hydrogen peroxide was terminated the reaction and the final color was became to yellowish brown. The obtained nGO was collected by ultracentrifugation for 5 min. For the best result, it is suggested that the precipitate was washed with hydrochloric acid and water.

2.2. Characterization of nGO

Transmission electron micrographs (TEM) was taken by Zeiss EM 900 electron microscope operating at 100 kV. A T80+ UV-Vis spectrometer (PG instruments, Australia) was applied for recording UV-Vis spectra. KBr pellet of graphene oxide was prepared to take Fourier transform infrared (FT-IR) spectra by a FT-IR spectrometer (Perkin Elmer, UK). Atomic force micrographs (AFM) were obtained in the contact mode using Nano wizard II (JPK, Germany). Dynamic light scattering and zeta potential was calculated by zetacheck and nanoflex (microtrac, Germany). The AvaSpec-ULS 3648 High-resolution Raman Spectrometer (Avantes, Netherland) with 532 nm diode laser was used for Raman spectroscopy.

2.3. Attachment of collagenase to graphene oxide

The enzyme collagenase type I obtained from *Clostridium histolyticum* (EC: 3.4.24.3, Cat. No. C9891) was purchased from Sigma Chemical Company, St. Louis, MO, USA. Its loading on nGO was achieved by mixing protein in an aqueous media containing nGO (1:1 w/w) at room temperature for 24 h. After centrifugation and removing unbound collagenase, the concentration of protein was evaluated using UV spectrophotometer at a wavelength of 595 nm according to method of Bradford protein assay. The presence of bounded collagenase on nGO was analyzed by SDS-PAGE. Collagenase loading efficacy (LE) was achieved by Equation 1.

$$LE = \left[\frac{(\text{total concentration of Collagenase} - \text{concentration of free Collagenase})}{\text{total Collagenase}} \right] \times 100 \quad \text{Eq. 1}$$

2.4. Collagen preparation

A primary stock of Type I collagen was extracted from rat tail tendons by the method of Rajan *et al.* (11). Firstly, this crude collagen was

dissolved in 0.2% (v/v) acetic acid at $-20\text{ }^{\circ}\text{C}$, then lyophilized and stored at $-80\text{ }^{\circ}\text{C}$. At the moment of use, the final preparation was dispersed in acetic acid on ice to obtain a sterile solution for experimental applications

2.5. Preparation of collagen containing microplate

A total of $50\text{ }\mu\text{L}$ collagen ($700\text{ }\mu\text{g/mL}$) in a neutralizing buffer (100 mM Tris-HCl, 200 mM NaCl, 0.04% (w/v) NaN_3 , pH 7.8) was transferred to a 96 well microplate and incubated at $30\text{ }^{\circ}\text{C}$ for 48 h. The air humidity was set in a humidified atmosphere for 24 h and then in a dry atmosphere for another 24 h. Then, the wells were washed by distilled water (DW) three times and maintained at room temperature to dry.

2.6. Collagenase assay

A total of $100\text{ }\mu\text{L}$ of nGO loaded collagenase enzymes, in different concentrations ranging from 15–480 ng, were added to collagen-coated microwells. The microplate was placed in a humidified incubator at $37\text{ }^{\circ}\text{C}$ for 24 h. Then the contents of the wells were discarded and the wells were washed with washing buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl_2 , 0.02% NaN_3 , pH 7.5) three times and twice with DW. A total of 100 mM of staining solution, composed of 0.25% Coomassie brilliant blue (CBB), 10% acetic acid and 50% ethanol, was added to wells and the plate incubated at $37\text{ }^{\circ}\text{C}$ for 25 min and then was removed. The microplate was washed again with DW and left to dry at room temperature. Subsequently, the absorption of each well was read via an ELISA plate reader (PowerWave™ X52, BioTek Instruments Inc., Woburn, MA, USA) at a wavelength of 595 nm.

3. Results and discussion

3.1. Characterizations of GO and GO-CS

In this study, a Hummer method was applied for nGO synthesis in which graphite powder was peroxidized to form graphene oxide nanosheets. Because of this chemical reaction, the color of GO became yellowish brown (Figure 1). The obtained nGO was dispersed in aqueous solution and was characterized by UV-Vis spectrophotometer ranging from 200 to 500 nm. As shown in



Figure 1. The appearance of GO.

Figure 2, there was an intense peak at 230–235 nm and a small shoulder at around 300 nm that contributed to $\pi-\pi^*$ transition of C=C and the $n-\pi^*$ transition of C=O, respectively (12, 13).

In the FT-IR spectra, there are characteristic peaks at 3418 cm^{-1} , 2912 cm^{-1} , 1731 cm^{-1} and 1056 cm^{-1} which are related to OH stretching vibration, C-H stretching, COOH stretching, primary alcohol C-O stretching vibration, respectively (Figure 3). In comparison, the oxygen-containing groups were not observed in the graphite spectra.

The study of nGO layers was assessed by Raman analysis. As demonstrated in Figure 4, there are three characteristic peaks at 1358 cm^{-1} , 1595 cm^{-1} and 2696 cm^{-1} in Raman spectra of nGO which are contributed to D, G and 2D bands, respectively. The previous studies showed that the presence of G and 2D bands at 1585 and 2679 represented a single or two-layer GO sheets. Also, the intensity ratios of D/G and 2D/G in our work were 0.53, which are related to bilayer GO. Thus, bilayer sheets of nGO were synthesized in

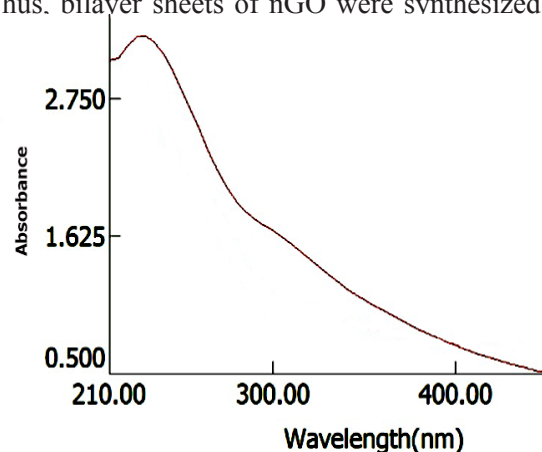


Figure 2. UV/vis spectra of GO.

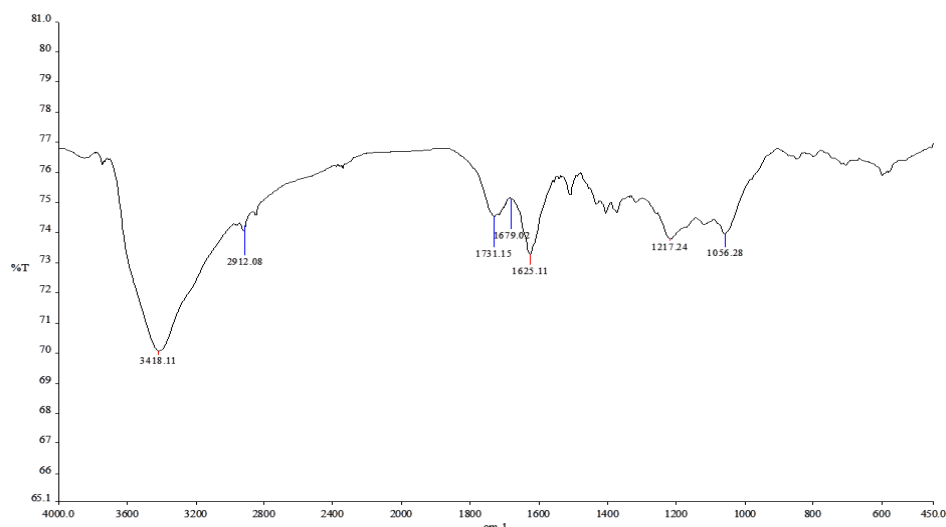


Figure 3. FT-IR spectra of GO.

this study.

In order to further study the properties of nGO nanosheets, TEM and AFM techniques was applied for morphological evaluations. TEM image of nGO indicated an extended thin sheet with a wrinkled surface which provides a larger surface area (Figure 5). This large area is useful for preventing the collapse back of layers to graphitic structure.

The figure 5 is AFM image of nGO which characterized the thickness of layers. In the figure,

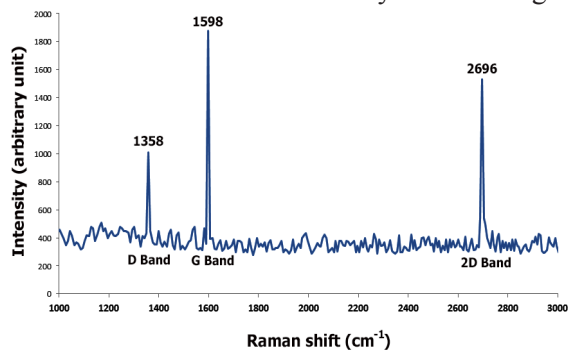


Figure 4. Raman spectra of GO.

the sharp edges of the sheets are clearly visible. The total thickness of nGO nanosheets was in the range of 0.6-10 nm calculated by AFM-contributed software.

The DLS analysis showed that the average size of nGO is 150 nm with the surface net charge of -98.

3.2. Collagenase assay

Figure 6 shows that the optical absorption

of collagen at 590 nm is proportional to the amount of collagen that is related to amount of substrate over the range of 1 to 30 μg. Solubilization of collagen is measurable directly by optical absorption in the range 0.1 to 1.6 (7).

Figure 7 shows the results of collagenase assay calculating by the described method using nGO. It was done after incubation different concentration of collagenase enzyme with 25 μg collagen in the wells for 24 h. As revealed in the figure, this method could detect the activity of collagenase enzyme in very low concentrations up to about 15 pictogram. Thus, this method clarify the using of graphene oxide nanosheet and CBB in collagenase assays. However, previous studies revealed the use of CBB in microplates (6, 7), this paper is emphasis on using an enzyme protecting agent for collagenase. The major advantage of this method in comparison of other previous collagenase assays

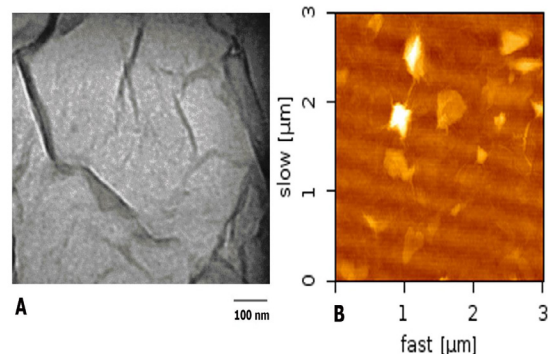


Figure 5. A) TEM images of GO with a wrinkled surface and thin sheet-like structure, and B) AFM images of GO with sharp edges and flat surfaces; the thickness range is about 4 nm.

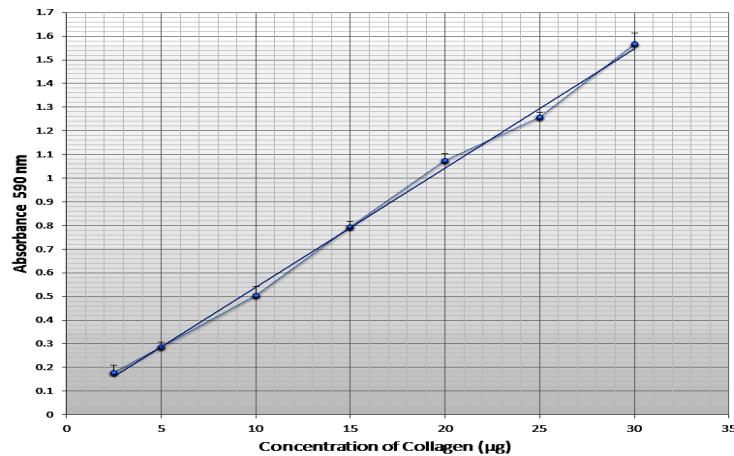


Figure 6. Optical absorption of collagen in different concentration measured by CBB staining spectrophotometric assay.

is protecting collagenase against other proteases and also enzymatic auto-digestion. This protease enzyme in addition to digest collagen fibres and are disaggregate connective tissues, could degrade some of its domains (14, 15). Therefore, many of common assays that used for other proteins is not applicable for collagenase. Also, the routine collagenase assays is done only few minutes. The mentioned technique in this paper is a simple, safe, inexpensive and relatively fast method for evaluation of collagenase enzyme and could be applicable in experimental or commercial purposes. This method could also be extended to other protease assays in which the insoluble substrates is undertaken. However, it should be noted that there are

some limitation using this spectrophotometric assay. Interfering collagen binding stained proteins is always a problem in all spectrophotometric assays of collagenase (16). The results of this study showed the protecting ability of nGO helps to significantly decrease this problem. Also, the spectrophotometric assays using common apparatuses is limit in counting absorbance ranges to 3.0. The higher optical absorption may not be measurable or along with device errors. It seems that an appropriate solution is to sets up a serial dilutions of sample which are in the accurate range of apparatus.

In conclusion, this new colorimetric assay could be used in research projects and industrials

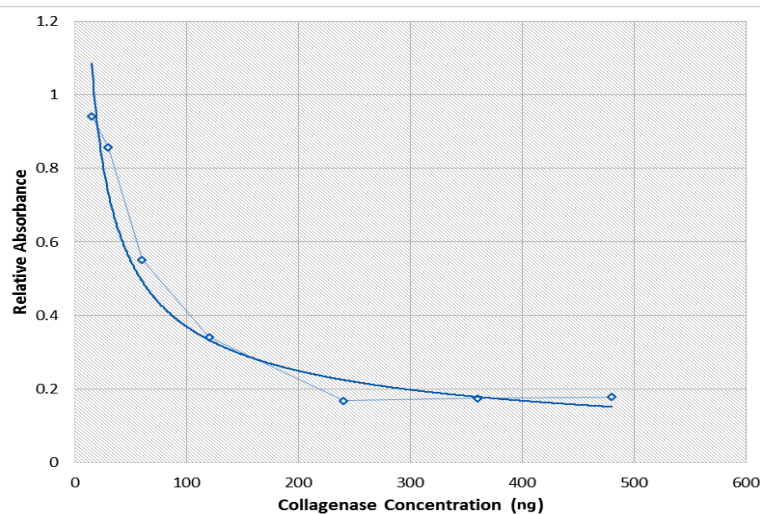


Figure 7. Measuring concentration of collagenase enzyme by CBB using nGO. Collagenase enzyme were incubated with 35 µg collagen at 35 °C for 24 h. The resultant A 590 values were averaged and expressed relative to the absorbance control.

for identification and evaluation of collagenase activity in a relatively rapid, sensitive and inexpensive manner. This method can measure collagen concentration 1 to 30 µg in a linear order. Also this cost-effective method would provide more opportunity for finding better biological collagenase source than current pathogenic microorganisms.

4. References

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Further studies are required to determine the exact amount of the enzyme in higher concentration and effects on inhibitors on the results of this method.

Conflict of Interest

None declared.

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