Article

Journal of Nanoscience and Nanotechnology
Vol. 20, 5333–5337, 2020
www.aspbs.com/jnn

Poly-γ-Glutamic Acid/Chitosan Hydrogel Nanoparticles Entrapping Glucose Oxidase and Magnetic Nanoparticles for Glucose Biosensing

Hye Su Kim, Ji Sun Lee, and Moon Il Kim*

Department of BioNano Technology, Gachon University, Seongnam, Gyeonggi 13120, Republic of Korea

We have developed hydrogel nanoparticles made of poly-γ-glutamic acid (PGA) and chitosan, which entraps both glucose oxidase (GOx) and magnetic nanoparticles (MNPs) within the hydrogel matrix. The preparation of poly-γ-glutamic acid/chitosan hydrogel nanoparticles (PGA/CS NPs) entrapping GOx and MNPs begins with the mixing of GOx and MNPs with PGA solution followed by their dropwise addition into chitosan solution to induce rapid ionic gelation. The glucose sensing relies on the generation of H_2O_2 through the entrapped GOx-mediated catalysis in the presence of glucose, which consequently activates the peroxidase-like activity of MNPs to convert an employed chromogenic substrate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), into a green colored product. Using this strategy, the target glucose was successfully detected over a wide linear range, from 5 to 100 μM with a lower detection limit of 3 μM, which is sufficient to diagnose high level of glucose (hyperglycemia) in human blood. The hydrogel nanoparticle-based glucose biosensor also showed high stability with magnetic reusability. Since any oxidative enzymes could be incorporated within the PGA/CS NPs, we expect that the hydrogel-based biosensor would be highly beneficial for the detection of various other clinically important target molecules.

Keywords: Hydrogel Nanoparticles, Glucose Detection, Poly-γ-Glutamic Acid (PGA), Chitosan.

1. INTRODUCTION

Since diabetes mellitus has become a worldwide public health concern, glucose detection technology providing high sensitivity, selectivity, and reliability has been intensively studied and attracted a lot of attention in biomedical science and food industry [1]. Among diverse glucose detection strategies, glucose biosensors employing glucose oxidase (GOx), which catalyzes the oxidation of glucose to produce gluconic acid and hydrogen peroxide (H_2O_2), play a leading role due to their high selectivity, sensitivity, and convenience to use [2]. Since horseradish peroxidase (HRP) could utilize H_2O_2 to produce diverse kinds of optical or electrochemical responses, HRP is generally coupled with GOx to develop glucose biosensors [3]. However, the intrinsic limitations of natural enzymes, such as low stability in harsh conditions, limited operating conditions, and high production/purification cost, have still hindered their widespread use in biosensing [4, 5]. Therefore, the development of highly efficient glucose biosensors to produce stable signals in diverse environments is critically needed.

In recent days, nanomaterials with enzyme-like characteristics, so called nanozymes, have been reported as potent alternatives to natural enzymes, due to their distinct superiorities such as high stability and robustness even in harsh conditions, low-cost production by facile scale-up, and possibility to further improve the catalytic features to exceed those of corresponding natural enzymes [4]. Until now, diverse kinds of nanostructures, such as iron oxide magnetic nanoparticles (MNPs), cerium oxide nanoparticles, platinum nanoparticles, gold nanoparticles, graphene oxide, and carbon nanotubes, have been reported as nanozymes having the ability to mimic natural enzymes including HRP [6–12]. MNPs are recognized as the most widely studied peroxidase-mimicking nanozymes that catalyzes the oxidation of peroxidase substrates such as 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) in the presence

*Author to whom correspondence should be addressed.

of H_2O_2 [13–16]. Until now, the peroxidase activity of MNPs have been widely utilized to detect various biomolecules, such as cells, proteins, and carbohydrates [17–19]. Cascade reaction composed of GOx and MNPs, which act as peroxidase, was also reported to construct glucose biosensors [17]. Although these examples demonstrate the potential of MNPs to replace natural HRP in biosensing applications, further investigations are still required to make them more compatible with other enzymatic systems and facilitate their easy separation and reuse.

To address this need, we have developed hydrogel nanoparticles made of poly-γ-glutamic acid (PGA) and chitosan (PGA/CS NPs), which consist of GOx as well as MNPs simultaneously entrapped within the hydrogel matrix. Through the dropwise addition of PGA solution containing GOx and MNPs into chitosan solution, hydrogel particles having sizes about several hundreds of nanometers were produced, with GOx and MNPs entrapped within the hydrogel matrix. Various characteristics of PGA/CS NPs entrapping both GOx and MNPs, such as specificity, sensitivity, operational stability, and magnetic reusability were investigated.

2. EXPERIMENTAL DETAILS

2.1. Materials

Glucose oxidase from *Aspergillus niger* (GOx), acetic acid, sodium acetate, phosphate buffered saline (PBS), iron(III) chloride hexahydrate, iron(III) chloride tetrahydrate, ABTS, glucose, fructose, lactose, urea, ascorbic acid were all purchased from Sigma-Aldrich (Milwaukee, WI) and used as received. PGA and chitosan (MW = 50 kDa) were purchased from Vedan (Taichung, Taiwan) and Axoin (China), respectively. All other reagents were of analytical grade.

2.2. Preparation of PGA/CS NPs Entrapping Both GOx and MNPs

MNPs were first synthesized via co-precipitation of Fe^{3+} and Fe^{2+} aqueous salt solutions (2:1 molar ratio) through the addition of a base [20]. 1 M sodium hydroxide solution was added to a mixture of 0.25 M of $FeCl_2$ and $FeCl_3$ ($Fe^{3+}/Fe^{2+} = 2$) in water until the pH changed to 10, then the mixture was heated at 80 °C for 40 min while stirring. The obtained MNPs were washed with DI water three times and ethanol three times, and dried at 70 °C under vacuum.

To encapsulate GOx and MNPs within PGA/CS NPs, PGA was first dissolved in DI water at a concentration of 1 mg/mL. Chitosan was also dissolved in 1% acetic acid solution. GOx (1 mL, 10 mg/mL) and MNPs (1 mL, 2 mg/mL) were thoroughly mixed with 2 mL of PGA solution at RT. Then, the mixtures were dropwisely added into the chitosan solution (1 mg/mL) with stirring to induce ionic gelation, and further reacted for 30 min at RT. The prepared hydrogel nanoparticles were analyzed by transmission electron microscopy (TEM) on a Jeol EM-2010 microscope (Jeol Co.) and energy dispersive X-ray spectrometer (EDS).

2.3. Colorimetric Detection of Glucose Using PGA/CS NPs Entrapping GOx and MNPs

To investigate the cascade reaction activity for the synthesized PGA/CS NPs entrapping GOx and MNPs for the detection of glucose, colorimetric assay was performed using ABTS substrate in the presence of glucose. Typically, synthesized NPs (50 μL, 2 mg/mL), glucose at diverse concentrations (50 μL), and colorimetric substrate (ABTS, 50 μL, 60 mM) were added in sodium acetate buffer (350 μL, 100 mM, pH 4.0), and incubated at RT for 30 min. After reaction, the NPs were separated by enforcing external magnet for 2 min, and the supernatant was transferred to the designated well of a transparent 96-well plate. The well-plate was used to capture color images representing the progress of the reaction, and the absorption intensity was also measured using a microplate reader in either scanning mode or at 417 nm (Synergy H1, Biotek, VT). To detect glucose using free GOx and MNPs, free GOx (50 μL, 5 mg/mL) and free MNPs (50 μL, 1 mg/mL) were used rather than PGA/CS NPs and the other procedures were the same as described above.

2.4. Stability and Magnetic Reusability

The stability of PGA/CS NPs entrapping GOx and MNPs was evaluated by measuring the glucose-detecting activity when incubating with 1 mM glucose in sodium acetate buffer (100 mM, pH 4.0) at RT with shaking (200 rpm). As a control, PGA/CS NPs entrapping only MNPs were incubated with free GOx (1 mg/mL), and the residual glucose-detecting activity was assessed as described above, at each time point. Reusability of PGA/CS NPs entrapping GOx and MNPs was also assessed after cycles involving the typical reaction with glucose (1 mM), simple separation with external magnet, and three washing steps with aqueous buffer. In both experiments, the relative activity (%) was calculated according to the ratio between initial and residual activity, in which the initial activity was set up as 100%.

3. RESULTS AND DISCUSSION

The construction of PGA/CS NPs entrapping both GOx and MNPs begins with the mixing of GOx and MNPs in PGA solution, followed by their dropwise addition into chitosan solution. This process results in PGA/CS hydrogel NPs entrapping both GOx and MNPs through the rapid ionic gelation (Fig. 1). We envisioned that the composite obtained by entrapping GOx and MNPs within hydrogel matrix would serve as an efficient biosensor capable of being used for colorimetric glucose detection. Specifically, in the presence of glucose, the catalytic action of

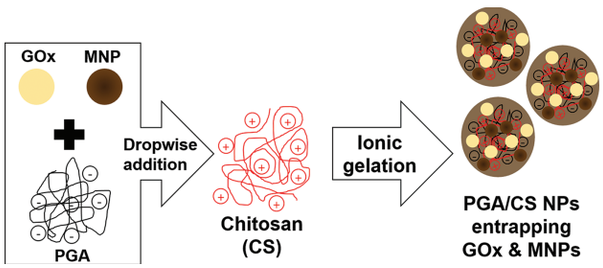

Figure 1. Schematic illustration of the PGA/CS NPs entrapping GOx and MNPs.

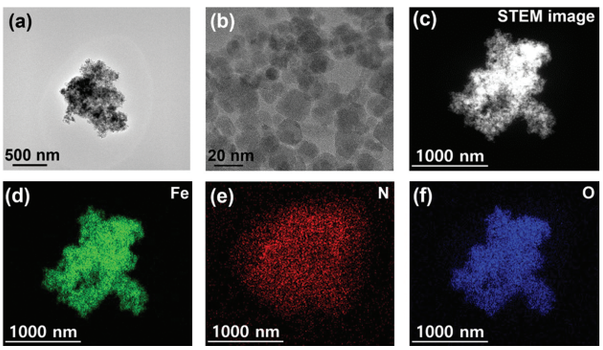

Figure 2. TEM images of (a) PGA/CS NPs entrapping GOx and MNPs, (b) entrapped MNPs, (c) STEM images of the cross-section of (a), and the corresponding EDS maps of (d) Fe, (e) N, and (f) O elements.

GOx is expected to generate H_2O_2, which should activate peroxidase-mimicking MNPs to convert the employed substrate, ABTS, into green colored product. To first obtain structural insights for the synthesized PGA/CS NPs entrapping GOx and MNPs, TEM analyses coupled with EDS elemental mapping were performed (Fig. 2). The TEM images revealed that MNPs with an average diameter of ∼15 nm were incorporated within the hydrogel NPs. Elemental mapping images of Fe, N, and O elements, which are the main components of MNPs, GOx, PGA, and chitosan, show their concentrated distribution region, demonstrating that MNPs and GOx are homogeneously positioned throughout the hydrogel matrix.

Glucose sensing capability of the hydrogel NPs was investigated. With free GOx and MNPs, target glucose was specifically detected through the generation of green color by the respective ABTS oxidation, whereas negligible signal was observed in a glucose-free control sample, indicating that glucose could be detected via GOx-MNPs cascade reaction (Fig. 3(a)). PGA/CS NPs entrapping GOx and MNPs were then employed for the glucose detection, yielding a green color corresponding to the oxidized ABTS (Fig. 3(b)). This result reveals that a specific colorimetric reaction of only the target glucose is promoted by our hydrogel NPs. Under the typical experimental conditions, glucose could be spectrometrically determined at concentrations as low as 3 μM with a linear range from 5 to 100 μM (Fig. 4). The limit of detection values are among the best results of those recently reported describing colorimetric measurement of glucose [7, 13].

Selectivity of PGA/CS NPs entrapping GOx and MNPs was explored by monitoring the interfering effects of four common substances (urea, ascorbic acid, fructose, and lactose), present in human blood, on the colorimetric detection of glucose (Fig. 5(a)). As a result, negligible signal is observed in the control solutions containing interfering species, proving the excellent selectivity of our hydrogel NPs for glucose biosensing applications.

In addition, we assessed stability and reusability of our system, both of which are important for practical applications. PGA/CS NPs entrapping both GOx and MNPs retained almost all of their original activity during 1 day operation with shaking, whereas the control sample consisting of PGA/CS NPs entrapping only MNPs with free GOx showed continuous decrease in their catalytic activity (Fig. 5(b)). After 1 day operation, the control sample lost ∼40% of its original activity for glucose detection. This result clearly demonstrates that the entrapment of GOx within the hydrogel matrix is essential to preserve original activity. The reusability was also assessed by

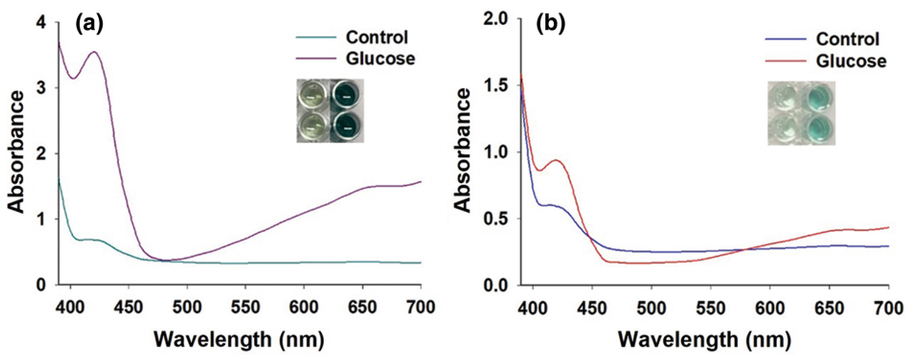

Figure 3. Absorption spectra of the reaction solution obtained via cascade reaction of (a) free GOx with MNPs, and (b) PGA/CS NPs entrapping GOx and MNPs, in the presence of glucose. A picture of the reaction solutions is shown in the inset. For the control, DI water was used instead of glucose.

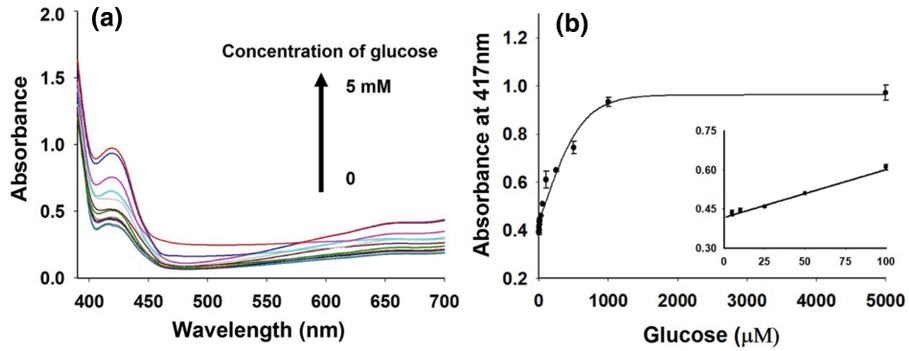

Figure 4. Absorption spectra of the reaction solution obtained via cascade reaction of (a) free GOx with MNPs, and (b) PGA/CS NPs entrapping GOx and MNPs, in the presence of glucose. A picture of the reaction solutions is shown in the inset. For the control, DI water was used instead of glucose.

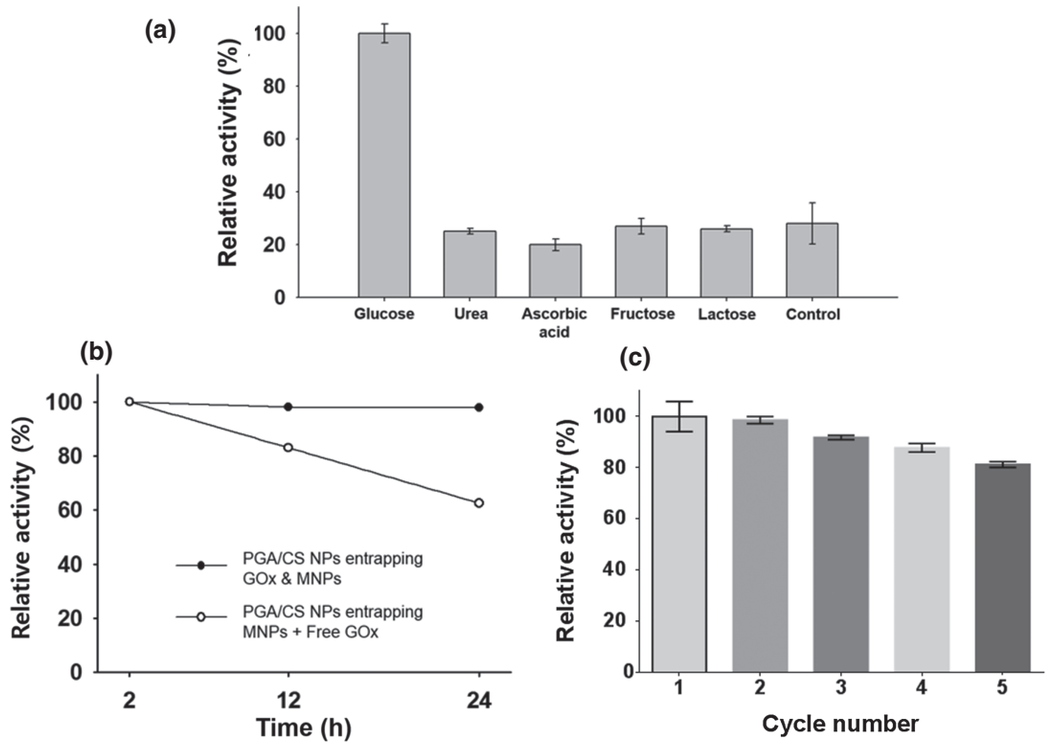

Figure 5. (a) Selectivity evaluation of PGA/CS NPs entrapping GOx and MNPs for the determination of target glucose by measuring the interfering effects of molecules commonly found in human blood. Considering their general levels in human blood, 1 mM glucose was tested with urea (0.67 mM), ascorbic acid (0.08 mM), fructose (0.02 mM), and lactose (0.67 mM). (b) Operational stability for the determination of glucose at RT with shaking condition (200 rpm). (c) Magnetic reusability after iterative cycles of glucose sensing involving facile magnetic separation and washing steps.

repeating cycles involving the typical glucose sensing, simple separation by external magnet, and three washing steps. As shown in Figure 5(c), over 80% of the initial catalytic activity was maintained after 5 cycles, indicating that our PGA/CS NPs entrapping GOx and MNPs could be used repeatedly. The marginal loss of the catalytic activity could be caused by the possible leaching of GOx or MNPs during the washing procedures.

4. CONCLUSIONS

We have developed a colorimetric biosensor based on nanocomposites entrapping GOx and peroxidase-like MNPs within PGA/CS hydrogel matrix. The hydrogel composites were simply prepared but yielded excellent sensitivity and selectivity for target glucose detection, along with high stability and magnetic reusability. Based on these results, we expect that the hydrogel-based

multi-catalytic nanostructures would be useful for convenient and highly efficient detection of biologically important target molecules, particularly for point-of-care testing within facility-limited environments, through the use of nanozymes rather than unstable natural enzymes.

Acknowledgments: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (Ministry of Science and ICT (NRF-2017R1C1B2009460)), and by the Gachon University research fund of 2018 (GCU-2018-0667).

References and Notes

1. Newman, J.D. and Turner, A.P., **2005**. Home blood glucose biosensors: A commercial perspective. *Biosensors and Bioelectronics, 20*(12), pp.2435–2453.
2. Wang, K., Xu, J.J., Sun, D.C., Wei, H. and Xia, X.H., **2005**. Selective glucose detection based on the concept of electrochemical depletion of electroactive species in diffusion layer. *Biosensors and Bioelectronics, 20*(7), pp.1366–1372.
3. Veitch, N.C., **2004**. Horseradish peroxidase: A modern view of a classic enzyme. *Phytochemistry, 65*(3), pp.249–259.
4. Wei, H. and Wang, E., **2013**. Nanomaterials with enzyme-like characteristics (nanozymes): Next-generation artificial enzymes. *Chemical Society Reviews, 42*(14), pp.6060–6093.
5. Shin, H.Y., Park, T.J. and Kim, M.I., **2015**. Recent research trends and future prospects in nanozymes. *Journal of Nanomaterials, 756278*, pp.1–11.
6. Gao, L.Z., Fan, K.L. and Yan, X.Y., **2017**. Iron oxide nanozyme: A multifunctional enzyme mimetic for biomedical applications. *Theranostics, 7*(13), pp.3207–3227.
7. Kim, M.I., Shim, J., Li, T., Lee, J. and Park, H.G., **2011**. Fabrication of nanoporous nanocomposites entrapping Fe_3O_4 magnetic nanoparticles and oxidases for colorimetric biosensing. *Chemistry—A European Journal, 17*(38), pp.10700–10707.
8. Kim, D.H., Hur, J., Park, H.G. and Kim, M.I., **2017**. Reagentless colorimetric biosensing platform based on nanoceria within an agarose gel matrix. *Biosensors and Bioelectronics, 93*, pp.226–233.
9. Kim, M.S., Kwon, S.H., Cho, S., An, S.S.A., Kim, M.I., Doh, J. and Lee, J., **2017**. Pt-decorated magnetic nanozymes for facile and sensitive point-of-care bioassay. *ACS Applied Materials and Interfaces, 9*(40), pp.35133–35140.
10. Luo, W., Zhu, C., Su. S., Li, D., He, Y., Huang, Q. and Fan, C., **2010**. Self-catalyzed, self-limiting growth of glucose oxidase-mimicking gold nanoparticles. *ACS Nano, 4*(12), pp.7451–7458.
11. Kim, M.S., Cho, S., Joo, S.H., Lee, J., Kwak, S.K., Kim, M.I. and Lee, J., **2019**. N and B co-doped graphene: A strong candidate to replace natural peroxidase in sensitive and selective bioassays. *ACS Nano, 13*(4), pp.4312–4321.
12. Song, Y., Wang, X., Zhao, C., Qu, K., Ren, J. and Qu, X., **2010**. Label-free colorimetric detection of single nucleotide polymorphism by using single-walled carbon nanotube intrinsic peroxidase-like activity. *Chemistry—A European Journal, 16*(12), pp.3617–3621.
13. Shin, H.Y., Kim, B.G., Cho, S., Lee, J., Na, H.B. and Kim, M.I., **2017**. Visual determination of hydrogen peroxide and glucose by exploiting the peroxidase-like activity of magnetic nanoparticles functionalized with a poly(ethylene glycol) derivative. *Microchimica Acta, 184*(7), pp.2115–2122.
14. Liu, Y., Yuan, M., Qiao, L. and Guo, R., **2014**. An efficient colorimetric biosensor for glucose based on peroxidase-like protein-Fe_3O_4 and glucose oxidase nanocomposites. *Biosensors and Bioelectronics, 52*, pp.391–396.
15. Kim, M.I., Ye, Y., Won, B.Y., Shin, S., Lee, J. and Park, H.G., **2011**. A highly efficient electrochemical biosensing platform by employing conductive nanocomposite entrapping magnetic nanoparticles and oxidase in mesoporous carbon foam. *Advanced Functional Materials, 21*(15), pp.2868–2875.
16. Shin, H.Y., Cho, S. and Kim, M.I., **2017**. Enzyme-free colorimetric detection of glucose using a composite entrapping gold and magnetic nanoparticles within an agarose gel matrix. *Journal of Nanoscience and Nanotechnology, 17*(11), pp.7971–7977.
17. Cheon, H.J., Adhikari, M.D., Chung, M., Tran, T.D., Kim, J. and Kim, M.I., **2019**. Magnetic nanoparticles-embedded enzyme-inorganic hybrid nanoflowers with enhanced peroxidase-like activity and substrate channeling for glucose biosensing. *Advanced Healthcare Materials*, *8*(9), p.1801507.
18. Kim, M.I., Kim, M.S., Woo, M.A., Ye, Y., Kang, S.K., Lee, J. and Park, H.G., **2014**. Highly efficient colorimetric detection of target cancer cell utilizing superior catalytic activity of graphene oxide-magnetic-platinum nanohybrids. *Nanoscale, 6*(3), pp.1529–1536.
19. Kim, M.I., Ye, Y., Woo, M.A., Lee, J. and Park, H.G., **2014**. A highly efficient colorimetric immunoassay using a nanocomposite entrapping magnetic and platinum nanoparticles in ordered mesoporous carbon. *Advanced Healthcare Materials, 3*(1), pp.36–41.
20. Mehta, R., Upadhyay, R., Charles, S. and Ramchand, C., **1997**. Direct binding of protein to magnetic particles. *Biotechnology Techniques, 11*(7), pp.493–496.

Received: 18 May 2019. Revised/Accepted: 11 June 2019.