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Nanobeads-based assays. The case of gluten detection

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Abstract

In order to verify if the use of nanobeads of poly[phenylacetylene-(co-acrylic acid)] (PPA/AA) in the ELISA test would affect the immune-activity of the antibodies (Ab) and/or the activity of the enzymes used to label the Ab anti-rabbit IGg, in this work we immobilized the horse liver peroxidase labelled Ab anti-rabbit IGg onto PPA/AA nanobeads. The gluten test was chosen as the model to demonstrate the usefulness of these nanobeads in immunoassays. The synthesis of PPA/AA nanobeads was performed by a modified emulsion polymerization. Self-assembly of nanospheres with mean diameter equal to 200 nm was achieved by casting aqueous suspensions. The materials were characterized by traditional spectroscopic techniques, while the size and dispersion of the particles were analysed by scanning electron microscopy (SEM) measurements. The obtained results show that the immobilization process of the Abs onto PPA/AA did not affect either the immune-response of the Abs or the functional activity of the peroxidase suggesting the usefulness of PPA/AA for the design of advanced nanobeads-based assays for the simultaneous screening of several analytes in complex media.

Abbreviations

PPA/AA	poly[phenylacetylene-(co-acrylic acid)]
SEM	Scanning electron microscopy

1. Introduction

A large effort is currently devoted in research to develop chemical approaches which allow the preparation of materials with mesoscale dimensions. It is now recognized that nanoparticles have the potential to regulate cellular processes such as protein–protein interactions, protein–nucleic acid interactions and enzyme activity. Scaffolds with large surfaces are of particular interest for biomolecular recognition.

In this field, organic materials (e.g. polystyrene, polyacrylates) have the advantage of greater synthetic flexibility in comparison with inorganic ones (e.g. titanium, silicon) [1, 2]. Among the wide number of literature reports, some examples will be cited. Cellular uptake of polystyrene and PLGA (poly(lactic-co-glycolic acid)) nanoparticles for oral delivery of anticancer drugs was successfully demonstrated [3], as well as the enhancement of recombinant protein production induced by PLGA nanospheres [4]. Highlights on the up to date research efforts and paradigms concerning protein–nanoparticle interactions appeared recently [5].

Micro and nanospheres of uniform size and shape play a dramatic role in biomolecule–nanoparticle activity and performance. Generally, emulsion polymerization or co-polymerization of monomers [6] are the most suitable chemical methods to prepare micro-nanospheres. Since many applications and properties of these particles are significantly influenced by the morphology and surface properties of the particles, interest has been increasingly focused on the control of the particle size and its distribution and the control of the distribution of functional groups. In this framework, π conjugated polymers such as polyphenylacetylene (PPA) and

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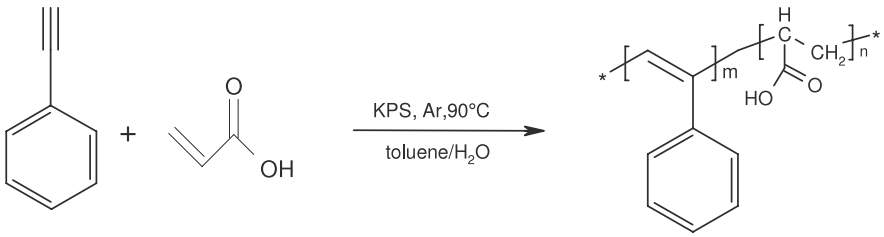

Figure 1. Modified emulsion synthesis of PPA/AA.

its copolymers are of particular interest because PPA is a semiconductor luminescent polymer and it is a candidate for cell and protein immobilization due to its biocompatibility [7].

Our research has been focussed on the synthesis of a novel material, i.e. poly[phenylacetylene-(co-acrylic acid)] (PPA/AA), at the micro and nanoscale, which is a material suited for the investigation of scientific aspects related to biotechnology because the functional groups are expected to strongly interact with biomolecules such as antibodies, enzymes and nucleic acids.

In this work we immobilized horse liver peroxidase labelled Abs anti-rabbit IGg onto PPA/AA nanobeads and we checked their functional properties before and after the immobilization process. The obtained data demonstrate that the immobilized biomolecules retain their functional properties suggesting their utilization for the design of new nanobeads-based assays.

2. Experimental details

2.1. Instruments and materials

Deionized water was obtained with Millipore-Q RG(CPMQ00 4R1) and degassed for 30 min with argon before use; phenylacetylene (PA) (Aldrich 99% pure) was distilled under reduced pressure before use; acrylic acid (AA) (Aldrich 99% pure) and potassium persulfate (KPS) (Aldrich 99.99% pure), were used as received; other solvents and materials were reagent grade (Aldrich).

FTIR spectra were recorded as nujol mulls or as films deposited from $CHCl_3$ solutions by using CsI cells, on a Bruker Vertex 70 spectrophotometer. UV–vis spectra were carried out on a Varian Cary100 spectrophotometer; the samples were analysed as solutions in common organic solvents.

The morphology and the diameter of the beads and their poly-dispersity were determined by an SEM-LEO1450VP instrument on metallized samples; nanobead dimensions were calculated from the SEM images of films deposited by casting, using an image analysis software tool (Scion Image for Windows, Scion Corp, Beta 4.0.2) and the poly-dispersity index (PI) was obtained using the formula:

$$\mathrm{PI} = (d_{\max} - d_{\min})/d_{\text{average}},$$

where d is the particle diameter in nm.

2.2. Synthesis of polymeric nanobeads

Poly[phenylacetylene-(co-acrylic acid)] (PPA/AA) nanospheres were prepared by a modified emulsion synthesis. The PPA/AA nanobeads were prepared by following this typical procedure: 50 ml of deionized water, 1 ml of toluene, 1 ml (0.936 g, 0.01 mol) of PA and 0.2 ml (5.255 g, 0.07 mol) of AA, were degassed for 15 min and then stirred in an Argon atmosphere, at 90 °C for 1 h; then 5 ml of KPS water solution (20 mg ml^{-1}) was added and the reaction was refluxed under vigourous stirring in an Argon atmosphere for 20 h; polymerization was stopped by opening the flask (yield ∼70% of crude product) and the light yellow emulsion was filtered, redispersed and centrifuged with deionized water seven times, in order to remove the unreacted chemicals.

UV–vis spectra in a $CHCl_3$ solution showed continuous absorption in the λ range 200–400 nm. IR (cm^{-1}): 3050 ($\nu_{\text{aromatic CH}}$), 1670 (ν_{COOH}), 1597 ($\nu_{\text{aromatic C=C}}$), 770 ($\nu_{\text{CH}}$). SEM images show mono-dispersed spheres with diameter 200–210 nm and PI 0.8.

2.3. ELISA test

We used the following procedure. (1) We applied a sample of known concentration of gliadin to the plate surface. The plate walls were then coated with specific antibodies anti-gliadin, diluted into the same buffer used for the antigen. A concentrated solution of non-interacting protein, such as bovine serum albumin (BSA) was added to all plate walls. The plate was washed, and a detection antibody specific to the antigen of interest was applied to all plate walls. The plate was washed to remove any unbound detection antibody. After this wash, only the antibody–antigen complexes remained attached to the wall. Secondary antibodies, previously immobilized onto PPA/AA nanobeads were added to the walls. These secondary antibodies were conjugated to the substrate-specific enzyme. The plate was washed so that the excess of unbound enzyme–antibody conjugates was removed. The substrate was added to the walls and a chromogenic signal was detected at 620 nm.

3. Results and discussion

Poly[phenylacetylene-(co-acrylic acid)] nanospheres were synthesized by using a modified emulsion technique, as reported in figure 1.

In analogy to synthetic procedures for the preparation of alike copolymers [8], the KPS initiator, PA/toluene and PA/initiator ratios and the reaction time were optimized in order to achieve monodisperse nanospheres with a mean diameter of 200 nm, whose SEM image is reported in figure 2.

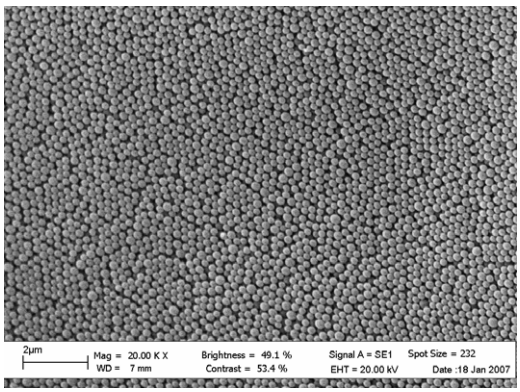

Figure 2. SEM image of PPA/AA nanobeads, mean diameter 200 nm.

We used these beads as a solid support to immobilize the horse liver peroxidase labelled Abs anti-rabbit IGg. After rinsing the PPA/AA beads in distilled water several times at room temperature, the PPA/AA nanobeads were soaked in a 10 mM PBS buffer, pH 7.4. These nanobeads were incubated with a solution 20 μg ml^{-1} of horse liver peroxidase labelled Abs anti-rabbit IGg at room temperature for 1.0 h. After incubation, the nanobeads were washed several times with a PBS buffer, pH 7.4 and used for the last step of the ELISA test. In the discarded washing solutions we did not find an appreciable amount of Ab. This result suggested that almost all the Ab molecules were attached to the nanobeads. For this experiment, we decided to use a well known Ab–Ag system used in the detection of gliadin in food for coeliac patients. We choose gluten as the model since we already worked on this system by using different detection methodologies [9–11].

The ELISA tests were performed by using the commercial secondary antibodies attached to the PPA/AA nanobeads or free in solution. The goal of this experiments was to understand if the immobilization of secondary antibodies onto the PPA/AA nanobeads could affect the binding features of the Abs and/or the enzyme activity of the horse liver peroxidase.

The results obtained show that there is no difference for the detection of gluten when we use secondary Abs immobilized onto PPA/AA nanobeads or secondary Abs in free solution.

The results obtained by using different concentrations of Ab anti-gliadin and secondary Ab immobilized onto the nanobeads are shown in figure 3. The results obtained indicate that the immobilization process of horse liver peroxidase labelled Ab anti-rabbit IGg onto PPA/AA nanobeads does not perturb the functional features of the Abs as well as the activity of the enzyme, suggesting a more general utilization of these nanobeads in ELISA tests.

4. Conclusions

In conclusion, the results obtained in this work show that there is a strong interaction between the nanobeads of PPA/AA and anti-gliadin antibodies and that the immobilized antibodies retain their immunological activity. These results suggest a potentially wider utilization of the PPA/AA nanobeads for the design of new assays that allow a simultaneous screening of different analytes that are present in complex media.

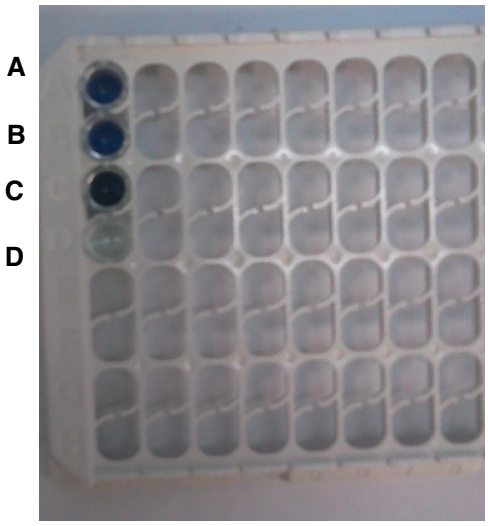

Figure 3. ELISA test performed by using secondary Abs immobilized onto the nanobeads PPA/AA. (A) Gliadin (25 μg ml^{-1}) + Ab anti-Gliadin dil.1:100 000; (B) Gliadin (25 μg ml^{-1}) + Ab anti-Gliadin dil.1:50 000; (C) Gliadin (25 μg ml^{-1}) + Ab anti-Gliadin dil.1:10 000; (D) negative control-Gliadin (25 μg ml^{-1}) + Ab-Gliadin dil.1:10 000.
(This figure is in colour only in the electronic version)

Acknowledgments

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