Aging effect and antibody immobilization on —COOH exposed surfaces designed for dengue virus detection

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ABSTRACT

Polymethylmethacrylate-co-methacrylic acid, poly(MMA-co-MAA) coatings were produced with different initial molar ratios of monomers (MMA and MAA) in free-radical polymerization reaction. Polymeric platforms were specifically designed with controlled concentration of surface-exposed carboxyl (—COOH) groups that can be used as a desirable functionality for protein immobilization. Spin-coated chips were used for antibody (Ab) immobilization in order to investigate the influence of —COOH surface concentration on dengue virus detection efficiency in enzyme-linked immunosorbent assay (ELISA) experiment. Successful immobilization of Ab was achieved by two different techniques: (1) physical adsorption; and (2) covalent immobilization by carbodiimide coupling between the surface —COOH groups and amine functionalities of dengue Ab molecules. Produced polymer coatings were characterized with surface spectroscopy techniques (Raman and X-ray photoelectron spectroscopy, XPS) and water-in-air contact angle (WCA) measurements. In particular, this research concentrated on the aging effect on the availability and activity of surface —COOH groups. For that reason, WCA and Ab immobilization (ELISA) experiments were repeated on coated biochips after 3, 6 and 9 months of storage. Results in this paper describe the robust and sustainable functionalized polymeric platform that can be used effectively for protein activation and development of novel biosensors.

1. Introduction

Enzyme-linked immunosorbent assay (ELISA) has found many applications in the field of food industry, determination of peptides, proteins, hormones and drug allergens. Possibly the most common and important clinical application of ELISA is detection of viruses in human blood [1]. Despite the standardization and commercialization of ELISA in clinical practice, the methodology has several drawbacks, such as laborious protocol, long incubation times, lack of reproducibility and high detection limits, which create serious problems in early diagnostics of viruses [2]. Since ELISA assay is based on heterogeneous processes that occur on interfaces between solid platforms (ELISA plates) and protein solutions (serum), there are interesting opportunities to use engineered polymer substrates to improve the assay. Well-designed substrates would enable increased solid-liquid contact surface area (i.e., more exposure of surface-tethered antibodies) and higher binding affinity between the polymer surface and proteins [3,4].

One of the major drawbacks that have been ignored is the performance of polymer surfaces such as polystyrene (PS) and polymethyl metacrylate (PMMA) as ELISA substrates. Although PS and PMMA are cost-effective and suitable for mass production, both are inert materials and do not contain reactive functional groups such as hydroxyl, amines or carboxylic acids. For that reason, such commercial analytical kits do not provide a surface that is particularly promoting adsorption of proteins. In order to overcome mentioned drawbacks of conventional ELISA, research efforts have been focused on the development of functionalized polymers with high degree of control over surface properties such as chemistry and morphology [5–8]. In that perspective, poly(acrylate)

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and poly(methacrylate) macromolecules have drawn a great deal of interest due to their unique properties such as low specific weight, high impact resistance, transparency and flexibility [2,9]. Different methods of surface modifications have been applied for generation of desirable functionality on the surface of such polymers. Specifically, generation of —COOH groups on PMMA has been reported by variety of techniques such as wet chemical treatments [2,10–12], UV exposure [13] and different plasma treatment techniques [14–19]. Although mentioned methods show relative effectiveness in generation of surface —COOH groups, such functionalities do not last for a long period of time as the treated polymeric platform "relaxes" back to the untreated status. In such cases surface functional groups re-orient themselves as they naturally tend to occupy lower energy levels, thus losing their reactivity [20]. This phenomenon so called "aging effect" causes serious problems in terms of polymer shelf-life and availability for diagnostic devices on clinical scale for reasonable periods of time.

As a new approach and a replacement for relatively uncertain surface modification methods that can generate desirable but not stable functional groups on PMMA polymer, we have produced different compositions of co-polymers synthesized by methyl methacrylate (MMA) and methacrylic acid (MAA) in different molar ratios of monomers. With this method the presence of —COOH groups at material's surface is ascertained [21]. The major aim of this paper is to address the significant concerns about aging effect of functionalized polymer surfaces, produced for diagnostic applications. Variety of monomer concentrations (MMA/MAA) in reaction mixture is the key point to achieve the optimum number of surface—COOH groups. High density of surface functional groups might cause steric hindrance between immobilized biomolecules and subsequent loss of bioactivity. This phenomenon further causes the loss of proteins' ability to recognize and bind complementary molecules from a supernatant solution. Moreover, in the presence of many surface-bound —COOH groups, multiple binding of any biomolecule to the surface is likely to occur, leading to aberrant conformations, partial loss of recognition and capacity to bind complementary solutes [20,22]. Similar to high surface concentration of functional groups, insufficient number of surface functionalities could also cause protein deactivation in close proximity to the polymer surface. For those reasons, the essential criteria for potential biosensor platforms require high level of control over polymer surface properties.

In our study we have utilized protein immobilization experiments of dengue antibody (Ab) molecules and subsequent detection of isolated dengue virus (DV). Dengue infection is a widespread mosquito-borne viral disease which initially appears with dengue fever (DF). This virus which is wide-spread in tropical and subtropical areas may also result in fatal manifestations such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [23–25]. In order to produce sustainable and robust polymer surfaces that can provide high sensitivity for DV detection, we have analyzed poly(MMA-co-MAA) coatings (Fig. 1) by different polymer and surface characterization techniques. This study demonstrates a straightforward technique for improvement of polymeric materials that could find a wide application as diagnostic devices in terms of early detection of all kinds of viruses including dengue.

2. Materials and methods

2.1. Chemicals and reagents

MMA, MAA, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), bovine serum albumin (BSA), monosodium phosphate (NaH_2PO_4), Tween 20, disodium hydrogen phosphate (Na_2HPO_4) and pH buffer solutions (pH 4, 7 and 10) were purchased from Sigma–Aldrich, US. MMA monomer was distilled at atmospheric pressure prior to use. The other materials were used as received. Phosphate buffer saline (PBS) was purchased from Thermo Fisher Scientific, US. Diced silicon OFET substrates (20 mm × 20 mm) were purchased from Ossila, UK. The free-radical initiator azobisisobutyronitrile (AIBN) was purchased from Friedemann Schmidt Chemical, Germany. Tetrahydrofuran (THF, Sigma–Aldrich) was used as solvent in polymerization and processing procedure of coatings.

2.2. Poly(MMA-co-MAA) synthesis and processing

Different molar ratios of MMA/MAA have been used in free-radical polymerization reaction in order to produce four compositions of poly(MMA-co-MAA): pure PMMA, poly(MMA-co-MAA-9:1), poly(MMA-co-MAA-7:3) and poly(MMA-co-MAA-5:5). Detailed polymerization procedure was previously reported [21]. The chemical structure of the copolymer is displayed in Fig. 1a. For fabrication of the biochips polymer solutions of different compositions (5% polymer solutions in THF) were spin-coated on silicon wafers (20 mm × 20 mm) using spinning time of 55 s (3000 rpm) by Laurell, WS-650MZ-23NPP spin coater (Fig. 1b–d) [21]. Coated surfaces were cut in dimension of 4 mm × 4 mm to fit into the ELISA well-plates.

2.3. Polymer analysis by Raman spectroscopy

Coated surfaces of different compositions have been analyzed with Raman spectroscopy (Renishaw, model inVia). The spectral data were collected with resolution of 1 cm^{-1} using the 520 nm line of a helium–neon laser. The incident laser beam was focused on the specimen surface through a 50 × objective lens, forming a laser spot of approximately 5 μm in diameter. Three coated samples of each copolymer composition have been analyzed.

2.4. Water-in-air contact angle (WCA) measurement

WCA of coated surfaces was measured with droplets of acidic (pH 4), neutral (pH 7) and alkaline (pH 10) buffer solutions, deposited on the solid surfaces of coated substrates. The measurement has been performed at room temperature applying sessile drop method for samples of different aging periods after coating deposition: immediate, 3, 6 and 9 months. Dataphysics instrument (OCA) was used for contact angle measurement. The WCA values were measured in first minutes after each droplet of water (0.1 μL) was placed on the polymer surface. Reported values are the average of five different measurements, on the center and four corners of each sample. Standard deviations were calculated for 6 samples of each polymer composition.

2.5. X-ray photoelectron spectroscopy (XPS)

The XPS measurements were performed by using Quantera SXMtm from Ulvac-PHI (Q1). The measurements were done using monochromatic AlKα-radiation and a take-off angle θ of 45° at which the information depth is approximately 7 nm. A spot size of 300 × 500 μm was chosen for the sample analyses. Wide-scan measurements have identified the presence of the elements on the surface. Accurate quantification and precise identification of chemical states was performed by using narrow-scans. Standard sensitivity parameters were used to convert peak positions to atomic concentrations. Therefore, the concentrations might be deviated from reality in the absolute sense (relatively not more than 20%).

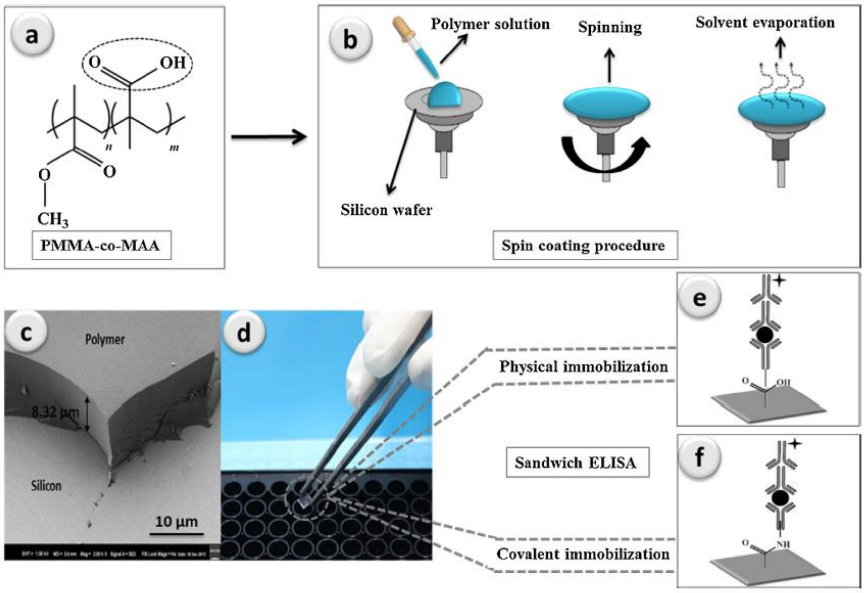

Fig. 1. Summary of the experimental procedure: (a) poly(MMA-co-MAA) chemical structure prepared via free-radical polymerization reaction; (b) spin-coating procedure by using poly(MMA-co-MAA) solutions of different compositions deposited on silicon wafers; (c) SEM cross-section image of the coated silicon wafer (bar = 10 μm); (d) developed samples being cut into the chips with dimensions of 4 mm × 4 mm to fit 96 well-plate; (e) physical attachment of dengue Ab to the coated surface; (f) covalent immobilization of dengue Ab by means of carbodiimide chemistry.

2.6. Protein dengue antibody immobilization methods on poly(MMA-co-MAA) coatings

The coated silicon chips with the coating thickness of ~10 μm (Fig. 1c) were cut into the dimension of 4 mm × 4 mm (Fig. 1d) in order to fit inside the 96-well plates (SPL, life science, Korea). The total number of 12 samples from each composition were examined for detection of DV. Sandwich ELISA protocol was performed by using colorimetric assay in order to evaluate the efficiency of polymer coated platforms for DV detection. Apart from conventional ELISA which was performed as a standard method (control), two sets of experiments with coated chips have been conducted: (1) physical adsorption of Ab on poly(MMA-co-MAA) coatings (untreated surfaces, Fig. 1e) and (2) covalent immobilization of Ab through carbodiimide chemistry (Fig. 1f) by incubation of the chips in EDC/NHS solution (0.155 g of EDC and 0.115 g of NHS in 200 ml of PBS) for 1 h. For covalent immobilization, samples were taken out and thoroughly washed with PBS prior to placement in 96-well plate and subsequent Ab immobilization.

2.7. Dengue virus propagation in mosquito cells and titration

A clinical isolate of dengue serotype 2 from a patient's serum sample (DENV2-isolate Malaysia M2, Gen Bank Toxonomy No.: 11,062) was used for virus propagation by a single passage on C6/36 mosquito cells. The dengue-infected cells with obvious cytopathic effects (CPE) were lysed by freeze and thaw cycle. The culture medium was centrifuged at RCF = 871 G for 10 min to remove cell debris, filtered (0.2 μm), portioned into aliquots, and stored at −80 °C until used. The viral titer of the dengue suspension was established by serial dilutions on Vero cells using plaque assay. In brief, a 10-fold serial dilution of medium supernatant was added to new Vero cells grown in 24-well plate (1.5×10^5 cells) and incubated for 1 h at 37 °C. The cells were then overlaid with DMEM medium containing 1.1% methylcellulose. Viral plaques were stained with naphthol blue-black dye after 5 days of incubation. Virus titers were calculated according to the following formula: titer (p.f.u/ml) = number of plaques / volume of diluted virus added to the well × dilution factor of the virus used to infect the well in which plaques were enumerated. The titer of DV that was used in the following experiments of colorimetric ELISA was 3.5×10^7 p.f.u/ml and the serial dilutions were prepared in PBS. In order to eliminate the cross- reactions due to the host cell proteins, the cell supernatant of non-infected cells was used as a control in the ELISA assay. The values that represent a possible non-specific reaction between the host cell proteins and dengue Ab were subtracted from the original values to increase the ELISA sensitivity.

2.8. Sandwich colorimetric ELISA assay on poly(MMA-co-MAA) coatings

Each well of the ELISA well-plate was charged with 100 μl of capture Ab (Goat IgG anti DV 2 (D-15): SC-325014) which was diluted (1:300) in coating buffer (0.85 g of NaCl, 0.14 g of Na_2HPO_4 and 0.02 g of NaH_2PO_4 in 100 ml of PBS). Incubation was carried on for 2 hours in 37 °C. Washing step was performed with 200 μl per well of washing buffer (0.05% Tween 20 in PBS) at room temperature. ELISA kits of both, empty and including polymer chips were washed 3 times (each time 5 min) by using shaker with shaking speed of 1000 rpm. The exact same washing procedure was performed between each two steps of the ELISA assay. In order to achieve high selectivity and to avoid non-specific bindings, blocking procedure was conducted by adding 100 μl of blocking buffer (1 g of BSA in 100 ml of washing buffer) to each well (37 °C for 1 h). Dengue enveloped virus was diluted (serial dilution) in variety of concentrations in coating buffer. Depending on the application,

different concentrations of the virus have been used in the assay. Different concentrations in the range between 3.5×10^{-6} p.f.u/ml to 3.5×10^{2} p.f.u/ml were chosen for determination of the calibration curves (by using Log scale). Detection range was studied by performing sandwich ELISA assay in the concentration range of 3.5×10^{3} p.f.u/ml to 3.5×10^{-6} p.f.u/ml. In particular, concentration 3.5×10^{-2} p.f.u/ml was chosen for detection comparison as this DV concentration has resulted in the highest detection signal. Each well was charged with 100 μl of the virus solution and incubation was carried out overnight at 4 °C. Primary Ab solution was prepared by diluting mouse IgG anti DV ICL2: SC-65725 (1:300) in diluting buffer (0.4 g BSA, 4 ml PBS buffer and 120 μl of Trintonx-100 in 36 ml of distilled water). Each well has received 100 μl of Ab solution and ELISA kits were placed in incubator for 2 h at 37 °C. For accuracy of the judgment, chips were taken out from the analytical kit, washed and placed in the new ELISA kit prior to further steps of experiment. The incubation of 30 min was done at 37 °C by adding 100 μl of anti-mouse IgG (Fc specific) as secondary Ab which was diluted (1:200) in diluting buffer. Wells were thoroughly washed (as it was described) and filled with 100 μl of mixed substrate (Alkaline phosphatase blue microwell substrate components A and B). Eventually reaction was stopped after 10–15 min by adding 50 μl of alkaline phosphatase stop solution (A585, Sigma) and signal intensity was recorded by using Bio-Rad (model 680) at the wavelength of 570 nm. The ELISA detection results were plotted after subtraction of the cut-off values from the raw data obtained from the assay. Only those samples that resulted in optical density (OD) above cut-off values (calculated as twice of the mean values of negative controls) were considered as positives [25]. Negative controls were measured as a result of running the assay in the absence of antigen ($n = 8$). Positive detection results were investigated as the average value of 12 replicates ($n = 12$). It is also noteworthy that the risk of cross reactivity was less than 2%. Total numbers of 512 samples have been used in sandwich ELISA (via physical and covalent immobilization) to assess the potential of the developed platforms in biological analysis. Precise calculations of sensitivity and specificity were defined by 384 positive samples with predetermined DV concentration and 128 negative controls where utterly non-infected samples were analyzed. Moreover, the accuracy of the assay has also been calculated from negative and positive readings (true and false) in comparison to the total number of the actual samples which have been examined. Limit of detection (LoD) for each separate platform was calculated from the slopes of the calibration curves and standard deviations [26].

3. Results and discussion

3.1. Polymer analysis by Raman spectroscopy

Copolymers of different compositions have been analyzed by Raman spectroscopy and results are shown in Fig. 2. Detected wavelengths of the most dominant bands in spectra are marked in the figure. The peak in the range of 2800–3000 cm^{-1} is the most prominent since it originates from combination of different types of C—H stretching vibrations [27,28]. This spectral band consists of three overlapping peaks, which agrees well with data from the literature [27,28]. In brief, this combination band includes vibrations such as ν (C—H) of O—CH_3, α-CH_3 and —CH_2 [27]. Results in Fig. 2 show systematic decrease in intensity which is attributed to decrease in MMA content upon going from PMMA to poly(MMA-co-MAA 5:5) (Fig. 2b–d). Other peaks also showed the similar pattern of decrease in intensity (bands at 815 and 1456 cm^{-1}) due to systematic replacement of —$COOCH_3$ with —COOH groups, generated from MMA and MAA, respectively. Carbonyl (C=O) band detected at 1729 cm^{-1} for PMMA is a sharp peak in comparison to broad bands

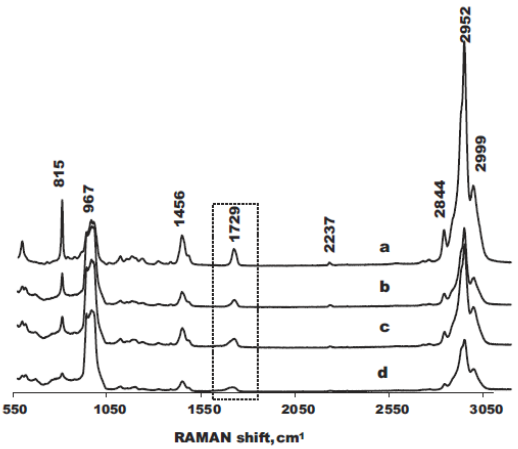

Fig. 2. Raman spectra of produced polymer coatings: (a) PMMA; (b) poly(MMA-co-MAA-9:1); (c) poly(MMA-co-MAA-7:3); (d) poly(MMA-co-MAA-5:5).

at the same wavelength, detected for other poly(MMA-co-MAA) coatings (highlighted section, Fig. 2) [29–31]. The exposed —COOH groups engaged in hydrogen bonding, cause the appearance of a broad bands in the same spectral position [31,32]. This is a well-known phenomenon which clearly shows the presence of pendant —COOH groups in developed polymer systems. The concentration of —COOH is controlled by tuning the polymerization reaction parameters; in current case this is the variation of MMA/MAA initial molar ratio in reaction mixture. Inherent —COOH groups (generated from MAA monomers) are also expected to be exposed at the outmost surface layer of the coatings. All our samples demonstrated high level of consistency in terms of coating thickness and surface morphology (Fig. 1) as reported previously [21].

3.2. Surface analysis with XPS

The chemical compostition at the surface of the four copolymer structures of the copolymer was analyzed with XPS and results are displayed in Table 1. Peak positions in eV and the chemical assignments are indicated in the top row ("–"denotes that the concentration is less than the detection limit: <0.1 %). Results demonstrated a decrease for the C1s peak (286.4 eV) which is attributed to carbon atoms in the —O—CH_3 groups (typical for the MMA building blocks). Logically, the intensity of this signal decreases upon increasing the concentration of MAA segments from PMMA to poly(MMA-co-MAA 5:5), (Table 1). This is naturally causing an increase in —COOH group's concentration on the surface of poly(MMA-co-MAA) coatings. An obvious increase in aliphatic carbon concentration (detected at 284.8 eV) can be observed from

Table 1
Surface concentrations (in %) of the investigated poly(MMA-co-MAA) samples by XPS (small concentrations of Na, K, Cl and some organic Si were neglected in presented data).

Peak	C 1s			N1s	O1s
Binding energy (eV)	284.8	286.4	288.7	400	530
Peak assignment	—CH	—CO	O—C=O		
PMMA (%)	42	16	12	0.2	26.5
Poly(MMA-co-MAA-9:1)	44	15	13	0.5	26
Poly(MMA-co-MAA-7:3)	45	13	12.5	0.1	27
Poly(MMA-co-MAA-5:5)	48	10.5	11.5	0.2	26.5

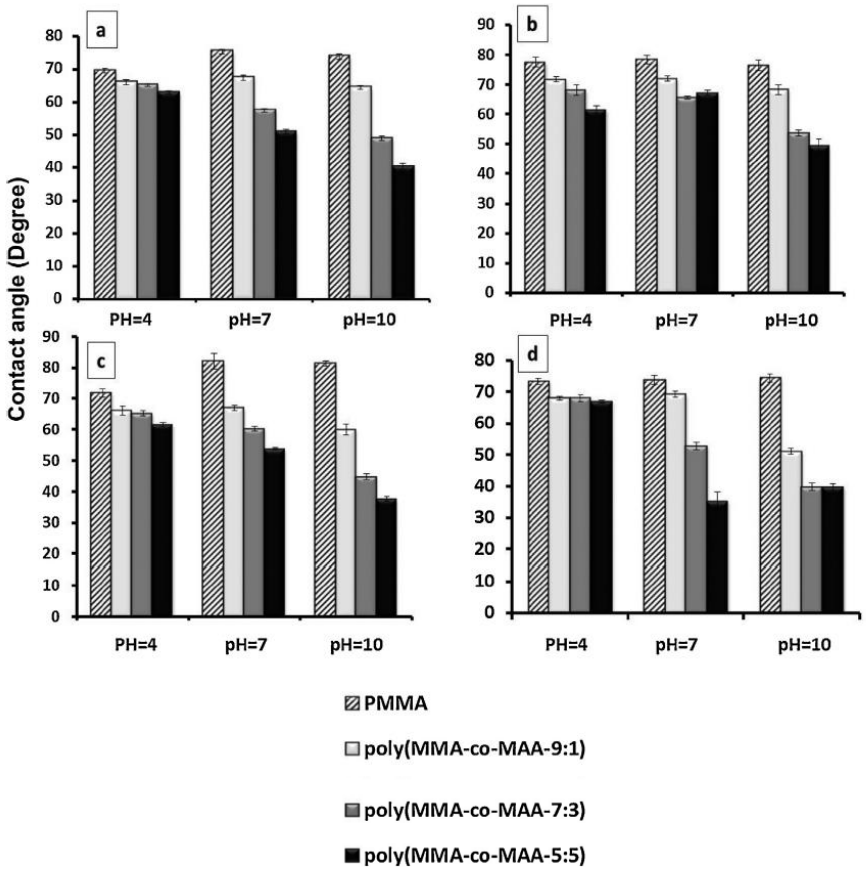

Fig. 3. Influence of pH on water-in-air contact angle measured for poly(MMA-co-MAA) coatings after different aging periods: (a) immediate coatings; (b) 3 months; (c) 6 months; (d) 9 months.

Table 1 as the concentration of MAA increases. This result could be predicted as the number of aliphatic carbons gradually increases in comparison to the total number of carbon atoms in poly(MMA-co-MAA) chains. However, it can be seen from Table 1 that the values for O—C=O peak (288.7 eV) and O 1s peak (530 eV) have not shown considerable changes. A very small amount of nitrogen (400 eV) on the surface was also recorded which is a consequence of unavoidable surface contamination and sensitivity of XPS technique. In general, XPS analysis confirms that the content of MAA increases upon changing the polymer structure in ordered form. The —COOH groups do exist on the outermost surface layer of poly(MMA-co-MAA) coatings and their concentration gradually increases with concentration of MAA segments in polymer structures [21].

3.3. Contact angle analysis and aging effect

WCA experiment was conducted in order to determine the relative surface hydrophilicity of polymer coatings. Furthermore, the experiment was repeated on the exact same coated samples in different shelf-life periods (3, 6, and 9 months) in order to analyze the possibility of aging effect [16]. As a control and for the accuracy of the method, WCA was measured for pure PMMA as well. The results have shown the WCA measured in the range of 72–82° for PMMA which is in agreement with previously reported data [15,33]. Gradual changes in WCA from PMMA to different molar ratios of poly(MMA-co-MAA) copolymers clearly indicates that chemical changes had occurred on the surface due to the different concentrations of monomers used in polymerization reaction (Fig. 3). The relative increase in MAA segments has resulted in more hydrophilic surfaces, as a consequence of —COOH surface groups exposure (Fig. 4) [21]. As expected, the lowest contact angles have been measured at the surfaces of poly(MMA-co-MAA-5:5) regardless of the age of the samples.

In order to study the response of the coated surfaces to different environments, detailed behavior of the samples have been analyzed by contact angle measurements, but in a slightly different manner as usual. By depositing droplets of different mediums such as pH 4 (acidic), pH 7 (neutral) and pH 10 (alkaline), the interaction between coated surfaces and droplets have been carefully studied (Figs. 3 and 4). In the case of acidic environment (pH 4), and assuming that the —COOH groups at the surface have a dissociation rate comparable to acetic acid ($pKa = 4.76$), it can be readily calculated that approximately 85 % of the surface acid groups might not be dissociated (i.e., neutral, —COOH), while approximately 15 % of them can be dissociated (i.e., negatively charged, $—COO^-$). Hence, it was predicted that the poly(MMA-co-MAA) surfaces exhibit hydropho-

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