Preprint Title	Colorimetric Based Analysis Using Clustered Superparamagnetic Iron Oxide Nanoparticles for Enhanced Glucose Detection
Authors	Wonseok Choi and Jaehong Key
Publication Date	14 Sep. 2020
Article Type	Full Research Paper
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Colorimetric Based Analysis Using Clustered Superparamagnetic Iron Oxide Nanoparticles for Enhanced Glucose Detection

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Abstract

Superparamagnetic iron oxide nanoparticles (SPIONs) are approved by the Food and Drug (FDA) in the United States. SPIONs are used in magnetic resonance imaging (MRI) as contrast agents and for target delivery in nanomedicine using external magnet sources. These can also act as an artificial peroxidase (i.e. nanozyme), and a reaction between SPIONs and peroxides was regarded as highly stable in various pH conditions and temperatures.

In this study, we report a nanozyme ability of the clustered SPIONs (CSPIONs) coated with biocompatible poly(lactic-co-glycolic acid) (PLGA) and the results based on colorimetric changes. The synthesized CPSIONs had an average size of 120.1 nm, zeta-potential (ζ-potential) of -61.7 mV (n=3), and the clustered shape was identified by taking transmission electron microscopy images. We hypothesize that the CSPIONs can have more catalytic effects toward H_2O_2 than single SPIONs not clustered. As a result, CSPIONs were shown to oxidize a 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) commonly used as a substrate for hydrogen peroxidase in the presence of H_2O_2, leading to a change the color of the substrate. We also utilized a colorimetric assay at 417 nm covering various glucose concentrations from 5 mM to 1.25 μM that considered the glucose condition of diabetes patients in physiological fluids. This study demonstrated that the absorbance value increases along with increasing the glucose level. It suggests that the particles can detect the glucose after SPIONs were clustered. The results were highly repeated at concentrations below 5 mM (standard deviations were presented as < 0.03). Moreover, the sensitivity and limit of detection (LOD) were 1.50 and 5.44 μM, respectively, indicating CSPIONs are more responsive to glucose compared to the SPIONs crystals.

In conclusion, this study proposes that glucose can be detected more sensitive *in vitro* when SPIONs are clustered. The CSPIONs have the potential to be used for glucose detection in diabetic patients using a physiological fluid such as ocular, saliva, and urine.

Keywords

Iron Oxide Nanoparticles; Clustered Iron Oxide Nanoparticles; Artificial Peroxidase; Nanoenzyme; Colorimetric Detection

Introduction

Superparamagnetic iron oxide nanoparticles (SPIONs) are used in the innumerable biomedical fields such as target delivery with external sources, T_2-MRI contrast agents, and hyperthermia treatment for cancers[1-7]. In a few decades, the SPIONs, including any other metallic particles, have been reported to exhibit an artificial peroxidase activity (i.e. nanozyme), and those have been widely studied at the earliest time. SPIONs mediated nanozyme activity has been regarded as high stable in various temperatures and pH, also can be synthesized in bulk quantities at the comparative cost compared to the naturally expressed peroxidase[8,9]. For these advantages, SPIONs have been focused on substituting peroxidases in diverse fields, especially detecting real glucose. The glucose can be oxidated by glucose oxidase (GO_X), succeeding in the release of hydrogen peroxides (H_2O_2) in an aqueous solution. The SPIONs can catalyze a peroxidase sensitive chromogenic substrate (i.e. 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS)), leading to the change of color in the presence of H_2O_2. Several studies of the glucose detection based on the colorimetric change were

reported [9-12] and this procedure can be performed in a simple, easy, low-cost, and observed by our eyes intuitionally [13].

Gao et al. firstly demonstrated that Fe^{2+} ions in the SPIONs can play the significant role of the nanozymes, presenting this activity were originated from instinct SPIONs property [8]. YU, Faquan, et al. investigated the effect of six differently coated SPIONs on glucose detection and shown the results were very different depending on the used substrate and core coating materials [9]. They discussed that improving electrostatic interaction between the chromogenic substrate and SPIONs is essential to yielding higher catalytic activity and sensitivity. However, few kinds of research of engineered SPIONs, in which the particles were not modified in the whole structure or surface themselves, have existed. The modification of nanocrystals characters must be done in synthesized stages and is not easy to be changed once these were made. One of the most used in the synthesis of high qualities SPIONs, the thermal decomposition method, a process that allows the generation of particles with excessive and extremely uniform size, is very important to expose the reaction mixture at a specific temperature and time but also to add a ligand to get specific characters [14]. Even if it is not a modification of the core of SPIONs, in reporting the utilization of engineered particles for nanozymes, several strategies to enhance the sensitivity and limit of detection (LOD) to glucose were tried by grafting ligands to the structure.

Focusing on the fact that the major role of SPIONs for nanozyme is Fe ions, we imaged a bulk enzyme design that can easily be made without further surface modification and promote the oxidation of the chromogenic substrate widely used in peroxidase studies. In this study, we synthesized spherical-shaped clustered SPIONs (CSPIONs). Because SPIONs consists of numerous Fe_3O_4 and CSPIONs also consists of many primary SPIONs, we hypothesize that CSPIONs are more sensitive toward H_2O_2 and glucose. For that, the glucose levels were set in conditions of real

diabetes contained in physiology fluids [15,16]. We evaluated the possibility of CSPIONs nanozyme ability to H_2O_2 and also H_2O_2 produced from the oxidation of glucose by GO_X to measure the glucose level in the colorimetric method. The sensitivity of CSPIONs to glucose and LOD were explored and compared to the SPIONs reported before [9-11].

Results and Discussion

Synthesis and Characterization of Clustered Iron Oxide Nanoparticles (CSPIONs)

We used the oil-in-water (O/W) method to cluster the iron oxide nanoparticles (SPIONs), and it was shown in **Figure 1a**. The mixture contained distilled water, SPIONs, and PLGA were emulsified with vortexing and sonication. PLGA is an amphiphilic polymer, and it was used for clustering the SPIONs that are highly hydrophobic materials in the water-abundant environment. The characterization of CSPIONs was shown in **Figure 2**. The CSPIONs were synthesized in highly monodisperse (PDI=0.124), and the average size and zeta-potential (ζ-potential) were measured as 120.1 nm and -61.7 mV, respectively (n=3, **Fig. 2a**). The CSPIONs were shown bigger size than used SPIONs whose average size is 10 ± 1 nm (provided by the manufacturer). The transmission electron microscopy (TEM) image shows that the synthesized particles have a spherical shape and successfully clustered within the PLGA (**Fig. 2b**).

Because the catalysis process depends on the Fe ions on the SPIONs surface [8], it is crucial to figure out how many ions were existed in the particles. In order to quantify the Fe in CSPIONs, the ferene-s assay was performed. This assay is widely used for quantification of SPIONs in nanostructure fastly and accurately [17-19]. The

representative standard curve was regressed in y=0.4671x+0.0649 (R^2=0.996), and the back-calculated quantities of SPIONs were found in 100.73μg/100μl, indicating that the ratio of mass and volume was about 1:1 (**Fig. 2c**). About 20% of SPIONs were clustered into the nanostructure (500 μg SPIONs were used in this synthesis) and others were stuck in the surface of the vial due to the instinct hydrophobic property of SPIONs. The ferene-s assay was regularly performed for the quantification of SPIONs when the CSPIONs were synthesized.

The Artificial Peroxidase Activity of CSPIONs

Given that CSPIONs have responsibilities of a nanozyme, it can oxidate the substrate through a Fenton reaction. The Fenton reaction is a catalytic process, leading to converting hydrogen peroxide into a hydroxyl free radical as described in (1) and **Figure 1b**.

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad (1)$$

The results of the oxidated substrate ABTS in the presence of H_2O_2 were shown in **Fig. 3** depending on the increased concentration of CSPIONs. The absorbance was increased along with increasing the amount of H_2O_2 in each group and reached the plateau at about 100 mM. The plotting figure between absorbance and concentration of H_2O_2 was tended to shift upward according to increasing the concentration of particles at the fixed substrate, indicating the more chromogenic substrate was oxidated by rapid reaction of CPSIONs and H_2O_2. This result demonstrated the particles act as a nanozyme and shown to be similar in the relationship between substrate and enzyme.

In similar to this work, the different shape of nanocrystals (clustered sphere, octahedra, and triangular) was investigated in the study of peroxidase-like activities.

Liu, Shanhu, et al. demonstrated different structure can affect the peroxidase of SPIONs and the cluster spherical shape as shown in high activity against chromogenic substrate [20]. Zhou et al. reported that the catalytic mechanism can roughly be divided into two groups; structure-insensitive and structure-sensitive. They suggested that decreasing the fewer crystals planes and increasing the more reactive planes were required to enhance the catalysis effect [21]. Although further studies for the precise mechanism of the enhanced nanozyme activity of cluster shape are required, based on the previous reports, either clustering in nucleation and growth stage or structure modification of nanoparticles can increase the activity toward the chromogenic substrate. Besides, it may be related to their preferential exposure of catalytically active iron atoms or crystals planes.

The Artificial Peroxidase Activity About H_2O_2 Derived from Glucose

In order to investigate the performance of the CSPIONs for glucose detection, the degree of oxidated substrate ABTS in the presence of different glucose concentrations was shown in **Figure 4**. There is no chemical mechanism that SPIONs detect the glucose, so we measured the H_2O_2 originated from glucose oxidase (GO_X) and glucose indirectly. It was also shown as substrate-enzyme affinity, presenting the absorbance was increased with glucose level increased. The results were highly accruable (standard deviation of all groups was less than 0.03) and demonstrated that the glucose was detected by using CSPIONs.

The Sensitivity and Limit of Detection of CSPIONs to Glucose and Comparison with Reported SPIONs

The sensitivity and LOD are presented in **Figure 5**. The absorbance and glucose concentration were correlated with each other linearly. The regressed line of the slope

represents the sensitivity of CSPIONs to glucose. The sensitivity was calculated in 1.50. Multiplying the y-intercepts of the regression line by 3 and dividing by the slope of the line can be considered as the LOD of CSPIONs to glucose. The LOD was presented as 5.44 μM.

In order to compare the synthesized CSPIONs with others reported SPIONs, the artificial peroxidase ability of modified in nanocrystals' structure or surface was summarized in **Table 1**. The CSPIONs were shown more sensitive to glucose compared to the case SPIONs used. The LOD was also lower than that of SPIONs, indicating that even small amounts of CSPIONs can oxidize the chromogenic substrate and successfully detect the degree of oxidation in the colorimetric based system.

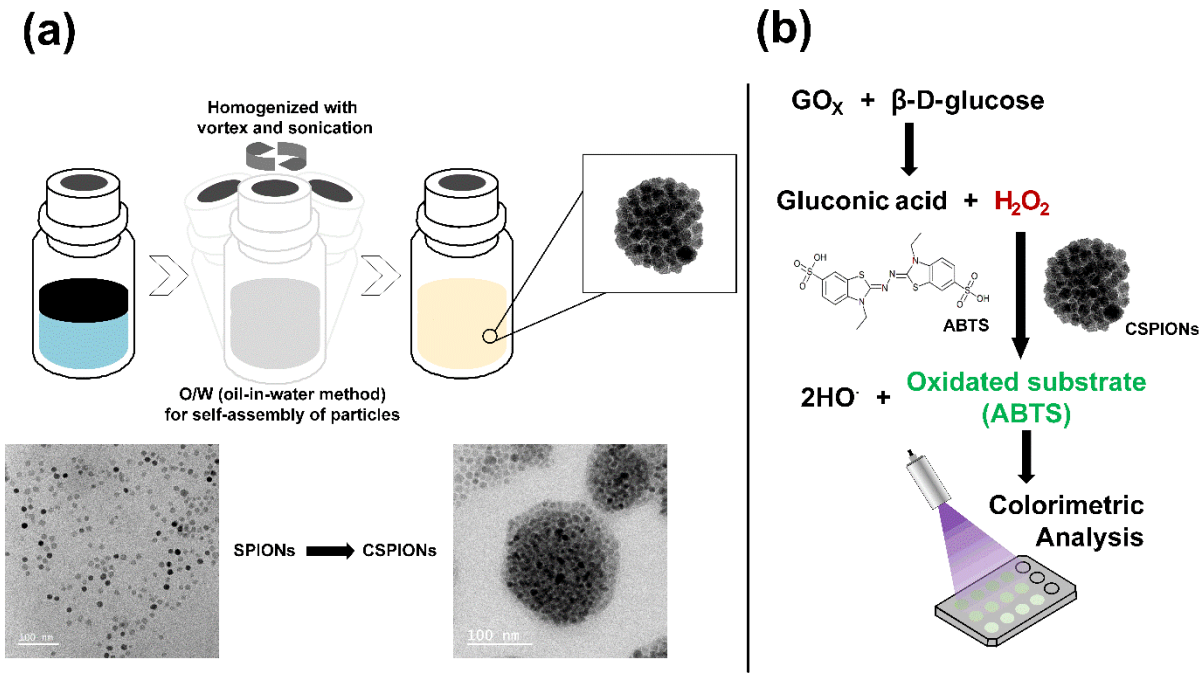

Figure 1: The schematic diagram of the synthesis and the artificial peroxidase process of clustered iron oxide nanoparticles (CSPIONs). **(a)** CSPIONs were synthesized via O/W (oil-in-water method) by homogenizing with high energy sources. The SPIONs (bottom, left) were clustered into the spherical shape (bottom, right). **(b)** One of the products of the reaction between GO_X and β-D-glucose is H_2O_2 and a chromogenic substrate, ABTS, can be oxidated in the presence of the CSPIONs by Fenton reaction leading to the change of color. We analyzed the ABTS assay at 417 nm wavelength.

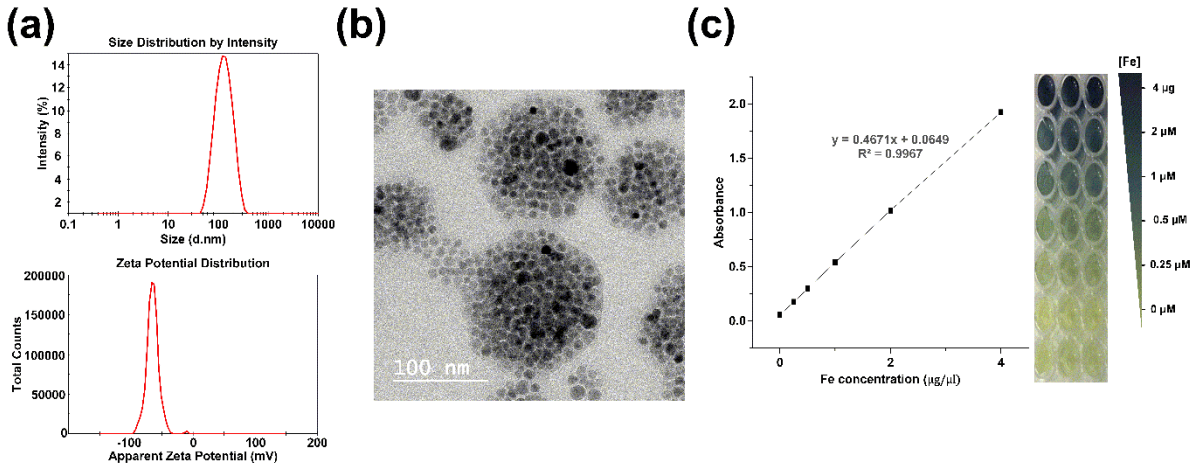

Figure 2: The characterization of CSPIONs. (a) The size (top) and zeta-potential (ζ-potential, bottom) of the particles were 120.1 nm and -61.7 mV, respectively (average value, n=3). (b) The transmission electron microscopy (TEM) image demonstrated the CSPIONs were clustered into PLGA. (c) The ferene-s assay was performed to quantify SPIONs in the clustered structures. The concentration of particles was exploited into 100.73µg/100µl considered as same in the ratio between mass and volume.

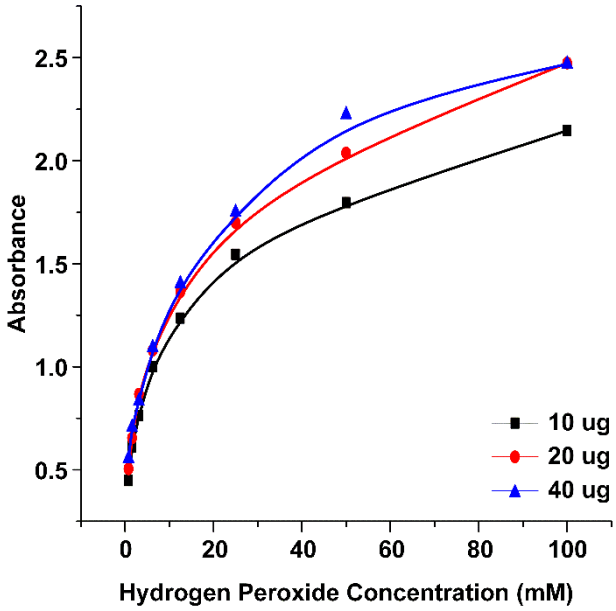

Figure 3: The artificial peroxidase activity of CSPIONs. The degree of oxidation of ABTS was increased along with the concentration of particles, and the results were saturated at 100 mM of H_2O_2. This suggests that the CSPIONs maintain the nanozyme ability after it was clustered in the polymeric material.

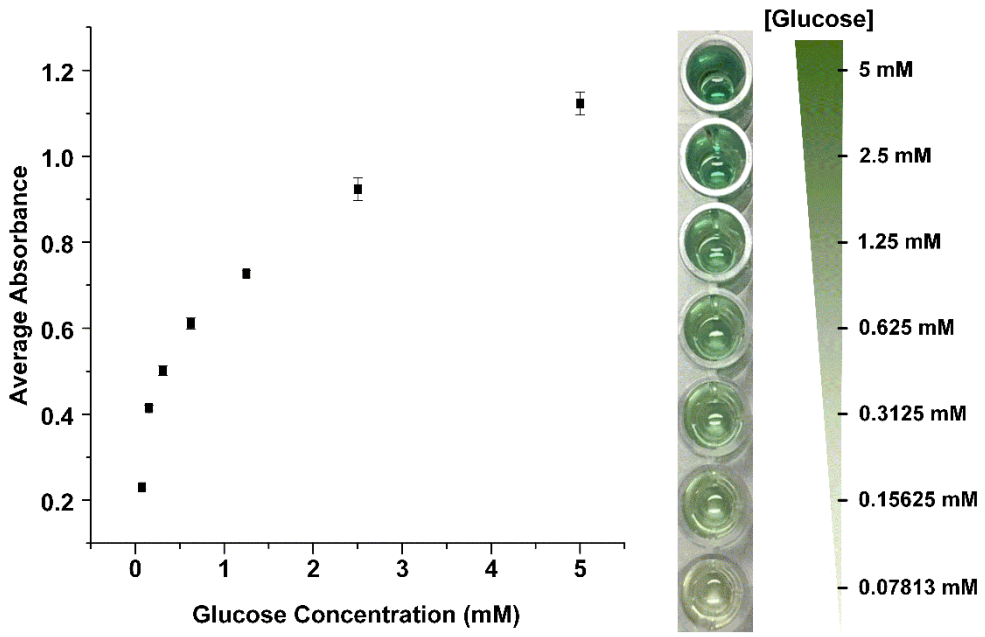

Figure 4: The glucose detection using CSPIONs. The mechanism of detection was based on the sensing of derived H_2O_2 from the reaction between GO_X and glucose. It also was shown for particles to have an artificial peroxidase in the complex whose environment contained real enzymes and abundant monosaccharides, and the results were highly repeatable (standard deviation of all samples < 0.03).

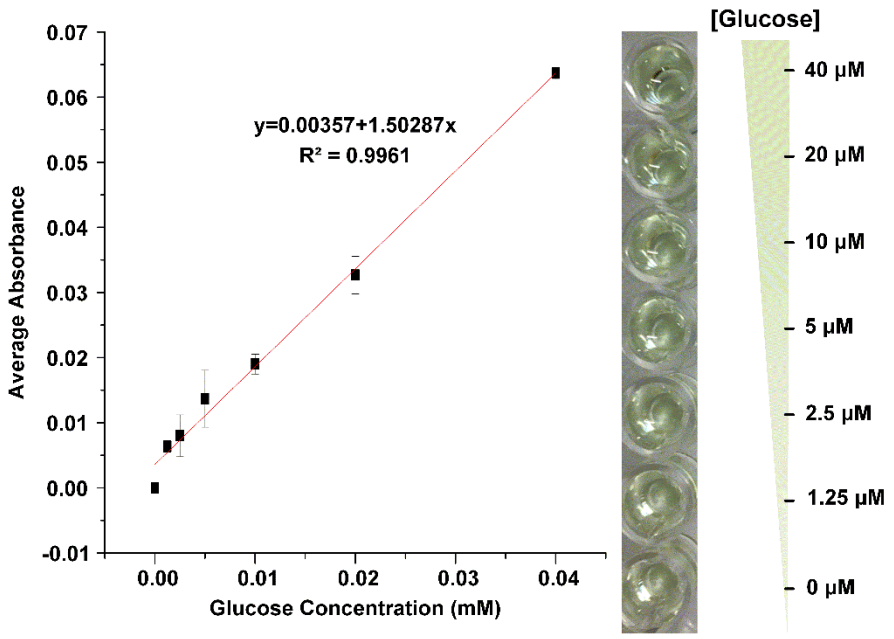

Figure 5: Investigation of the sensitivity and limit of detection (LOD) of CSPIONs to glucose. The sensitivity and LOD were shown as 1.50 and 5.44 µM.

Table 1: The artificial peroxidase in SPIONs.

Nanoparticles	Sensitivity	Limit of Detection	Substrate	[Fe]
Glycine-coated SPIONs [9]	1.251	8.5 µmol/L	ABTS	325 µg
Heparin-coated SPIONs [9]	0.910	15.8 µmol/L	ABTS	325 µg
GO_X-IONPs cellulose nanocrystals [10]	1.1304	83 µmol/L	ABTS	None
Citrate-coated SPIONs [11]	None	50 µmol/L	TMB	30 µg
Clustered iron oxide nanoparticles (CSPIONs)	1.50287	5.44 µmol/L	ABTS	50 µg

Conclusion

The CSPIONs were synthesized via the engineered bottom-up method. The particles kept the peroxidase ability after SPIONs are clustered in the amphiphilic PLGA polymer. The various range of glucose test (below 5 mM) demonstrated the CSPIONs detect the H_2O_2 from the reaction between glucose and GO_X in high repeatable (Standard Deviation < 0.03). The comparison with the reported SPIONs showed CSPIONs were more sensitive to glucose and had lower LOD despite using a substrate that is not proper to negatively charged particles. The results show that CSPIONs may serve as a glucose sensor. For future works, non-invasive and color-based glucose measurements using CSPIONs will be investigated.

Experimental

1. Chemicals and reagents

All materials were purchased and used without further modification and treatment. Iron oxide nanoparticles (SPIONs, 5 mg/ml, avg size: 10 nm), poly(lactic-co-glycolic acid) (PLGA), β-D-glucose, glucose oxidase (i.e. GOX, from Aspergillus niger), L-Ascorbic acid, TraceCERT Iron Standard for ICP, hydrogen peroxide solution, sodium acetate, and ferene-s 0.5 M were purchased from Sigma-Aldrich CO. Ltd. (St.Louis, MO, USA). 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from TCI Co. Ltd. (Tokyo, Japan). Acetic acid, glacial was bought from Fisher Science (Massachusetts, USA). Acetonitrile was purchased from Honeywell CO. Ltd. (North Carolina, USA).

2. The Synthesis of Clustered Superparamagnetic Iron Oxide Nanoparticles (CSPIONs)

The CSPIONs were synthesized using a bottom-up and oil-in-water (O/W) method. The solution containing 3 mL of deionized water, 500 μg of SPIONs, and 100 μg of PLGA dissolved in acetonitrile (1 mg/ml) was prepared. The SPIONs and PLGA in the organic solvent were added as a drop-wise manner into the deionized water. Vortexing and sonication procedure was required to make the reaction solution emulsified. After the homogenization, the solution was stirred at 400 RPM during 6 hrs on a magnetic stirrer (PC-420D, Corning, USA) to evaporate the organic solvent. The remaining solution was collected and first centrifuged at 2,700 RPM for 10 min (CF-10, DAIHAN Scientific, Korea), following gathering the supernatant carefully. The supernatant was centrifuged at 13,500 RPM for 10 min to purify CSPIONS from the solution.

3. The Quantification of SPIONs in CSPIONs

A ferene-s assay was performed. This assay can be used for accurate SPIONs quantification, and also regarded as reasonable compared to inductively coupled plasma mass spectrometer (ICP-MS) [17-19]. Briefly, a 1x working solution consists of 2.27 mM L-ascorbic acid in 2 M acetate buffer (pH ≈ 4.0), 12 mM ferene-s, and deionized water in a total volume of 10 ml. The standards of SPIONs were established using TraceCERT Iron Standard for ICP reagent from 0 µg to 4 µg. 50 µl of the sample (2 µl of CSPIONs plus 48 µl of deionized water) was added into 950 µl 1x working solution directly and spilled 300 µl per 96-well plate well for calculating the average absorbance of triplicates. The mixture was incubated in a heated oven at 37 ℃ for 20 hrs and then measured optical density [4] at 595 nm. The standard curve was exploited using average ODs of standard and sample concentration in 2 µl of CSPIONs can be assessed in reverse estimation.

4. The Characterization of CSPIONs

The study of the size, zeta-potential (ξ-potential), and shape of CSPIONs were done to characterize the CSPIONs. The sample was prepared into 1 ml volume (100: 1=deionized water: CSPIONs, volume ratio) in size (DTS0012, Malvern Panalytical Ltd, UK) and ξ cuvette (DTS1070, Malvern Panalytical Ltd, UK), respectively. The size and ξ-potential were measured by using Zetasizer (ZS90, Malvern Panalytical Ltd, UK). The size and morphology were determined by Cs-corrected STEM (JEM-ARM 200F, JEOL, USA).

5. Assessment of Artificial Peroxidase Ability of CSPIONs

To assess the artificial peroxidase ability of CPSIONs, we utilized colorimetric based 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) assay in the presence of H_2O_2. Briefly, 24 µl of 60 mM ABTS, 185 µl of 0.2 M acetate buffer (pH ≈ 4), and different amounts of CSPIONs (10 µg, 20 µg, and 40 µg) were used in this assessment. The H_2O_2 was half-diluted from 100 mM to 0.7813 mM (i.e. 781.3 µM) using PBS and the mixture was reacted at 45 ℃ for 10 min. After centrifuging the mixture at 13,500 RPM to remove precipitated CPSIONs, the supernatant of the solution was divided into 300 µl per well to determine average absorbance. Control groups were conducted using only PBS (no glucose in the sample).

6. Measurement of Glucose Using CSPIONs

Similar to the procedure of the assessment of artificial peroxidase, it was also based on the chromogenic substrate's oxidation. 40 µl of GO_X (10 mg/ml), 360 µl of 4 mM ABTS, and 50 µg of CSPIONs were added into various concentrations of β-D-glucose. The β-D-glucose was diluted from 5 mM to 0.078125 mM (i.e. 78.125 µM) in PBS and the blend was incubated at 45 ℃ for 45 min, following centrifuging the resulting solvent at 13,500 RPM to remove thrown CPSIONs. The supernatant was analyzed the same as the above paragraph.

7. The assessment of the sensitivity and limit of detection (LOD) to the glucose of CSPIONs

The sensitivity and LOD were identified below 40 µM glucose of concentration. The glucose level was half-diluted from 40 µM to 1.25 µM and analyzed ODs at 417 nm. The sensitivity and LOD were defined as (2) and (3), respectively.

$$\text{Glucose Sensitivity} = \frac{\Delta\text{Absorbance}}{\Delta\text{Glucose Concentration}} \approx \text{Coefficient of regressed } 1^{\text{st}} \text{ function} \quad (2)$$

$$\text{Limit of Detection (LOD)} = \frac{3 \times \text{Standard Deviation of y-intercepts of Regression Line}}{\text{Corresponding Slope of Regression Line}} \quad (3)$$

Acknowledgments

This research was funded by the National Research Foundation of Korea (NRF), grant numbers 2018R1D1A1B07042339, and 2019K2A9A2A08000123. Following are the results of a study on the “Leaders in Industry-university Cooperation +” Project, supported by the Ministry of Education and National Research Foundation of Korea

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