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Cytotoxicity assessment of a gold nanoparticle-chitosan nanocomposite as an efficient support for cell immobilization: comparison with chitosan hydrogel and chitosan-gelatin

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ABSTRACT: Cell-based biosensors have become a research hotspot in the biosensors and bioelectronics fields. The main feature of cell-based biosensors is immobilization of living cells on the surface of transducers. Different types of polymers which are used as scaffolds for cell growth should be biocompatible and should have reactive functional groups for further attachment of biomolecules. In this work, cell attachment and proliferation on chitosan hydrogel, chitosan-gelatin and gold nanoparticle-chitosan nanocomposite membranes was studied. Characterization of the membranes was performed using scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR). Cytotoxicity assessment on HEK293 cells was carried out for all membranes using the MTT assay. Cell morphology and viability were assessed to evaluate cell attachment and proliferation. Regarding cell studies, the findings revealed that none of the membranes induced cytotoxic effects. However, the data showed that gold nanoparticle nanocomposite membranes improved HEK293 attachment and adhesion more than other membranes, indicating that it provides an effective surface for immobilizing cells for sensing applications.

Introduction

During the last decade, cell-based biosensors have become a research hotspot in the biosensors and bioelectronics fields since they can detect the functional information of biologically active analytes (Wang *et al.,* 2012; Wang *et al.,* 2005). Because cell-based biosensors have numerous advantages (such as long-term recording in noninvasive ways, fast response, and easy construction), they have extensive applications, including pharmaceutical screening, cellular physiological analysis, toxin detection, peripheral nerve regeneration, and environmental monitoring, as well as *in vivo* recordings (Wang *et al.,* 2012).

The main feature of cell-based biosensors 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 efforts in cell-based biosensors research. It is a significant challenge to find adequate coupling between the cells and the substrate and to obtain accurate signals from living cells (Park and Shuler, 2003; Kiilerich-Pedersen and Rozlosnik, 2012).

If efficiently coupled to an electronic readout device, cells can 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 (CHI), which offers distinctive advantages such

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as good biocompatibility, nontoxicity, remarkable affinity to proteins, and excellent gel-forming abilities (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 superior crosslinking ability due to the presence of an amino (-NH2) group (Rohindra *et al*., 2004; Han *et al*., 2004; Venault *et al.,* 2011). Because of its numerous biological properties, chitosan both 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 backbone chain are one of its amazing features, and one that enables the possibility to form 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 fundamental 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). The incorporation of GNPs into polymer matrices has attracted increasing interest in improving the stability and biocompatibility of GNPs and enhancing their capability for immobilization (Alexandridis, 2010; Huang *et al.,* 2004; Sedeño and Pingarrón, 2005).

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

Previous research by Ding *et al.* (2007) has shown that a GNP-CHIT nanocomposite is useful for immobilization of cells and electrochemical cell-based biosensors. In the present study we attempted to survey capability of chitosan supports (chitosan hydrogel, chitosan-gelatin and CHI-GNP nanocomposite) for immobilization and proliferation of HEK293 cells. We have compared the biological response (attachment and proliferation) of HEK293 cells seeded onto three kinds of chitosan membranes.

Materials and Methods

Materials

$AuCl_3HCl.4H_2O$ (Au% > 48%) and medium molecular weight Chitosan was purchased from Sigma-Aldrich. The degree of deacetylation was between 75-85%. Phosphate buffer saline (PBS) (pH 7.4) containing NaCl 137 mM, KCl 2.7 mM, $Na_2HPO_4.12H_2O$ 87.2 mM, and KH_2PO_4 14.1 mM and Type A Gelatin from porcine skin, ~175 g Bloom, was purchased from Sigma-Aldrich. Fourier transform infrared spectroscopy was carried out on a a NICOLET IR-100 spectrometer. The morphology of dried film was observed under a EM3200 Scanning Electron Microscope.

Cell culture

A HEK293 cell line was kindly provided by the Affiliated Dr. Hosseinkhani, Faculty of Biological Sciences, Tarbiat Modares University, 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.

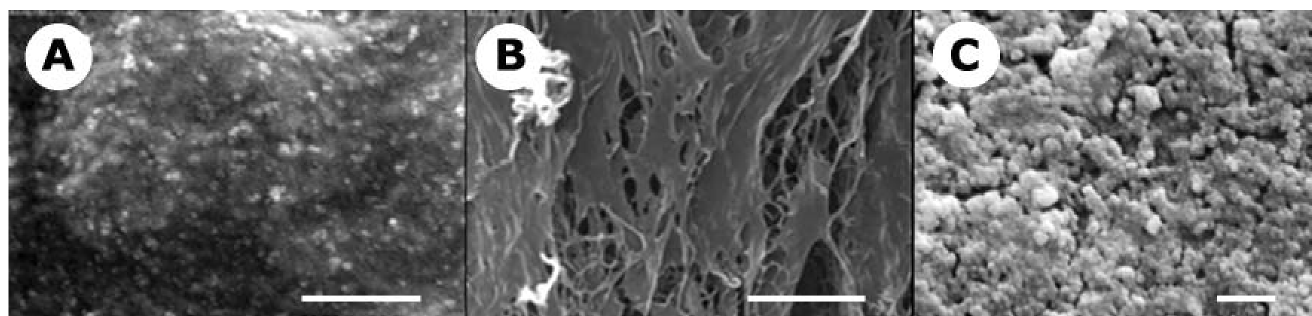

FIGURE 1. SEM images of the,chitosan /gelatin(a), chitosan/hydrogel(b) and GNP-CHIT nano composite(c) films (scale bars represent 1μm ,20 and 1 μm, respectively.

Cell number was determined using a neobar lam and light microscopy.

Preparation of Gold Nanoparticles-Chitosan Nanocomposite, chitosan hydrogel and chitosan/gelatin membranes

Gold nanoparticles were prepared using chitosan as reducing/stabilizing reagent according to a modified method used by Ding *et al.* (2007). All glassware was briefly cleaned and rinsed with aqua regia solution ($HCl:HNO_3$ 3:1) and H_2O prior to use. 1.32 mL of a 0.33% $HAuCl_4$ solution was added dropwise to 10 mL of a solution of 1% chitosan (1% chitosan solution in 1% acetic acid) under magnetic stirring, and the mixture was then heated to 80 °C using a water bath and allowed to stay for 2 h, after which a red GNPs-CHIT solution was obtained. To prepare the GNPs-embedded chitosan nanocomposite gel (GNPs-CHIT gel), a vial containing 0.25 mL of resultant GNPs-CHIT solution diluted with 2.25 mL of 1% chitosan 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-CHIT solution was then taken out of the vial and dialyzed to remove ammonium acetate.

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 the chitosan solution until pH of the solution reached 10-12. The obtained hydrogel was then dialyzed against distilled water until the outer solution was neutralized. After the dialysis, the chitosan hydrogel was separated by centrifugation. The water content of the chitosan hydrogel was 97.3% (w/w). The appropriate mass of gelatin was added to the appropriate volume of deionized water at 60 °C for three hours. Ultimately, the solution is prepared for immediate use. Then, the chitosan solution was mixed with the gelatin solution and agitated at 50 °C in the ratios 0.5:0.5. The chitosan solution was added to gelatin before filtration. The mixed chitosan/gelatin solution was then produced for the preparation of chitosan/gelatin membranes. Chitosan solutions (chitosan/gelatin, chitosan hydrogel and GNP-CHIT nanocomposite) were filtered through a paper filter to remove the water. The resultant membranes were pressed under vacuum pressure and dried at room temperature for a day.

Cell attachment studies

The chitosan/gelatin, chitosan hydrogel and GNP-CHIT nanocomposite membranes 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 for 1 h. After the pre-treatment, the samples were carefully placed in 96 well plates and glassy carbon electrodes 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. To prepare 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 sputter coated with platinum and the cell morphology was examined using SEM.

MTT assay

MTT assays were tested according to protocols. For this test, the membranes were immersed in culture medium for 24 h at 37 °C. The filtered membranes were placed onto 96-well plates and glassy carbon electrodes in contact with HEK293 cells for 5 days. Then, the cell viability was evaluated by MTT assay. For the 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 × 104 cell/mL and seeded onto 96-well plates.

Results

Morphology studies

The SEM images of the chitosan/gelatin, chitosan hydrogel and GNP-CHIT nanocomposite films are shown in Fig. 1.

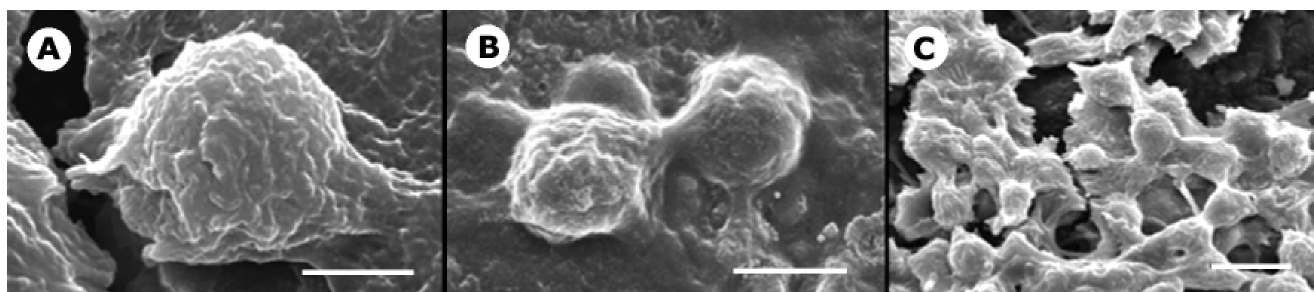

FIGURE 2. HEK293 cells attachment on GNP-CHIT nano composite membranes at (a,b) 8 h and (c) 48 h after seeding (scale bars represent 3,10 and 20 μm, respectively.

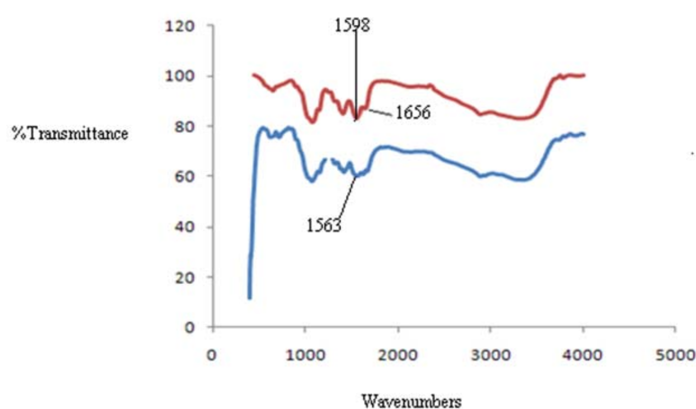

FIGURE 3. FTIR transmission spectra o, GNPs-CHIT solution and GNPs-CHIT nanocomposite.

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

Cell attachment studies

Fig. 2 shows scanning electron microscopy of the HEK293 cell attachment on GNP-CHIT nanocomposites and optical microscopy after 8 h (a), 24 h (b) and 48 h (c). It was found that, in comparison to other membranes, cells adhered and spread completely on the surface of the GNP-CHIT nanocomposite.

GNPs-CHIT Nanocomposites Solution/Gel formation

Evidence for the neutralization of -NH3 + and formation of the GNP-CHIT nanocomposites was observed by IR spectroscopic measurement (Fig. 3). The films prepared with both chitosan and GNPs-CHIT solutions showed the strong absorption of -NH3+ deformation at 1563 cm-1, which related to amide I and amide II absorption of CHIT. With the gradual neutralization of -NH3 +, the absorption at 1563 cm-1 disappeared and peaks at 1656 cm-1 and 1598 cm-1 for amide I and amide II of CHIT were observed, which verified the formation of the nanocomposites.

Discussion

The appearance of the GNPs implied that they were surrounded by chitosan to form GNPs-CHIT 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 the solution. The solution formation process could be roughly divided into two stages: initially, gaseous ammonia diffused in the acidic nanocomposites solution and neutralized the H+; secondly, with the 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 nanocomposite solution.

A critical parameter for immobilization of cells is roughness of the surface. In comparison with the other two films, the rough surface of the GNPs-CHIT gel film could serve as a better medium for cell immobilization. Moreover, the porous property of the nanocomposite on the electrode made the redox probe more accessible to the electrode surface, thus facilitating electron transfer.

Cytotoxicity assessment

The extracts obtained from membranes did not induce changes in cell morphology and cell growth (data not shown), revealing a normal cell morphology and proliferation patterns, similar to those of the control, showing the noncytotoxic effect of the membranes. The results obtained in the MTT test showed, in general, that none of the membranes prepared from chitosan exhibited any cytotoxic effect. However, in comparison, the GNP-CHIT 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 nanocomposites produced a good polymer network with moderate porosity and roughness. The cell membrane itself is not absolutely smooth, having transmembrane proteins, surface antigens, receptors, and so on. Neither too smooth nor too rough a surface is suitable for cell adherence. It

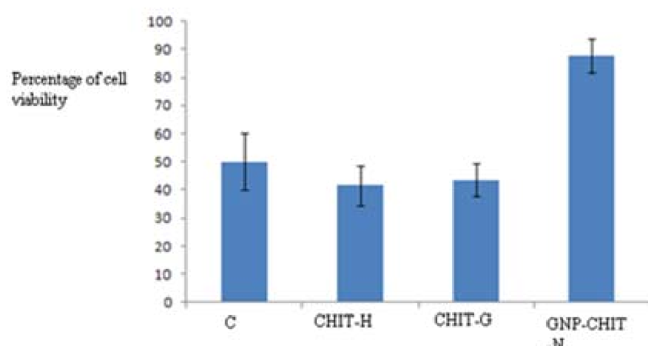

FIGURE 4. Percentage of cell viability (MTT assay) of HEK293 cells. C (control), chitosan hydrogel (CHIT-H), chitosan/gelatin (CHIT-G) and GNP-CHIT nano composite (GNP-CHIT-N).

should match the surface roughness of the membrane, and this should be chiefly considered in biosensor designs. Nevertheless, no cells in contact with any of the membranes showed significant morphological changes when compared to the negative control, showing no signs of any cytotoxic effects.

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 nanocomposite. In fact, surface-treated GNP-CHIT nanocomposite membranes exhibited the formation of a cell monolayer on the surfaces with significant confluency after 5 days. In the SEM pictures (Fig. 2 and 3) it is possible to observe the HEK293 cells spreading onto the surface of the membranes and exhibiting a flattened morphology that demonstrated a good adherence to the surface. In contrast, in other membranes the HEK293 cells exhibited a round morphology, showing low cell adherence and proliferation (data not shown).

As mentioned before, the formation of GNP-CHIT nanocomposites 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 nanoparticles may explain our results, which demonstrate an enhanced cell spread and proliferation after embedding in chitosan solution to produce GNP-CHIT nanocomposite membranes. The GNPs residing in chitosan polymer attenuate and moderate the groves. It can be important in cell attachment and cell growth because the presence of GNPs can increase acidity of the surface.

In our study, the incorporation of possible GNPs 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 the GNP-CHIT nanocomposite for cell attachment and proliferation. This result can be associated to the monopolar basic nature of 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 membranes may additionally contribute for better adhesion of HEK293 cells on the surface.

The results obtained also demonstrated that the GNP-CHIT nanocomposite produced the best surface for cell proliferation for all the culture periods studied. These results indicate that medium molecular weight chitosan 1% and 0/33% Au solution is enough to produce improvement in the cell growth on chitosan-based membranes after a few days of culture.

Conclusion

All chitosan membranes promote cell attachment and proliferation. Cytotoxic assay showed that none of the membranes induced any cytotoxic effects. SEM and optical microscope observations showed a clear enhancement of cell attachment and proliferation using GNP-CHIT nanocomposite membranes as compared to other chitosan membranes. GNP-CHIT nanocomposite membranes seem to produce better results for cell attachment and proliferation. Overall, the results showed GNP-CHIT nanocomposite membranes to be a useful surface for treating chitosan-based materials leading to enhanced cell adhesion and proliferation. This kind of surface seems to be useful for sensing applications and may enhance the biological response of membrane and cells.

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