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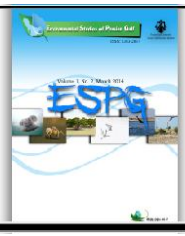

Cytotoxity Assessment of Gold Nanoparticle-Chitosan Hydrogel Nanocomposite as an Efficient Support for Cell Immobilization: toward Sensing Application

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ABSTRACT

Cell-based biosensors have become a research hotspot in biosensors and bioelectronics fields. The main feature of cell-based biosensors is the immobilization of living cell on the surface of transducers. Different types of polymers which are used as scaffolds for cell growth should be biocompatible and have reactive functional groups for further attachment of biomolecules. In this work, the cell attachment and proliferation on the chitosan hydrogel, chitosan-gelatin and gold nanoparticle–chitosan nanocomposite membranes were investigated. Characterization of the membranes was performed using Scanning Electron Microscopy and Infrared spectroscopy. Cytotoxicity assessment was carried out for all membranes and controlled using MTT assay on HEK293 cells. Cell morphology and viability were assessed to evaluate the cell attachment and proliferation. Regarding cell studies, the findings revealed that chitosan hydrogel and gold nanoparticle–chitosan hydrogel nanocomposite membranes did not induce cytotoxic effects while chitosan-gelatin's effect was very low on cell proliferation. Moreover, the data showed that gold nanoparticle-chitosan hydrogel nanocomposite membrane improved HEK293 attachment and adhesion more than other membranes. Overall, our data demonstrated that gold nanoparticle-chitosan hydrogel nanocomposite membrane constitutes an effective surface for immobilizing cells toward sensing application.

1 INTRODUCTION

For a decade, cell-based biosensors have become a research hotspot in biosensors and bioelectronics fields because they can detect the functional information of biological active analysts (Wang et al., 2012; Wang et al., 2005). Because cell-based biosensors have lots of advantages (e.g. Long-term recording in noninvasive ways, fast response, and easy fabrication), they have extensive applications including pharmaceutical screening, cellular physiological analysis, toxin detecting, peripheral nerve regeneration, and environment monitoring, as well as in vivo recordings(Wang et al., 2012).

The main feature of cell-based biosensors

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is that they employ living cells as receptors in contrast to the other types of biosensors that contain only materials extracted from living things. Picking up, isolating, and immobilizing the living cell on the surface of transducers, are the main concerns of cell-based biosensors research. It is a big challenge to find good coupling between cells and substrate and get accurate signals from the living cells (Park and Shuler, 2003; Kiilerich-Pedersen and Rozlosnik, 2012).

If efficiently coupled to an electronic readout device, cells could function as versatile biosensors in a variety of applications. The materials should fulfill their biological functions within their natural biological media. The living cells must be immobilized around or on the surface of the transducer without limiting their biological functions (Park and Shuler, 2003; Xu et al., 2006).

Different types of polymers have been used as scaffolds for cell growth. Ideally, this polymeric material should be biocompatible and have reactive functional groups for further attachment of biomolecules. One such natural polymer is chitosan that offers distinctive advantages such as good biocompatibility, nontoxicity, remarkable affinity to proteins, and excellent gel-forming ability (Hossler et al., 2004; Ding et al., 2007; Feng et al., 2009). Chitosan mostly comes from chitin, a natural biopolymer extracted from the shells of crustaceans. Chitosan has been found to be a good chemical entity for synthesizing hydrogels because of its greater crosslinking ability due to the presence of amino (–NH2) group (Rohindra et al., 2004; Han et al., 2004; Venaultc et al., 2011).Because of its numerous biological properties, chitosan alone and in association with other polysaccharides, in natural and cross-linked forms has been extensively studied (Shingh et al., 2005; Potara et al., 2012).

Gelatin is made up of a mixture of peptides and proteins produced by partial hydrolysis of naturally occurring collagen. The carboxyl groups on its chain backbones is one of its amazing features that creates the possibility of forming hydrogen bonds with chitosan for a well-mixed hybrid (Nagahama et al., 2008; Ahmad et al., 2011). On the other hand, a natural biopolymer such as chitosan is currently receiving a great deal of attention for medical and pharmaceutical applications.

With unique chemical and physical properties, gold nanoparticles (GNPs) have shown widespread use in basic research particularly in biological and sensing applications (preparation of nontoxic support for immobilization of cells, construction of cell-based sensors and electrochemical study of cells on surface). Incorporation of GNPs into polymer matrices has attracted increasing interest in improving the stability and biocompatibility of GNPs and also enhancing their capability for immobilization (Alexandridis, 2010; Huang et al., 2004; Sedeno and Pingarron, 2005).

Human Embryonic Kidney cells (HEK293) HEK 293 cells are very easy to grow and transfect very readily. In addition, they have been widely used in cell biology research for many years. As an experimentally transformed cell line, HEK 293 cells are a particularly good model for studying the effects of substrate on cell attachment and growth, and also studying the effects of drugs on cell receptors.

Previous study performed by Ding et al. (2007) has shown that GNP-CHIT nanocomposite is useful for the immobilization of cells and electrochemical cell-based biosensor. In this study, we tried to survey the capability of supports of chitosan (chitosan hydrogel, chitosan-gelatin and chitosan hydrogel-GNP nanocomposite) for the immobilization and proliferation of HEK293 cells. We compared the biological response (attachment and proliferation) of HEK293 cells

seeded onto three kinds of chitosan membrane.

2 MATERIALS AND METHODS

2.1 Materials

AuCl3HCl.4H2O (Au% > 48%) and Medium molecular weight Chitosan were purchased from Sigma-Aldrich. The degree of deacetylation was between 75-85%. Phosphate buffer saline (PBS) (pH 7.4) contain NaCl 137 mM, KCl 2.7 mM, Na2HPO4.12H2O 87.2 mM, and KH2PO4 14.1 mM. Type A Gelatin from porcine skin, ~175 g Bloom, was purchased from Sigma-Aldrich. Fourier-transform infrared spectroscopy (FTIR) was carried out on a NEXUS 870 FTIR. The morphology of dried film was observed under a 1530VP Scanning Electron Microscope.

2.2 Cell line and cell culture.

HEK293 cell line was kindly provided by the Affiliated Dr. Hosseinkhani's faculty of biological science at Tarbiat Modares University in Tehran, Iran. HEK293 cells were cultured in a flask in DMEM high glucose medium (GIBCO) supplemented with 10% fetal bovine serum (FBS, Sigma), penicillin, and streptomycin (sigma) at 37 °C in a humidified atmosphere containing 5% CO2. After 72 h, the cells were collected and separated from the medium by centrifugation at 1200 *g* for 5 min. The sediment was resuspended in the PBS to obtain a homogeneous cell suspension. Cell number was determined using a neobar lam and light microscopy.

2.3 Preparation of Gold Nanoparticles Chitosan hydrogel nanocomposites

Gold nanoparticles were prepared using chitosan hydrogel as reducing/stabilizing reagent according to a modified method used by ding et al. (2007). Briefly, all glassware was cleaned and rinsed with aqua regia solution (HCl: HNO3 3:1) and H_2O prior to use. 1.32 mL of a 0.33% HAuCl4 solution was added drop wise to 10 mL of a solution of chitosan hydrogel under magnetic stirring, and then the mixture was heated to 80 °C using a water bath and allowed to stay for 2 h, at which a red GNPs-CHIT hydrogel solution was obtained. To prepare the GNPs-embedded chitosan hydrogel nanocomposites gel (GNPs-chitosan hydrogel gel), a vial containing 0.25 mL of resultant GNPs- chitosan hydrogel solution diluted with 2.25 mL of chitosan hydrogel solution was placed in a glass reactor containing 20 mL of 1% ammonia solution . The sample was allowed to stand for 24 h in the reactor. The obtained GNPs-chitosan hydrogel sol was then taken out of the vial and dialyzed to remove the ammonium acetate.

2.4 Preparation of chitosan hydrogel and chitosan/gelatin membrane

A mass of 1.0 g chitosan was suspended with Blender in 1.0 L of 2.0% (w/v) acetic acid solution. Then, 10.0% (w/v) sodium hydroxide was slowly added into chitosan solution till pH of the solution reached to 10–12. After that, the obtained hydrogel was dialyzed against distilled water until the outer solution was neutralized. Following the dialysis, the chitosan hydrogel was separated by centrifugation. The water content of chitosan hydrogel was 97.3% (w/w).The appropriate mass of gelatin was added to the appropriate volume of deionized water at 60 ºC for three hours. Ultimately, the solution was prepared for the immediate use. Then, the chitosan solution was mixed with the gelatin solution and agitated at 50^0C in the ratios 0.5:0.5. The chitosan solution was added with gelatin before the filtration. Next, the mixed chitosan/gelatin solution was fabricated for the preparation of chitosan/gelatin membranes. Chitosan solutions (chitosan/ gelatin, chitosan hydrogel and GNP-CHIT hydrogenl nanocomposite) were filtered through a paper filter to remove the water resultant membranes pressed under vacuum pressure and

dried at room temperature for a day.

2.5 Cell attachment studies

The chitosan/gelatin, chitosan hydrogel and GNP-CHIT hydrogel nanocomposite membrane were used for the cell study. Prior to cell culture work, the samples were surface sterilized by immersing in 70% alcohol for 30 min and kept under UV for 1 h. The samples were then pre-treated by immersing in Phosphate Buffer Saline–EDTA (PBS–EDTA) for 1 h and kept immersed in DMEM 1 h. After the pre-treatment, the samples were carefully placed in 96 well plates and glassy carbon electrode and the cells were seeded at a density of 5000 viable cells/well. The morphology of the cells seeded on the membranes was investigated with a scanning electron microscope. For preparing SEM analysis, the samples were placed in PBS–EDTA solution and rinsed quickly. The samples were subsequently fixed using 4% gluteraldehyde in PBS for 1 h and dehydrated through a graded series of alcohol (20%, 40%, 60%, 80% and 100%) for 10 min each and air-dried. The samples were later sputter-coated with platinum and the cell morphology was examined using SEM.

2.6 MTT assay

MTT assay were tested according to protocols. For this test, the membranes were immersed in culture medium for 24 h at 37 ^{0}C. The filtered membrane was placed onto 96-well plates and glassy carbon electrode in contact with HEK293cells for 5 days. Then, the cell viability was evaluated by MTT assay. For MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenylte trazolium bromide – Sigma), a cell suspension was prepared with a concentration of approximately 6×10^4 cell/mL and seeded onto 96-well plates.

3 RESULTS

3.1. Morphology studies

The SEM images of the chitosan/gelatin, chitosan hydrogel and GNP-CHIT hydrogel nano composite films are shown in Fig. 1. It was found that the surface morphology of the chitosan/gelatin membranes and chitosan hydrogel membrane were relatively smooth. This smooth morphology was due to the presence of chitosan hydrogel/gelatin in the membranes.

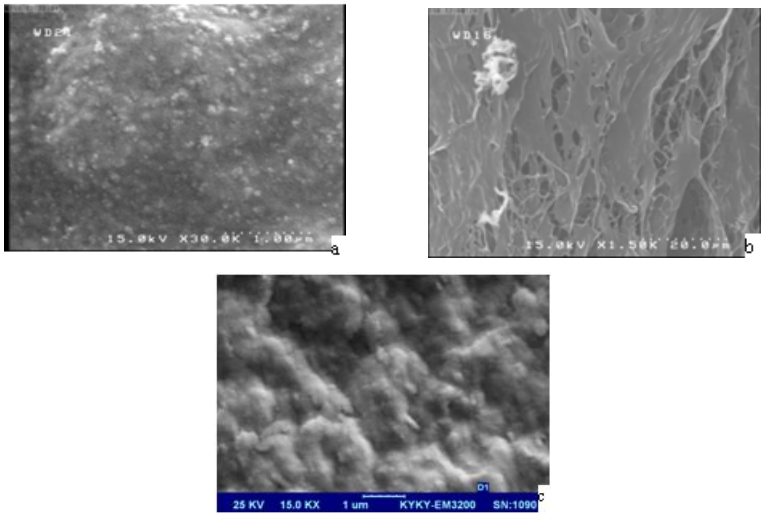

Fig. 1. The SEM images of the, chitosan/gelatin(a), chitosan/hydrogel(b) and GNP-CHIT hydrogel nanocomposite(c) films.

The SEM images showed a polymer network of the gel different from those of other films. The GNPs-CHIT hydrogel nanocomposite comprised of nanometer-scaled spheres that were aggregated to form a rough, porous surface attributable to the formation of polymer network accompanying the neutralization, while the films prepared with other membranes exhibited flat and featureless morphology. The gold particles could not be observed because they had been embedded in chitosan network.

3.2. Cell attachment studies

Fig. 2 shows scanning electron microscopy of the HEK293cell attachment on GNP-CHIT hydrogel nanocomposite and optical microscopy after 8h(a), 24 h(b) and 48h(c). It was found that in comparison to other membranes, cells adhered and completely spread on the surface of GNP-CHIT hydrogel nanocomposite.

3.3. GNPs-CHIT hydrogel nanocomposites solution/gel formation

Evidence for the neutralization of -$NH3^+$ and GNP-CHIT hydrogel nanocomposite formation could be observed from an IR spectroscopic measurement (Fig. 3). The films prepared with both chitosan hydrogel and GNPs-CHIT hydrogel solutions shows the strong absorption of -$NH3^+$ deformation at 1563 cm^{-1}, which is related to amide Iand amide II absorption of CHIT. With the gradual neutralization of -$NH3^+$, the adsorption at 1563 cm^{-1} disappeared; thus the peaks at 1656 and 1598 cm^{-1} for amide I and amide II of CHIT could be observed which verified the formation of the nanocomposites.

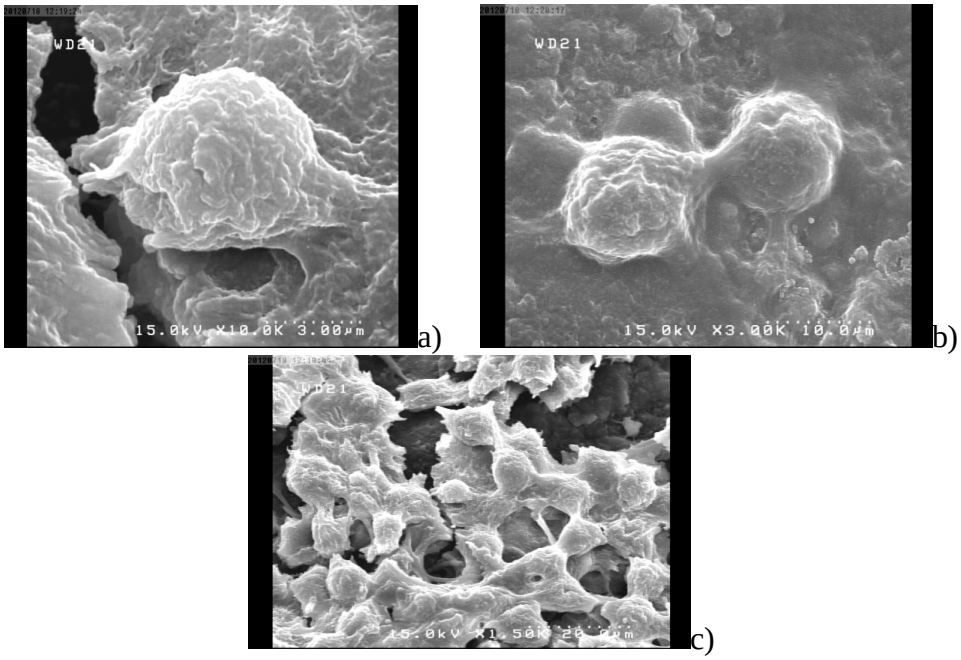

Fig. 2. HEK293 cells attachments of GNP-CHIT hydrogel nanocomposite membranes of (a, b) 8 h and (c) 48 h after seeding the cells.

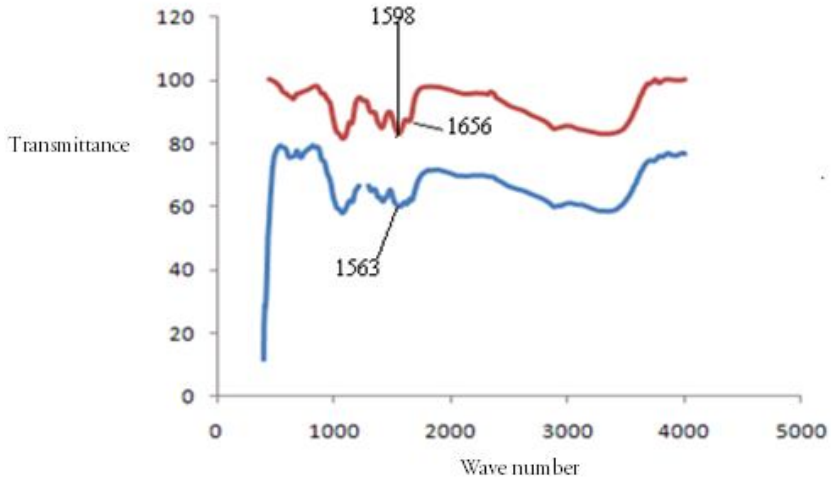

Fig. 3. FTIR transmission spectra of, GNPs-CHIT hydrogel solution and GNPs-CHIT hydrogel nanocomposite.

4 DISCUSSION

The appearance of the GNPs implied that they were surrounded by chitosan to form GNPs-CHIT hydrogel nanocomposites due to the electrostatic attraction between protonized amino group of chitosan and negatively charged GNPs. Gaseous ammonia was used as a neutralization reagent to induce the formation of sol. The sol formation process could be roughly divided into two stages: initially, gaseous ammonia diffused in the acidic nanocomposites solution and neutralized the H^+; second, by increasing pH the charge density of chitosan decreased, which made the polymer chains more flexible. This allowed the occurrence of chain entanglement and generation of physical junctions, which ultimately led to the formation of an extensive polymer network and the GNPs-CHIT hydrogel nanocomposites sol.

One critical parameter for the immobilization of cells is the roughness of the surface.In comparison with the other two films, the rough surface of the GNPs-CHIT hydrogel gel film could serve as a better medium for the cell immobilization.

4.1. Cytotoxity assessment

The extracts obtained from chitosan hydrogel and gold nanoparticle–chitosan hydrogel nanocomposite membranes did not induce changes in the cell morphology and cell growth (data not shown), while chitosan-gelatin's effect was very low on cell proliferation, demonstrating the noncytotoxic effect of chitosan hydrogel and gold nanoparticle–chitosan hydrogel nanocomposite membranes. However, in comparison, the GNP-CHIT hydrogel nanocomposite showed significant differences in cell viability (Fig. 4). This behavior can be explained with regard to scanning electron microscopy which shows that GNP-CHIT hydrogel nanocomposite produced a good polymer network with moderate porosity and roughness. A cell membrane itself is not absolutely smooth, but with transmembrane proteins, surface antigens, receptors, and so on. Neither too smooth nor too rough is suitable for cell adherence. It should match the surface roughness with the membrane, and this should be chiefly considered in biosensor designs.

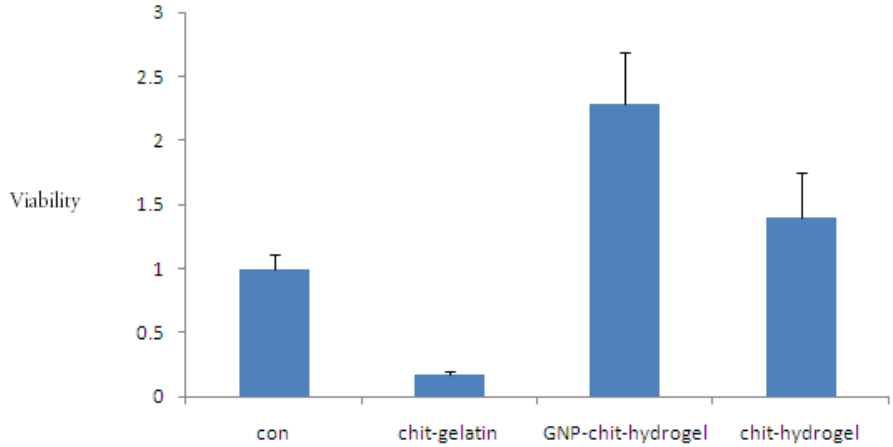

Fig. 4. Percentage of cell viability, obtained from the MTT assay, of the HEK293 cells. C (control) , chitosan-gelatin (Chit-Gelatin), GNP-Chit-Hydrogel and Chit-Hydrogel.

Nevertheless, the cells in contact with chitosan hydrogel and gold nanoparticle–chitosan hydrogel-nanocomposite membranes did not show significant morphological changes when compared with the negative control, demonstrating no signs of any cytotoxicity effect.

In general, the SEM pictures after different culture periods, revealed a higher cell adhesion and proliferation of the HEK293 in contact with the GNP-CHIT hydrogel nanocomposite. In fact, surface-treated GNP-CHIT hydrogel nanocomposite membrane exhibited the formation of a cell monolayer on the surfaces with significant confluency after 5 days. In the SEM pictures (Figs 2 and 3) it is possible to observe the HEK293 cells spreading onto the surface of the membranes exhibiting a flattened morphology that demonstrated a good adherence to the surface.

As mentioned before, the formation of GNP-CHIT hydrogel nanocomposite offers a method of altering the surface characteristics of materials without affecting the material's physical properties. Previous studies showed that the gold nanoparticles are widely used to improve hydrophilicity, biocompatibility and porosity of polymers.The presence of gold nanoparticle may explain our results, which demonstrate and enhance cell spread and proliferation after embedding in chitosanhydrogel solution to produce GNP-CHIT hydrogel nanocomposite membrane. The GNP residing in chitosan polymer, attenuate and moderate the groves. It can be important in the cell attachment and cell growth because the presence of GNP can increase acidity of the surface.

In our study, the incorporation of possible GNP onto chitosan membranes surface enabled high cell proliferation and cell attachment in a few days. In contrast, other chitosan membranes showed not to be as favorable as GNP-CHIT hydrogel nanocomposite for cell attachment and proliferation. This result can be associated to the monopolar basic natureof chitosan, which does not interact well with the bipolar extracellular matrix proteins present in the bovine serum proteins of the culture medium. Moreover, the roughness in the GNP-CHIT hydrogel membranes may additionally contribute to a better adhesion of HEK293 cells on the surface.

The results obtained also demonstrated that the GNP-CHIT hydrogel nanocomposite produced the best surface for cell proliferation for all the culture periods studied. These results indicate that medium molecular weight chitosan

and 0.33% Au solution is enough to produce improvement in the cell growth on chitosan-based membranes after afew days of culture.

5 CONCLUSION

All chitosan membranes promote cell attachment and proliferation. Cytotoxic assay showed that chitosan hydrogel and gold nanoparticle–chitosan hydrogel nanocomposite membranes induced no cytotoxic effect. SEM and optical microscope observations showed a clear enhancement of cell attachment and proliferation using GNP-CHIT hydrogel nanocomposite membranes as compared to other chitosan membranes. GNP-CHIT hydrogel nanocomposite membranes seem to produce better results for cell attachment and proliferation. Overall, the results showed GNP-CHIT hydrogel nanocomposite membranes to be useful surface to treat chitosan-based material leading to enhanced cell adhesion and proliferation. This kind of surface seems to be useful for sensing application. They enhance the biological response of membrane and cells.

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