

Research paper

Surface modification of PS microtiter plate with chitosan oligosaccharides by ^{60}Co irradiation



Shixin Han, Huashan Wang*, Zhipeng Sun, Huiqi Zhao, Pai Zhang

School of Chemical Engineering and Material Science, Tianjin University of Science and Technology, Tianjin 300457, China

ARTICLE INFO

Keywords:

Microtiter plate
Irradiation grafting
Chitosan-oligosaccharides
Protein adsorption

ABSTRACT

By ^{60}Co - γ irradiation method, the Chitosan-oligosaccharide (COS) was grafted onto the inner surface of the polystyrene (PS) microtiter, which was soaked with COS solution before the irradiation. To evaluate the effect of COS concentration on the properties of the PS microtiter, FTIR, XPS, AFM, Contact angle tester and enzyme-linked analyser was used to measure the surface properties and BSA adsorption of PS-COS plates. The results show that, with the increase of COS concentration, the contact angle clearly decreased at the dose of 12 kGy. The absorbance variances of the COS modified plate is less than 5% while the BSA adsorption is higher than the PS plates. The COS-modified microtiter has the potential applications in biochemical analysis.

1. Introduction

Enzyme-linked immunosorbent assays (ELISA) methods are mainly based on the reaction or adsorption between biomolecules and polystyrene microtiter plates (Eckert, Grobe, & Rothe, 2000; Mcclung, Clapper, Hu, & Brash, 2001; Werning et al., 2014). Usually the surface functional groups of modified polystyrene microtiter plates directly affect the results of ELISA. Lysine, the ester of methacrylic acid, acrylic acid and crotonic acid are mostly used as active monomers in the surface modification of polystyrene microtiter plates by free radical polymerization or vapor deposition (Eckert et al., 2000; Goddard & Hotchkiss, 2007; Larsson, Johansson, Hult, & Gothe, 1987). The adsorption capacity of modified polystyrene microtiter plates are obviously improved, but the impurities, such as initiator and cross-linking agent, were left behind. In comparison with free radical polymerization or vapor deposition, the method of irradiation grafting has many advantages, for example, the free radicals can be initiated by irradiation without initiator, which has no chemical pollution in the reaction system (Bhattacharya, 2000; Jin, Yang, Yang, Chen, & Chen, 2014; Kim, Saito, & Furusaki, 1991; Tsuneda, Saito, Furusaki, Sugo, & Okamoto, 1991; Wu, Sang, Liu, & Shen, 2004). Hence, the irradiation surface modification has become a research hotspot (Chmielewski, Haji-Saeid, & Ahmed, 2005; Guven, 2017). Chitosan was grafted onto poly (3-hydroxybutyrate), glycidyl methacrylate (GMA) was grafted onto microcrystalline cellulose (MCC) and a new kind of non-fluorin-based organic hybrid superhydrophobic cotton fabric was prepared by irradiation grafting have been reported (Gao et al., 2016; Madrid & Abad, 2015; Torres et al., 2015).

Chitosan (CS) is usually used as biocompatible macromolecule which has the advantages of hydrophilicity, pliability, plasticity, stability (Anitha et al., 2014; Choi, Nam, & Nah, 2016; Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Padilla-Rodriguez et al., 2015). Even though CS has shown various functional properties, the poor solubility limits its application in surface grafting. So, to overcome this issue, soluble chitosan derivatives have been prepared by irradiation. It has been reported that irradiation grafted acrylic acid (AA) and acrylamide (AAm) onto chitosan would control drug release, and irradiated chitosan would improve its antioxidant activity and reduce the radiation damage to the radiation workers or radiation cured patients (Chmielewski, 2010). Unlike CS, COS are readily soluble in aqueous solutions due to their smaller molecular weight and free amino groups. COS possesses the biocompatibility, surface positive charge, physico-chemical properties and water/acid solubility (Zou et al., 2016). Electrostatic interactions between positively charged COS and negatively charged cells carrying negative charge, and the reaction between the amino groups of COS and the carboxyl groups of protein, are beneficial for biological detections. Owing its physical and chemical properties, COS has potential applications at a cellular or molecular level in many aspects. Murata, Nakano, Tahara, Tozuka, and Takeuch (2012) reported that COS was used as surface modifier to increase the cellular association of liposomes. The stearic acid (SA) and poly-lactic-co-glycolic acid (PLGA) grafted chitosan oligosaccharides tripolymer can be used as a carrier for hydrophobic drugs (Zhou et al., 2010). Grafting of polystyrene onto chitin and chitosan using ^{60}Co γ -irradiation at room temperature was investigated (Liu, Zhai, & Wu, 2001).

Based on above description and discussion, it can be seen that there

* Corresponding author.

E-mail addresses: whs@tust.edu.cn, whs2002cn@sohu.com (H. Wang).

is the feasibility in theories and practices to modify the PS-microtiter with COS by irradiation method. In this work, we have expected to obtain a novel microtiter plate which could adsorb the BSA homogeneously, stability. The COS was selected to prepare PS-COS microtiter plates by ^{60}Co - γ irradiation method. For investigating the effect of COS concentration on the physical and chemical properties of PS-COS microtiter plate, the surface properties, water contact angle, absorbance variances and the BSA adsorption properties were evaluated.

2. Experiment

2.1. Materials

Microtiter plates (126mm \times 86 mm, 96 wells) were purchased from Haimen San Ye plastics factory (Jiangsu, China). Chitosan (average molecular weight 1.82×10^5 Da; degree of deacetylation DDA = 95%) as initial material was supplied by Laizhou Hali biological product Co., Ltd. (Shandong, China) and was purified in HCl aqueous solution (1%, v/v) and NaOH aqueous solution (5%, v/v). COS (Mn = $1.5\text{--}2.0 \times 10^3$; DDA = 65%) was obtained by enzymatic hydrolysis according to the method described previously (Qin, Du, Xiao, Li, & Gao, 2002).

Bovine serum albumin (BSA, purity $\geq 98\%$) was purchased from Sigma Aldrich. Acetic acid (AR, 99%) was supplied by Jiangtian Chemical Industry Technique Co., Ltd. (Tianjin, China). Phosphate buffer saline (PBS, C0221A–500 mL), Tetramethylbenzidine (TMB, P0209–100 mL) was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). All aqueous solutions were used with redistilled water.

2.2. Methods and procedures

Preparation of PS-COS microtiter plates. Microtiter plates were washed with anhydrous ethanol and distilled water successively and then dried in a vacuum oven at 50 °C for 12 h to remove water. After that, these microtiter plates were soaked with an acetic acid/water (1%, v/v) solution of various COS concentration (0, 0.2, 0.4, 0.6, 0.8, 1.0%, w/w) about 200 μL per pore and quickly sealed with films respectively. The samples were irradiated by ^{60}Co γ -ray to initiate the grafting reaction at room temperature. In the process of irradiation, the selected irradiation dose was 12 kGy and the dose rate was 60 Gy/h. Thereafter, the samples were washed with acetic acid/water solution by ultrasonic washing to remove homo-polymers and the unreacted monomers until the residual is completely removed, and dried at room temperature. Fig. 1 shows a possible mechanism in grafting COS on microtiter plates via γ -ray irradiation. Through the irradiation, COS and the surface of polystyrene microtiter plates were able to generate living free radicals (Depan & Singh, 2015; Gryczka et al., 2009; Pasanphan & Chirachanchai, 2008; Shen, Hu, Wang, & Qu, 2011). Eventually the COS could be grafted onto polystyrene microtiter plates by free radicals combination. Finally, we have obtained the PS-COS microtiter plates.

Surface Properties. FT-IR spectra were recorded as KBr pellets using a Bruker (TENSOR 37) spectrometer in the range 4000–400 cm^{-1} . The powder was obtained from the inner surface of the PS and PS-COS microtiter plates. XPS spectra were obtained on an EDAX-GENESIS 60S instrument using Al K_{α} (1486.6 eV) excitation at pass energies of 200.0 eV for survey and 50.0 eV for high-resolution core-level spectra of C1s, O1s, N1s. Atomic Force Microscope (AFM) was used to ascertain and compare the morphology of the original plates, the acetic acid modified plates and COS-grafted plates. The measurements were performed using AFM (JSPM-5200) working on tapping mode. The microtiter plates were cut and the flat inner surface were measured using digital image analysis. Water contact angle measurements were carry out by JY-150 contact angle measuring instrument (Chengde Testing Machine Co., Ltd, China). The enzyme-linked analyzer (Multiskan Mk3, Thermo Fisher Scientific) was used to measure the absorbance value of

the microtiter plates at 450 nm wavelength.

Protein Adsorption. The bovine serum albumin (BSA) solution was prepared to calibrate the concentration via Ultraviolet Spectrophotometer, and the equation is:

$$C_{Pr} = 1.45 \times A_{280} - 0.74 \times A_{260} \text{ (mg/mL)}.$$

Where, C_{Pr} is the concentration of BSA; A_{280} and A_{260} are the values of the ultraviolet absorption, respectively.

The BSA solution ($[C] = 20, 40, 80, 120, 160, 200$ ng/mL in PBS at pH = 7.4) were added to the unmodified microtiter plates, about 100 μL per pore, and incubated 1 h at 37 °C. A row of unmodified microtiter plates as blank were blocked by PBS and washed (wash buffer: 0.05% Tween20, PBS; pH = 7.4) up to 6 times latter. Next the dilution BSA-HRP (1:1000, v/v) antibody was added to each pore and incubated for 30 min at 37 °C. After the plates were washed with buffer solution until unbound protein were removed, and the enzyme activity with TMB was tested. The reaction was terminated after 2 min with 50 μL H_2SO_4 (2 mol/L) and the absorbance were measured at the wavelength of 450 nm in ELISA meter. After the procedures were performed at least three times, the standard curve was obtained.

The modified microtiter plates were soaked with BSA ($[C] = 3800, 5000$ ng/mL in phosphate buffer saline at pH = 7.4), about 100 μL per well, and incubated 1 h at 37 °C. After the mixing, 10 μL supernatants were added to 90 mL PBS of the unmodified microtiter plates and mixed with mini shaker, and incubated for 1 h at 37 °C. Then the previous steps of how to get standard curve were repeated. The protein adsorption would be obtained by comparing with the standard curve.

Statistical Analysis. The statistical analysis for both the surface analysis and protein adsorption data was performed with Origin (v. 8.1.3490, Origin Lab Corp., USA). As indicated earlier, all experiments were carried out in triplicate and repeated twice. All graphs in this study are shown as mean \pm standard deviation (SD). Differences were considered significant for $p < 0.05$.

3. Results and discussion

3.1. Surface characterization

As illustrated in Fig. 2, the characteristic vibrations of PS displayed vibration of =CH₂ stretch at 2922 cm^{-1} , 2849 cm^{-1} and –CH stretch peak at 3025 cm^{-1} . These peaks all appeared in the spectrum PS-COS. In addition, the OH stretch peak at 3440 cm^{-1} was belonged to COS (Fig. 2(a)). For COS, the characteristic bands at 1657 cm^{-1} , 1522 cm^{-1} correspond C=O in amide group, NH bending of amine (II) at 1562 cm^{-1} and 1552 cm^{-1} , C–N stretch peak at 1424 cm^{-1} , and vibration of –CH stretch at 1380 cm^{-1} , 1360 cm^{-1} (Fig. 2(b)). Otherwise, the peaks at 1778 cm^{-1} , 1739 cm^{-1} correspond to the C=O of carboxyl groups. There is no obvious change in bands at 1200–800 cm^{-1} , which indicated that the pyranose ring opening (Fig. 2(c)). This result is in accordance with Fig. 1.

In order to further confirm that the COS was grafted onto the surface of microtiter plates, the XPS were used to quantify the surface concentration of carbon, oxygen and nitrogen groups. The oxygen and nitrogen content were all increased than the untreated PS microtiter plates (Fig. 3 and Table 1). However, the C/N ratio of PS-COS plates were decreased, which is due to the structure of COS molecular contains O and N, changed the C content.

Fig. 4 shows the surface morphology images of microtiter plates. Comparison with the original microtiter plate, the surface of the PS-COS microtiter plate is rougher and exhibits a large amount of protuberances (Fig. 4(b)). The protuberances display discontinuity, which contributed to the protein well adsorbed. In addition, grafting reaction in acetic acid was displayed in Fig. 4(c). The results indicated that the COS was well grafted onto the surface of PS microtiter plate.

Fig. 5 shows that the concentration of COS effects on water contact

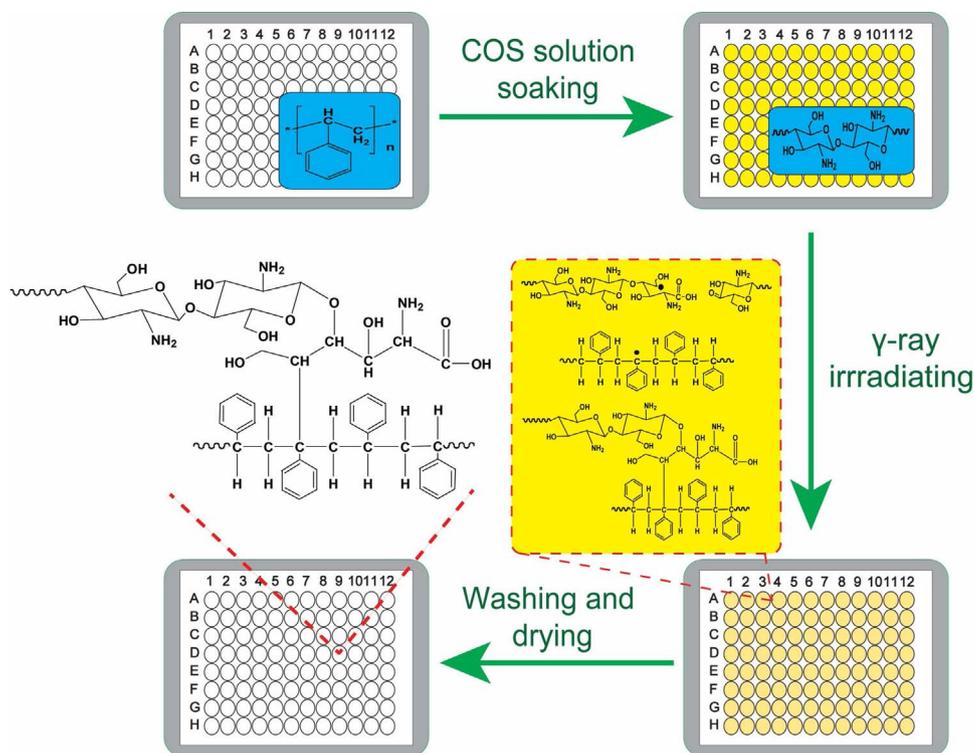


Fig. 1. Scheme of the COS modification of PS microtiter plate by irradiation grafting.

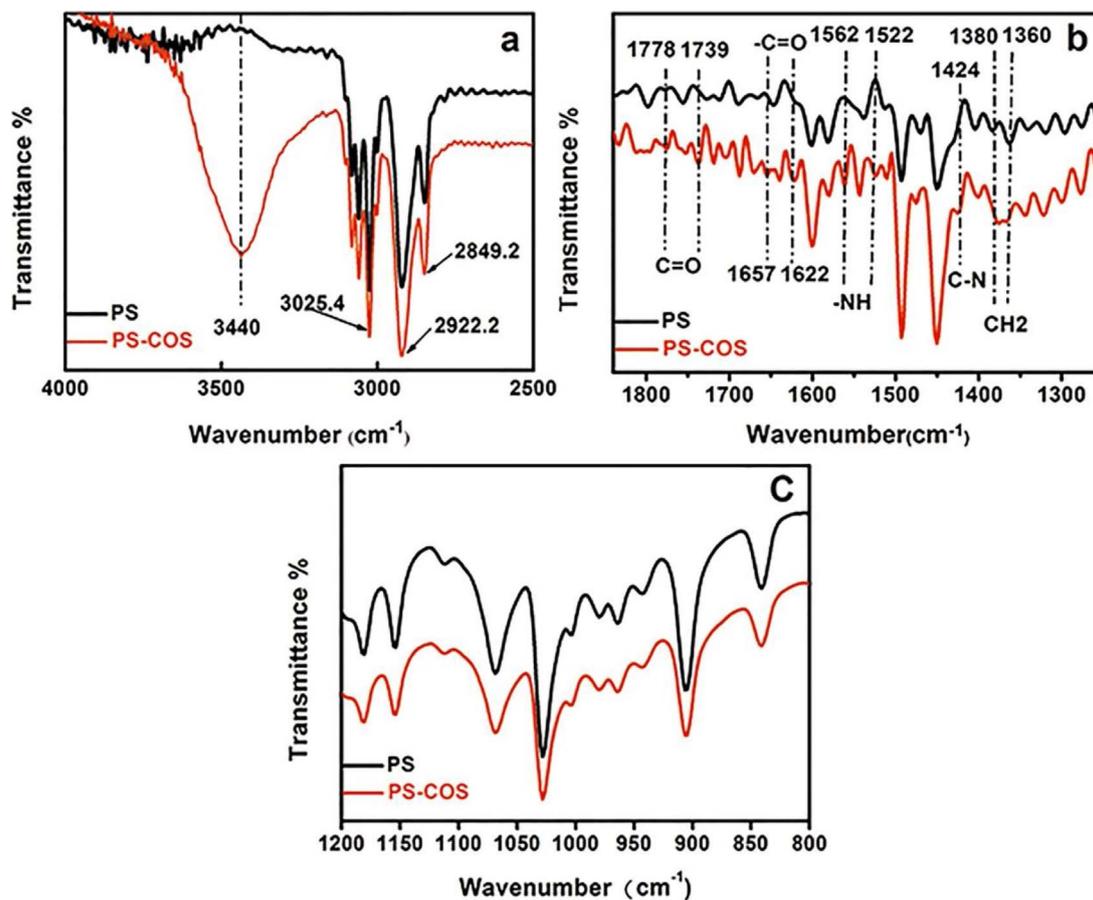


Fig. 2. FT-IR spectra of PS and PS-COS in the (a) 4000–2500 cm⁻¹, (b) 1800–1300 cm⁻¹, (c) 1200–800 cm⁻¹ region.

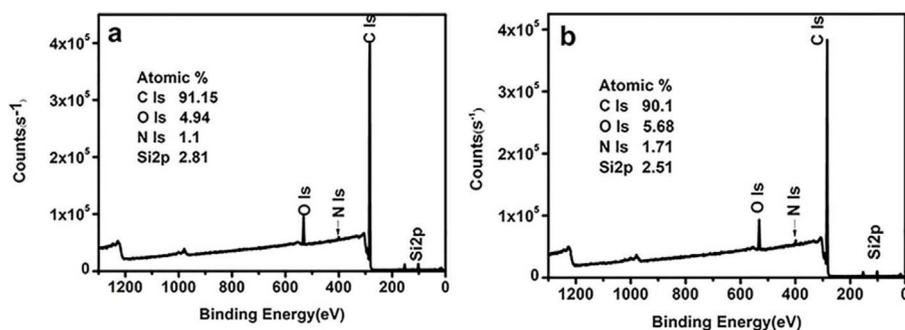


Fig. 3. XPS spectrum of (a) Original microtiter plates, (b) PS-COS microtiter plates.

Table 1
The elemental composition of original and grafted COS microtiter plates

Samples	Element composition(%)			
	C	O	N	C/N
Original microtiter plate	91.15	4.94	1.10	82.86
PS-COS microtiter plate	90.10	5.68	1.71	52.69

angle. A rapid decrease of contact angle is observed up to 0.8% and then the contact angle decrease slows down gradually. It suggested that with the increase of COS concentration, the hydrophilicity of the PS-COS plates increase, due to the surface polarity effect (Kozbial et al., 2014). The wettability indicated that the COS grafted onto the inner surface of the PS microtiter plate.

3.2. Absorbance of PS-COS microtiter plate

The relationship between the absorbance variances and the concentration of COS were described as Fig. 6. The increasing trend was obviously observed as the concentration of COS increased. When the concentration of COS changed from 0.6 wt% to 1.0 wt%, the increase rate of absorbance decreased. This implied that with the concentration of COS increasing, the grafting amount of COS would increase. However, higher grafting ratio affects the transmittance of microtiter plates and the sensitivity for biochemical analysis. Therefore, the concentration of COS should be lower.

3.3. BSA adsorption of PS-COS microtiter plate

Protein adsorption is the first and the foremost step in the biomaterials applications (Depan & Singh, 2015; Gagliardi, 2012). BSA is one of the most widely used blocking agents for ELISA, which has amino (–NH₂) and carboxyl (–COOH) groups (Guo, Yuan, & Zeng, 2014; Wangkam et al., 2012). COS has reactive amino and hydroxyl groups that provide many possibilities for combination with BSA. It has been reported that the protein adsorbed CS promotes the adhesion and proliferation of human endothelial cells (Chung, Lu, Wang, Lin, & Chu, 2002).

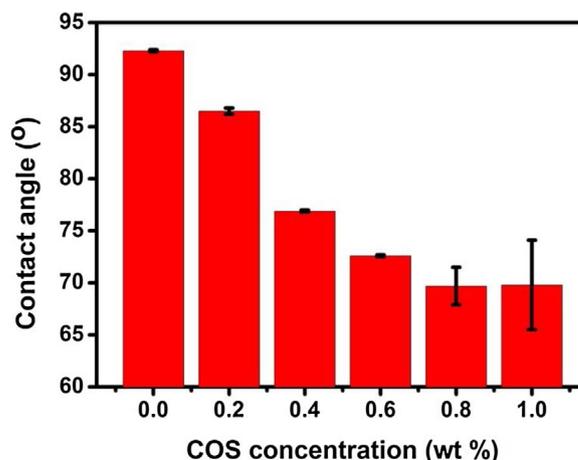


Fig. 5. Effect of COS concentration on the contact angle of the PS-COS surface.

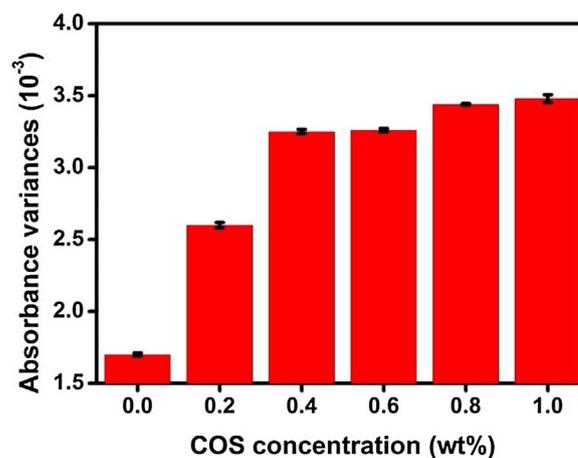


Fig. 6. Effect of COS concentration on absorbance variances.

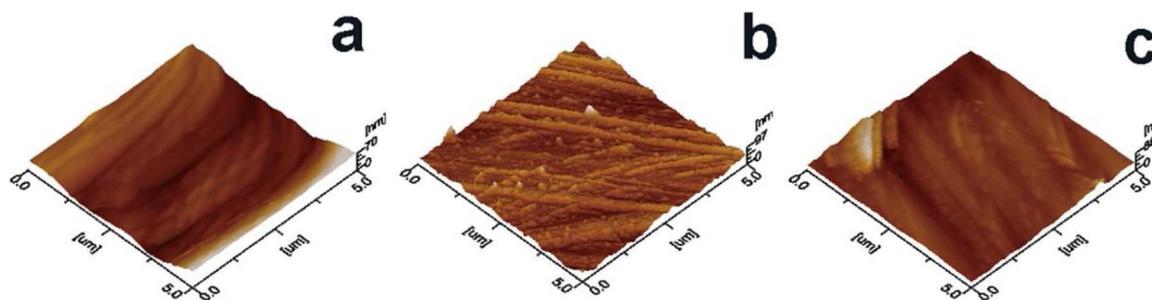


Fig. 4. AFM images of (a) original microtiter plate, (b) PS-COS microtiter plate, (c) acetic acid microtiter plate.

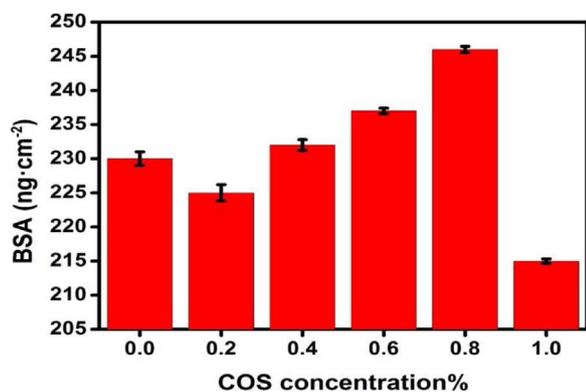


Fig. 7. Effect of COS concentration on the BSA adsorption.

Fig. 7 shows the effect of COS concentration on BSA adsorption. The increase of BSA adsorption is observed up to 0.8% and then decrease with the increase of COS concentration. The results indicated that the existence of COS onto the plates and with the increase of COS concentration the adsorption increased, due to the electrostatic interaction (Yoon, Kim, & Kim, 1999). Moreover, the BSA adsorption closely relates to hydrophobic interaction. With the increase of COS concentration, the BSA adsorption decrease. The probable reason why the decrease of adsorption at the COS concentration higher than 0.8% is that the adsorption is controlled by surface wettability of PS-COS (Fig. 5). The hydrophilicity results in a low level of adsorption. Hence it can be concluded that the modified PS-COS microtiter plates has the capacity to adsorb BSA, and the maximum adsorption capacity was higher than original microtiter plate (230 ng cm^{-2}). The data of the water contact angle and the BSA adsorption indicated that the optimized concentration of COS is 0.8 wt%. The mean coefficient of variation (CV) was lower than 5% eventually. It turned out the PS-COS plates adsorb the BSA homogeneously and stability.

4. Conclusions

In this work, the surface properties of microtiter plates were improved by a direct $^{60}\text{Co-}\gamma$ ray irradiation method. When the original PS microtiter was soaked by 0.8 wt% COS water solution first and then irradiated with 12 kGy dose at ambient temperature, both the water contact angle and the BSA adsorption of the modified microtiter plates reached to the optimum. The value of absorbance variances with repeated measures is less than 5%, which demonstrated that the PS-COS microtiter plates can adsorb the BSA homogeneously. Comparison with the original PS microtiter plates, the grafted COS and its polymer demonstrated higher protein adsorption. The obtained PS-COS microtiter plates are promising for ELISA applications.

Acknowledgment

This work was supported by the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period of China [2015BAK45B01].

References

- Anitha, A., Sowmya, S., Sudheesh Kumar, P. T., Deepthi, S., Chennazhi, K. P., Ehrlich, H., et al. (2014). Chitin and chitosan in selected biomedical applications. *Progress in Polymer Science*, 39, 1644–1667.
- Bhattacharya, A. (2000). Radiation and industrial polymers. *Progress in Polymer Science*, 25(3), 371–401.
- Chmielewski, A. G., Haji-Saeid, M., & Ahmed, S. (2005). Progress in radiation processing of polymers. *Nuclear Instruments & Methods in Physics Research*, 236(1–4), 44–54.
- Chmielewski, A. G. (2010). Chitosan and radiation chemistry. *Radiation Physics & Chemistry*, 79(3), 272–275.
- Choi, C., Nam, J. P., & Nah, J. W. (2016). Application of chitosan and chitosan derivatives as biomaterials. *Journal of Industrial and Engineering Chemistry*, 33, 1–10.

- Chung, T. W., Lu, Y. F., Wang, S. S., Lin, Y. S., & Chu, S. H. (2002). Growth of human endothelial cells on photochemically grafted Gly-Arg-Gly-Asp (GRGD) chitosans. *Biomaterials*, 23, 4803.
- Dash, M., Chiellini, F., Ottenbrite, R. M., & Chiellini, E. (2011). Chitosan—A versatile semi-synthetic polymer in biomedical applications. *Progress in Polymer Science*, 36, 981–1014.
- Depan, D., & Singh, R. P. (2015). Surface modification of chitosan and its implications in tissue engineering and drug delivery. In V. K. Thakur, & A. S. Singha (Eds.), *Surface modification of biopolymers* (pp. 20–44). John Wiley Sons Inc.
- Eckert, A. W., Grobe, U., & Rothe, D. (2000). Surface-modification of polystyrene-microtitre plates via grafting of glycidylmethacrylate and coating of poly-glycidylmethacrylate. *Biomaterials*, 21, 441–447.
- Gagliardi, M. (2012). In vitro haematic proteins adsorption and cytocompatibility study on acrylic copolymer to realise coatings for drug-eluting stents. *Materials Science & Engineering C*, 32(8), 2445–2451.
- Gao, Q., Hu, J., Li, R., Pang, L., Xing, Z., Wang, M., et al. (2016). Preparation and characterization of superhydrophobic organic-inorganic hybrid cotton fabrics via gamma-radiation-induced graft polymerization. *Carbohydrate Polymers*, 149, 308–316.
- Goddard, J. M., & Hotchkiss, J. H. (2007). Polymer surface modification for the attachment of bioactive compounds. *Progress in Polymer Science*, 32, 698–725.
- Gryczka, U., Dondi, D., Chmielewski, A. G., Migdal, W., Buttafava, A., & Fautitano, A. (2009). The mechanism of chitosan degradation by gamma and e-beam irradiation. *Radiation Physics & Chemistry*, 78(7–8), 543–548.
- Guo, Q., Yuan, J., & Zeng, J. (2014). Binding of dihydromyricetin and its metal ion complexes with bovine serum albumin. *Biotechnology & Biotechnological Equipment*, 28, 333–341.
- Guyen, O. (2017). Applications of ionizing radiation in materials processing. In Y. X. Sun, & A. G. Chmielewski (Eds.), *Established and emerging applications of radiation-induced graft polymerization* (pp. 355–373). Warszawa: Institute of Nuclear Chemistry and Technology.
- Jin, W., Yang, L., Yang, W., Chen, B., & Chen, J. (2014). Grafting of HEMA onto dopamine coated stainless steel by $^{60}\text{Co-}\gamma$ irradiation method. *Radiation Physics & Chemistry*, 105, 57–62.
- Kim, M., Saito, K., & Furusaki, S. (1991). Adsorption and elution of bovine γ -globulin using an affinity membrane containing hydrophobic amino acids as ligands. *Journal of Chromatography*, 585, 45–51.
- Kozbial, A., Li, Z., Conaway, C., McGinley, R., Dhingra, S., Vahdat, V., et al. (2014). Study on the surface energy of graphene by contact angle measurements. *Langmuir*, 30, 8598–8606.
- Larsson, H., Johansson, S. G., Hult, A., & Gothe, S. (1987). Covalent binding of proteins to grafted plastic surfaces suitable for immunoassays I. Binding capacity and characteristics of grafted polymers. *Journal of Immunological Methods*, 98(1), 129.
- Liu, P., Zhai, M., & Wu, J. (2001). Study on radiation-induced grafting of styrene onto chitin and chitosan. *Radiation Physics and Chemistry*, 61, 149–153.
- Madrid, J. F., & Abad, L. V. (2015). Modification of microcrystalline cellulose by gamma radiation-induced grafting. *Radiation Physics and Chemistry*, 115, 143–147.
- McClung, W. G., Clapper, D. L., & Hu, S. P. (2001). Lysine-derivatized polyurethane as a clot lysing surface: Conversion of adsorbed plasminogen to plasmin and clot lysis in vitro. *Biomaterials*, 22, 1919–1924.
- Murata, M., Nakano, K., Tahara, K., Tozuka, Y., & Takeuchi, H. (2012). Pulmonary delivery of elcatonin using surface-modified liposomes to improve systemic absorption: Polyvinyl alcohol with a hydrophobic anchor and chitosan oligosaccharide as effective surface modifiers. *European Journal of Pharmaceutics and Biopharmaceutics*, 80, 340–346.
- Padilla-Rodriguez, A., Hernandez-Viezcas, J. A., Peralta-Videa, J. R., Gardea-Torresdey, J. L., Peraltes-Perez, O., & Roman-Velazquez, F. R. (2015). Synthesis of protonated chitosan flakes for the removal of vanadium (III, IV and V) oxyanions from aqueous solutions. *Microchemical Journal*, 118, 1–11.
- Pasanphan, W., & Chirachanchai, S. (2008). Polyethylene film surface functionalized with chitosan via γ -ray irradiation in aqueous system: An approach to induce copper(II) ion adsorptivity on PE. *Reactive & Functional Polymers*, 68(8), 1231–1238.
- Qin, C., Du, Y., Xiao, L., Li, Z., & Gao, X. (2002). Enzymic preparation of water-soluble chitosan and their antitumor activity. *International Journal of Biological Macromolecules*, 31, 111–117.
- Shen, K., Hu, Q., Wang, Z., & Qu, J. (2011). Effect of ^{60}Co irradiation on the properties of chitosan rod. *Materials Science & Engineering C*, 31(5), 866–872.
- Torres, M. G., Munoz, S. V., Rosales, S. G. S., Castro, M. D. P. C., Munoz, R. A. E., Gonzalez, R. O., et al. (2015). Radiation-induced graft polymerization of chitosan onto poly(3-hydroxybutyrate). *Carbohydrate Polymers*, 133(3), 482–492.
- Tsunedo, S., Saito, K., Furusaki, S., Sugo, T., & Okamoto, J. (1991). Metal collection using chelating hollow fiber membrane. *Journal of Membrane Science*, 58, 221–234.
- Wangkam, T., Yodmongkol, S., Disrattakit, J., Sutapun, B., Amarit, R., Somboonkaew, A., et al. (2012). Adsorption of bovine serum albumin (BSA) on polystyrene (PS) and its acid copolymer. *Current Applied Physics*, 12(1), 44–52.
- Werning, M. L., Perez-Ramos, A., Palencia, P. F. D., Mohedano, M. L., Duenas, M. T., Prieto, A., et al. (2014). A specific immunological method to detect and quantify bacterial 2-substituted (1,3)- β -D-glucan. *Carbohydrate Polymers*, 113, 39–45.
- Wu, S., Sang, H., Liu, Z., & Shen, J. (2004). Effect of ultraviolet irradiation on structure and properties of HDPE and HDPE/STC blends. *Journal of Macromolecular Science Part A Pure and Applied Chemistry*, 41, 1311–1319.
- Yoon, J. Y., Kim, J. H., & Kim, W. S. (1999). The relationship of interaction forces in the protein adsorption onto polymeric microspheres. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 153, 413–419.
- Zhou, Y., Du, Y., Wang, L., Yuan, H., Zhou, J., & Hu, F. (2010). Preparation and pharmacodynamics of stearic acid and poly (lactic-co-glycolic acid) grafted chitosan oligosaccharide micelles for 10-hydroxycamptothecin. *International Journal of Pharmaceutics*, 393, 144–152.
- Zou, P., Yang, X., Wang, J., Li, Y., Yu, H., Zhang, Y., et al. (2016). Advances in characterisation and biological activities of chitosan and chitosan oligosaccharides. *Food Chemistry*, 190, 1174–1181.