In Vivo Properties of an *In Situ* Forming Gel for Parenteral Delivery of Macromolecular Drugs

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Purpose. This study characterizes the *in vivo* properties of an *in situ* forming gel, comprising an IPC of water-soluble polymers, PMA and PEG, for sustained release of macromolecular drugs.

Methods. 40, 50 or 60% w/v formulations were injected subcutaneously in a rat model either alone, or containing model macromolecules, 3A2-ATG-psODN or REV-psODN, to (i) determine the approximate gelling and residence time of the gel at the site of injection, (ii) assess the biological efficacy of the formulation using a MZ sleep time model, and (iii) demonstrate specificity of the sequence and selectivity of the psODNs by measuring changes in microsomal enzyme levels and urine volumes.

Results. A sol to gel transition requires 15 min *in vivo*, and the 60% w/v IPC gel remains at the site of injection for up to 72 hr. The MZ sleep times and CYP3A2 expression due to 3A2-ATG-psODNs released from the gel are significantly different compared to that of REV-psODNs.

Conclusions. The IPC solutions exhibit phase transformation *in vivo*, and demonstrate no evidence of toxicity. The pharmacological effects observed from the of release of 3A2-ATG-psODNs suggest that the formulation can entrap, protect, and sustain the delivery of macromolecules.

KEY WORDS: *in situ* gel; poly(methacrylic acid); poly(ethylene glycol); macromolecular drugs; sustained-delivery.

INTRODUCTION

Conventional methods of drug delivery have been unsuccessful in effectively delivering clinically useful macromolecules, such as proteins/peptides or oligonucleotides (ODNs), primarily due to their high molecular weights and short half-lives (1). When delivered orally most macromolecules are susceptible to physicochemical degradation in the gastrointestinal tract or are poorly absorbed across the gastrointestinal mucosa. The current practice involves parenteral delivery of macromolecules that requires frequent dosing and constant patient monitoring, thereby increasing treatment expenses. However, a sustained-release system delivered parenterally will circumvent repeated injections, reduce treatment costs, and efficiently deliver therapeutic doses over a period of time improving patient compliance.

An inter-polymeric complex (IPC) of water-soluble polymers, poly(methacrylic acid) (PMA) and poly(ethylene glycol) (PEG), present in equimolar proportions, is being considered as a drug-carrier to improve parenteral delivery of macromolecular drugs. It has been previously reported that: (i) an IPC of PMA and PEG was the main component of a composite polymer carrier developed into an oral controlled release formulation approved for medical use in Russia (2), and (ii) graft copolymers of PMA and PEG are being used to prepare hydrogels for controlled release (3).

In this study, it was hypothesized that a solution of the IPC in a biocompatible cosolvent system can transform, *in situ*, from a sol to a gel at physiological pH to entrap, protect and sustain the delivery of macromolecular drugs, such as proteins, peptides or oligonucleotides. This postulation is based on previous studies that confirmed the *in vitro* transformation of an IPC solution into a gel at physiological pH (4). However, this solution was reported to contain up to 50% ethanol, a cosolvent, that may be toxic or undesirable in higher concentrations in a formulation intended to deliver macromolecular drugs. Therefore, N-methyl pyrrolidone (NMP), a biocompatible solvent used in some implant-delivery systems, was considered to replace all or part of ethanol maintaining the phase transition properties of the *in situ* forming gel (5,6).

It was proposed that an *in situ* forming gel formulation would have the following benefits: (i) intramuscular or subcutaneous injection would eliminate the need for either surgical insertion or removal, (ii) the frequency of administration would be decreased compared to the conventional parenteral dosage forms and, (iii) the macromolecules may be protected while entrapped in the formulation, to be subsequently released in an active form.

The goal of this study was to preliminarily assess the *in vivo* properties of the *in situ* forming gel including the determination gel formation and residence time, evaluate the toxicity and the ability to provide sustained release of pharmacologically active macromolecules. We selected a phosphorothioate oligonucleotide, an antisense inhibitor to CYP3A2 protein, as a model macromolecule (3A2-ATG-psODNs), to demonstrate the ability of the formulation to release pharmacologically active compounds. The 3A2-ATG-psODN targets the CYP3A2 mRNA translation start site to inhibit CYP3A2 expression in the liver (7). Midazolam (MZ) metabolism is considered a specific *in vivo* pharmacological marker of CYP3A2 enzymatic activity (8,9). It has been previously demonstrated that MZ sleep times in Sprague Dawley rats can be used as a measure of the *in vivo* CYP3A2 enzymatic activity (7). Additionally, measurement of changes in *in vitro* microsomal enzyme activities provided an insight into the ODNs specificity or potential toxicity of the formulation.

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ABBREVIATIONS: IPC, inter-polymeric complex; PMA, poly (methacrylic acid); PEG, poly(ethylene glycol); ODNs, oligonucleotides; 3A2-ATG-psODNs, antisense phosphorothioate oligonucleotides to rat CYP3A2-ATG mRNA translation site; REV-psODNs, the 5′ to 3′ reverse sequence of 3A2-ATG-psODN; C-MYC-psODNs, antisense phosphorothioate oligonucleotides to primate C-MYC mRNA translation start site; FAM, 5′ carboxyfluorescein phosphoramidite; ERDEM, erythromycin demethylation; PNP, p-nitrophenol; EROD, ethoxyresorufin O-dealkylase; PROD, pentoxy-reorufin O-delkylase; CYP3A2, 2E1, 2B1/2, 1A1/2, cytochrome P450 3A2, 2E1, 2B1/2, 1A1/2; pmol, picomoles.

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MATERIALS AND METHODS

Oligonucleotide Syntheses

Syntheses were performed on an Applied Biosystems Model 380B DNA synthesizer (Foster City, CA) with 1 mmol column supports. The psODNs were prepared by the cyanoethyl approach via phosphoramidite chemistry as described by ABI User Bulletin, No. 58 (1991). Table 1 lists the sequence of the antisense to CYP3A2-ATG mRNA translation site (3A2-ATG-psODN), antisense to primate C-MYC mRNA translation start site (C-MYC-psODN) and CYP3A2-REV, the 5′ to 3′ reverse sequence of 3A2-ATG-psODN (REV-psODN). The 3A2-ATG-psODN was then labeled with FAM Amidite, a single-isomer, 5′ carboxyfluorescein phosphoramidite, that enables direct labeling of synthetic oligonucleotides on any ABI synthesizer. Purification was carried out with the Oligonucleotide Purification Cartridge, OPC. It is demonstrated previously that the FAM-label on the 5′-end remains intact *in vivo* (10).

Preparation of the Formulation

The IPC of PMA (sodium salt, mol wt 15,000, Polysciences, Inc.) and PEG (modified PEG, mol wt 20,000, Polysciences, Inc.), present in equimolar proportions, instantly formed at pH < 5.6, was dissolved in a cosolvent system comprising a 1:1:2 ratio of NMP (EM Sciences), ethanol (Sigma Chemicals) and aqueous buffer, pH 7.2 + 0.2, respectively to obtain 40, 50 and 60% w/v solutions (based on the wet weight of the IPC). One mg of FAM-labeled 3A2-ATG-psODN, REV-psODN, a non-complementary control or normal saline was reconstituted in 1 ml of this solution prior to administration.

Animal Treatment

All *in vivo* studies were conducted using the rat subcutaneous model in accordance with the guidelines set by the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human services) and were approved by the Institutional Animal Care and Use Committee, University of Nebraska Medical Center, NE. Male Sprague Dawley rats (Sasco, Kingston, NY) weighing 180–200 g were used for the study and housed in the University's AAALAC-approved animal resource facility, exposed to a 12 hr light/dark cycle and allowed access to Purina rat chow and tap water *ad libidum*.

In Vivo Gelling and Gel Residence Time

Four groups of rats were anesthetized with methoxyflurane (METOFANE^R, Mallinckrodt Veterinary, Mundelein, IL) and injected subcutaneously on the right side of the mid dorsal line below the scapulas with 1 ml each of 40, 50 and 60% w/v formulations via an 18 G needle. Three rats from each group were sacrificed at each time point, 15 min, 6, 24, 48 and 72 hr. An incision was made at the site of injection and the area photographed to visually detect the presence of the formulation or assess any damage caused to the surrounding tissue. Additionally, the surface area of the gel, organ weights and urine output were recorded every 24 hr. In a similar experiment, 0.5 ml of a 60% w/v formulation was administered subcutaneously and intramuscularly into two groups of rats to compare the effect of the routes of injection on gel formation.

Delivery of ODNs in the Formulation

Three groups of rats, each received 1 ml of a 5 mg/kg subcutaneous dose of FAM-labeled 3A2-ATG-psODN, REV-psODN or normal saline contained in the formulation (Table 1). Sleep times were evaluated at periodic intervals of 0, 24, 48 and 72 br following administration of these formulations. The sleep time, defined as the time for the rat to gain its righting reflex after being placed on its back (7), was measured following an intraperitoneal injection of 50 mg/kg midazolam (Hoffman-La Roche, Nutley, NJ). Four rats from each group were sacrificed at the end of 24, 48 and 72 hr, specific organ weights were recorded, and liver or small intestinal microsomes prepared as described by Franklin and Estabrook, 1971 (11). The microsomal pellet was resuspended in an equal volume buffer (10 mM Tris-acetate, 1 mm EDTA, 20% glycerol; Sigma Chemicals) and frozen at −80°C. Protein concentrations were determined by a Bradford assay (12) followed by determination of the microsomal enzyme activity. In a similar experiment, 1 ml of a 60% w/v formulation, that contained no ethanol, was injected subcutaneously into a group of rats to compare the effect of ethanol.

Continuous Infusion of ODNs

Alzet pumps Model 2002 (Alza Corp., Palo Alto, CA), containing 3A2-ATG-psODN or C-MYC-psODN, as the sequence control, were surgically implanted subcutaneously below the mid-dorsal line of rats. A dose of 0.343 mg/day of each ODNs was administered in saline for the 14 day duration of the study, using two groups of rats consisting of 3 animals each. The sleep times were measured at periodic intervals and microsomes prepared at the end of the study (data not shown) similar to the procedures following administration of the *in situ* gel forming formulation.

Microsomal Enzyme Assays

A specific assay involving erythromycin demethylation (ERDEM) was used to measure the CYP3A2 enzymatic activity (13,14) in terms of the mmol of formaldehyde liberated per mg protein/min. The activities for CYP 1A1/2 and 2B1/2 were

Table 1. Oligonucleotide Sequences Used in the Study

Oligonucleotide	Sequence
CYP3A2-ATG antisense phosphorothioate (3A2-ATG-psODN)	5′-TGAGAGCTGAAAGCAGGTCCAT-3′
REV-CYP3A2-ATG antisense phosphorothioate (REV-psODN)	5′-TACCTGGACGAAAGTCGAGAGT-3′
C-MYC antisense phosphorothioate (C-MYC-psODN)	5′-ACGTGAGGGGCATCGTCG-3′

determined by ethoxy- and pentoxy-resorufin O-dealkylase activities (EROD or PROD), respectively (15) and recorded as pmol of resorufin per mg protein/min. The CYP2E1 activity was determined by the p-nitrophenol (PNP) activity in terms of O.D./mg protein/min (16).

Urine Output and Urinalysis

Urine volumes were measured every 24 hr and aliquots were analyzed to determine the amount of macromolecule excreted and analyzed for the FAM-labeled psODN content using a spectrophotofluorimeter at I_{Ex} 495 nm and I_{Em} 535 nm. Urinalysis was performed using LABSTIX^R (Bayer Corporation, Elkhart, IN) to detect any differences in pH and the levels of glucose, protein, ketone and blood in the urine.

Histology

The tissue samples were fixed in 10% neutralized formalin (Sigma Diagnostics, St Louis, MO), embedded in paraffin, and sectioned in the Eppley Institute Histology core facility, (University of Nebraska Medical Center, Omaha, NE). Sections were stained with hematoxylin and eosin for microscopic evaluation. Samples were photographed on Olympus U-CA microscope with Kodak ISO 400 print film.

Statistical Analysis

All data were reported as mean ± SEM. The p-values were calculated by one-way ANOVA, Tukey Multiple Comparison test or the Student's t-test using Prism v 2.0 (GraphPad, San Diego).

RESULTS

In Vivo Gelling and Gel Residence Time

A complete transformation from solution to gel takes approximately 15 min following a subcutaneous injection of 1 ml solution via an 18 G needle. The photographs (Fig. 1) and the measurements of the gel surface area (Table 2) show that the gel formed from a 60% w/v formulation resides at the site of injection up to 72 hr. However, the 40% and the 50% w/v formulations disappear within 48 hr. When 0.5 ml of the formulation was injected either subcutaneously or intramuscularly, traces of the gel were seen at the subcutaneous site after 24 hr while no gel was visible at the intramuscular site.

Evaluation of Acute Toxicity

One-way ANOVA analysis was performed to show that there are no significant differences, $p > 0.05$, in organ weights and urine output between control (saline-treated) and those treated with 40, 50 or 60% w/v IPC solutions (data not shown). Results from the urinalysis indicated that none of the treated groups were outside the normal levels of glucose, protein, ketone, and blood or the normal pH of the urine (data not shown). Following injection, a slight discoloration of the tissue surrounding the injection site appeared but disappeared after 48 hr. No inflammation was noticed. All animals demonstrated typical grooming behavior, normal posture, and activity, although, scratching was noticed immediately after injection up to 8 hr.

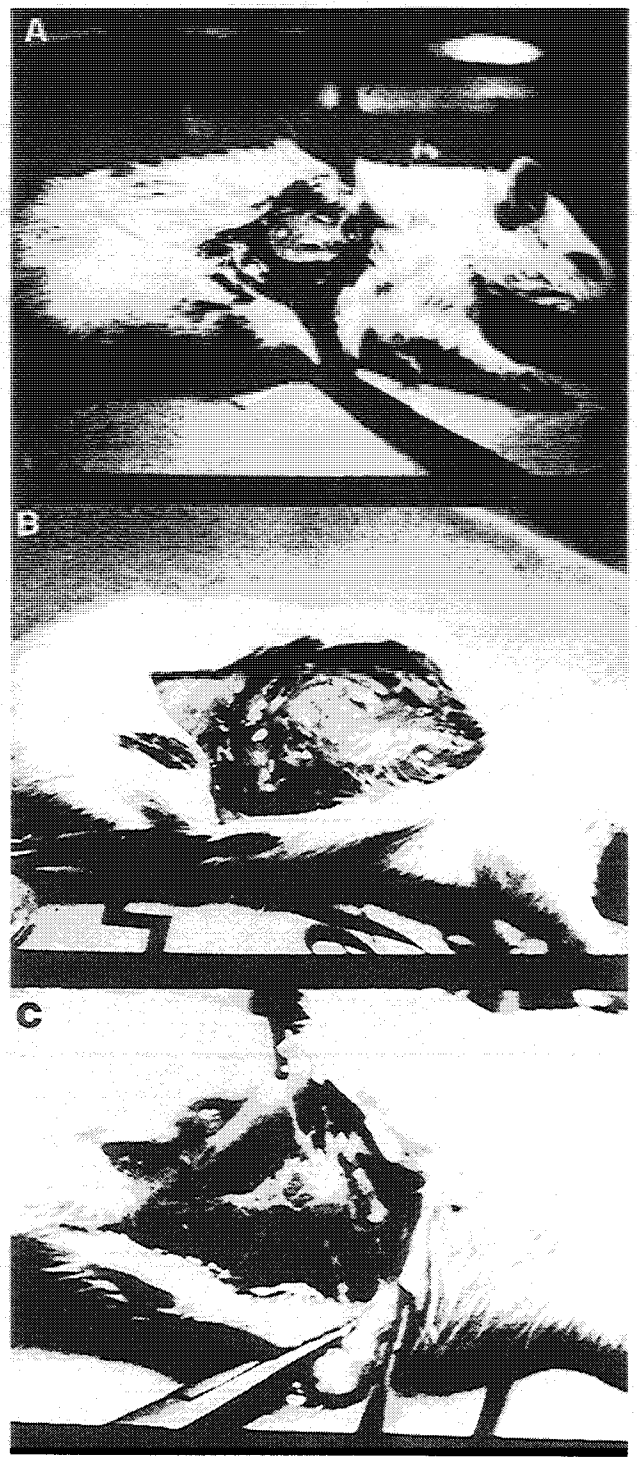

Fig. 1. Photographs of gel residence at the s.c. site of injection following administration of 1 ml of 60% w/v formulation in a Sprague Dawley rat model, after (A) 15 min, (B) 24 hr, and (C) 48 hr.

MZ Sleep Time

The MZ sleep time in the rats treated with 3A2-ATG-psODN-formulation (Fig. 2), administered subcutaneously,

Table 2. Surface Area[a] of Gel Remaining After s.c. or i.m. Administration of the IPC Formulations in a Rat Model

Volume and route of injection	Surface area remaining (cm^2) as a function of time				
	IPC formulation	15 min	24 hr	48 hr	72 hr
1 ml s.c	40% w/v	30.0 ± 2.2	18.1 ± 1.3	9.5 ± 1.8	0.0
1 ml s.c	50% w/v	25.6 ± 3.2	17.5 ± 2.1	7.5 ± 2.4	0.0
1 ml s.c	60% w/v	21.8 ± 1.2	18.7 ± 1.0	11.2 ± 1.0	traces
0.5 ml s.c	60% w/v	11.3 ± 0.9	traces	0.0	0.0
0.5 ml i.m	60% w/v	7.5 ± 0.9	0.0	0.0	0.0

[a] Calculated using surface area of the sphere expressed as mean area ± SEM, n = 3.

were significantly lower, $p < 0.05$, than sequence controls. Similarly, MZ sleep times in rats treated with Alzet pumps for continuous infusion of 3A2-ATG-psODNs (Fig. 3) were significantly lower, $p < 0.05$, than C-MYC-psODN-treated controls. There were no significant differences, $p > 0.05$, in MZ sleep times between formulation controls and saline controls.

Selectivity for the Target

The rats treated with 3A2-ATG-psODN-formulation significantly inhibited, $p < 0.05$, ERDEM activity in the liver (Table 3) while significantly induced, $p < 0.001$, this activity in the small intestine (Table 3) compared to the saline or formulation controls. The ERDEM activity in the saline-control or formulation-control rats was not significantly different, $p > 0.05$, than in rats treated with REV-psODN-formulation. Therefore, this shows that 3A2-ATG-psODN was selective in affecting CYP3A2 activity. Similarly, there were no significant differences, $p > 0.05$, in the PNP activity between rats treated with REV-psODN-formulation, 3A2-ATG-psODN-formulation or formulation controls, all of which contained ethanol as a part of the cosolvent (Table 3). However, the PNP activity following treatment with a formulation containing no ethanol were significantly different, $p < 0.05$, than rats treated with the formulation containing ethanol suggesting that ethanol induces CYP2E1 expression. Finally, no significant differences, $p > 0.05$, were evident in EROD and PROD activities between various treatments (Table 3).

Urinary Excretion and Urine Output

The cumulative percent of 3A2-ATG-psODNs recovered in the urine per day after 24, 48 and 72 hr following subcutaneous administration of their gel formulation was 7.75%, 16.87% and 48.17%, respectively (Fig. 4). Therefore, over the sampling period at least 48% of the drug was recovered in the urine. The gel surface area also decreased by 50% in 72 hr. The rats treated with 3A2-ATG-psODN had a twofold increase in the average urine output per day compared to the rats treated with sequence controls (Table 4). The urine output from the formulation-controls were not significantly different, $p > 0.05$, from the rats treated with REV-psODN-formulation.

Specificity for the ODN Sequence

Significant changes in MZ sleep times following subcutaneous administration of 3A2-ATG-psODNs in the gel formulation or implants, compared to their respective sequence controls

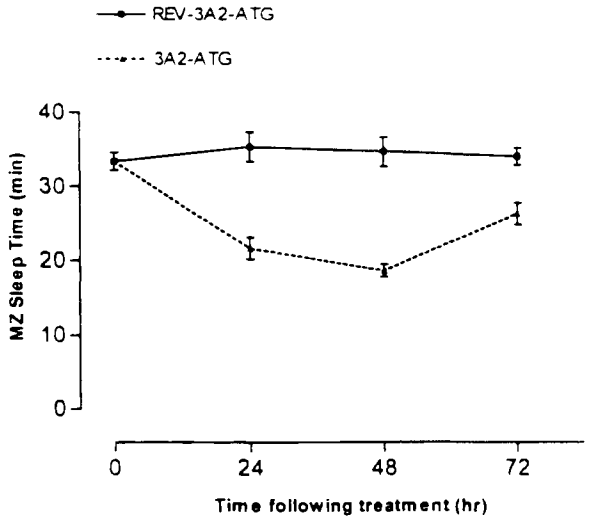

Fig. 2. MZ sleep times expressed as mean ± SEM, for n = 3, determined at various times following s.c. administration of 3A2-ATG-psODNs or REV-psODNs in a 60% w/v IPC solution in a Sprague Dawley rat model.

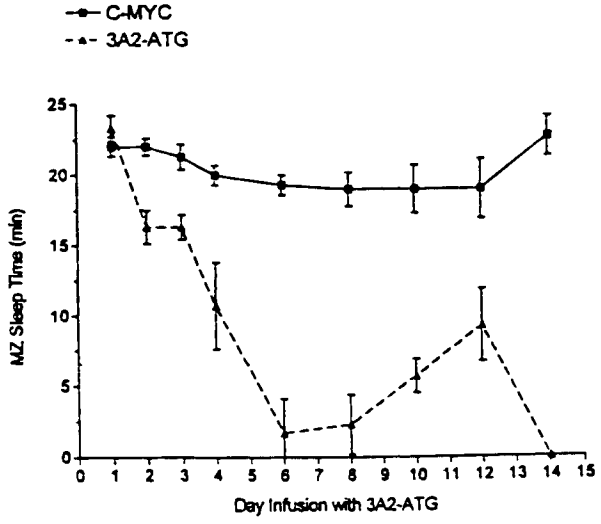

Fig. 3. MZ sleep times expressed as mean ± SEM, for n = 3, determined at various times following s.c. a continuous infusion of 3A2-ATG-psODNs or C-MYC-psODNs via Alzet pumps in a Sprague Dawley rat model.

Table 3. Midazolam Sleep Times and Microsomal Protein Activities Determined After 48 hr[a] in a Rat Model Following s.c. Injection of Various 60% w/v Formulations

		Microsomal protein activities				
	MZ sleep time (n[b])	ERDEM (CYP3A2) (liver)	ERDEM (CYP3A2) (small intestine)	PNP (CYP2E1)	PROD (CYP2B1/2)	EROD (CYP2A1/2)
Saline only	35 ± 10.0 (8)	93.2 ± 2.4	12.1 ± 1.5	0.8 ± 0.1	48.6 ± 0.9	35.4 ± 0.9
formulation	34 ± 0.9 (7)	98.3 ± 3.7	13.6 ± 3.1	0.9 ± 0.0	40.3 ± 1.3	34.8 ± 1.0
formulation (no ethanol)	36 ± 2.2 (3)	91.6 ± 2.7	11.6 ± 1.8	0.7 ± 0.0	49.7 ± 0.9	33.6 ± 1.0
3A2-REV reconstituted formulation	31 ± 2.4 (6)	99.5 ± 4.6	13.6 ± 3.1	1.0 ± 0.1	49.7 ± 0.7	30.4 ± 1.0
3A2-ATG reconstituted formulation	19 ± 3.0 (5)	70.5 ± 1.6	77.9 ± 11.4	1.0 ± 0.1	49.6 ± 2.1	31.5 ± 0.5

Note: MZ—midazolam, the sleep time expressed in min; ERDEM—erythromycin demethylase, μmol formaldehyde/mg protein/min; PNP—p-nitrophenol hydroxylase, O.D./mg protein/min; PROD—pentoxyresorufin O-dealkylase, pmol/mg protein/min; EROD—ethoxy resorufin O-dealkylase, pmol/mg protein/min.

[a] The data at 24 and 72 hr not shown.

[b] The variability in the n value was because some animals failed to loose their righting reflex due to improper injection of MZ.

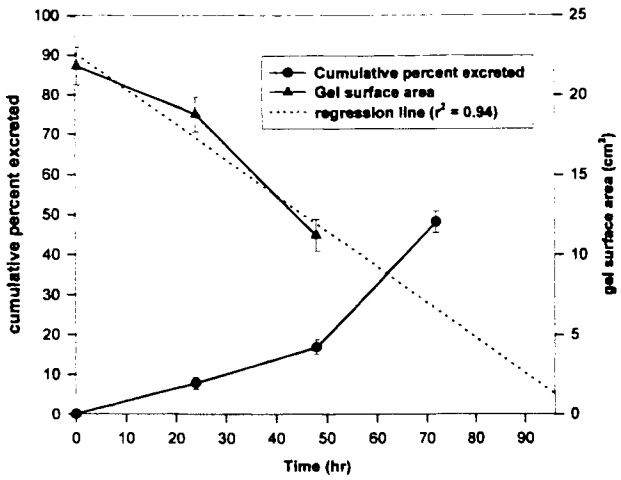

Fig. 4. Cumulative percent of 3A2-ATG-psODNs excreted in urine or the gel surface area remaining at the site of administration following a s.c. injection of psODN in 60% w/v IPC solution. Each data point represents mean ± S.E.M., for n = 3.

Table 4. Average Urine Output/Day After s.c. Administration of Oligonucleotides in a Rat Model

	Urine output in ml (mean ± SEM)			
Treatment	Formulation	C-MYC	REV-3A2-ATG	3A2-ATG
s.c. implants (Alzet pumps)	—	8.9 ± 0.5	—	13.2 ± 1.4
s.c. injection (*in situ* forming gel)	9.1 ± 2.8	—	7.8 ± 2.5	13.6 ± 3.5

demonstrated the sequence specificity of the 3A2-ATG-psODN, confirmed by the selectivity of 3A2-ATG-psODNs. However, the difference in the baseline values obtained for the MZ sleep times in rats between the two sequence controls, REV-psODNs and C-MYC-psODNs, may be attributed to the variation in individual judgment of the righting reflex in rats following MZ injection. The MZ sleep times were reproducible for these controls within an experiment performed by a single investigator. A significant increase, $p < 0.05$, in urine output in rats treated with 3A2-ATG-psODNs compared to those treated with the respective sequence controls, emphasize the specificity of 3A2-ATG-psODNs in their response.

Histology

Mid-zonal toxicity after 24 hr was detected in a few randomly selected livers from the rats treated with various formulations. However, none of the samples showed toxicity after 48 hr. These observations are consistent with the changes observed in PNP activity and suggest a response to acute ethanol toxicity.

DISCUSSION

Water-soluble polymers, PMA and PEG, interact in an equimolar ratio to form a water-insoluble complex that dissolves in certain non-aqueous solvents, such as ethanol or N-methyl pyrrolidone. The IPC is primarily hydrogen bonded but other interactions such as, hydrophobic or ionic interactions also appear to be involved in its formation (17). Previously it has been demonstrated that a solution of the IPC has the ability to undergo a reversible, sol ↔ gel, phase transformation at physiological pH (3). The current studies demonstrate this phenomenon to be potentially useful in the development of a sustained-release drug-delivery system. Thus, the gel phase can entrap pharmacologically active macromolecules, protect them and ultimately release them in an active form as the gel dissolves. This gel is an erodible system made up of water-soluble

polymers that are physically bonded, and therefore the dissolution of the gel becomes the rate limiting step in the release of macromolecules embedded in the matrix (18). The key considerations of these studies included the *in vivo* transformation of sol ↔ gel and the ability of the formulation to deliver pharmacologically active macromolecules in a sustained release manner.

In the rat model, a complete transformation from a sol to a gel phase occurs in a period of 15 min following a subcutaneous injection of 1 ml via an 18 G needle. However, this transformation is initiated by the formation of an outer membrane probably due to faster diffusion of ethanol that confines the solution to the site of administration and may prevent the burst release of the entrapped macromolecule, if any. The reversible transition from a gel to a sol may be correlated to the residence time of the gel, *in vivo*. The residence time of the gel increases with the concentration of the IPC in the solution because 40 or 50% w/v formulations disappear within 48 hr and a 60% w/v formulation resides at the site for up to 72 hr (Table 2). Further, within 24 hr the gel disappears rapidly from the intramuscular site whereas traces of the gel remain at the subcutaneous site probably due to the differences in the constitution and physiological activities at the two sites. A faster disappearance from the site of injection may be compensated by increasing the IPC content of the solution, but above a concentration of 60% w/v, the formulation is highly viscous and cannot be injected via an 18 G needle. Following a subcutaneous injection, a slight discoloration of the tissue surrounding the subcutaneous site of injection appears but disappears after 48 hr. However, during the course of the study there were no changes in the specific organ weights including liver, spleen, kidney and heart, or in the daily urine output. Further, the pH of the urine and levels of glucose, protein, ketone and blood in the urine were normal. The absence of toxicity imply that the formulation has the potential for a sustained-release drug delivery.

The formulation was able to deliver pharmacologically active 3A2-ATG-psODN, for 72 hr as was evidenced by the changes in MZ sleep times and microsomal enzyme activities. The prolonged lowering of MZ sleep time following administration of 3A2-ATG-psODNs in the *in situ* forming gel (Fig. 2) was similar to that observed when the ODNs were administered via an Alzet pump (Fig. 3) indicating both the delivery systems, are capable of providing constant concentrations of the circulating ODNs required to invoke this response.

The pharmacological activity of the administered 3A2-ATG-psODNs was further confirmed by alteration in microsomal enzyme activity. A decrease in the MZ sleep time following administration of the 3A2-ATG-psODNs, either in the *in situ* forming gel or via the Alzet pumps, was accompanied by an increase in the CYP3A2 expression measured *in vitro* as the ERDEM activity and an increase in the volume of urine. In contrast, when a 1 mg bolus dose of 3A2-ATG-psODN was injected intraperitoneally into a rat model there was an increase in MZ sleep time and a decrease in the ERDEM activity (7). Decrease in ERDEM activity is a consequence of CYP3A2 inhibition caused by the presence of 3A2-ATG-psODNs. Both CYP expression and glucocorticoid secretion follow a circadian rhythm in rats (19,20) that could lead to an accumulation of glucocorticoids in the plasma. Since glucocorticoids induce CYP3A2 (21), a higher glucocorticoid plasma concentration could cause a rebound induction of CYP3A2. Clearly, a bolus injection of a short duration is unable to escalate the levels of plasma glucocorticoids that result in a rebound induction of CYP3A2, whereas a continuous infusion or a sustained-delivery system may be capable of doing so thereby resulting in increased CYP3A2. Further, a rebound induction of CYP3A2 leads to increased excretion of sodium and water in urine and explains increased volume of urine output that was observed. Conclusively, the formulation sustains the delivery 3A2-ATG-psODNs similar to a continuous infusion and unlike a bolus injection.

In a rat model liver is a major site for CYP3A2 expression and is the rate limiting organ in the clearance of MZ (22,23). However, the ERDEM activity in the small intestinal microsomes was higher than in the liver following treatment with 3A2ATG-psODN-formulation. This was probably due to the distribution of the 3A2-ATG-psODNs, the antisense inhibitors to CYP3A2 protein. Therefore, a greater accumulation of the antisense inhibitors in the liver may attenuate the induction response of CYP3A2 to endogenous glucocorticoids. In contrast, the small intestine, which also contributes significantly to the MZ clearance, does not accumulate the antisense inhibitors and responds favorably to endogenous glucocorticoids. A higher inhibitor concentration in the liver than in the small intestine is responsible in reversing the roles of the two organs. Consequently, the small intestine becomes the rate limiting organ for clearance of MZ.

Finally, the cumulative percent of the administered 3A2-ATG-psODNs excreted in the urine correlated with the changes gel surface areas (Fig. 4). The gel surface area at the site of administration decreased by 50% and 48% of the drug was excreted in 72 hr. Conclusively, the changes in MZ sleep times, modulation in the CYP3A2 expression and differences in urine output were the result of the release of 3A2-ATG-psODNs from the formulation. This indicates the formulation can entrap, protect and deliver pharmacologically active macromolecules in a sustained manner.

CONCLUSIONS

The results from this study confirm the potential of the *in situ* forming gel as a sustained-release delivery system for macromolecular drugs. Firstly, the results demonstrate that a formulation comprising an IPC has the ability to undergo *in situ* phase transformation from sol ↔ gel, *in vivo*. Secondly, preliminary evidence is provided for the efficient delivery of pharmacologically active macromolecules. Finally, these promising results invite further exploration of the other applications of the *in situ* forming gel in drug-delivery technology.

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REFERENCES

1. T. Tice and S. Tabibi. Parenteral Drug Delivery: Injectables. In A. Kydonieus (eds), *Treatise on Controlled Drug Delivery*, Marcel Dekker, Inc., 1992, pp. 319–336.
2. A. Kharenko and V. Kemenova. Controlled release from oral formulations based on interpolymeric polymethacrylic-polyethylene glycol complex. *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* **22**:232 (1995).
3. C. Bell and N. Peppas. Poly(methacrylic acid-g-ethylene glycol) hydrogels as pH responsive biomedical materials. *Mat. Res. Soc. Symp. Proc.* **331**:199–204 (1994).
4. B. Haglund, R. Joshi, and K. Himmelstein. An in situ gelling system for parenteral delivery. *J Control. Rel.* **41**:229–235 (1995).

5. M. Shively, B. Coonts, W. Renner, J. Southard, and A. Bennett. Physico-chemical characterization of a polymeric injectable implant delivery system. *J Control. Rel.* **33**:237–243 (1995).
6. W. Lambert and K. Peck. Development of an *in situ* forming biodegradable polylactide-co-glycolide system for the controlled release of proteins. *J. Control. Rel.* 189–195 (1995).
7. J. Desjardins and P. Iversen. Inhibition of the rat cytochrome P450 3A2 by an antisense phosphorothioate oligodeoxynucleotide *in vivo*. *J. Pharmacol. Exp. Ther.* **275**:1608–1613 (1995).
8. K. Thummel, D. Shen, T. Podoll, K. Kunze, W. Trager, P. Hartwell, V. Raisys, C. Marsh, J. McVicar, D. Barr, J. Perkins, and R. Carithers Jr. Use of midazolam as a human cytochrome P450 3A probe. *J. Pharmacol. Exp. Ther.* **271**:549–556 (1994).
9. P. Watkins. Non-invasive test of CYP3A enzymes. *Pharmacogenetics* **4**:171–184 (1994).
10. J. Desjardins, B. Sproat, B. Beijer, M. Blaschke, M. Dunkel, W. Gerdes, J. Ludwig, V. Reither, T. Rupp, and P. Iversen. Pharmacokinetics of a synthetic, chemically modified hammerhead ribozyme against the rat cytochrome P-450 3A2 mRNA after single intravenous injections. *J. Pharmacol. Exp. Ther.* **278**:1419–1427 (1996).
11. M. Franklin and R. Estabrook. On the inhibitory action of mersalyl on microsomal drug oxidation: A rigid organization of the electron transport chain. *Arch. Biochem.* **143**:318–329 (1971).
12. M. Bradford. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
13. F. Gonzalez. The molecular biology of the cytochrome P450s. *Pharmacol. Rev.* **40**:243–287 (1989).
14. T. Nash. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**:416–421 (1953).
15. M. Burke, S. Thompson, C. Elcombe, J. Halpert, T. Haaparanta and R. Mayer. Etoxy-, Pentoxy-, and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochrome P450s. *Biochem. Pharmacol.* **34**:3337–3345 (1985).
16. L. Reinke and M. Moyer. P-nitrophenol hydroxylation a microsomal oxidation which is highly inducible by ethanol. *Drug Metab. and Disp.* **13**:548–552 (1985).
17. J. Nishikawa, T. Yabe, A. Kast, and H. Albert. Circadian rhythm of the liver of male rats pre-treated with phenobarbital-II. Hexabarbital sleeping times and lipid contents in liver and serum. *Chronoiol. Inter.* **4**:175–182 (1987).
18. P. Robel, E. Baulieu, M. Synguelakis, and F. Halberg. Chronobiologic dynamics of Δ_5-3b-hydroxysteriods and glucocorticoids in rat brain and plasma and human plasma. *Prog. Clin. Biol. Res.* **227A**:451–465 (1987).
19. D. Simmons, McQuiddy, and C. Kasper. Induction of the hepatic mixed-function oxidase system by synthetic glucocorticoids. *J. Biol. Chem.* **262**:326–332 (1987).
20. M. Paine, D. Shen, K. Kunze, J. Perkins, C. March, J. Mc Vicar, D. Barr, B. Gillies, and K. Thummel. First-pass metabolism of midazolam by the human intestine. *Clin. Pharmacol. Ther.* **60**(1):14–24 (1996).
21. K. Thummel, D. O'Shea, M. Paine, D. Shen, K. Kunze, J. Perkins, and G. Wilkinson. Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin. Pharmacol. Ther.* **59**(5):491–502 (1996).
22. M. Paine, D. Shen, K. Kunze, J. Perkins, C. March, J. McVicar, D. Barr, B. Gillies, and K. Thummel. First-pass metabolism of midazolam by the human intestine. *Clin. Pharmacol. Ther.* **60**(1):14–24 (1996).
23. K. Thummel, D. O'Shea, M. Paine, D. Shen, K. Kunze, J. Perkins, and G. Wilkinson. Oral first-pass elimination of midazolam involves both gastrointestinal and hepatic CYP3A-mediated metabolism. *Clin. Pharmacol. Ther.* **59**(5):491–502 (1996).