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Chemical oxidative synthesis of polypyrrole particles and functionalization by proteins

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Polypyrrole particles (Ppy) were chemically synthesized by H_2O_2 and $K_2Cr_2O_7$ induced polymerization. We discuss the dependence of several oxidizing agents on the synthesis of polypyrrole particles and covalent immobilization of proteins. During modification of Ppy particles by secondary horseradish peroxidase (HRP) labeled antibodies we observed that additional oxidation of Ppy particles with $K_2Cr_2O_7$ increased the efficiency of protein immobilization 1.4 and 3.8 times for H_2O_2 and $K_2Cr_2O_7$ initiated particles, respectively. The possibility to apply Ppy particles modified by the bovine leukemia virus (BLV) protein *gp*51 to detect specific antibodies in the blood serum of BLV-infected animals was investigated and is presented in this paper.

INTRODUCTION

Recently, nanoscience and nanotechnology have become topical in different fields of research such as physics, chemistry, biology and medicine. Nanoscale materials are not only smaller, but also exhibit entirely different physical and chemical properties if compared with the macroscopic ones. Nanotechnologies have enabled the development of entirely new drugs and altered the properties of already marketed drugs to create potentially safer, more directed, and effective pharmaceuticals. The ability to deliver drugs locally is of great benefit in the treatment of many diseases. Polymeric nano- and microparticles can be applied as drug carriers that allow achieving targeted and precisely controlled drug delivery [1, 2]. A conducting polymer, polypyrrole is a potential candidate to be applied in the design of drug carriers. On the other hand, among various conducting polymers, polypyrrole has been the most widely studied material for potential biomedical applications [3], mainly because of its relatively high environmental stability, versatile electrical properties, and because this polymer can be easy synthesized chemically in the form of micro- and even nanoparticles as well as electrochemically synthesized in the form of thin micro- and nanofilms that can be deposited on various electrodes from aqueous media [4, 5]. Moreover, electrically deposited Ppy might be doped with various dopants to advance its physical, chemical and electrical properties [6–8]. Polypyrrole-based nanoparticles belong to a unique class of materials with potential application in optical / visual immunodiagnostic assays because of their deep black colour and intense optical absorbance. The facile preparation of polypyrrole in aqueous media and its surface modification by charged functional groups makes this polymer particularly suitable for the adsorption of proteins and interesting for biomedical applications [9–11]. Moreover, Ppy nanoparticles can be used for biosensor design [12].

In this paper, we describe the influence of some oxidizing agents on the synthesis and properties of polypyrrole particles for the efficiency of covalent modification by proteins.

MATERIALS AND METHODS

Reagents and equipment

Pyrrole (Acros Organics, Geel, Belgium) was purified additionally prior to polymerization by passing through

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a 5 cm long Al_2O_3 filled column until it became colorless, and then was stored at 4˚C in the dark before use. The other chemicals were of analytical grade and were used as received. All solutions were prepared using deionized water purified with a Millipore S.A. water purification system (Molsheim, France). Polypyrrole (Ppy) particles were chemically synthesized using two different oxidation agents, such as hydrogen peroxide (H_2O_2) and potassium bichromate ($K_2Cr_2O_7$). The first polymerization solution was based on 250 mM pyrrole and 150 mM H_2O_2 thoroughly dissolved in 0.05 M acetate phosphate (1:1) buffer, pH 2.0. The second polymerization solution was based on 250 mM pyrrole and 100 mM $K_2Cr_2O_7$ thoroughly dissolved in 0.05 M acetate phosphate (1:1) buffer, pH 2.0. The polymerization was performed at 23 ˚C for 24 h. The formed black precipitates based on chemically synthesized Ppy particles were separated from supernatant by centrifugation at 9300g for 2 min and thoroughly washed three times with 0.05 M phosphate buffer, pH 7.0, and with 0.5 M NaCl. The bovine leukemia virus protein *gp*51 (BIOK, Kursk, Russia) was used for the modification of Ppy particles. Anti-*gp*51 specific antibodies (BIOK, Kursk, Russia) and secondary antibodies labeled with enzyme horseradish peroxidase (Pourquier Institute, Paris, France) were applied for complex formation with *gp*51 and / or for determination of protein immobilization efficiency.

Preparation of Ppy particles and optical evaluation

*Modification of Ppy particles by secondary labeled antibodies***.** Two different types of Ppy particles were formed by application of different oxidators: (i) H_2O_2 and (ii) $K_2Cr_2O_7$. Then aliquots of each type of particles were additionally oxidized with 100 nm of $K_2Cr_2O_7$ solution at 37 ˚C for 15 min, and the aliquots of each type of particles were left for control experiment without any additional oxidation. During optimization of protein immobilization protocol, the secondary labeled antibodies diluted with phosphate buffer pH 7.0 (0.6 mg protein/ml) were added to all particles for 30 min at room temperature for covalent attachment on the surface of each type of Ppy particles. Then the particles were washed two times with 1 ml of phosphate buffer, collected by centrifugation and suspended in 1 ml of phosphate buffer pH 7.0. Later the particles were treated with 1 M ethanolamine hydrochloride for 30 min to achieve deactivation of the possibly formed during Ppy oxidation carbonyl groups that after immobilization of protein remained on Ppy surface. After this step, the particles were separated by centrifugation, washed and dispersed in phosphate buffer.

*Modification of Ppy particles by bovine leukemia virus (BLV) protein gp*51. Ppy particles synthesized with $K_2Cr_2O_7$ and additionally oxidized prior to immobilization of proteins were used for covalent protein *gp*51 immobilization. The solution of *gp*51 in 0.05 M phosphate buffer, pH 7.0 (10 mg/ml), was added and allowed to react for 30 min at room temperature (Figure, step 1). For the deactivation of the remaining active carbonyl groups on Ppy surface, 1 M ethanolamine hydrochloride was used (step 2). The anti-*gp*51 antibodies containing blood serum (10 mg/ml) (step 3) and secondary labeled antibodies diluted with 0.05 M phosphate buffer, pH 7.0 (0.6 mg/ml) (step 4) were added and allowed to interact with the corresponding affinity agents immobilized and/or formed on the surface of Ppy at room temperature for 30 min. After each step the particles were quickly washed, collected by centrifugation and dispersed in phosphate buffer. A schematic diagram consecutively showing protein *gp*51 immobilization and further specific interaction steps is presented in Figure.

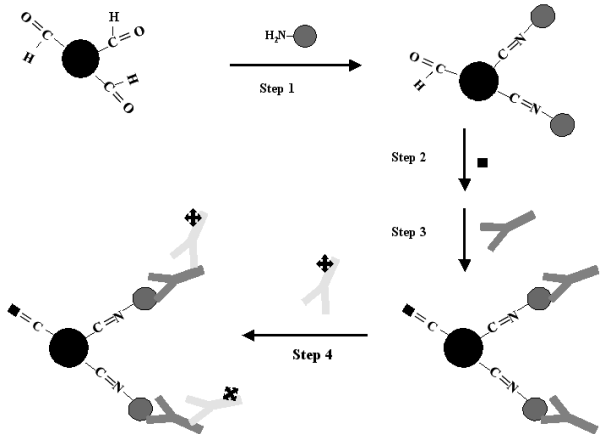

Figure. Schematic diagram showing the protein gp 51 immobilization and further specific interaction with specific and secondary labeled antibodies (protein gp 51– H_2N--●; ethanolamine – ■; specific antibody – Y; secondary labeled antibody – $Y_{\diamond}$)

To determine HRP activity, 100 µl of tetramethylbensidine solution (Pourquier Institute, Paris, France) was added to 100 µl of particle suspension and allowed to react for 20 min in the dark at room temperature. The reaction was stopped with 100 µl 0.5 M H_2SO_4 solution. Probe and control particles-containing suspensions were investigated. Measurements of optical density at $\lambda = 450$ nm were performed, the ratio of optical densities of the probe and control suspensions was calculated.

RESULTS AND DISCUSSION

Polypyrrole particles can readily be prepared in aqueous solutions by polymerization initiated by chemical oxidative agents $FeCl_3$, $Fe(NO_3)_3$, $(NH_4)_2S_2O_8$. The Ppy particles appear in size ranging from 200 nm up to 450 nm (13 ,14). In our study, polypyrrole (Ppy) particles were chemically synthesized using two other strong oxidation agents possessing different oxidation potential, hydrogen peroxide (exhibiting a lower oxidation capacity) and potassium bichromate (exhibiting a higher oxidation capacity). The chemical synthesis of Ppy particles initiated with $K_2Cr_2O_7$ took only a few minutes: the solution became very dark and no spectrophotometric studies could be applied for Ppy formation monitoring. The size of

polypyrrole particles depends on the polymerization time (15). However, a lower polymerization rate allows a better size control of the formed particles; H_2O_2 is not so dangerous for biological objects if compared with $K_2Cr_2O_7$ and might be even exploited as a bactericide agent during the storage of such particles. If any type of particle is applied for biomedical purposes, it is very desirable to immobilize affinity-exhibiting biomolecules. Since we planned to apply Ppy nano- and/or microparticles for bioanalytical purposes (determination of BLV infection), we have used BLV protein *gp*51 as an agent specifically recognizing anti-*gp*51 antibodies. Immobilization of agents exhibiting an affinity towards the analyte is the crucial point, and therefore covalent binding to the carrier surface was selected. This method has an advantage if compared with physical adsorption, which can under certain circumstances result in the leaching of *gp*51 proteins. On the other hand, covalent attachment allows to eliminate the non-specific sorption of other proteins, since a tight bond between Ppy and *gp*51 allows applying the optimal stringing conditions for a sufficient formation of the antigen-antibody complex and minimal non-specific sorption.

If the surface of Ppy particles was not additionally oxidized prior to the modification by proteins, then following the incubation with HRP-labeled antibodies we detected a very low efficiency of antibody immobilization, which was determined by addition of HRP substrates and detection of the optical density of the enzymatic reaction products at $\lambda = 450$ nm. After additional oxidation of Ppy particles synthesized by initiation with H_2O_2 or $K_2Cr_2O_7$ covalent attachment of HRP-labeled antibodies was more successful, because we observed an increase in the enzymatic activity of HRP, which was 1.4 and 3.8 times higher, respectively. This means that HRP-labeled antibodies were successfully immobilized on the surface of the Ppy particles. To achieve a better efficiency of protein covalent immobilization, additional oxidation of Ppy particles with by $K_2Cr_2O_7$ is desirable. Moreover, Ppy particles synthesized by initiation with $K_2Cr_2O_7$ and additionally oxidized are more suitable for protein-covalent immobilization.

In our further experiment, for protein *gp*51 immobilization we used $K_2Cr_2O_7$-synthesized and prepared Ppy particles. After protein *gp*51 immobilization and the further specific steps of interaction (Figure) with specific antibodies and secondary labeled antibodies, we observed a 1.6-fold increase in the enzymatic activity of HRP if compared with the control. Thereby, the possibility to use thus synthesized and modified by *gp*51 protein Ppy particles for detecting the specific antibodies in the serum of leukemia-infected animals might be predicted. In this way we observed a lower increase in the enzymatic activity of HRP because of a non-covalent binding of HRP-labeled antibodies and a significantly longer interaction chain: the secondary HRP-labeled antibodies interact only with the *gp*51-anti-*gp*51 antibody complex, which was formed during incubation of *gp*51-modified Ppy particles in blood serum containing anti-*gp*51 antibodies.

CONCLUSIONS

Ppy particles were chemically synthesized in aqueous media using two different oxidation agents: H_2O_2 and $K_2Cr_2O_7$. Our results have shown that Ppy particles synthesized with $K_2Cr_2O_7$ and additionally oxidized with $K_2Cr_2O_7$ prior to immobilization are more suitable for protein covalent immobilization if compared with those not additionally oxidized with $K_2Cr_2O_7$. Our results imply the possibility of applying Ppy particles modified by the bovine leukemia virus protein for detecting specific antibodies in the serum of infected animals.

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References

1. Chauvierre C, Vauthier C, Labarre D et al. Cell Mol Biol 2004; 50(3): 233–9.
2. Zeng Y, Pitt WG J Biomater Sci Polym Ed 2005; 16(3): 371–80.
3. Tessier D, Dao LH, Zhang Z et al. Biomater Sci Polym Ed 2000; 11(1): 87–99.
4. Ramanaviciene A, Ramanavicius A. Biosens Bioelectron 2004; 20(6): 1076–82.
5. Wang X, Gu X, Yuan C et al. J Biomed Mater Res A 2003; 68(3): 411–22.
6. Masuda H, Asano DK, Synth Metals 2003; 43: 135–6.
7. Li CM, Sun CQ, Song S et al. Front Biosci 2005; 10: 180–6.
8. Ramanaviciene A, Ramanavicius A. in: Advanced Biomaterials for Medical Applications (ed. D.W. Thomas), Kluwer Academic Publishers, Dordrecht / Boston / London, 2004: 111-25.
9. Azioune A, Slimane AB, Hamou LA et al. Langmuir 2004; 20(8): 3350–6.
10. Benabderrahmane S, Bousalem S, Mangeney C et al. Polymer 2005, 46(4): 1339–47.
11. Ramanavicius A, Habermüller K, Razumiene J et al. Prog Colloid Polym Sci 2000; 116: 143–48.
12. Ramanavicius A, Kausaite A, Ramanaviciene A. Sens Actuators B 2005; 111–112: 532–9.
13. Markham G, Obey TM, Vincent B, Colloids and Surfaces 1990, 51: 239–54.
14. Saoudi B, Jammul N, Chehimi MM et al. Spectroscopy 2004, 18(4): 519–35.
15. Ramanaviciene A, Schuhmann W, Ramanavicius A. Colloids Surf. B: Biointerf 2006; 48: 159–66.

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CHEMINĖ OKSIDACINĖ POLIPIROLO DALELIŲ SINTEZĖ BEI JŲ MODIFIKAVIMAS BALTYMAIS

S a n t r a u k a

Naudojant H_2O_2 ir $K_2Cr_2O_7$ buvo susintetintos polipirolo (Ppy) dalelės. Šiame straipsnyje apžvelgiame polipirolo sintezę inicijuojančio oksidacinio agento poveikį tolesniam polipirolo dalelių kovalentiniam modifikavimui baltymais. Nustatyta, kad po papildomos dalelių oksidacijos su $K_2Cr_2O_7$ susintetintos dalelės yra tinkamesnės baltymų imobilizavimui. Parodyta galimybė Ppy daleles, modifikuotas galvijų leukemijos viruso baltymais, taikyti nustatant specifinius antikūnius infekuotų galvijų serume.