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Pulse radiolysis and spectrophotometric studies on the binding of organic cations with heparin

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HIGHLIGHTS

- We present the spectroscopic and radiolytic study of heparin–cations interactions.
- The same formalism can be applied to absorption and rate constants changes.
- The number of binding sites and the equilibrium constants were determined.

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ABSTRACT

Here we present the spectroscopic and pulse radiolysis studies of the interactions of heparin and some organic cations:methylene blue (**MB**), 1-methylnicotinamide (**MNA**$^+$), and its dimer 1,3-bis(1-methylnicotinamide)propane (**bis(MNA**$^+$**)**).

The interaction between heparin and some cationic dyes (e.g. methylene blue) in aqueous solution leads to the changes in the absorption spectra of those dyes. This effect, called metachromasia, has been successfully used to study the interactions of a number of cationic dyes with heparin and other glycosaminoglycans.

Using methylene blue, as a model compound, we have shown that the changes in the rate constant for the reaction of solvated electrons with cationic dye in the presence of heparin (pulse radiolysis) are well correlated with the accompanying changes in the absorption spectra (metachromasia). The same mathematical formalism was applied to absorption and rate constants changes. Using such formalism, we have determined the total number of binding sites per heparin molecule and the equilibrium constants for **MNA**$^+$ and **bis(MNA**$^+$**)**.

1. Introduction

Heparin (**Hep**), a linear polyanionic polysaccharide consisted mainly of trisulfated disaccharide repeating units, is the most negative charge dense natural polymer. Since 1930s, it is extensively used clinically, as an effective anticoagulant (Rabenstein, 2002; Rabenstein et al., 1992). As a result of its anticoagulant activity and its clinical importance, there is a great interest in the development of appropriate heparin sensors, that can be used for the determination of heparin content in the serum. Due to negative charge of heparin the mechanism of probes action is usually based on the interaction of heparin with organic cations. Over the past few decades the interaction of organic cationic dyes with heparin has been extensively studied, and some of such dyes have been proposed as a suitable probes for the detection and quantitation of heparin, even in such highly competitive media, as serum (Szelke et al., 2010; Wang and Chang, 2008; Bromfield., 2013). The interactions between anionic polymers (e.g. heparin and other glycosaminoglycans, **GAG's**) with some cationic dyes (e.g. methylene blue, **MB**) in aqueous solution lead to the changes in the absorption spectra of those dyes. This effect, called metachromasia, has been successfully used to study the interactions of a number of cationic dyes with heparin and other **GAG's** (Zhou et al., 2002; Chen et al., 2005; Zhang et al., 2004). Jiao and co-workers have proposed simple mathematical formalism for the description of the equilibria in polyanion-metachromatic dye system (Jiao and Liu, 1999; Jiao et al., 1999). However, this method cannot be used to study the heparin binding of organic cations without metachromatic properties.

Abbreviations: **bis(MNA**$^+$**)**, 1,3-bis(1-methylnicotinamide)propane; **$(CH_3)_3COH$**, *tert*-butanol; **COX-2**, cyclooxygenase 2; e_{aq}, solvated electron; **GAG's**, glycosaminoglycans; **H**$^\bullet$, hydrogen atom; **H_2O_2**, hydrogen peroxide; **Hep**, heparin; **KSCN**, potassium thiocyanate; **MB**, methylene blue; **MNA**$^+$, 1-methylnicotinamide; **N_2O**, dinitrogen monoxide; **$^\bullet$OH**, hydroxyl radical; **PGI_2**, prostacyclin

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In the past the pulse radiolysis technique has been also used to study the interactions of cations with heparin and other anionic polymers. The method was based on the measurements of the rate constant for the reaction of solvated electrons with organic cations in the presence and absence of polyanions (Zielonka et al., 2006; Balazs et al., 1968c, 1968d, 1968a; Davies et al., 1969; Moore et al., 1970; Jooyahdeh et al., 1974; Edwards et al., 1978).

Here we present the spectroscopic and pulse radiolysis studies of the interactions of heparin and some organic cations. Using methylene blue we show that the changes in the rate constant for the reaction of solvated electrons with cationic dye in the absence and presence of heparin are correlated with the accompanying changes in the absorption spectra. In fact, the same mathematical formalism can be applied to absorption and rate constants changes. Using such formalism, we have determined the values of equilibrium constant for the interaction of heparin with methylene blue, 1-methylnicotinamide (**MNA**$^+$) and its dimer 1,3-bis(1-methylnicotinamido)propan (**bis(MNA**$^+$**)**) (Fig. 1). The total number of binding sites per heparin molecule and the equilibrium constants have been determined for the studied cations.

Methylene blue (**MB**) was chosen as the reference compound for studies of the interactions of heparin as a model anionic polymer and organic cations since the metachromatic effect of this dye is well recognized and documented in the literature (Jiao and Liu, 1999; Jiao and Liu, 1999). Methylene blue—heparin system was also investigated by pulse radiolysis (Balazs et al., 1968c; Davies et al., 1969; Jooyahdeh et al., 1974; Moore et al., 1970; Balazs et al., 1968d, 1968a). The data analysis procedure tested with **MB** was then used for 1-methylnicotinamide, one of the major primary metabolites of nicotinamide. Over the years it has been regarded as an inactive biomarker of niacin status. However, at present, it is known that **MNA**$^+$ has antiinflammatory and antithrombotic activities (Gebicki et al., 2003; Sikora et al., 2008). The mechanism of **MNA**$^+$ action is dependent on endothelial COX-2/PGI_2 pathway (Chlopicki et al., 2007). It is likely that the ability of **MNA**$^+$ to modulate some endothelial functions is related to interaction of 1-methylnicotinamide with glycosaminoglycans. Our interest to study the interactions between **MNA**$^+$ and **GAG's** comes from the fact, that these interactions could play a significant role in mechanism of pharmacological activity of 1-methylnicotinamide and related compounds. It is likely that interaction of **MNA**$^+$ with **GAG's** increases the local concentration of **MNA**$^+$ and **GAG's** could be mediators of **MNA**$^+$ activity. The interactions of **MNA**$^+$ with **GAG's** can increase the shear forces and stimulate endothelium secretory functions, for example resulted in the increased production of endogenous prostacyclin. In this context structural modifications of **MNA**$^+$ by coupling two **MNA**$^+$ moieties could significantly change the interactions with **GAG's** and affect the pharmacological potential of such bicationic **MNA**$^+$ derivative. This can be helpful in understanding of a role of such interactions in **MNA**$^+$ biological activity.

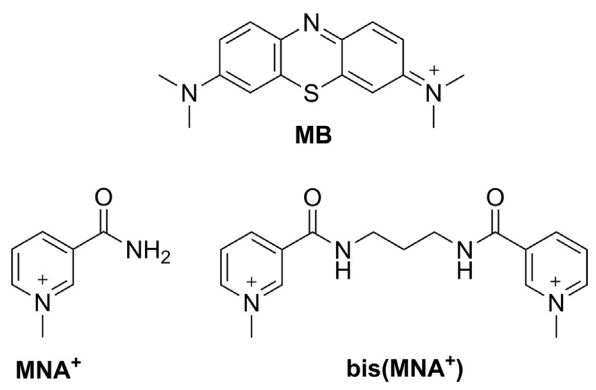

Fig. 1. Chemical structures of methylene blue (**MB**), 1-methylnicotinamide (**MNA**$^+$), 1,3-bis(1-methylnicotinamide)propane (**bis(MNA**$^+$**)**).

2. Experimental

2.1. Apparatus

The spectrophotometric study was carried out with Varian Cary 300 UV/Vis spectrophotometer and quartz cuvettes (1 cm path length). ELU-6 linear accelerator was used to generate high energy (6 MeV) 3 ns electron pulses. The dose absorbed per pulse (5–7 Gy) was determined with an aqueous solution of KSCN (0.01 M), N_2O-saturated to convert the hydrated electrons (e_{aq}) into hydroxyl radicals and remove oxygen. More specific information about the pulse radiolysis system and radiolysis of aqueous solutions is given elsewhere (Buxton et al., 1988; Karolczak et al., 1992). Water used in all experiments was purified by Millipore Milli-Q Integral 10 system.

2.2. Reagents

Heparin sodium salt from porcine intestinal mucosa was purchased from Sigma-Aldrich, and its molecular weight ranges from 17 to 19 kDa. The average molecular mass was assumed to be 18 kDa. Methylene blue (**MB**) was purchased from Sigma-Aldrich. The chloride salt of **MNA**$^+$ was obtained from IFOTAM (Poland).

2.2.1. Preparation of 1,3-bis(1-methylnicotinamide)propane, dichloride

A mixture of ethyl nicotinate (20 mmol) and 1,3-propylenediamine (9.5 mmol) was placed in a thick-wall glass ampule. The ampule was sealed and heated at 110 °C for 80 h. After cooling the reaction mixture was concentrated *in vacuo* (0.1 mbar) and crystalized from acetone-methanol to give 7.8 mmol (83%) of 1,3-bis (nicotinamide)propane as colorless prisms, m.p. 163–164 °C. This compound was then allowed to react with methyl iodide (11 mmol) in methanol (25 ml) solution. After 2 days at room temperature the solvent was removed on a rotary evaporator and the residue was crystalized from acetone. The resulting 1,3-bis(1-methylnicotinamide)propane diiodide salt was converted to dichloride salt by shaking its water solution with freshly precipitated silver chloride (12 mmol) for 8 h at room temperature. Silver salts were filtered off and the filtrate was concentrated on a rotary evaporator and dried under reduced pressure. The residue was crystalized from acetone-methanol (12:1, v/v) to give 1,3-bis (1-methylnicotinamide)propane, dichloride (7.3 mmol; 75%). Colorless crystals, m.p. 203 °C (decomposition).

^{1}H NMR (Bruker 250 MHz, D_2O), δ ppm: 1.99–2.06 (m, 2H, CH_2), 3.57 (t, 4H, J=7 Hz, CH_2), 4.49 (s, 6H, CH_3), 8.86 (d, J=8 Hz, 2H), 8.97 (d, J=8 Hz, 2H), 9.24 (s, 2H).

2.3. Methods

The pulse radiolysis of water or dilute aqueous solutions causes ionization of water molecules and the formation of three highly reactive species: the hydrated electron (e_{aq}), hydroxyl radical ($^{\bullet}$OH) and hydrogen atom ($^{\bullet}$H) (Reaction (1)). These products recombine with each other to produce less reactive H_2O_2 and H_2.

$$H_2O \rightarrow e_{aq} + {}^{\bullet}OH + H^{\bullet} + H_2 + H_2O_2 \quad (1)$$

Preparation of samples for radiolytic studies. The series of samples with constant concentration of methylene blue and changing concentration of heparin was prepared. The aqueous solutions contained: **MB** (15 μM), **Hep** (0–30 μM), *tert*-butanol (1 M). The addition of *tert*-butanol in all samples at final