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The quest for reusability: The facile and stable immobilization of papain on cysteine functionalized iron oxide nanoparticles activated glass surface

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The study displays the immobilization of papain on cysteine functionalized iron oxide nanoparticle coated glass beads. Glass beads were treated with (3-mercaptopropyl) trimethoxysilane and iron oxide nanoparticles capped with cysteine, to create a layer of cysteine on the surface of glass bead. This functionalized glass bead surface was further used to immobilize papain through glutaraldehyde treatment. The average size of cysteine capped iron oxide nanoparticles were found to be 50 nm. The binding of cysteine through the iron oxide nanoparticles was validated by Fourier transform infra-red spectroscopy. The activity of enzyme was found to be stable at variable temperature and pH conditions. The covalently immobilized enzyme on the glass bead sustained high enzyme activity and could be reused for 5 times without losing its activity. The immobilized papain retained 81% of its initial activity after 5 consecutive cycle. The storage stability analysis of the immobilized system revealed that it is stable for 6 months without loss in its activity. An average of 1.8 mg papain was successfully immobilized per gram of glass beads. In this case cysteine emerges as a new and effective medium for immobilizing biomolecules as it provides high efficiency towards immobilization.

Keywords: Cysteine, Iron oxide nanoparticle, Papain immobilization

Enzymes act as a catalysts, they are very specific in nature and require ambient pH and temperature conditions; thus are extremely useful in industrial application[1]. They have potential role in various biological fields. To increase the utility of enzymes in a much more economical way in numerous biomedical, bioanalytical and biochemical procedures, the enzymes need to be immobilized. This prevents the loss of enzyme during the process and facilitates its various usage in biotechnological fields. Immobilization can also tune the activity, stability and specificity of the enzyme. There are various methods that have been reported for enzyme immobilization to enhance their enzymatic activity, stability and reusability. The efficiency of immobilization depends on the method used for immobilization as well as the choice of matrix. Immobilization on glass beads were found to be an easy approach as it is very stable, provide better handling, non-toxic and can be retrieved easily from the reaction mixture and washed for further use[2]. Success and efficiency of biomolecule immobilization on glass surface can be enhanced by surface activation/ modification. Various reagents like sol-gel precursors (3-Aminopropyl) triethoxysilane, (3-mercaptopropyl) trimethoxysilane that contains alkoxy, thiol, silane groups can be utilized. These groups when coated on the inter surface of glass provide range of various substrate immobilization[3]. In past few decades metallic nanoparticles particularly iron oxide, zinc oxide, gold nanoparticles have received much attention as it provides strong chemical stability and decrease the toxicity which facilitate their use in various disease diagnosis, medicines, drug delivery and environmental applications[4-7]. As most of the iron nanoparticles poses magnetic property thus recovery of the immobilized system utilizing them as immobilization matrix is comparatively easier[8]. The amino acid cysteine is considered to be more suitable capping agent as it contains thiol group (-SH) which demonstrates higher affinity towards nanoparticles and other free groups present on the surface of biomolecules[9-12]. Enzyme L-lactate dehydrogenase isolated from strain *Bacillus stearothermophilus* reported to immobilize using cysteine residue. Formation of a bio-conjugate by coupling with cysteine thiol on solid supports is widely used process. The immobilization method using cysteine

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involves the formation of heterodisulfide linkage that provide site-specific reactions. On the other hand these thiol linkages are more susceptible to self-oxidation thus offers disulfides linkages very easily at certain pH ranges from 7 to 9 when the oxygen is present[3].

Therefore in this study the covalent immobilization method of cysteine functionalized iron oxide nanoparticles has been described. The cysteine functionalize nanoparticles were used as a linker between glass bead and papain. Glutaraldehyde treatment was used for the activation of $–NH_2$ functional group on the surface of cysteine. The glutaraldehyde mediated activation of the support matrix is one of the most common and olden method used for enzyme immobilization and it is still being used as a popular method due to its application in new areas[13,14]. The immobilization of papain and its efficiency in varying condition of pH and temperature was studied.

Material and Methods

Chemicals

Ferric chloride ($FeCl_3$), Sodium hydroxide (NaOH), Papain (from *Carica papaya*) (EC 3.4.22.2), *Camellia Sinensis*, L-cysteine, and glass beads were procured from Himedia. (3-Mercaptopropyl) trimethoxysilane (MPTS, 95% purity) was obtained from Sigma-Aldrich, glutaraldehyde (25% purity) was purchased from Loba Chemie.

Synthesis of cysteine capped iron oxide nanoparticles (CIONPs)

To synthesis CIONPs, green tea was used as the reducing agent[15]. The green tea (4gm) was boiled for 10 minutes in 50 mL of distilled water and then filtered with whatman filter paper No. 1. The filtrate was subjected to centrifugation at 5000 rpm for 15 min. In 50 mL of 0.01 M $FeCl_3$ 1 mM cysteine was added. 50 mL extract of green tea was added into the solution with constant stirring at 60°C for 30 min. The solution's pH was maintained at 11 by adding 10 mM NaOH. The color change of the reaction mixture from brown to black indicates the formation of iron oxide nanoparticles. The separation of nanoparticle from solution mixture was done by centrifugation at 8000 rpm for about 30 min with further drying at 50°C for 2 h. The structure of the CIONPs were analyzed from transmission electron microscopy (TEM M/s JOEL JEM 2100). The capping of L-cysteine on the surface of iron oxide nanoparticles was confirmed by Fourier transform infrared (FT-IR Bruker Alpha) spectroscopy by taking the transmittance in the range of 500 to 4000 cm^{-1}.

Immobilization of papain on glass bead

Papain immobilization steps on activated glass bead surface using CIONPs as a linker has been diagrammatically represented in (Fig. 1) and the description of the same as follows.

Silanization of glass beads

The glass beads (1 g) were first washed with methanol and HCl solution (1:1 volumetric ratio) at room temperature for 30 min. The beads were dipped in a piranha solution prepared by mixing H_2SO_4 and H_2O_2 in 3:1 volumetric ratio at 70°C for 30 min. The beads were carefully washed with DI water. Silanization was carried out by placing the washed beads in 5% MPTS solution for 2 h.

Coupling of L-cysteine capped iron oxide nanoparticles

The silanized beads were washed with methanol and further drying was done at room temperature. The beads were incubated overnight in the solution of freshly prepared cysteine capped iron oxide nanoparticle solution synthesized by following the method explained in section 2.2. On the successive

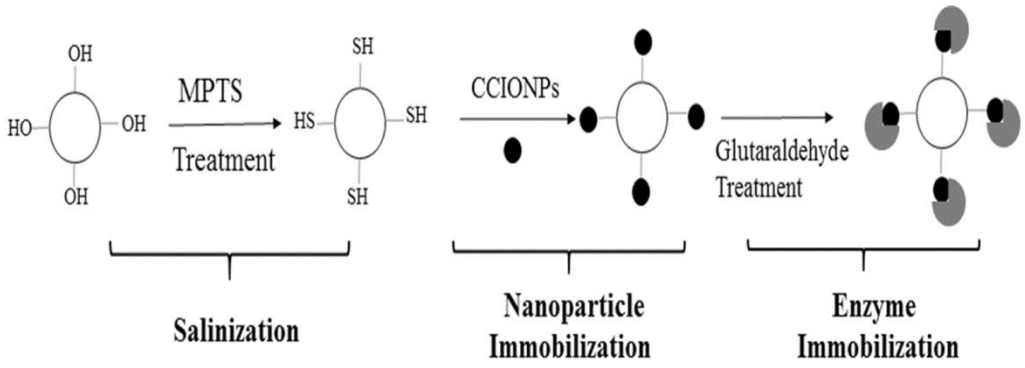

Fig. 1 — Schematic representation of papain immobilization on the glass bead's surface of glass

day the beads were retrieved and washed with DI water and methanol.

Immobilization of enzyme

Immobilization of enzyme was carried out in two sequential steps. First the nanoparticle coated beads were subjected to glutaraldehyde treatment to activate amino-group[16,17]. Beads were soaked in 1% glutaraldehyde solution for 2 h with constant stirring at 100 rpm. Glutaraldehyde treated beads were washed thoroughly with DI water. For the immobilization of papain the glutaraldehyde activated nanoparticles coupled glass beads were incubated with enzyme solution (30000 U/mg, 10 mL) for 24 h at 4°C in phosphate buffer (50 mM, pH 7). After the incubation period was completed the beads were removed from the enzyme solution and washed with ice-cold phosphate buffer (50 mM, pH 7). The immobilized papain thus obtained were subjected to enzyme activity assay using casein (0.6%) as the substrate. Activity of papain was estimated by measuring the amount of tyrosine released during the reaction and it is the estimation of the activity of papain. Per unit enzyme activity of papain was defined as amount of enzyme which liberated 1 µM of tyrosine per minute under the optimum assay conditions. The standard curve was plotted using Lowry method, taking bovine serum albumin (BSA) protein and the amount of protein immobilized on the beads was calculated by taking the difference between the initial concentration loaded and the amount of protein left after immobilization in the enzyme buffer solution[18].

Reusability and stability analysis

The study of the reusability of the papain coated beads were assayed by studying the enzyme activity of the same beads for 5 continuous cycle with the interval of 24 h between successive assay. After every assay the beads were washed and stored in the phosphate buffer at 4°C. The stability of the soluble and immobilized enzyme was studied by measuring their enzyme activity in various temperature and pH ranges. Storage stability of the immobilized enzyme system was analyzed for a period of 6 months, during the analysis the papain coated beads were stored in phosphate buffer (50 mM, pH 7) at 4°C and assayed periodically every month. All the experiments were carried out in the triplicates (n=3) the mean and standard deviation of the data was calculated and represented by Origin Pro version 2018b.

Results and Discussion

Synthesis of cysteine capped iron oxide nanoparticles

The CIONPs were synthesized using green tea as a reducing agent to reduce the salt of $FeCl_3$ in the presence of L-cysteine. L-cysteine was added to the solution and green tea was used as a reducing agent for the synthesis of nanoparticles at alkaline pH[11]. This results in the formation of cysteine capped iron oxide nanoparticle synthesis. To analyze the structural morphology the synthesized nanoparticles were subjected to TEM analysis. TEM analysis (Fig. 2) shows that the nanoparticles obtained were spherical in shape with the average size of 50nm. Further the cysteine capping on the surface of CIONPs was confirmed by FTIR. The characteristic peak of SH group at 2500 cm^{-1} was observed in the cysteine was disappeared in the synthesized iron oxide nanoparticles. This disappearance of thiol group indicates the binding of cysteine with iron oxide nanoparticles *via* thiol group and eventually indicates formation of CIONPs (Fig. 3). Various reports regarding disappearance of thiol group in case of functionalization of nanomaterials with cysteine have been reported[3,19]. However two bands at 1742 cm^{-1} and 1576 cm^{-1} position which corresponds to carbonyl (C=O) and C=C stretches have slightly shifted from their positions in CIONPs spectra that gives a clear indication of the attachment of cysteine on iron oxide nanoparticle' surface[20].

Immobilization of enzyme

The work represented here describes the role of CIONPs to increase the efficiency of papain immobilization on the glass bead. The CIONPs creates a bridge between glass bead and papain. The CIONPs enhances the loading capacity of the enzyme as it has high surface to volume ratio. The glass beads needs to be activated before loading the nanoparticles and enzyme to generate the functional groups on its surface. The glass beads were first silanized by (3-mercaptopropyl) trimethoxysilane (MPTS) that generates thiol groups on the surface. The silanized glass beads were analyzed by SEM for its surface characterization (Fig. 4). The images indicates that after salinization of the glass bead continuous layer of MPTS was present instead of non-treated glass bead's surface.

The silanized glass beads surface create the even layer of nanoparticles[21]. Furthermore the glass bead surface bounded with CIONPs was subjected to

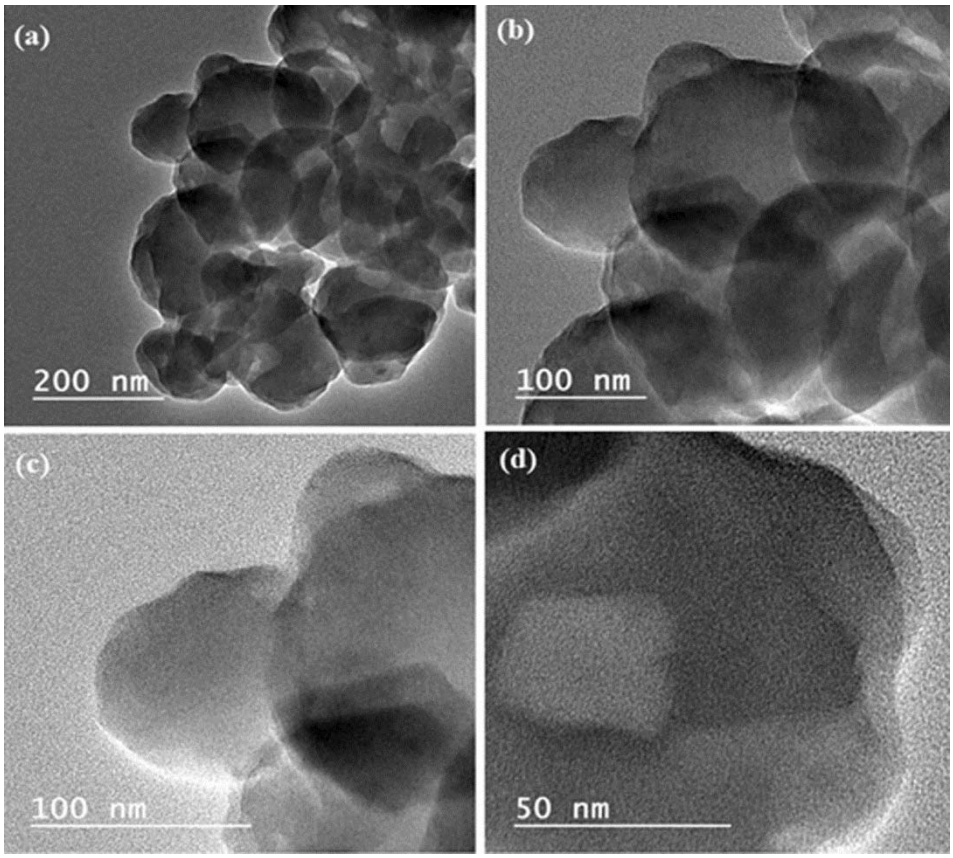

Fig. 2 — TEM images of CIONPs, with scale bar corresponds to (A) 200 nM; (B) 100 nM; (C) 100 nM; and (D) 50 nM

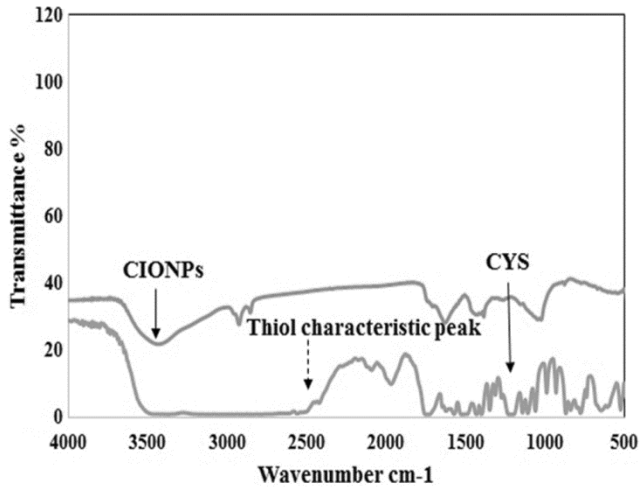

Fig. 3 — FTIR spectrum indicates the surface modification of iron oxide nanoparticles by cysteine

glutaraldehyde treatment. Glutaraldehyde treatment activates the amino group of cysteine residue present on surface of nanoparticle attach to glass bead. Similar studies has been reported regarding the attachment of silver nanoparticle on the glass surface by Upadhyay and Verma (2014)[3].

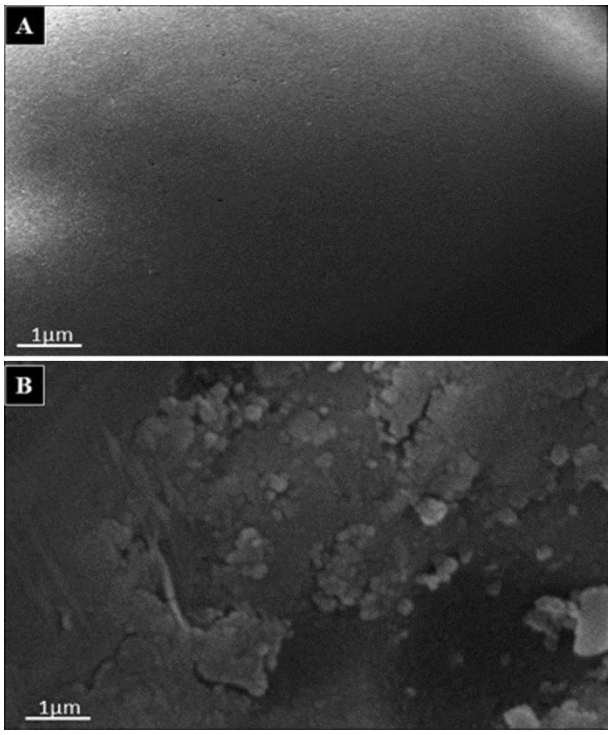

Fig. 4 — SEM images of (A) without MPTS salinization; and (B) MPTS salinized glass beads

Table 1 described the immobilization efficiency of immobilization of papain on glass beads. It was observed that the papain was successfully immobilized on the glass bead with the increment in the enzyme activity and stability. The concentration of immobilized protein and activity on the surface of glass bead was calculated to be 0.09±0.007 mg/bead and 2699.02±72.3 U/bead respectively. To test the stability of the immobilized enzyme with soluble enzyme the experiments were conducted at different pH and temperature ranges from 5-11 and 20-60°C respectively. The pH of the buffer system changes the charge in the solution thus affects the structure and activity of the papain. The pH profile to establish pH optima for soluble and immobilized papain is represented in (Fig. 5) and it was found to be 7 and 9 for soluble and immobilized enzyme respectively. It was observed that immobilized papain was more stable in a range of pH 5-11 as compare to soluble papain. A shift in the pH optima was also achieved from 7 (soluble) to 9 (immobilized).

The change in temperature of reaction mixture can alter the rate of enzyme catalyzed reactions. The increase in the temperature of the reaction mixture can increase the reaction rate to a certain point further rise in the temperature alter the structure of enzyme thus eventually decrease the activity. The Figure 6 represents and explains the temperature profile and effect of temperature on the activity of soluble enzyme and immobilized. The maximum activity of both the soluble and immobilized enzymes were found to be at 40°C but the immobilized enzyme showed significant activity up to a temperature of 60°C also which was found to be higher than the activity observed in soluble enzyme counterpart.

Immobilized systems are designed in such way that the biological element can be utilized again and again without losing their activity and can be stored for a longer time period. Therefore reusability and stability are the major concern and advantage of immobilized systems. The reusability of the immobilized biological component depends on various factors which includes the immobilization matrix, the storage condition etc. The immobilization of papain via CIONPs as linker was done by non-reversible linkage formation that provides strong support and binding of the enzyme on the glass beads. To check the reusability of the immobilized enzyme the surface of glass bead its activity was tested for 5 successive days. After each test the bead was washed with phosphate buffer (50 mM, 7pH) and stored at 4°C. Figure 7A displayed that the beads has retained 81% of its activity after 5 successive uses. Thus it shows that immobilization has provided the reusability of the papain enzyme. Further the analysis of storage stability was done and the immobilized papain was found to be extremely stable up to 6 months. As the Figure 7B describes that there is very less decrease in the activity was observed

Table 1 — Immobilization efficiency of papain on glass bead

Method	Initial concentration (mg)	Immobilized enzyme concentration (mg/bead), (mg/g bead)	Enzyme activity (U/bead)
Covalent immobilization	20 ±0.02	0.09±0.007, 1.8±0.009	2699.02±72.3

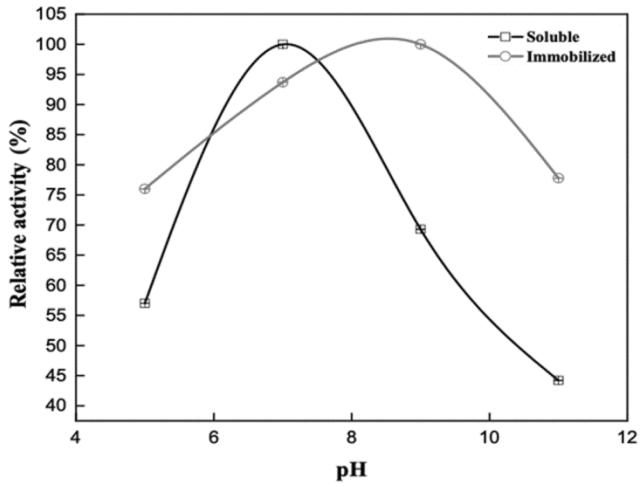

Fig. 5 — Effect of pH on the activity of soluble and immobilized papain enzyme

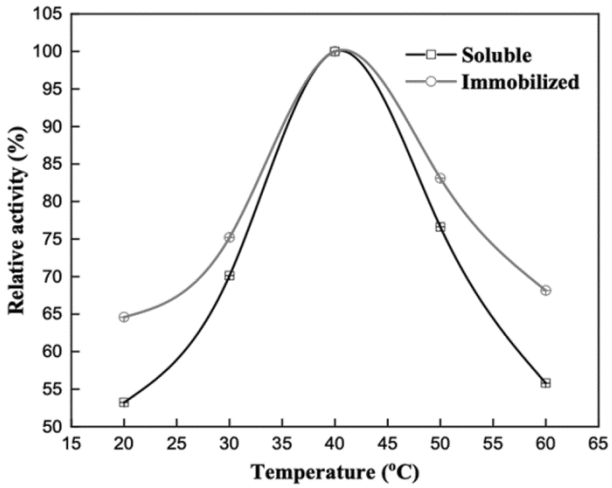

Fig. 6 — Effect of temperature on the activity of soluble and immobilized enzyme

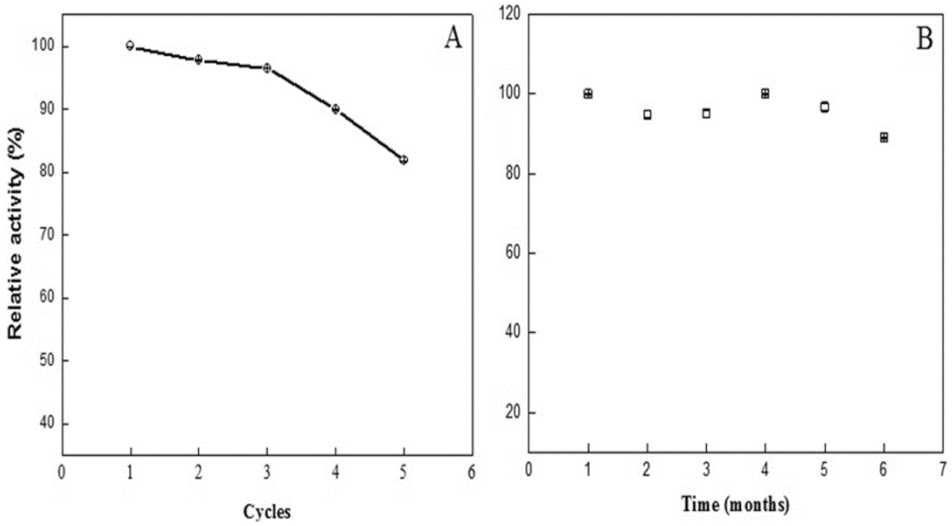

Fig. 7 — (A) Change in the activity of immobilized papain for 5 cycles; and (B) Stability analysis of the immobilized papain for 6 months

after keeping it for 6 months, thus this bioconjugate can be stored for a longer time. This enhanced the shelf life of enzyme papain that was not observed in the soluble form.

Conclusion

The study defined a suitable approach for immobilization of papain on surface of glass beads employing CIONPs as linker. Glass beads were first functionalized with (3-mercaptopropyl) trimethoxysilane to activate thiol group and it was further subjected to CIONPs to create inter connecting layer. The free amino group on CIONPs was activated by glutaraldehyde treatment for immobilizing papain. The immobilization methods adopted provides stability to the papain on glass bead and it retained 81% of its activity after 5 times of reuse and was stable up to 6 months under appropriate storage conditions. From the study it can be concluded that enzyme was efficiently immobilized and it's also resulted into increase in durability of the papain at high pH and temperature. This approach can be utilize to develop more immobilized systems with different biomaterials.

Conflict of interest

All authors declare no conflict of interest.

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