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COLORIMETRIC DETERMINATION OF MUCIN DISPERSIONS AND ITS APPLICATION FOR BIOADHESIVE EVALUATION OF PEGYLATED NANOPARTICLES

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Summary. The aim of the present study was to evaluate the possibility for quantitative colorimetric determination of mucin by using Micro BCA reagent. Series of mucin dispersions (concentration range 100-500 μg/ml) were prepared in distilled water, acidic and phosphate buffers. The results obtained in the three media showed good linearity and a possibility for quantitative mucin determination by colorimetry (measurement at 570 nm). Further, the capacity of pegylated poly(anhydride) nanoparticles to interact with mucin was evaluated applying the quantitative determination of mucin fractions adsorbed on the nanoparticles. Different amounts of mucin were adsorbed on the various types of the nanoparticles (modified with PEG and mPEG), which was attributed to the different surface properties of the particles.

Key words: mucin dispersions, nanoparticles, colorimetry, bioadhesion

КОЛОРИМЕТРИЧНО ОПРЕДЕЛЯНЕ НА МУЦИНОВИ ДИСПЕРСИИ И ПРИЛОЖЕНИЕ НА МЕТОДА ЗА ОЦЕНКА НА БИОАДХЕЗИВНИТЕ СВОЙСТВА НА ПЕГИЛИРАНИ НАНОЧАСТИЦИ

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Резюме. Целта на настоящата работа бе да се проучи възможността за количествено колориметрично определяне на муцин чрез използване на Micro BCA реагент. Серии от муцинови дисперсии (концентрации 100-500 μg/ml) бяха приготвени в носител дестилирана вода, солно-кисел или фосфатен буфер. Получените резултати показаха добра линейност и за трите вида дисперсии и очертаха възможността за прилагането на количественото колориметрично определяне на муцин (λ = 570 nm). Капацитетът на пегилирани полианхидридни наночастици да взаимодействат с муцин бе изследван посредством колориметрично количествено определяне на адсорбираните на повърхността на наночастиците муцинови фракции. Резултатите показаха, че наночастиците, модифицирани с различни видове полиетилен гликол (PEG или mPEG), адсорбират различни фракции муцин, което би могло да се обясни с разлика в морфологията на наночастиците.

Ключови думи: муцинови дисперсии, наночастици, колориметрия, биоадхезия

Introduction

Mucin is the main compound in the gel layer covering mucosal epithelial surfaces. Diffusion of the drug delivery systems (e.g., micro- and nanoparticles) through the mucus layer depends on their behaviour in the presence of mucin. Since many routes of nanoparticle administration are associated with the penetration through the mucus (e.g., oral, ocular, nasal), determination of the eventual interactions between mucin and nanoparticles is very important. The interactions could be presupposed on the base of any changes in the physicochemical properties of the particles. Vandervoort et al. have observed changes in the surface charge of poly(lactide-co-glycolide) nanoparticles during their incubation in mucin dispersion [5].

Mucin adsorption on nanoparticle surfaces was determined by quantitative colorimetric method using

periodic acid and Shiff reagent [3]. However, this method requires primary preparation of both reagents including long incubation of the Shiff reagent stock solution. Thus, the aim of the present study was to examine simple colorimetric technique for quantitative determination of mucin in dispersion using Micro BCA reagent. In addition, the interac-tion between pegylated poly(anhydride) nanoparticles and mucin was investigated applying the colorimetric technique.

Materials and methods

Materials

Pig gastric mucin (Type II: crude) was provided by Sigma (St Louis, USA) and Micro BCA Protein Assay Reagent Kit from Pierce (Rockford, USA). Poly(ethylene glycol) (Mw 1000 or 2000) and monomethyl ether of PEG 2000 (mPEG) were supplied by Fluka (Switzerland). Poly(methyl vinyl ether-co-maleic anhydride) (PVM/MA) (Gantrez AN 119; Mw of 200 kDa) was a gift from ISP (Barcelona, Spain).

Preparation of standard mucin dispersions

Mucin was dispersed in different concentrations (concentration range 100-500 μg/ml) in distilled water under stirring (4 h). Then, the dispersions were centrifuged (17 000 rpm, 20 min) and 150 μl of each supernatant was mixed with Micro BCA (150 μl) in a test plate. The plates were incubated for two hours at 37°C. After equilibration, the absorbance of mucin was measured by colorimetry at wavelength of 570 nm. The same procedure was applied when the standard curve was prepared in acidic (0.1N HCl, pH = 1.2) or phosphate buffer (pH = 7.4).

Preparation of poly(anhydride) pegylated nanoparticles

PVM/MA copolymer (100 mg) and 25 mg of poly(ethylene glycol) (PEG 1000, PEG 2000 or mPEG) were dissolved and stirred in acetone (5 ml) for 1 h. After their incubation, 10 ml of a hydroalcoholic mixture (1:1 v/v) was added to the organic phase. The solvents were eliminated under reduced pressure (Buchi R-144, Switzerland). The nanoparticles were purified by twice centrifugation at 17000 rpm for 20 min (Sigma 3K30, Germany) and finally lyophilized (Genesis 12EL, Virtis, USA) using sucrose as cryoprotector (5% w/v).

Characterization of nanoparticles

The nanoparticle size and zeta-potential were determined by photon correlation spectroscopy and electrophoretic laser doppler anemometry using a Zetamaster analyzer (Malvern Instruments, UK). Samples were diluted with 0.05M phosphate buffered saline (pH 7.4) and measured at 25°C.

The quantity of PEG attached to the nanoparticles was calculated by the ratio between peak areas of the protons of ethylene units (3.51 ppm) detected in the spectra of pegylated nanoparticles (5 mg/0.5 ml DMSO) and in the spectra of free PEGs (5 mg/0.5 ml DMSO), respectively.

In vitro interaction between mucin and nanoparticles

The interaction was studied by incubation of mucin and pegylated nanoparticles (1:4 weight ratio) in distilled water. The incubation was carried out under stirring (700 rpm) at 37°C (Variomag, Germany). The dispersions were centrifuged at predetermined time (17 000 rpm, 20 min) and 150 μl of each supernatant was placed in a test plate. Micro BCA Protein Assay Reagent Kit (150 μl) was added to the supernatants, the plate was incubated for two hours at 37°C, and the absorbance of mucin was finally measured by colorimetry (570 nm). The amount of the mucin adsorbed to the nanoparticles was determined as a difference between its initial concentration and the concentration found in the dispersion after incubation and centrifugation.

Results and discussion

The aim of the present study was to develop simple quantitative method for determination of mucin. The colorimetric technique was considered appropriate taking in account the capacity of the amino-groups of mucin to form a color complex with Micro BCA reagent.

The standard series of mucin dispersions were investigated in distilled water, acid and phos-phate buffers. Figure 1 represents the standard curves of mucin in distilled water and in both buffers. As shown, good linearity in a large concentration range was observed. Comparing the curves, the lowest regression coefficient was found for the curve prepared in a phosphate buffer. This fact suggested, that under the experimental conditions, the behavior of mucin was influenced by the presence of phosphate ions and the higher pH value of the buffer. In fact, the mucin solubility could be affected at pH 7.4 due to the ionization of the mucin sialic residues (pKa = 2.6). In all cases, the results in the three media demonstrated the utility of the colorimetric method for quantitative determination of mucin.

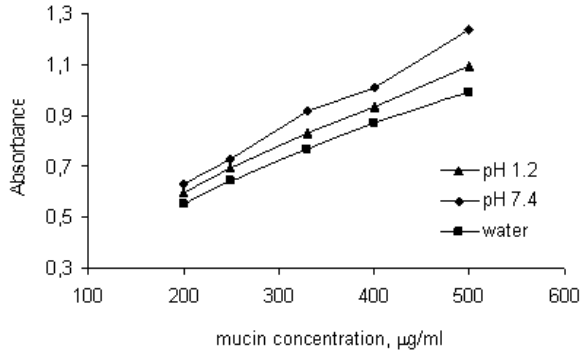

Fig. 1. Standard calibration of mucin in distilled water (r = 0.995), acidic buffer (r = 0.998) and phosphate buffer (r = 0.994)

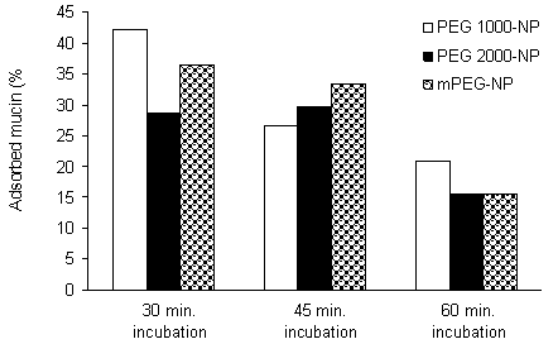

Fig. 2. Incubation of nanoparticle series in aqueous mucin dispersion (4:1 weight ratio) at 37°C

Mucin determination, quantitative or qualitative, is a useful approach when characterization of any bioadhesive drug delivery systems is needed. The interaction between formulation and mucus layer is definitive for the biodistribution and bioavailabilty of the inserted drug. Regarding nanoparticle formulations, previous studies have demonstrated better transport of surface modified nanoparticles through intestinal, ocular and nasal mucosa [1, 6, 7]. In the present study, the capacity of pegylated poly(anhydride) nanoparticles to interact with mucin was investigated by colorimetric determination of the adsorbed mucin fractions. Pegylated nanoparticles were used as model, because they could establish specific bioadhesive interac-tions with gastrointestinal mucosa [2, 4, 8]. The results from the quantitative determination of the mucin fractions adsorbed on the nanoparticle surface are illustrated in Figure 2. As shown, the quantitative determination of mucin allows in vitro estimation of the nanoparticle behavior in the presence of mucin. It is evident that an interaction between nanoparticles and mucin occurred more intensively in the beginning of the study and declined with time. The largest amount of mucin was adsorbed on the surface of nanoparticles modified with PEG 1000, while the lowest was adsorbed on the nanoparticles modified with PEG 2000. The comparison of the physicochemical characteristics of the resulted nanoparticles showed that the size and zeta-potential of the different series were almost similar (Table 1). These results suggested that both the amount of the associated PEG and the conformation of PEG-chains forming the coating layer were important for the different nanoparticle behavior. In addition, the types of PEGs investigated in the study (PEG 1000, PEG 2000 or mPEG 2000) formed probably coating layers with different density and thickness. Hence, the properties of the coating layer influenced the amounts of mucin adsorption and the potential of the nanoparticles for bioadhesive interactions.

Table 1. *Physicochemical properties of poly(methyl vinyl ether-co-maleic anhydride) nanoparticles modified with various poly(ethylene glycols)*

Nanoparticles	Size (nm)	Zeta-potential (mV)	Associated PEG (μg/mg)*
PEG 1000 – NP	271 ± 10	-50.4 ± 2.2	19.8 ± 2.05
PEG 2000 – NP	299 ± 22	-44.1 ± 4.0	30.2 ± 4.0
mPEG 2000 – NP	272 ± 17	-48.6 ± 0.5	36.6 ± 2.9

*The amount of PEG bound to the nanoparticles (μg/mg) was determined by ^{1}H-NMR spec-troscopy (n_s = 6400)

Conclusion

The quantitative colorimetric determination of mucin in dispersions was characterized with simplicity and good linearity. The method allowed determination of mucin fractions adsorbed on nanoparticles during their incubation. The latter could be considered important step in the evaluation of bioadhesive properties of the drug delivery systems, in particular micro- and nanoparticles.

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