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Release of DNA from cryogel PVA-DNA membranes

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Abstract. Poly(vinyl alcohol) (PVA) hydrogels have been used for numerous biomedical and pharmaceutical applications, as a consequence of their non-toxic, non-carcinogenic and bioadhesive properties. In this communication the effect of different factors, such as type of electrolyte, ionic strength, temperature (ranging from 20 to 40°C) and cationic surfactants on the distribution coefficients (α) and release rate constants (k_R) of deoxyribonucleic acid (DNA) from PVA-DNA blend gel matrices (of a sheet shape), will be presented and discussed. The release kinetic constant and the distribution coefficient of DNA are quite sensitive to the surrounding matrix media (e.g., k_R ranges from $1.5\cdot10^{-8}$ to $4.7\cdot10^{-7}$ s^{-1}). The analysis of the temperature dependence on k_R shows that the activation energy for the DNA desorption to an aqueous solution is equal to 21.2 kJ/mol. These results constitute a step forward towards the design of controlled DNA release PVA-based devices.

***Keywords:** polymer gels, polymers membranes, poly(vinyl alcohol), deoxyribonucleic acid, release kinetics*

1. Introduction

Hydrogels are polymeric materials with a three-dimensional network structure that can imbibe water, buffered or physiological solutions. Hydrogels show high water content, soft and rubbery consistency and low interfacial tension with water or biological fluids [1]. The ability of molecules of different size to diffuse into (drug loading), and out of (drug release) hydrogels, allows the use of hydrogels as delivery systems [2, 3].

In recent years, there has been considerable work performed in the development of cross-linked polymeric networks which are sensitive to their surrounding physiological environment and therefore will be desirable systems for site-specific drug-delivery [4–9].

Poly(vinyl alcohol) (PVA) is commonly used as a component to form hydrogels. PVA and its copolymers have been widely employed in controlled drug release systems [10]. PVA is hydrophilic and easily swells upon hydration [11]. Furthermore, PVA is non-toxic, non-carcinogenic, shows bioadhesive characteristics and is easily processed [12]. These properties make it ideal for biomedical uses, especially in drug delivery systems. PVA hydrogels can be cross-linked through the use of bifunctional agents, by using electron beam or γ-radiation, or by a 'freezing-thawing' process [13]. The later process addresses toxicity issues; furthermore, these physically cross-linked materials also exhibit higher mechanical strength and elasticity than PVA gels prepared by other methods [13]. Characterization and properties of the so-called PVA-based cryogels have been summarized by V. I. Lozinsky [14, 15].

Recently we have reported the encapsulation of deoxyribonucleic acid (DNA) into PVA hydrogels, obtained by a technique of repeated freezing and thawing [16]. The obtained cryogels were chemically and physically characterized, and show a good mechanical resistance and a white and opaque

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appearance due to a heterogeneous porous structure. Furthermore, the encapsulated DNA molecules can be compacted or extended in the PVA matrix by tailoring the crystallinity degree of the PVA network.

In this paper the effect of different factors, such as electrolytes, ionic strength, cationic surfactants and temperature (ranging from 25 to 50°C) on the distribution coefficients (α) and release rate constants (k_R) of DNA from PVA-DNA blend gel matrices (of a sheet shape), will be presented and discussed. The kinetics of release will be evaluated by a reversible first-order kinetic law equation, developed by Reis *et al.* [17], based on the assumption that the release of a solute from a hydrogel is treated as a partition phenomenon.

2. Experimental

2.1. Reagents and materials

Poly(vinyl alcohol) (PVA) (molecular weight (M_w) 72 000, degree of polymerization ~1600, degree of hydrolysis 97.5–99.5 mol%) was supplied from Fluka (Steinheim, Germany). The sodium salt of deoxyribonucleic acid (DNA) from salmon testes of an average degree of polymerization of about 2000 base pairs was purchased from Sigma (Steinheim, Germany) and used as received. Sodium bromide, NaBr, (Merck, Darmstadt, Germany), sodium iodide, NaI (Merck, Darmstadt, Germany), sodium nitrate, $NaNO_3$ (Riedel-de Haën, Seelze, Germany), and sodium chloride, NaCl (Riedel-de Haën, Seelze, Germany) all of *pro analysis* grade, were used as received.

The fluorescence dye *N*,*N*,*N′*,*N′*-tetramethylacridine-3,6-diamine (acridine orange (AO)) was purchased from Molecular Probes (Invitrogen, Eugene, OR, USA).

These reagents were used without further purification. All solutions were prepared using Millipore-Q water.

2.2. Surfactant synthesis

The 1,12-dibromododecane (Aldrich, Steinheim, Germany), (10 mmol) was dissolved in 20–25 ml dry ethanol and 200 mmol of amine (triethylamine or an ethanolic solution of trimethylamine, 31–35%) were added. The reaction mixture was refluxed until the alkylbromide was consumed, usually 48 h, as monitored by TLC. The solvent and the excess amine were evaporated under reduced pressure and the residue was crystallized several times from the appropriate solvent or solvent mixtures.

Dodecane-1,12-bis(trimethylammonium bromide), $C_{12}Me_6$: Crystallized in ethanol, 93% yield.

Dodecane-1,12-bis(triethylammonium bromide), $C_{12}Et_6$: Crystallized in CH_2Cl_2/ethyl acetate, 85% yield.

2.3. Preparation of PVA-DNA gel matrices

A PVA solution of 14 wt% concentration was prepared by dissolving the appropriate amount of PVA into distilled water at 80°C under continuous stirring for three hours. An accurate amount of DNA (ca. 1% w/w), using a Scaltec SBC22 (Göttingen, Germany) balance with a resolution of ±0.01 mg, was added to 1 g of PVA solution, at room temperature, and mixed, under continuously stirring, during 4 hours. After that, the solution was casted into cylinder flasks and submitted to freezing for 12 hours at –20°C and, after that, thawed for 12 hours at +25°C. The cycles of freezing and thawing were repeated three times. After that, the blend gel membrane, of 2.20(±0.04) mm thickness – measured by a digimatic micrometer Mitutoyo (Kawasaki, Japan) with a resolution of 0.001 mm – shows a good mechanical resistance and a white and opaque appearance.

2.4. Desorption kinetics of DNA

DNA desorption kinetics were performed by immersing a PVA-DNA gel membrane sample (as it was synthesised) in 100 ml of liquid (water, salt or surfactant solution). Experiments have been carried out at 20°C. The effect of temperature on the release kinetics of DNA has been studied in the 20 to 40°C temperature range. In all experiments, temperature was kept constant by using a thermostatic bath Multistirrer 6 from Velp Scientifica (Milan, Italy). During DNA release experiments gel-containing solutions were stirred at ca. 220 rpm. At defined intervals, aliquots of the supernatant were collected. The amount of substance of DNA released from polymeric matrices, $n_{R,t}$, to the supernatant solution was determined by UV-vis spectrophotometry, by measuring the absorbance at 260 nm with a Jasco V-530 (Essex, UK) spectrophotome-

ter, and using the extinction coefficient of 6600 $M^{-1}\cdot cm^{-1}$ [18, 19].

2.5. Fluorescence microscopy

Freshly prepared membranes or supernatant solutions derived from the DNA release studies were stained with *N*,*N*,*N′*,*N′*-tetramethylacridine-3,6-diamine (acridine orange, AO), a nucleic acid selective fluorescent cationic dye, used to confirm the presence of DNA in the membranes. In addition, using AO, information about the secondary structure of the nucleic acid in the membranes has been obtained [20].

Stained samples were immediately examined with an Olympus BX51M (Hamburg, Germany) microscope equipped with a UV-mercury lamp (100W Ushio Olympus) and a filter set type MNIBA3 (470–495 nm excitation and 505 nm dichromatic mirror). The PVA-DNA membranes were observed using an Olympus 4×/0.10 objective lens (∞/–/FN22); supernatant solutions were observed using an Olympus 100×/1.30 oil-immersed objective lens (Hamburg, Germany).

Images were digitized on a computer through a video camera (Olympus digital camera DP70) and were analyzed with an image processor (Olympus DP Controller 2.1.1.176, Olympus DP Manager 2.1.1.158 – Olympus, Hamburg, Germany). All observations were carried out at 20°C.

Figure 1 shows modification on the surface morphology of PVA gel matrices in the absence and presence of DNA. It is possible to observe that DNA is distributed throughout all matrix, confirming the effectiveness of the mixing method.

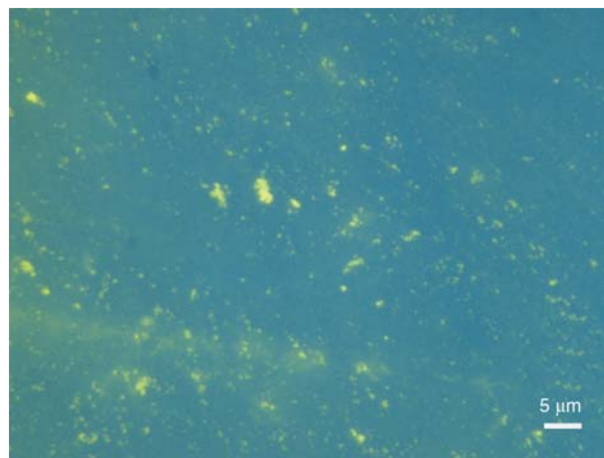

Figure 2. Fluorescence microscopy photograph of an aqueous supernatant solution of water in contact with PVA-DNA gel membrane

The secondary structure of DNA desorbed from PVA-DNA gel was also checked by fluorescence microscopy. Based on the observation of green or red fluorescence, acridine orange has been used to differentiate native, double-stranded DNA from denatured, single-stranded DNA [20]. Although the size of the observed objects suggests that the delivery of DNA is in the form of aggregates, no evidences of denaturation of DNA have been found (Figure 2).

3. Mathematical model

The quantification of the release kinetics of DNA has been done taking into account the initial and border conditions of a non-steady state diffusion transport occurring in a stirred solution of limited volume [21]. Recently, Reis *et al.* [17] developed a set of equations to model the release of dyes from

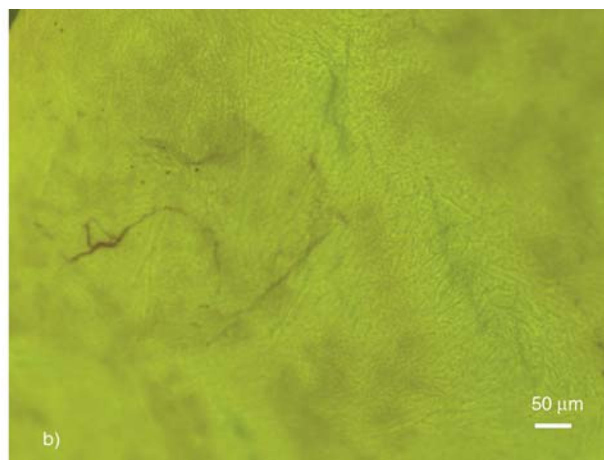

Figure 1. Fluorescence microscopy images for PVA blend matrix in the absence (a) and presence (b) of DNA

poly(*N*-isopropylacrylamide)–polyacrylamide taking into account the previous mentioned conditions, and treating the release of a solute from the gel as a partition phenomena, where the partitioning of such solute occurs between a solvent (or solution) phase and the hydrogel.

This behaviour can be quantified through the distribution coefficient, α, which characterizes the physical chemical affinity of the solute for both phases (Equation (1)):

$$\alpha = \frac{F_{R,\max}}{1 - F_{R,\max}} \qquad (1)$$

where $F_{R,\max}$ is the maximum fraction of the released solute, and F_R is given by Equation (2):

$$F_R = \frac{C_{R,t}}{C_0} \qquad (2)$$

where $C_{R,t}$ is the concentration of the solute released, at time t, and C_0 is the initial concentration of the loaded solute inside gel matrix.

When $t > 0$, the diffusion of the solute between the hydrogel and the solution phase occurs, and the process of release and absorption of solute occurs simultaneously.

Assuming a first order kinetic process, changes of solute concentration in solution at a given time t, can be expressed as Equation (3):

$$\frac{\mathrm{d}C_{R,t}}{\mathrm{d}t} = k_R(C_0 - C_{R,t}) - k_A(C_{R,0} - C_{A,t}) \qquad (3)$$

where $C_{R,0}$ and $C_{R,t}$ are the concentration of the release solute at $t = 0$ and at time t, C_0 is the initial concentration of DNA in the gel and $C_{A,t}$ is the concentration of absorbed solute at specific time t. k_R and k_A are the rate constants for the release and absorption processes, respectively.

From the kinetic law equation, Equation (3), and taking into account considerations reported elsewhere [17], the release kinetics of DNA can be treated by using Equation (4):

$$F_R = F_{R,\max}\left(1 - e^{-(k_R / F_{R,\max})t}\right) \qquad (4)$$

where $F_{R,\max} = C_{R,\max}/C_0$, and $C_{R,\max}$ is the maximum concentration of solute in solution released from the gel. By fitting Equation (4) to experimental data (e.g., Figure 3) it is possible to calculate the following parameters: $F_{R,\max}$ and k_R, and so to characterise the release not only in terms of kinetics, but also in terms of the partition coefficient.

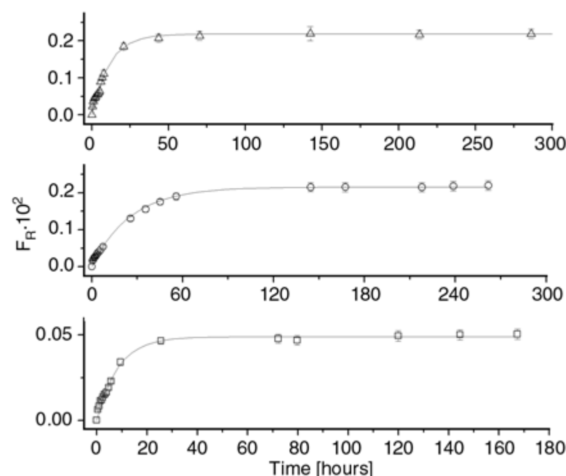

Figure 3. Desorption kinetics of DNA from PVA-DNA membranes to cationic surfactant solution: (□) $C_{12}TAB$, (o) $C_{12}Me_6$, (Δ) $C_{12}Et_6$. Solid lines represent the fraction of release DNA predicted by Equation (4).

It is worthwhile to note that the release of DNA, in all systems mentioned in this communication, follows a first-order kinetic law. The experimental data were analyzed using a nonlinear least-squares fitting procedure (Origin 8.0), using a 95% confidence level. The uncertainty in the fit of Equation (4) to the data is in general smaller than 5%.

4. Results and discussion

4.1. Effect of symmetrical and unsymmetrical electrolytes

Table 1 shows the effect of sodium salts on the distribution coefficient (α) and released rate constants (k_R) of DNA release from PVA-DNA gel membranes in different electrolyte media.

It is possible to observe that comparing the release of DNA to sodium salts, at a given concentration, there is a decrease of both α and k_R in the order: NaI > $NaNO_3$ > NaCl > NaBr. The order of anions does not differ much from that involved in their water structure modifying effects or of the Hofmeister series [22, 23]; that is, with the capacity of different aqueous solutions to solubilise DNA. Lozinsky *et al.* [24] have also reported that the reinforcing ability of the electrolyte with respect to the PVA cryogel strength has been shown to be in agreement with the position of these ions in the

Table 1. Values of different kinetic and equilibrium parameters for the release of DNA, from PVA cryogels, to different electrolyte solutions, at 25°C

	$\alpha/10^{-3}$	$k_R \cdot 10^{-7}$ [s^{-1}]
[NaI] [mM]		
10	4.7 (± 0.1)	4.8 (± 0.3)
25	4.6 (± 0.1)	1.33 (± 0.09)
50	3.33 (± 0.06)	1.07 (± 0.04)
[$NaNO_3$] [mM]		
10	3.43 (± 0.08)	1.92 (± 0.09)
25	3.31 (± 0.05)	1.19 (± 0.03)
50	2.51 (± 0.07)	4.66 (± 0.02)
[NaCl] [mM]		
10	2.81 (± 0.06)	1.27 (± 0.05)
25	2.54 (± 0.07)	0.66 (± 0.03)
50	2.08 (± 0.05)	0.40 (± 0.02)
[NaBr] [mM]		
10	2.37 (± 0.07)	0.83 (± 0.05)
25	2.24 (± 0.01)	0.462 (± 0.008)
50	1.05 (± 0.01)	0.128 (± 0.004)

Values inside brackets correspond to standard deviations.

lyotropic series. Among these salts, iodide solution has the strongest effect as water structure breaking, being the highest effective on the solubilisation of DNA [25]. Using similar arguments, NaBr is the less effective on the solubilisation of DNA in the aqueous phase [20]. We can hypothesise that such variation in the empirical sequence, when compared with the Hofmeister series, can be justified by the specific effect of bromide ions on the crystallinity of PVA and/or on the DNA structure. That is, the presence of bromide ions affects the PVA structure by decreasing its crystallinity due to the ability of NaBr as a hydrogen bond breaker [26]; consequently, it can be suggested that in more amorphous gels, due to the higher mobility of the PVA chains between the crystalline parts, DNA molecules evidenced stronger self interactions, generating aggregate structures and, thus, increasing the retention (α) of DNA inside the gel membrane. On the other hand, the effect of NaBr, on the DNA structure, in the gel membrane, is similar to that found by the addition of an azobenzene trimethylammonium bromide surfactant in DNA aqueous solution upon exposure to visible radiation [27]. Using the same arguments, the decrease in the rate constant of the DNA release with an increase of the retention degree can be attributed to a stabilization of DNA in the gel state. The last argument is also justified by the analysis of the effect of ionic strength on the partition coefficient and release rate constant of DNA from PVA membranes. The analysis of data (Table 1) suggests that an increase of the ionic strength leads to a higher retention of DNA inside gel, leading to an increase of DNA concentration gradient and, consequently, to a lower k_R. This can be justified by a salting-in phenomena [28], which contribute for a stabilization of the DNA inside the gel phase. Such stabilization occurs via aggregation of DNA. Consequently, the ability of DNA to diffuse out of gel decreases by steric hindrance [29] and/or by an increase of the resistance coefficient, which is a measure of the friction acting on a solute as it moves through a solvent (the friction acting on an aggregate is higher than that acting on a single molecule) [30].

4.2. Effect of temperature

The effect of temperature on the release of DNA from PVA-DNA gels to unbuffered water was evaluated at 20, 30, 37 and 40°C. Figure 4a, shows the temperature dependency of drug release. It can be seen that the release is temperature-dependent, and α and k_R increase by increasing temperature. It is interesting to note that the rate and distribution coefficients obtained for the release of DNA at 20°C in water is similar to those found for a solution of sodium nitrate 10 mM. With such results we can hypothesise that the delivery DNA mechanism is the same even in the absence of salt.

The temperature dependence of the rate constant, k_R, can be described by the Arrhenius relationship – Equation (5):

$$k_R = k_0 e^{-(E_a/RT)} \tag{5}$$

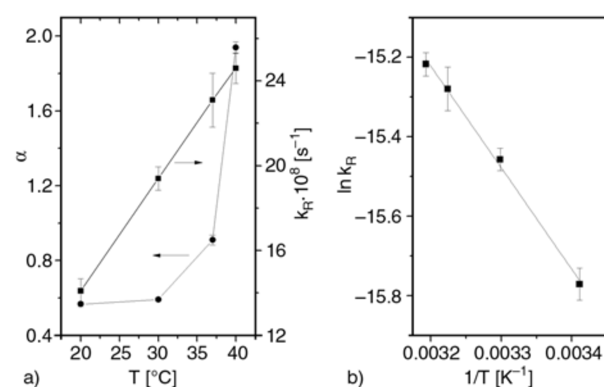

Figure 4. (a) Effect of temperature on the rate constants and distribution coefficients of DNA released from PVA-DNA membranes, (b) Arrhenius treatment of the temperature dependence of the rate constant, k_R

where k_0 is the preexponential factor, E_a is the activation energy, R is the universal gas constant, and T is the absolute temperature. One can determine the value of E_a from the slope of the linear relationship between ln$k$ and the reciprocal of absolute temperature as demonstrated in Figure 4b. It was found that E_a = 21.2(±0.8) kJ/mol. This value is in agreement with the previously reported activation energy for the mobility of DNA in cross-linked polyacrylamide slabs gels (ca. 16 kJ/mol for 700 bp DNA) [31].

4.3. Effect of cationic surfactants

Cationic surfactants interact strongly with DNA. Those interactions depend on the surfactant hydrophobic chain length, charge and headgroup [32, 33]. Figure 5 shows the effect of those factors on the release kinetics and distribution coefficient of DNA, from DNA-containing PVA gel matrices, to dodecyl-based cationic and di-cationic surfactants. All experiments have been carried out using surfactant solutions at pre-micelle concentrations.

The distribution coefficient of DNA in the presence of C_{12}TAB shows that almost all DNA is retained inside gel matrix (the cumulative DNA release is just 5%) [34]. The high retention of DNA inside cryogel, when in the presence of aqueous solutions of dodecyltrimethylammonium bromide, can be justified by the formation of a coating-like structure [35] on the PVA-DNA gel, formed as a consequence of strong interaction between the desorbed DNA and the sorbed surfactant. Upon surfactant charge increase the cumulative release of DNA also increase to around 20%. These values suggest that

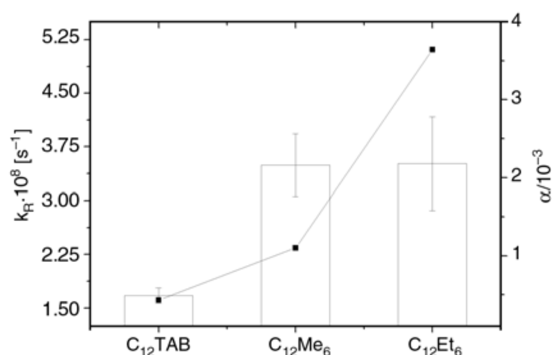

Figure 5. Distribution coefficients, α (columns), and rate constants, k_R (data points), for the release of DNA, from PVA-DNA membranes, to aqueous solutions of different surfactants at 20°C

interaction between C_{12}TAB and DNA is much stronger than with di-cationic surfactants, as has been reported elsewhere [16]. On the other hand, there is no effect of the bulkiness head group, of the bolaamphiphile surfactants, on the distribution coefficients of DNA [33].

However, it should be noted that the rate constants increase in the same order k_R(C_{12}TAB) < k_R($C_{12}Me_6$) < k_R($C_{12}Et_6$), suggesting that the interactions between surfactants and DNA will affect the kinetics mechanism of release.

The main conclusion is that the release of DNA to a surfactant solution is quite sensitive to the balance between hydrophobic/hydrophilic and electrostatic interactions. Further studies have been carried out in order to have a deeper insight on such release mechanisms.

5. Conclusions

The kinetics and equilibrium properties of the release of DNA from PVA-DNA cryogel membranes to different solutions and temperatures have been studied. The first order kinetic law equation was found to be an excellent model to describe the experimental release data. The kinetics of release and the maximum amount of DNA desorbed are quite sensitive to the media where the PVA-DNA gel matrix is immersed. The rate of DNA release from PVA-DNA gels increased with increasing temperature with activation energy (E_a) of 21.2 kJ/mol.

Kinetic and equilibrium parameters show that it will be possible to control the release of DNA, which, coupled with the very good mechanical stability of these gel blends, are promising results for the further development of more appropriated PVA matrices, loaded with DNA, for biomedical and pharmaceutical applications. Furthermore, our results show that a simple method can provide useful experimental information on the importance of ion-ion correlation effects in electrolyte-containing DNA solutions.

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