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# Preparation and characterization of diazeniumdiolate releasing ethylcellulose films

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**Abstract** A monolayer and trilayer membrane configuration of ethylcellulose were doped with a new synthesized diazeniumdiolate GAGS/NO (glutaraldehyde modification of glucosamine/NO adduct) and DETA/NO as the NO donor species, which can be used for altering the time course of nitric oxide donor release and targeting it to tissues with which the polymers are in physical contact. The NO donor release profiles show that the average release rate of DETA/NO can be controlled from 0.2 to $9 \times 10^{-10}$mol cm$^{-2}$ min$^{-1}$ for at least 7 day and up to 30 day under physiological condition. The average release rate of GAGS/NO is varied from 0.1 to $0.5 \times 10^{-10}$mol cm$^{-2}$ min$^{-1}$ for up to 94 day. The trilayer configuration effectively eliminates the burst release in the initial stage, and notably increases the NO donor release time. The trilayer films of DETA/NO can release 5% of the total NO donors over 69 h. In comparison, the trilayer films of GAGS/NO only release 2.5% of the total NO donors over 69 h. The results suggest that this nitric oxide donor releasing polymer may hold considerable promise for reducing the risk of restenosis following angioplasty and other interventional procedures for vascular repair.

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## 1 Introduction

The success of many intra-arterial medical devices depends upon favorable biocompatibility of the blood contacting surface of such implants. In many cases, the interaction between an artificial surface and blood results in protein deposition, platelet adhesion and activation, and initiation of the intrinsic coagulation cascade [1]. One possible solution to these thrombogenicity problems is the use of hydrophobic polymer materials that slowly release nitric oxide [2]. It is known that NO is produced by endothelial cells, where it serves to prevent platelet adhesion and activation, and to promote vasodilation of the surrounding blood vessels [3, 4]. It is a potentially ideal species for improving the thromboresistivity of polymeric materials intended for in vivo applications. Therefore, significant research has been carried out to developing polymeric materials with improved biocompatibility of medical devices [5].

To create polymers capable of releasing NO, various diazeniumdiolate NO donor molecules can be incorporated into polymer matrices. Smith and co-workers first reported incorporation of X[NONO]-(X is nucleophile) functional group into polymeric matrices that could be used for altering the time course of nitric oxide release and/or targeting it to tissues with which the polymers are in physical contact [6, 7]. They outlined three general structural types for preparing polymers containing the diazeniumdiolate moiety: (1) X[NONO]$^-$ non-covalently distributed throughout the polymeric matrix; (2) diazeniumdiolate groups covalently bound to pendent polymer side-chains; and (3) covalently bound diazeniumdiolate groups directly to the polymer backbone. Since this initial work, the preparation of new NO-releasing polymers and the application of these materials to biomedical devices have been studied. Several groups have embedded discrete diazeniumdiolates into

polymer matrixes and used these polymers in functional devices, such as diethylenetriamine diazeniumdiolate (DETA/NO) was embedded into ethylene-vinyl acetate [8, 9], SPER/NO into a biodegradable polymer [10] and DBHD/NO into polyurethane [11].

In order to prevent leaching of any potentially carcinogenic species from the polymer matrixes, NO-releasing polymers have been synthesized by covalent attachment of the diazeniumdiolate moiety. To this end, diazeniumdiolated polyethylenimines [7], silicone rubbers [12], polymethacrylates [13, 14], poly (vinyl chlorides) [2], polyurethanes [15] and hydrogels [16, 17] have all been synthesized to release NO.

Ethylcellulose (EC) is an inert, hydrophobic polymer and its properties such as minimum toxicity, excellent physiochemical stability and good film formability make it suitable for sustained release matrix. EC is widely used as an effective means for rate control of drug release, and such dosage forms are commonly termed as 'capsule-type controlled release system', 'diffusion-controlled reservoir devices' or membrane moderated controlled release systems [18]. Up to now, there is no paper has focus on the use of EC as a potential carrier for preparation of prolonged release formulations using diazeniumdiolate as a model drug. In this study, two NO donors, which have a prolonged half-life of NO release, GAGS/NO (glutaraldehyde modification of glucosamine/NO adduct) and DETA/NO were used as the model drugs, and EC was used to prepare the rate-controlling membrane to modulate the drug release for dosage form. A monolayer and trilayer membrane configurations doped with this two NO donors were prepared and effects of drug loading on NO donor release were studied.

## 2 Materials and methods

### 2.1 Materials

Ethylcellulose, glucosamine hydrochloride, glutaraldehyde, diethylenetriamine (DETA), Dibutyl phthalate (DBP) were purchased from Sinopharm group chemical reagent Co. Ltd (China). The viscosity grade of ethylcellulose is 100 cp and has an ethoxy content of 48.0–49.5%. Nitric oxide (99%) was purchased from Foshan Kedi Gas chemical industry Co., Ltd (China). All other chemicals and solvents were of analytical reagent grade.

### 2.2 Preparation of DETA/NO

DETA/NO was prepared and characterized as previously described by Hrabie [19]. A solution of diethylenetriamine (5.00 g, 48.5 mmol) in 150 ml of $CH_3CN$ was placed in a high-pressure reactor. Nitrogen was passed through the apparatus and bubbled through the solution for 30 min and then degassed under vacuum. Fresh NO gas was introduced to the compounds at 5 atm for 24 h. After the reaction was completed, the reactor was again flushed with Nitrogen. The product was filtered, washed with the reaction solvent and then with ether, and dried at room temperature under vacuum. It was stored in an airtight container in desiccators at −20°C.

### 2.3 The synthesis of glutaraldehyde modification of glucosamine/NO adducts (GAGS/NO)

20.24 g (0.05 mol) of a 25% (w/w) glutaraldehyde aqueous solution was added to a slurry of glucosamine hydrochloride (10 g, 0.05 mol) in 100 ml of methanol at room temperature under nitrogen. The mixture was stirred for 24 h. A solution of NaBH4 (3.83 g, 0.1012 mol) in 25 ml of water was added dropwised to the mixture and stirred at 60°C for 6 h, the resulting, transparent solution was cooled back to room temperature and the precipitate was removed by filtration. Acetone (30 ml) was added to the filtrate to make a precipitate 8.12 g, and the precipitate was re-dissolved by methanol and filtered. Then, the filtrate is evaporated and dried at room temperature under vacuum. The final product is 7.0 g.

For the synthesis of the GAGS/NO adduct, aliquots (5.0 g, 0.02 mol) of the products was dissolved in the solvent prepared from methanol (100 ml) and sodium methoxide (4.3 g, 0.04 mol) at the molar ratio of $[Na^+]/[NH] = 2$ in the high-pressure reactor. Nitrogen was passed through the apparatus and bubbled through the solution for 30 min and then degassed under vacuum. Fresh NO gas was introduced to the compounds at 5 atm for 3 days. After the reaction was completed, the reactor was again flushed with Nitrogen. The product was filtered, washed with the reaction solvent and then with ether, and dried at room temperature under vacuum. About 6 g of amorphous voluminous white powder was obtained and stored in an airtight container in desiccators at −20°C.

### 2.4 Preparation of films containing dispersed diazeniumdiolates

Single-layer ethylcellulose films containing DETA/NO and GAGS/NO were prepared by solvent evaporation. About 5 g of ethylcellulose was dissolved in 100 ml of toluene under stirring at 50°C, Dibutyl phthalate (DBP) was mixed with the EC solution at 6% (w/w) of the polymer. After the polymer dissolution, 14–114 mg of diazeniumdiolates (5–30 wt% of ethylcellulose) was added into 5 ml of polymer solution. The final solution was stirred for 30 min at room temperature. A volume of 1 ml of each of the polymeric solutions was transferred to the cavity of a glass

plate and allowed to evaporate at room temperature to cast the films. After solvent evaporation, the films were further dried at room temperature under vacuum prior to the analysis.

Ethylcellulose trilayer films were prepared by dividing the ethylcellulose toluene solution into three separate portions and each layer was cast sequentially. Prior to casting the middle layer of doped polymers, 114 mg (30 wt%) of DETA/NO or GAGS/NO was added and a fine dispersion was obtained by sonication. After Solvent evaporation at room temperature, a third ethylcellulose solution was cast onto the second film as a top layer and the solvent was evaporated again. All trilayer films were further dried at room temperature under vacuum prior to the analysis.

### 2.5 NO donor release measurements

NO donor diffusion from the EC films was measured by soaking the films in PBS at 37°C for extended periods of time. Because of the short half-life of NO in aqueous solution and its reaction with oxygen in water to yield nitrite, the total nitrite concentration in the buffer solution was taken to be equivalent to the NO decomposed from NO donor during each time period. One mole of NO donor can release two moles of nitric oxide. The nitrite concentration was determined via the Griess spectrophotometric method [20]. Griess reagent **I** is prepared from a 1% sulfanilamide in 5% phosphoric acid, Griess reagent II is a 0.1% p-naphthylethylenediamine dihydrochloride aqueous solution. The standard curve was generated using 0–100 μM sodium nitrite in PBS solution. About 20 mg EC films were cut from the parent films and measured its area, then dispersed in 50 ml PBS and stirred in a sealed vial at 37°C in a water bath; at appropriate time intervals, 20 ml of the upper layer of the soaking solution was removed and 1 ml of it combined with Griess reagent I(1 ml) and Griess reagent II(1 ml). 20 ml fresh PBS was added to the vial in order to replace the aliquot which has been removed (In order to simulate the dilution to which NO is subjected when released in the blood flow, 20 ml of the solution was removed in order to remarkably change the concentration of solution). Then, the 3 ml of solution was incubated for 15 min at room temperature, protected from light. A purple/magenta color forms immediately. The maximum absorbance was read at 540 nm on a UV/Vis spectrophotometer. The total amount of drug release was calculated as follows:

$$T_1 = C_1 \times 0.05$$

$$T_2 = C_2 \times 0.05 + C_1 \times 0.02$$

$$T_3 = C_3 \times 0.05 + C_2 \times 0.02 + C_1 \times 0.02$$

$$T_4 = C_4 \times 0.05 + C_3 \times 0.02 + C_2 \times 0.02 + C_1 \times 0.02$$

……where T is the total amount of drug release; C is the concentration of drug during each time period.

### 2.6 FT-IR spectroscopy

FT-IR spectra of the samples were obtained with a Perkin-Elmer 1600× spectrometer with a resolution of 4 $cm^{-1}$ and 32 accumulations. KBr pellets were prepared in the usually way by using about 1 mg of the sample and 100 mg of KBr. Spectral scanning was done in the range between 4000 and 500 $cm^{-1}$.

## 3 Results and discussion

### 3.1 GAGS/NO synthesis and characterization

Diazeniumdiolate, i.e. compound containing the anionic $[N(O)NO]^-$ functional group, is one of the most important groups of NO donors, which is typically synthesized by the reaction of nucleophile (secondary amines) with NO at elevated pressure [21, 22]. They spontaneously generate NO under physiological conditions, which results in a wide variety of NO release rates and physical forms. However, some problems such as the toxicity and biocompatibility of the nucleophile are usually associated with these types of NO donors. Some diazeniumdiolate carriers such as diethylenetriamine are found to be harmful for the body, and free polyethylenimine has been found to induce widespread cell death [23, 24]. Therefore it is important to seek for new materials as diazeniumdiolate carriers, which can spontaneously degrade into harmless byproducts after releasing the NO.

Glucosamine, 2-amino-2-deoxy-D-glucose, is an amino monosaccharide that is an essential component of mucopolysaccharides and chitin. Glycosaminoglycans are large complexes of negatively-charged carbohydrate chains that are incorporated into mucous secretions, connective tissue, skin, tendons, ligaments and cartilage. Glucosamine supplements are widely used to relieve arthritic complaints [25]. The primary amine functional group on glucosamine can be modified to form Glucosamine derivatives bearing secondary amine. These secondary amines are available for further modification by nitric oxide to form NO donor compounds. In this study, glutaraldehyde modification of glucosamine/NO adduct was synthesized and its chemical structure and NO release property was investigated.

Figure 1 is the FT-IR spectra of glutaraldehyde modification of glucosamine (GAGS). A new strong band appeared at 2937 $cm^{-1}$ and 2878 $cm^{-1}$, which is assigned to the symmetric and asymmetric stretching of $CH_2$ of the long alkyl chain $(CH_2)_5$ of GAGS. 1615 and 1537 $cm^{-1}$ as

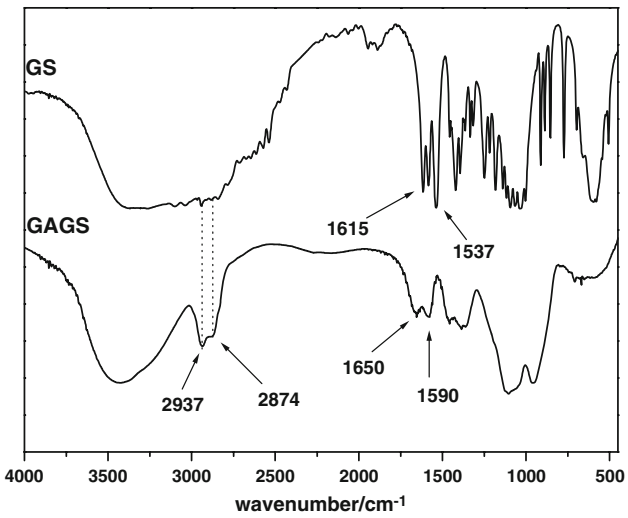



**Fig. 1** FT-IR spectra of GAGS and GS

the characteristic bands of $NH_3^+$ of glucosamine hydrochloride shift to 1650 and 1590 $cm^{-1}$ suggestive of NH deformation. It is indicated that secondary amine NH is formed after glucosamine react with glutaraldehyde.

There are two possible chemical structures of GAGS, as shown in Fig. 2. The elemental analysis indicates that the synthesized products are composed of 49.26% C, 7.92% H and 5.24% N, which is in agreement with the calculated results of 49.81% C, 8.68% H and 5.28% N. It is demonstrated that the molecular structure of the synthesized product is form A.

The GAGS/NO adduct was prepared according to the methods as previously described by Smith et al. [26]. The procedure for NO reacting with GAGS is shown in Scheme 1. The UV spectrum is the best method for characterization of N-diazeniumdiolates. Typical UV spectra of diazeniumdiolates measured in basic solution show an absorption maximum at 250 nm with molar extinction coefficients in the range from 7.2 to 9.4 $Mm^{-1}$ [27]. The UV spectra of GAGS/NO adduct were performed in 0.2 M phosphate buffer saline (PBS) solution (pH7.4) and the result is shown in Fig. 3. A characteristic absorption at 262 nm indicates the existence of the $[NONO]^-$ group. The absorption intensity at 262 nm decreases with time in PBS solution, the characteristic absorptions of nitrite at 210 nm and 329 nm increases simultaneously. It is in agreement with the property of diazeniumdiolate, i.e. this NO adduct decomposes to regenerate its precursors (nucleophile and NO) and NO reacts with oxygen in water to yield nitrite [28]. From the UV release data shown in Fig. 4, regression analysis of GAGS/NO suggests that NO release plot obeys the first order kinetics with a calculated half-life ($t_{1/2}$) of 46 h. This new NO donor exhibits a lower release rate and a longer half-life compare with other diazeniymdiolate ions, except DETA/NO ($t_{1/2}$ = 20 h, 37°C; $t_{1/2}$ = 56 h,

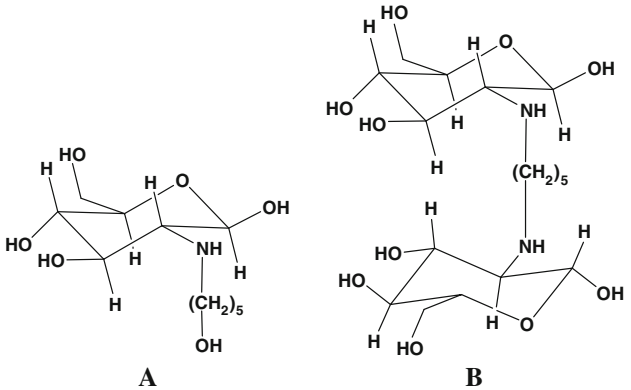



**Fig. 2** Two possible structures of glutaraldehyde modification of glucosamine

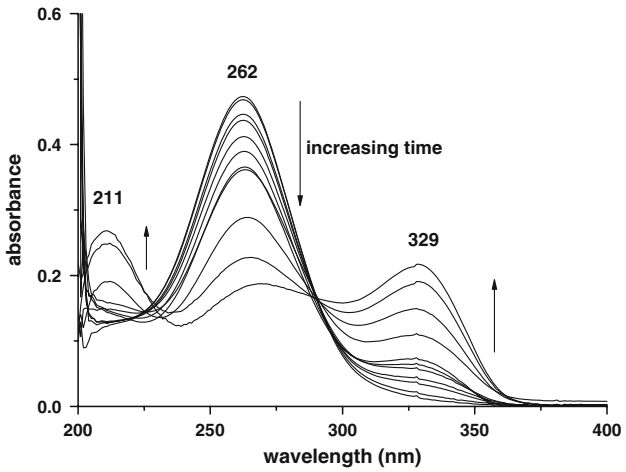
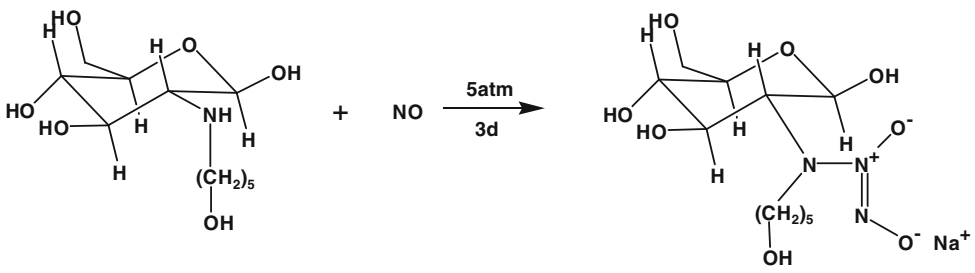



**Fig. 3** UV spectra of GAGS/NO in PBS (pH7.4) at 20°C

**Scheme 1** The procedure for NO reacting with GAGS



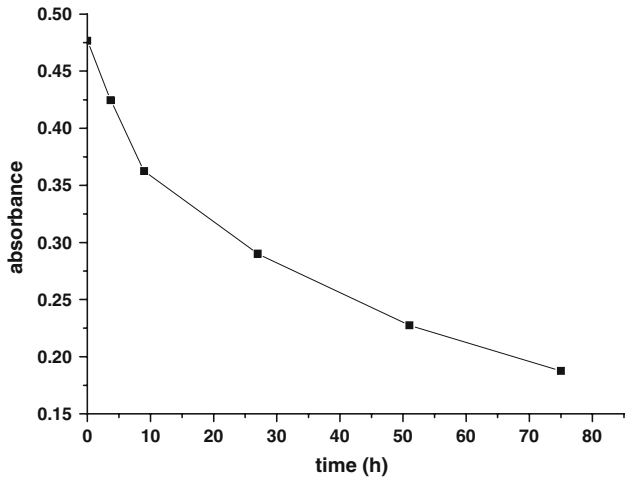



**Fig. 4** UV release data for GAGS/NO in PBS (pH7.4) at 20°C

22°C), which is known as the longest half-life of all ionic diazeniumdiolates studied to date [19, 28].

### 3.2 NO donor release study of EC films

Ethylcellulose as a water-insoluble polymer can dissolve in many organic solvents including ethanol, acetone, isopropanol, benzene and alkyl halide. One of important factors affecting the films properties is the type and amount of solvent used. In our study, several types of solvents were used to prepare EC films, and the results indicated that high boiling point solvents such as toluene and Butanol have been shown to increase the viscosity of EC solution and formed a transparent and uniform EC films, whereas the low boiling point solvents such as alcohol, acetone and isopropanol have been shown to reduce the viscosity of EC solution and formed a opaque brittle film. Therefore, toluene or binary solvent system (toluene and alcohol) is used to prepare EC films in our experiments.

Controlled release may be defined as the process by which one or more active agents or ingredients are made available at a desired site and time and at a specific rate. The interactions between dissolution media, polymer and drug are the primary factors in release control, various formulation variables influence drug release rate to a greater or lesser extent [29]. In this paper, the effects of drug loading on the NO donor release properties were preliminary investigated.

The NO donor release profiles of DETA/NO doped single layer EC films are shown in Fig. 5. DETA/NO has an aqueous solution half-life of 20 h at 37°C and is insoluble in organic solvents. The films are opaque and have many pits or craters following incubation in aqueous solution. Mowery et al. [2] have studied the mechanism of NO generation from the MAHMA/NO-doped hydrophobic polymer films and he argued that the majority of the donor diffuses from the polymer before decomposing to produce the biologically active NO. It can be seen from Fig. 5, the kinetics curves of NO donor diffusion are in agreement with Mowery's study. The drug on the surface of films rapidly dissolved and diffused into the aqueous solution, which contributed to a burst release in the first 2 h of incubation. It can also be seen from Table 1, with increasing of drug concentration, the burst drug release increased from 5% to 22%, indicating that the increasing of water-soluble drug concentration promote water molecules diffusing into the EC films. The drug release rates remain approximately constant at the slower release period. The average NO donor release rates correspond to the slope of the kinetics curves at the slower sustained release. With increasing of drug concentration (from 5% to 30%), the average NO donor release rate over 34 h increased from

**Table 1** NO donor release properties of DETA/NO doped single layer EC films

| DETA/NO concentration (%) | The burst drug release/Total NO donor (%) | Average NO donor release rate ($\times 10^{-10}$mol cm$^{-2}$ min$^{-1}$) | Release time (d) |
|---|---|---|---|
| 5 | 5 | 0.2 | 30 |
| 10 | 6 | 0.7 | 29 |
| 20 | 10 | 2 | 21 |
| 30 | 22 | 9 | 7 |

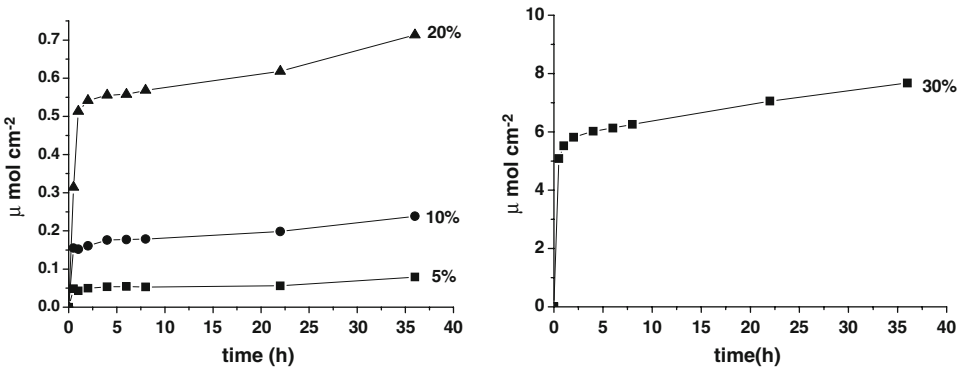



**Fig. 5** NO donor release profiles of different DETA/NO concentration of EC films in PBS at 37°C (pH7.4, DBP 6%)

0.2 to 9 $\times$ $10^{-10}$ mol $cm^{-2}$ $min^{-1}$. Rapid release occurred over the first 2 h followed by much slower, sustained release for at least 7 day and up to 30 day at physiological condition.

The NO donor release profiles of GAGS/NO doped single layer EC films are illustrated in Fig. 6. It is similar as the release profiles of DETA/NO. The NO donor release curves contained two stages. Rapid release occurred over the first hour followed by much slower, sustained release. With increasing of drug concentration (from 10% to 30%), the burst release of drug increased from 13% to 22%, the average release rates over 68 h increased from 0.1 to 0.5 $\times$ $10^{-10}$ mol $cm^{-2}$ $min^{-1}$ (Table 2). Compared with the release of DETA/NO, the amount of burst release increased apparently for lower drug loading, whereas the average drug release rate decreased prominently for higher drug loading in particular. Glucosamine has a similar glucose unit structure as ethylcellulose, and GAGS/NO has a long alkyl chain $(CH_2)_5$ can form a hydrophobic interacting with EC molecular chains. Therefore, a fine dispersion of GAGS/NO was obtained in EC solution. The water-soluble drugs on the surface of EC films result in an increase of burst release. After the rapid stage of drug release followed a slower period of release, the process of intact GAGS/NO molecules diffuse out of the EC film was inhibited by the strong inter-molecular interaction between ethylcellulose and GAGS/NO, such as hydrogen-bonding interaction of glucose unit and hydrophobic interaction between ethyl and amyl groups. This strong inter-molecular interaction contributes to a much longer sustained release time for at least 44 day and up to 94 day at physiological condition.

In order to eliminate the burst release of single-layer films, NO donor was dispersed in a trilayer configuration such that the NO donor was confined to a middle layer, shielded from direct exposure to the surrounding water. The NO release profiles of GAGS/NO and DETA/NO doped trilayer polymer films are shown in Fig. 7. As indicated, the trilayer configuration effectively eliminated the burst release in the initial stage. The release curve of DETA/NO obeys the first order kinetics and the release time of drug is greatly extended. The release kinetics of GAGS/NO is different and shows a lower and nearly constant drug release rate in the second release stage. The total release of DETA/NO is 5% of total drug loading over 69 h, whereas the GAGS/NO release is only 2.5% over 69 h, it is capable of releasing NO for several months. It is clear that the sustained release property of GAGS/NO in EC films is better than that of DETA/NO.

The release doses are therapeutically-significant, especially in vascular applications. Pro-inflammatory effects of NO include vasodilation, edema, cytotoxicity, and the mediation of cytokine-dependent processes that can lead to tissue destruction. Conversely, the production of NO by endothelial cells may serve a protective, or antiinflammatory, function by preventing the adhesion and release of oxidants by activated neutrophils in the microvasculature. Appreciation of this "double-edged sword" aspect of NO plays a pivotal role to any attempts at therapeutic NO modulation. Our results indicate that the average NO donor

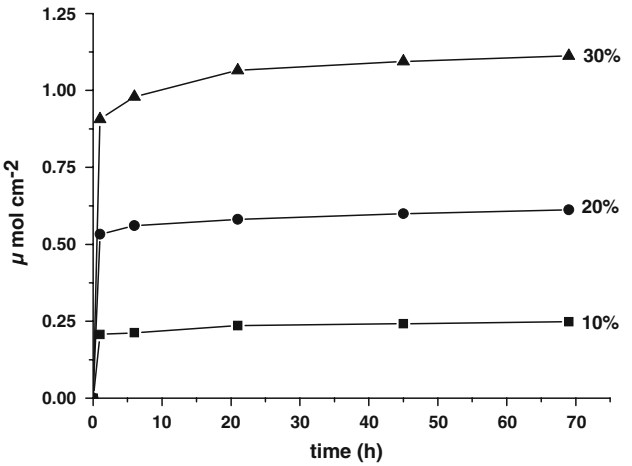



**Fig. 6** NO donor release profiles of different GSGA/NO concentration of EC films in PBS at 37°C (pH7.4, DBP 6%)

**Table 2** NO donor release properties of GAGS/NO doped single layer EC films

| GAGS/NO concentration (%) | The burst drug release/Total NO donor (%) | Average NO donor release rate ($\times 10^{-10}$mol $cm^{-2}$ $min^{-1}$) | Release time (d) |
|---|---|---|---|
| 10 | 13 | 0.1 | 94 |
| 20 | 18 | 0.2 | 85 |
| 30 | 22 | 0.5 | 44 |

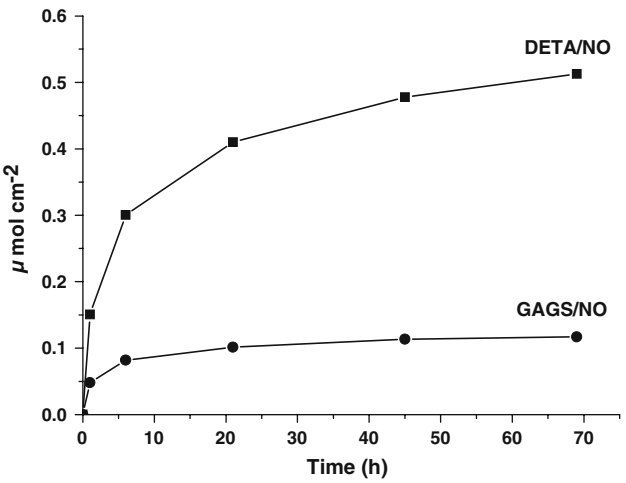



**Fig. 7** NO donor release profiles of NO donors doped trilayer films in PBS (pH7.4) at 37°C. (NO donor 30%, DBP 6%)

release rate is varied from 0.1 to $9 \times 10^{-10}$ mol $cm^{-2}$ $min^{-1}$, which is approximately to magnitude of the NO-flux from normal and stimulated endothelial cell ($1 \times 10^{-10}$mol $cm^{-2}$ $min^{-1}$) [30]. These results suggest that this nitric oxide donor-releasing polymer may hold considerable promise for reducing the risk of restenosis following angioplasty and other interventional procedures for vascular repair.

## 4 Conclusions

This study suggests that water-soluble diazeniumdiolated NO donor molecules can be incorporated into hydrophobic ethyl cellulose matrix and create polymers films capable of sustained releasing NO. The release of drugs from these ethyl cellulose films was a function of the drug/polymer ratio and chemical structure of NO donors. The release profiles of single layer EC films containing DETA/NO and GAGS/NO suggest that the release of NO donors with nearly zero-order kinetics after an initial burst release. The average release rate of DETA/NO can be controlled and maintained at various levels (from 0.2 to $9 \times 10^{-10}$ mol $cm^{-2}$ $min^{-1}$) for at least 7 day and up to 30 day at physiological condition; the average release rate of GAGS/NO varied from 0.1 to $0.5 \times 10^{-10}$ mol $cm^{-2}$ $min^{-1}$ for up to 94 day. The trilayer configuration effectively eliminates the burst release in the initial stage, the release curve of DETA/NO obeys first order kinetics and the release time is greatly extended. After this preliminary study, further work is in progress to evaluate the effect of different approaches for incorporating diazeniumdiolated species within EC matrices on the drug release properties.

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