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ePCL Electrospun Microfibrous Layers for Immune Assays: Sensitive ELISA for the Detection of Serum Antibodies Against HPV16 E7 Oncoprotein

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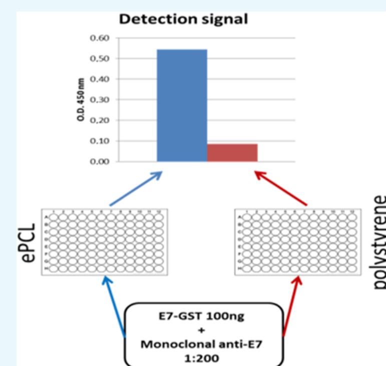
ABSTRACT: Human papillomavirus (HPV) type 16 is the etiologic agent of more than 50% anal/cervical cancers and about 20% oropharyngeal cancers. HPV16 E6 and E7 oncogenes favor the transformation and are essential for maintaining the transformed status. Serum anti-E6 and anti-E7 antibodies appear to have prognostic significance for HPV-associated cancers. However, most of the previous attempts to establish diagnostic tools based on serum detection of E6 and/or E7 antibodies have been unsuccessful, mainly due to the low accuracy of applied tests. This paper reports on a feasibility study to prove the possibility to easily immobilize HPV16 E7 onto electrospun substrates for application in diagnostic tools. In this study, poly(ε-caprolactone) electrospun scaffolds (called ePCL) are used to provide a microstructured substrate with a high surface-to-volume ratio, capable of binding E7 proteins when used for enzyme-linked immunosorbent assay (ELISA) tests. ePCL functionalized with E7 exhibited superior properties compared to standard polystyrene plates, increasing the detection signal from serum antibodies by 5−6 times. Analysis of the serum samples from mice immunized with HPV16 E7 DNA vaccine showed higher efficiency of this new anti-E7 ePCL-ELISA test vs control in E7-specific antibody detection. In addition, ePCL-E7-ELISA is prepared with a relatively low amount of antigen, decreasing the manufacturing costs.



INTRODUCTION

High-risk human papillomavirus (HR-HPV) is an etiological agent of cervical cancer.[1,2] In addition to cervical cancer, a substantial proportion of other cancers (penis, anus, vulva, vagina, and oropharynx) are associated with HPVs, mainly HPV16.[3,4] HPV16, indeed, is the causative agent of 50% cervical, 20% oropharyngeal, and a large majority of anal cancers.[4,5]

Viral E6 and E7 oncoproteins are necessary not only for malignant conversion but also for the maintenance of transformed cell phenotype. The expressions of the E6 and E7 proteins are markedly upregulated during cell transformation and pathological progression toward a higher grade of dysplasia, making these proteins important candidates as markers for persistent, as opposed to transient, HPV infections and for intraepithelial or invasive cervical neoplasia.[6] HPV infection leads to the production of antibodies against E6 and E7 viral oncoproteins that appear to have prognostic significance for HPV-associated head−neck and cervical cancers.[7,8] However, most previous attempts to establish diagnostic tools based on antibodies to either E6 or E7 have been unsuccessful, mainly due to the poor immunogenicity of these oncoproteins and the low accuracy of the applied tests. HPV serology is a challenging issue in particular for the development of reliable enzyme-linked immunosorbent assays (ELISAs). Indeed, ELISA tests, which use small, linear epitopes of the proteins for antibody detection, have low levels of sensitivity and specificity. Moreover, often the direct binding of protein antigens to hydrophobic polystyrene microwell plates can cause a partial or total denaturation of the bound protein.[9,10]

Electrospun fibers[11,12] have been generated for several applications such as wound dressings,[13] tissue engineering,[14,15] filtering processes,[16] biotechnological applications,[17] and many others from a number of synthetic and biopolymers.[18,19]

Their exceptional signature physicochemical characteristics include (e.g., about round 90% porosity), high surface-to-volume ratio, and compatibility with biological systems (PCL is FDA approved for clinical use), giving electrospun fibers a great potential in biotechnological applications. Nevertheless,

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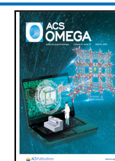

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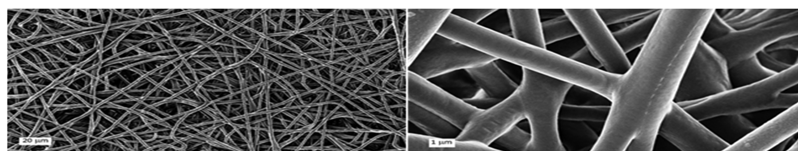

Figure 1. Scanning electron microscopy (SEM) micrographs of the ePCL electrospun layers at low and high magnification. Scale bar is indicated in each micrograph.

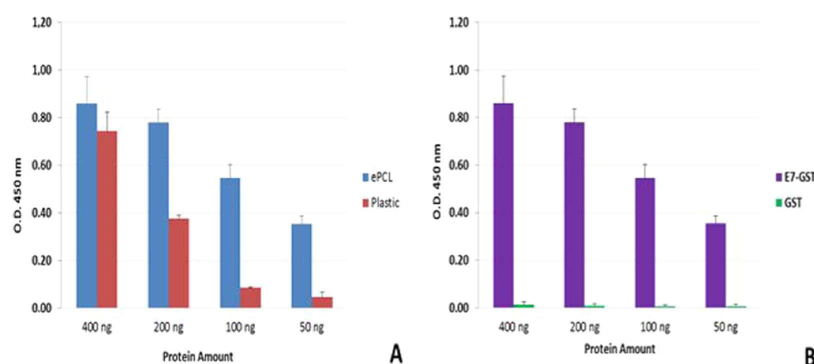

Figure 2. ePCL-ELISA. Panel A: comparison between ePCL and conventional polystyrene ELISA: microwell plates are coated with decreasing quantities of E7-GST on polystyrene and on ePCL, incubated with monoclonal anti-HPV16 E7 antibody, and thereafter with horseradish peroxidase-conjugated goat antimouse IgG. Absorbance at 450 nm is measured after a colorimetric reaction with 3,3′,5,5′-tetramethylbenzidine (TMB) substrate. Panel B: ePCL-ELISA specificity: ePCL-ELISA microwells are coated with decreasing concentrations of E7-GST or GST protein. Experimental procedures are the same as in panel A. Data are means of triplicate samples OD ± SD.

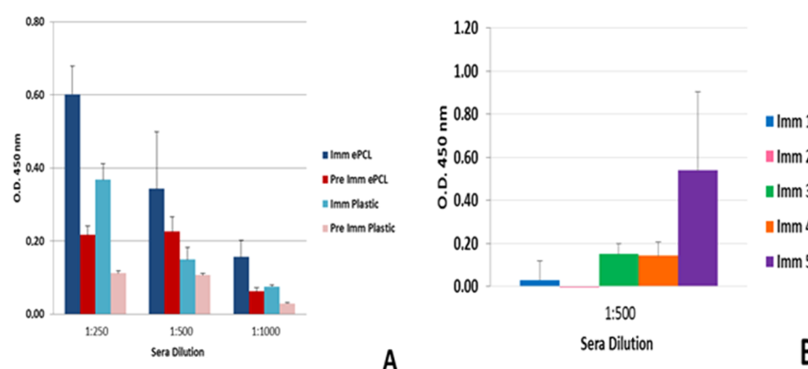

Figure 3. Analysis of mouse sera immunized with HPV16L2-E7 chimeric DNA vaccine. Panel A: comparison between ePCL and bare polystyrene: ELISA plates are coated with 100 ng of E7-GST protein either on bare polystyrene or on ePCL, incubated with decreasing dilution of preimmunized (Pre Imm) and immunized (Imm) mouse sera and thereafter with HRP conjugate goat antimouse. Absorbance at 450 nm is measured after a colorimetric reaction with TMB substrate. Data are means of triplicate samples OD ± SD. Panel B: ePCL-ELISA for E7 antibody detection in five different mouse sera (Imm): ePCL microwells are coated with 100 ng of E7-GST protein. Experimental procedures are the same as in panel A. Data are means of triplicate samples OD ± SD calculated after subtracting preimmunized serum value.

to date, only relatively few studies have explored their use in the medical diagnostic field and none of them in conjunction with E7 biomarker.

Polycaprolactone, like other aliphatic polyesters, is widely studied for biomedical and pharmaceutical applications. These fibers are produced by applying an electrical potential difference (high voltage) between the polymeric liquid and a collection target.[20]

Compared to PLA (poly lactic acid, another biocompatible FDA-approved polymer), PCL was preferred because prior studies from our group indicate that it undergoes more robust biodegradation, as observed in bacterial culture.[20]

Nowadays, the vast literature on electrospinning exists, and a useful reference specifically covering electrospinning of PCL is offered by the review of Cipitria et al.[21]

Usually, scaffolds produced by electrospinning are made of randomly oriented micro and nanometric fibers with an open porosity microstructure, which ensures a substrate of good mechanical properties and a high surface-to-volume ratio, thus providing a much greater active area compared to a flat solid substrate impervious to liquid. Therefore, adopting such a system for ELISA purposes is expected to deliver an electrospun PCL-ELISA test with significantly improved sensitivity and detection limits compared to the conventional ELISA systems.

Starting from this realistic hypothesis, the present report is a feasibility study to prove the possibility to easily immobilize HPV16 E7 antigen onto electrospun substrates for application in diagnostic tools.

Indeed, in the present work, we develop a more sensitive, rapid, reliable, and cheap diagnostic device for the detection of

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antibodies against E7 compared to conventional ELISA, exploiting the properties of electrospun PCL micro- and nanofibers. The activity of electrospun PCL (ePCL) fiber-coated ELISA is compared to that of the conventional ELISA on polystyrene wells in detecting serum antibodies against HPV16 E7 oncoprotein.

RESULTS AND DISCUSSION

The microstructure of the ePCL layers is displayed in Figure 1, showing a mean fiber diameter in the range of 1−3 μm, arranged with random orientations.

The ePCL microfibers have better efficiency than bare polystyrene surface when a monoclonal antibody specific for HPV16 E7 is tested with different amounts of the E7-GST fusion protein. Indeed, ePCL gives a signal significantly stronger compared to polystyrene for each given quantity of immobilized E7-GST. These results clearly highlighted that ePCLs work better than polystyrene, especially at low concentrations of immobilized protein, as shown in Figure 2 (panel A).

To determine whether the GST component of the fusion protein may interfere with binding signal, the corresponding amount of GST protein alone has been spotted on both ePCL and polystyrene. As shown in Figure 2 (panel B), the GST component does not affect the result. A secondary antibody without a primary one has been utilized as control and gives no signal, indicating that there are no unspecific bonds. Serial dilutions of the E7 monoclonal antibody are able to give signal until 1:100 000 dilution (corresponding to 2 pg/μL), indicating the sensitivity limit of ePCL-ELISA.

To validate this result, murine sera from HPV16-vaccinated animals were tested.[22] Comparison between pre- and immune sera at different dilutions, both on polystyrene and on ePCL using 100 ng of an immobilized E7-GST fusion protein, has been carried out. ePCL-ELISA gives a significantly higher signal than that obtained by polystyrene ELISA with the same quantity of antigen (Figure 3).

Thus, results with mouse serum antibodies substantially confirm those obtained with specific monoclonal antibodies. Best detection performance is obtained with 1:500 dilutions of mouse sera. This serum dilution is then utilized to test all (five mice) immunized animals separately. After subtracting the values generated by preimmune serum, different levels of E7-specific antibodies are raised by the different immunized mice, with, in particular, a mouse not showing any response (Figure 3, panel B). This result confirms those already published on these sera.[22]

To determine the specificity of the method in serum samples, where specific anti-E7 antibodies are in the background of unrelated antibodies, GST or E7-GST fusion protein is coated on ePCLs or polystyrene microwells. Dilutions of 1:500 of E7-immunized mouse sera do not recognize GST alone but are able to detect E7-GST-fused protein (Figure 4). This result is relevant for ePCL with no signal on the GST antigen alone. On the contrary, on the polystyrene surface, a signal is detected with GST alone. This result suggests that the ePCL surface has no unspecific binding activity on serum antibodies.

The ePCL-ELISA microwells are coated with 100 ng of E7-GST or GST protein, incubated with 1:500 dilutions of preimmunized or immunized mouse sera and thereafter with HRP conjugate goat antimouse antibodies. Absorbance at 450 nm is measured after a colorimetric reaction with the TMB substrate. Data are means of triplicate samples OD ± SD calculated after subtracting the preimmunized serum value.

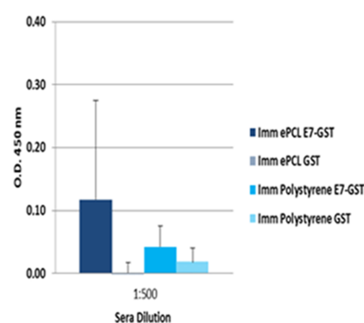

Figure 4. Specificity of the ePCL-ELISA test in serum samples.

To further explore the ePCL activity, decreasing amounts of E7-GST protein were immobilized on PCL microfibers. As shown in Figure 5, two mice (i.e., one showing no detectable serum response and one showing the highest serum response to HPV16 E7) were analyzed. After subtracting the values generated by the preimmune serum, the lowest amount of E7-GST antigen on ePCL (10 pg) is still able to give a colorimetric response within the range of assessment to the presence of E7-specific antibodies in the serum.

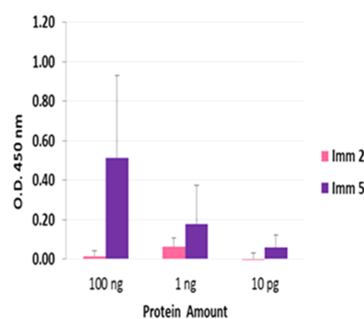

Figure 5. E7 antigen quantity on ePCL wells.

The ePCL-ELISA plate is coated with a decreasing concentration of E7-GST protein and incubated with preimmunized and immunized serum of mice 2 (Imm2) and 5 (Imm5) as in Experimental Section. Absorbance at 450 nm is measured after a colorimetric reaction with TMB substrate. Data are means of triplicate samples OD ± SD calculated after subtracting the preimmunized serum value.

CONCLUSIONS

The results indicate that the E7-ELISA test performed on electrospun PCL layers shows better effectiveness in comparison with standard polystyrene plate providing a higher (about 5−6 times) detection signal. The detection of E7 antibodies in immunized mice is more efficient with ePCL-ELISA confirming and validating the effectiveness of this new ePCL-E7-ELISA. Finally, the presented data highlight the greater efficiency of the new electrospun ePCL platform in E7 antibody detection and indicate that E7-ELISA can be prepared with very low quantities of purified protein (i.e., 10 pg), decreasing the costs of manufacturing. Analyses are in progress for the clinical validation of this ePCL-ELISA test with human sera because the prognostic value of anti-E7 titre in oropharyngeal and anal tumors has been demonstrated.[7,8] Further studies to explore the ePCL technology for the

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development of a new assay for the detection of E7 oncoprotein in clinical samples are in progress.

■ EXPERIMENTAL SECTION

Materials. PCL electrospun layers (ePCLs) is produced by electrospinning (NBARE protocol, Nanofaber srl) and sized for application into polystyrene 96-well plate (Costar 3596, Corning Incorporated).

GST beads (Glutathione SepharoseTM 4B, GE Healthcare) were utilized for protein purification.

pGEX/HPV16 E7 Recombinant plasmid[23] was utilized for bacterial production of E7-GST recombinant protein.

Mouse sera were collected and stored in a laboratory during a previous study of HPV DNA vaccines. Specifically, serum samples were taken before immunization and after the administration of a DNA vaccine containing a fusion gene consisting of plant signal sequence, which allows the compartmentalization of the antigen, linked to the HPV16 E7 oncoprotein fused to L2capsid protein, which is highly immunogenic. This chimeric DNA vaccine (pVAX ss-L21-200-E7*; Italian patent 102016000131935, PCT/IT2017/050008, WO 2018/122885 A1. European Patent Application 17826321.6, publication number EP 3562504; EP′504) was capable of inducing a high titer of anti-L2 and anti-E7 serum antibodies in mice as reported by Massa et al.[22]

Anti-E7 monoclonal antibodies are commercially available (HPV16 E7 ED17 SC-6981; Santa Cruz Biotechnology) and utilized at 1:200 dilution.

Horseradish-labeled goat antimouse IgG (H+L-HRP conjugate; BioRad) is utilized as the secondary antibody (1:1000 dilution). 3,3′,5,5′-Tetramethylbenzidine (TMB substrate kit, Thermo Scientific) is used as a substrate for colorimetric reaction.

Preparation and Characterization of Electrospun PCL Fiber (ePCL). The ePCL layers are prepared according to an industrial protocol developed by the producer Nanofaber (NBARE series), obtained from a 12% (w/w %) solution of medical-grade linear polycaprolactone polymer (50 000 MW and 80 000 MW; Perstorp, CAPA) dissolved in chloroform (99.2% purity, stabilized with 0.6% ethanol; VWR) and dimethylformamide (DMF) (100% purity; VWR). The electrospun fibers are collected on a flat collector under a total applied voltage of 29 KV, a flow rate of 6 mL/h, and a needle collector distance of 20 cm using an electrospinning station LE100 (Fluidnatek, Bioinicia, Spain).

The microstructure of the ePCL layers has been examined by scanning electron microscopy (SEM) using a field emission microscope (LEO 1530, Zeiss, Germany) operating at low voltage (e.g., 2 KV) to counter electronic charging effects. Higher-resolution imaging is carried out after sputtering a nanometric gold (Au) thin layer prior to SEM observation. The average fiber diameter and porosity is evaluated by analyzing micrographs with Image J (NIST).

Surface properties were evaluated in terms of the wettability of ePCL vs water (representative of a PBS solution) according to a standard method (UNI EN 15802; 2010) for the contact angle measurement, using a custom setup at ENEA. The results highlight the hydrophobicity of both sides (t = top; b = bottom) of the ePCL bare sheet. A water droplet of 10 μL was placed on both sides alternatively and allowed to settle for 10 s before collecting a digital image (Figure 6).

In addition, water fully penetrates the open porosity of ePCL through the thickness after soaking at the bottom of a deep well of a culture plate, thus enabling the diffusion of E7 through the thickness when protein is added. The surface roughness of ePCL was evaluated via atomic force microscopy (AFM) rendering a roughness of 0.81 μm root mean square (RMS) as shown in Figure 7, which has to be assumed as lower bounds for microscale roughness affected by error measurement (e.g., tip geometry and wear, z-range for cantilever). The sub-micrometer size of the fibers is at the basis of the hydrophobic behavior of the ePCL surface.

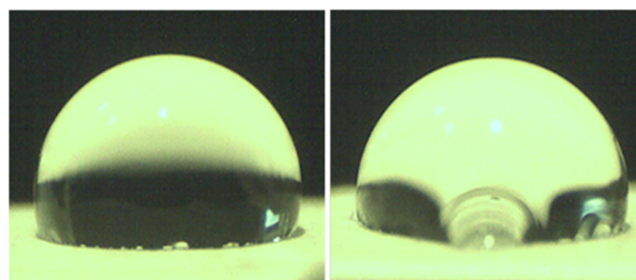

Figure 6. Droplet placed on the top surface and the bottom surface of ePCL, respectively: the contact angles of 105.4 and 110.8° are comparable, highlighting an initial superhydrophobic behavior. The contact angle was computed as $\vartheta = 2$ arc tg(2hw).

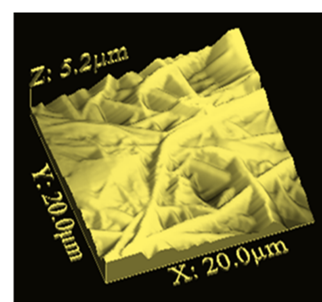

Figure 7. Surface roughness of ePCL: AFM 20 μm scan window (atomic force microscope: DI3100, Veeco).

Recombinant Protein E7-GST. The E7-GST fusion protein is produced from an *Escherichia coli* culture transfected with a recombinant pGEX/HPV16 E7 plasmid and purified using the GST beads technology, according to standard procedures.[24]

In brief, an overnight miniculture of transformed *E.coli* is diluted in a fresh LB broth (1:10) with ampicillin 50 μg/mL. Isopropyl-β-D-thiogalactoside (IPTG, 0.4 mM) is added to the culture when OD 600 nm reaches 0.5−0.7 and thereafter the culture is incubated for 4 h at 37 °C with vigorous shaking. Bacteria are collected by centrifugation (10 000 rpm for 30 min at +4 °C) and pellet lysed in 5 mL of NENT buffer with antiproteases (1 mM PMSF, 10 μg/mL leupeptin, 20 μg/mL aproteinin) plus 2% Sarkosil and sonicated. The cell lysate is then centrifuged (10 000 rpm for 10 min at +4 °C) and the supernatant containing E7-GST fusion protein stored at −80 °C until use.

The GST beads are equilibrated with NENT buffer (Tris 20 mM pH 8.0, NaCl 100 mM, EDTA 1 mM, 0.5% NP40) plus 0.5% no fat dry milk and then washed repeatedly with NENT with protease inhibitors (1 mM PMSF, 10 μg/mL leupeptin, 20 μg/mL aproteinin) and stored at +4 °C until use. The equilibrated GST beads are added to the E7-GST supernatants (2 mL/5 mL of the supernatant) for 3 h at 4 °C under rotation

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and then washed with 4 mL of NENT with antiprotease mix plus 0.7 M NaCl and thereafter with NENT without NaCl. The E7-GST fusion protein is eluted using 2 mL of TST buffer (Tris 50 mM pH 8.0, NaCl 150 mM, 0.1% Triton X100) with antiprotease plus 5 mM DTT and 20 mM reduced glutathione. All of the processes are carried out at 4 °C.

SDS-PAGE, comassie staining, and Western blotting were performed for quality control and quantification of E7-GST protein in comparison with known BSA concentrations according to standard procedures.

ePCL-ELISA. Precut electrospun disks made of ePCL are inserted "drop-in" into each well of a 96-well plate.

Different quantities of the E7-GST fusion protein and the GST protein (as control) in PBS are added to ePCL and polystyrene well plates (100 μL each well). After overnight incubation at +4 °C, the wells are washed four times with washing buffer (0.05% Tween 20/1× PBS): first washing is done with gentle shaking for 5 min and the remaining ones for 1 min.

Subsequently, 150 μL of the blocking buffer (3% no fat dry milk/1× PBS) is added and incubated for 1 h at 37 °C. Finally, wells are washed five times as above.

One hundred microliters of HPV16 E7 monoclonal antibody (1:200 in blocking buffer) or diluted sera (1:500 in blocking buffer) from different mice immunized with HPV16L2/E7 DNA vaccine is added to each well and incubated for 1 h at 37 °C. The wells for the nonspecific bound of the secondary antibody are filled with 100 μL of the blocking buffer.

After washings, 100 μL of polyclonal horseradish peroxidase-conjugated secondary antibody (1:1000 in blocking buffer) is added to each well and incubated for 1 h at 37 °C.

Wells are washed five times and 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) are added to each well and incubated for 30 min in the dark at room temperature. After adding 50 μL of stop solution (H_2SO_4 0.2 M), the absorbance is measured at 450 nm using a microplate reader (Labsystem Multiskan MS, DASIT).

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Notes

The authors declare no competing financial interest.

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