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(72) Inventeurs/Inventors:
MUMPER, RUSSELL, US;
JAY, MICHAEL, US
(73) Propriétaire/Owner:
UNIVERSITY OF KENTUCKY RESEARCH
FOUNDATION, US
(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : GELS FORMANT UNE PELLICULE MUCOADHESIVE SENSIBLE AU pH ET COMPOSITES CIRE-
PELLICULE CONNEXE

(54) Title: pH-SENSITIVE MUCOADHESIVE FILM-FORMING GELS AND WAX-FILM COMPOSITES

(57) **Abrégé/Abstract:**

The present invention relates to pH-sensitive mucoadhesive film-forming gels and wax-film composites suitable for topical and mucosal delivery of molecules of interest, namely active pharmaceuticals. The gels comprise a pharmaceutically acceptable pH-sensitive polymer that responds to a lowering of pH by precipitating into films when in contact with the skin or mucosal surface. The films also comprise an adhesive polymer that allows the film to remain in contact with the tissue for an extended period of time. The wax-film composites comprise a bi-layer film having both the said pH-sensitive mucoadhesive layer to promote strong adherence to the skin and mucosal surfaces as well as a specially bonded wax layer intended to extend the adherence of the film to tissues for a prolonged period of time. The invention also relates to the use of said pH-sensitive film-forming gels and wax-film composites to deliver molecules of interest, such as small molecules, peptides, proteins, and nucleic acids either locally to act at the site of administration or for the absorption of said molecules of interest across biological membranes into the systemic circulation.



pH-SENSITIVE MUCOADHESIVE FILM-FORMING GELS
AND WAX-FILM COMPOSITES

ABSTRACT

The present invention relates to pH-sensitive mucoadhesive film-forming gels and wax-film composites suitable for topical and mucosal delivery of molecules of interest, namely active pharmaceuticals. The gels comprise a pharmaceutically acceptable pH-sensitive polymer that responds to a lowering of pH by precipitating into films when in contact with the skin or mucosal surface. The films also comprise an adhesive polymer that allows the film to remain in contact with the tissue for an extended period of time. The wax-film composites comprise a bi-layer film having both the said pH-sensitive mucoadhesive layer to promote strong adherence to the skin and mucosal surfaces as well as a specially bonded wax layer intended to extend the adherence of the film to tissues for a prolonged period of time. The invention also relates to the use of said pH-sensitive film-forming gels and wax-film composites to deliver molecules of interest, such as small molecules, peptides, proteins, and nucleic acids either locally to act at the site of administration or for the absorption of said molecules of interest across biological membranes into the systemic circulation.

DESCRIPTION

PH-SENSITIVE MUCOADHESIVE FILM-FORMING GELS AND WAX-FILM COMPOSITES

FIELD OF THE INVENTION

The present invention relates to compositions and methods to treat the skin and mucosal surfaces with mucoadhesive film-forming gels and wax-film composites that are pH-sensitive. The invention also relates to using such pH-sensitive film-forming gels and wax-film composites to deliver molecules of interest, namely active pharmaceuticals. The invention also relates to the use of said pH-sensitive film-forming gels and wax-film composites to deliver molecules of interest, such as small molecules, peptides, proteins, and nucleic acids either locally to act at the site of administration or for the absorption of said molecules of interest across biological membranes into the systemic circulation.

BACKGROUND OF THE INVENTION

Pharmaceutical formulations intended to deliver drugs topically, either for local action at the site of administration or for absorption into the systemic circulation, have been described in the literature. These formulations are designed either for adherence of the dosage form to the skin (for dermal or transdermal delivery of drugs) or mucosal surface (for mucosal or transmucosal delivery of drugs). For skin delivery, formulations generally consist of dermal patches, pastes, band-aids, gels, lotions, sprays, or creams. For mucosal delivery, formulations generally consist of gels, creams, tablets, sprays, or films. In either case, one specific requirement is that the dosage form remains at the administration site for a sufficient amount of time so that the drug may function as needed. A second specific requirement is that the dosage form must consist of pharmaceutically acceptable materials. Many of the reported and commercially available delivery systems intended for topical and mucosal delivery are aqueous-based formulations comprising water-soluble excipients. However, these systems tend to be

easily and quickly washed away from the application site within minutes after application. This is very undesirable if the drug must remain at the application site for a prolonged period in order to be efficacious. In an attempt to prolong the residence time at the application site, researchers have described the use of water-insoluble excipients in the formulations. However, most often the use of water-insoluble excipients necessitates the use of non-aqueous solvents to dissolve the excipients. Excessive and repeated administration of non-aqueous solvents is not desirable.

Different types of formulations to deliver drugs topically are known in the art. Specific examples are illustrated below.

U.S. Patent 4,715,369 by Suzuki *et al.* relates to methods to treat the injured oral mucosa with a thin two-layer tablet comprised of both an adhesive layer and a nonadhesive layer. The adhesive layer is comprised of water-soluble cellulose-based polymers and polyacrylic acid while the nonadhesive layer is comprised mostly of materials with no adhesive properties. The Suzuki patent does not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the Suzuki patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 5,800,832 by Tapolsky *et al.* relates to a water-soluble, bioerodable device for the delivery of drugs, and specifically dyclonine, to mucosal surfaces. The device comprises an adhesive layer as well as a non-adhesive layer. Both the adhesive layer and the nonadhesive layer consist of water-soluble polymers such as cellulose-based polymers. The Tapolsky patent does not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the Tapolsky patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 5,955,097 by Tapolsky *et al.* relates to a non water-soluble gel, which adheres to mucosal surfaces and body tissues upon application and forms a film. The gel comprises at least one water-insoluble cellulose-based polymer, a non-aqueous solvent, and at least one active pharmaceutical. The patent teaches that upon application to the mucosal surface or skin, the non-aqueous solvent, primarily ethanol, evaporates, diffuses, or penetrates the surrounding tissue, resulting in precipitation of the non water-soluble polymers into films. The Tapolsky patent further teaches the use of 50 to 80% ethanol by weight in the pharmaceutical gel. The Tapolsky patent does not teach the use of film-forming gels containing at least 25%

water by weight and comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the Tapolsky patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 5,780,045 by McQuinn *et al.* relates to a transmucosal drug delivery device in the form of a sheet comprising an acid-containing particulate polymer dispersed in a polytetrafluoroethylene support matrix. The McQuinn patent does not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the McQuinn patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 4,552,751 by Inaba *et al.* relates to the preparation of multi-layered films of three, five, or seven layers for the administration of specific prostaglandins to mucosal sites. The Inaba patent does not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the Inaba patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 4,517,173 by Kizawa *et al.* relates to a film preparation consisting of at least three layers, including a pharmaceutical layer, a poorly water-soluble layer, and an intermediate layer. The pharmaceutical layer consists of predonisolone and allantoin together with a water-soluble cellulose-based polymer. The poorly water-soluble layer consists of shellack or fatty acids. The Kizawa patent does not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the Kizawa patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 5,192,802 by Rencher describes the use of a bioadhesive teething gel comprising benzocaine, sodium carboxy methyl cellulose; an agent selected from the group consisting of xanthan gum and sodium alginate and a diluent selected from the group consisting of polyethylene glycol and polyethylene glycol with glycerine. U.S. Patent 5,314,915 by Rencher and U.S. Patent 5,298,258 by Akemi *et al.* also describe the use of aqueous or oil-based bioadhesive gelling agents. These patents do not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to

changes in pH and/or desolvation of the polymers. Further, these patents do not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patents 5,081,157 and 5,081,158 by Pomerantz relate to a film-forming composition for topical application of medicaments to body tissues. The film-forming composition includes hydroxypropyl cellulose, a volatile solvent, and an esterification agent which reacts with the hydroxypropyl cellulose to form a reaction product which is soluble in the solvent. These patents do not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, these patents do not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

U.S. Patent 4,900,554 by Yanagibashi *et al.* relate to the use of a device for the delivery of drugs in the oral cavity. The device comprises an adhesive layer consisting of at least one acrylic acid polymer, a water-insoluble cellulose derivative, and a pharmaceutical preparation, and a water-insoluble or sparingly soluble backing layer. Yanagibashi *et al.* state that "it is impossible to achieve an adhesive device for application to body tissue without all three components, that is, acrylic acid polymer, water insoluble cellulose derivative and water insoluble or sparingly soluble backing layer". The Yanagibashi patent does not teach the use of film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the Yanagibashi patent does not teach the use of pH-sensitive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

As illustrated, the references described above appear to lack preferred compositions and properties for an efficacious and pharmaceutically acceptable bioadhesive delivery system. Namely, the references do not describe primarily aqueous-based film-forming gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers that form films due to changes in pH and/or desolvation of the polymers. Further, the references do not describe pH-sensitive mucoadhesive wax-film composites intended to remain adhered to the skin or mucosal surface for a prolonged period of time.

SUMMARY OF THE INVENTION

The invention is directed to a pharmaceutical gel composition comprising:

a solvent vehicle,

at least one water-insoluble swellable mucoadhesive polymer,

at least one pH-sensitive film-forming polymer, and
at least one molecule of interest.

The solvent vehicle may be comprised of at least 25 to 100 parts water or buffered water with 0 to 75 parts of ethanol, propylene glycol, glycerin, polyethylene glycol, or combinations thereof. The water-insoluble swellable mucoadhesive polymer may be polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol, wherein the water-insoluble swellable mucoadhesive polymer is preferably, Noveon or Carbomer. The water-insoluble swellable mucoadhesive polymer may be present at a concentration of from 0.1% to 20% by weight.

The pH-sensitive polymer is a copolymer of methacrylic acid and acrylic or methacrylic ester. Preferably, it is present at a concentration of from 0.05% to 10% by weight. More preferably, the polymer is a Eudragit polymer, or a chemical derivative thereof.

The molecule of interest may comprise an active pharmaceutical such as an antimicrobial, antiviral, antiinflammatory, antiseptic, antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent, a diagnostic agent, or combination thereof. Preferably, the molecule of interest is amlexanox, triclosan, peptide, protein, hirudin, plasmid DNA, lidocaine, benzocaine, dyclonine, or benzodiazepine drug or a derivative thereof.

The invention is also directed to a pharmaceutical gel which when applied to the skin or mucosal surface forms a film, said gel comprising a solvent vehicle, at least one water-insoluble swellable mucoadhesive polymer, at least one pH-sensitive film-forming polymer, and at least one molecule of interest, wherein said film is formed due to changes in pH and desolvation of the polymer, and wherein said film provides for the delivery of the molecule of interest to or through the application site. The solvent vehicle may be comprised of at least 25 to 100 parts water with 0 to 75 parts of ethanol, propylene glycol, glycerin, polyethylene glycol, or combinations thereof. The water-insoluble swellable mucoadhesive polymer may be polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol. Preferably, the water-insoluble swellable mucoadhesive polymer is Noveon or Carbomer. The water-insoluble swellable mucoadhesive polymer may be present at a concentration of from 0.1% to 20% by weight.

The pH-sensitive polymer may be a copolymer of methacrylic acid and acrylic or methacrylic ester. Preferably, it is present at a concentration of from 0.05% to 10% by weight. More preferably, the polymer is a Eudragit polymer, or a chemical derivative thereof.

The molecule of interest may comprise an active pharmaceutical such as an antimicrobial, antiviral, antiinflammatory, antiseptic, antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring

agent, a diagnostic agent, or combination thereof. Preferably, the molecule of interest is amlexanox, triclosan, peptide, protein, hirudin, plasmid DNA, lidocaine, benzocaine, dyclonine, or benzodiazepine drug or a derivative thereof. The application site may be the skin, mouth, vagina, nose, nasal cavity, or other accessible mucosal site.

The invention is also directed to a wax-film composite comprised of a pH-sensitive mucoadhesive layer and a water-insoluble wax layer. The pH-sensitive mucoadhesive layer may be present at a concentration of 20% to 90% by weight, and the water-insoluble wax layer may be present at a concentration of 10% to 80% by weight. The pH-sensitive mucoadhesive water-insoluble layer may be comprised of:

- at least one water-insoluble swellable mucoadhesive polymer,
- at least one pH-sensitive film-forming polymer, and
- at least one molecule of interest.

The water-insoluble swellable mucoadhesive polymer is polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol. Preferably, the water-insoluble swellable mucoadhesive polymer is Noveon or Carbomer. The water-insoluble swellable mucoadhesive polymer may be present in the pH-sensitive mucoadhesive layer at a concentration of from 0.1% to 20% by weight.

The pH-sensitive polymer may be a copolymer of methacrylic acid and acrylic or methacrylic ester. Preferably, it is present at a concentration of from 0.05% to 10% by weight. More preferably, the polymer is a Eudragit polymer, or a chemical derivative thereof.

The molecule of interest may comprise an active pharmaceutical such as an antimicrobial, antiviral, antiinflammatory, antiseptic, antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent, a diagnostic agent, or combination thereof. Preferably, the molecule of interest is amlexanox, triclosan, hirudin, plasmid DNA, lidocaine, benzocaine, dyclonine, or benzodiazepine drug or a derivative thereof. The wax-film composite may comprise at least one water-insoluble pharmaceutical wax having a melting point between 40°C and 100°C and at least one water-soluble or water-swellaable polymer.

The wax may be DENTSPLY® Utility Wax, beeswax, emulsifying wax, microcrystalline wax, carnauba wax, paraffin wax, white wax, yellow wax, or other suitable pharmaceutical wax. The water-soluble or swellable polymer may be present in the insoluble wax layer at a concentration of 0.05 to 10% by weight. The water-soluble or swellable polymer may be tragacanth, polyvinyl pyrrolidone, polyvinyl alcohol, cross-linked polyacrylic acid, polyethylene glycol, a cellulose polymer derivative, or other suitable pharmaceutical polymer that is water-

soluble or water-swellable. In the wax-film composite, the molecule of interest may be contained in and released from either the pH-sensitive mucoadhesive layer or the water-insoluble wax layer.

The molecule of interest comprises an active pharmaceutical compound such as an antimicrobial, antiviral, antiinflammatory, antiseptic, antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent, a diagnostic agent, or a combination thereof. The molecule of interest may comprise an active pharmaceutical such as an antimicrobial, antiviral, antiinflammatory, antiseptic, antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent, a diagnostic agent, or combination thereof. Preferably, the molecule of interest is amlexanox, triclosan, protein, peptide, hirudin, plasmid DNA, lidocaine, benzocaine, dyclonine, or benzodiazepine drug or a derivative thereof. Preferably, the hirudin may be complexed with a substance of opposite charge. More preferably, the substance of opposite charge may be chitosan or protamine. Alternatively, the molecule of interest may be a plasmid DNA or plasmid DNA complexed with a substance of opposite charge such as chitosan, protamine, or a cationic lipid.

The application site for the wax-film composite may be the skin, mouth, vagina, nasal cavity, or other accessible mucosal site. Preferably, the wax-film composite adheres to the application site for at least 1 hour. Preferably, the wax-film composite has a total thickness of less than 5 mm.

The invention is also directed to a method of making the above pharmaceutical gel composition, comprising:

- (i) adding a mucoadhesive polymer to a stirring water to form a solution that is clear and viscous,
- (ii) adding the pH-sensitive film-forming polymer to the solution formed in step (i) and measuring the pH of the solution,
- (iii) adding the molecule of interest to the solution of step (ii), and
- (iv) forming the final gel composition.

The invention is also directed to a method of making the above wax-film composite, comprising:

- (i) forming a homogeneous mucoadhesive film by fusing a mucoadhesive polymer and a pH-sensitive film-forming polymer,
- (ii) homogeneously coating one side of the mucoadhesive film of step (i) with a melted wax composition, and

(iii) drying the wax composition to form the wax-film composite, wherein the molecule of interest is added in either step (i) or (ii), or the molecule of interest is added to the mucoadhesive side after the wax-film composite is formed.

Furthermore, the invention is also directed to a method of treating a disease or an illness by administering to a person in need thereof, the gel or the wax-film composite as described above comprising a molecule of interest.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will become more fully understood from the detailed description given hereinbelow, and the accompanying drawings which are given by way of illustration only, and thus are not limitative of the present invention, and wherein;

Figure 1 shows a summary of the compositions of the invention

Figure 2 shows *in vitro* adhesion time of ¼ inch wax-film composites (n=3 each) on glass submerged in 40 mM KH₂PO₄/NaOH buffer, pH 6 at 37°C (stirred at 100 rpms). Each mucoadhesive layer contains from 1.2 mg to 11.2 mg of total polymer comprised of Noveon and Eudragit S100 in a weight ratio of 3:1. The wax layer consists of DENTSPLY® Utility Wax containing 1% w/w tragacanth polymer. See Table 2 and Example 9 for additional details.

Figure 3 shows a standard curve for plasmid DNA in solution as measured by the PicoGreen DNA Quantitation Kit (from Molecular Probes, Inc., Eugene, OR).

Figure 4 shows: (A) The reaction kinetics of Chromozym-TH and uncomplexed thrombin. Hirudin was added to excess thrombin and residual uncomplexed thrombin was assayed spectroscopically after the addition of Chromozym-TH. Hirudin was added to the 1.0 ml volume in the following amounts: 0 ng (□), 16.9 ng (●), 33.8 ng (Δ), 50.7 ng (▲), 67.6 ng (○), and 84.4 ng (■). (B) Calibration curve for released hirudin in solution. The figure was made by plotting the difference between the thrombin base value absorbance (thrombin in the presence of substrate only) and the hirudin sample values versus the concentration of hirudin standards from A.

Figure 5 shows an HPLC Standard Curve for Hirudin. Three standard curves for hirudin were generated using a C18 Nucleosil® 5 µm analytical column (4.6 x 250 mm). The mobile phase consisted of water:acetonitrile:trifluoroacetic acid (59.95:40:0.05 w/w/w). The flow rate was 0.6 mL/min. Each 50 µL sample of each standard curve was injected three times and the average AUC was plotted.

Figure 6 shows the Particle Size of Hirudin/Chitosan Complexes in water made with hirudin at a Concentration of 0.5 mg/mL.

Figure 7 shows the Zeta Potential of Hirudin/Chitosan Complexes in water made with Hirudin at a Concentration of 0.1 mg/mL. Zeta Potential values are reported as the mean and standard deviation of three measurements.

Figure 8 shows the stability of Hirudin/Chitosan Complexes (4:1 w/w) made in water as function of Hirudin concentration and storage time. All samples were stored at 4°C. For the Day 7 sample analysis, exactly 100 μ L of Hirudin/Chitosan complexes in water was diluted in 900 μ L ethanol to determine the stability of the complexes in ethanol.

Figure 9 shows the weight ratio of Noveon (N) and Eudragit S-100 (S) affected the adhesion time of the bilayer films to a glass matrix. The 3/8-inch bilayer films were prepared as mentioned in Materials and Methods. Films (n=5-9) were wetted with 2 μ L of phosphate buffered saline buffer, attached to microscope cover glass, and then submerged into 10 mM PBS, pH 6.0 maintained in a 37°C waterbath rotating at 100 rpm. The detachment time of the bilayer films from the cover glass was recorded and reported as the adhesion time (mean $\pm$ standard deviation). * Indicates that the adhesion time for the 3N:1S film was significantly longer than that of the others. Statistical analysis was completed using a two-sample t-test, assuming equal variances. A p value $\leq$ 0.05 was considered to be significant.

Figure 10 shows the release profile of pDNA from preloaded 3/8-inch bilayer films. Bilayer films (n=5) with pDNA (10 μ g/film) preloaded into the mucoadhesive layer were submerged in 10 mM PBS, pH 7.4 maintained in a 37°C waterbath rotating at 100 rpm.

Figure 11 shows the release profile of pDNA from postloaded 3/8-inch bilayer films. Bilayer films (n=5) with pDNA (10 μ g/film) postloaded on the mucoadhesive layer were submerged in 10 mM PBS, pH 7.4 maintained in a 37°C waterbath rotating at 100 rpm.

Figure 12 shows the effect of pDNA loading methods on the adhesion time of the bilayer films to glass matrix. Placebo 3/8-inch films and bilayer films, preloaded or postloaded with pDNA (10 μ g/film), were attached to glass microscope cover slips and submerged in 10 mM PBS buffer, pH. 6.0 maintained in a 37°C waterbath rotating at 100 rpm. The detachment time of the bilayer films from the cover glass was recorded and reported as the adhesion time (mean $\pm$ standard deviation, n=5). ANOVA test did not demonstrate any significant difference between the adhesion times of the three kinds of films (p = 0.43).

Figure 13 shows the release profile of postloaded β -galactosidase (Gal) protein (●) from bilayer films. Five hundred μ g of β -galactosidase was postloaded on the mucoadhesive layer of the films (n=5). β -galactosidase release was monitored in 10 mM PBS, pH, 7.4 maintained in a 37°C waterbath rotating at 100 rpm. β -galactosidase concentration was measured using the MicroBCA Protein Quantification Kit (Pierce). β -galactosidase (Gal) enzymatic activity (□) was

measured using a protocol from Edge BioSystem with modification. The enzymatic activity was reported as the percent of the control β -galactosidase. Data were reported as mean $\pm$ standard deviation (n=3).

Figure 14 shows rabbit total β -galactosidase specific IgG titer in sera after buccal immunization with bilayer films. In this preliminary study, New Zealand White rabbits (n=2) were dosed on day 0, 7, and 14 with either pDNA (100 μ g) (Black bars) or β -galactosidase protein (100 μ g) (White Bars), both adjuvanted with 100 μ g of Cholera Toxin (CT), via the buccal mucosa using postloaded bilayer films. As a positive control, two rabbits were also immunized with β -galactosidase (100 μ g) adjuvanted with CT (100 μ g) by subcutaneous injection (Gray Bars). Naïve rabbits received no treatment other than anesthesia. Blood was withdrawn at day 0, 7, 14, 21, and 28 via the ear vein. IgG titer in the sera was determined by ELISA and reported for individual rabbits.

Figure 15 shows rabbit total β -galactosidase specific IgG titer in sera after buccal immunization with bilayer films. In a second rabbit study, rabbits (n=4) were immunized with pDNA (100 μ g) adjuvanted with CT (100 μ g) via the buccal mucosa (White Bars). Three rabbits (n=3) were immunized with β -galactosidase protein (100 μ g) adjuvanted with 'Alum' (100 μ g) by subcutaneous injection (Black Bars). Naïve rabbits (n=3) received no treatment other than anesthesia. Rabbits were dosed on day 0, 7, and 14. IgG titer in the sera for individual rabbits was determined by ELISA and reported as mean $\pm$ standard deviation (n=3-4). * indicates that the mean titer after both routes of immunization are significantly lower those at all other time points by both routes of immunization. ** indicates that the IgG titer in after subcutaneous injection was significantly lower than that after buccal immunization. Statistical analysis was completed using a two-sample t-test, assuming unequal variances. A p value $\leq$ 0.05 was considered to be significant.

Figure 16 shows the adhesion time of placebo wax-film composites ("placebo WFC") and testosterone wax-film composites ("testosterone WFC") to glass as a function of both temperature (25°C or 37°C) and the presence of sodium lauryl sulfate (0% or 3%) in 10 mM phosphate buffer, pH 6.0. The mucoadhesive layer of both placebo WFCs and testosterone WFCs were wetted with 25 μ L water and adhered to the inside of a 50 mL dry glass beaker. Ten (10) mL of the medium was added and the beakers were rotated at 120 rpm using a water bath the temperature indicated. The adhesion times for circular 3/8 inch diameter WFCs were visually determined and recorded. The data reported are the mean $\pm$ standard deviation for five replicates (n=5).

Figure 17 shows the *in-vitro* release profile of testosterone from testosterone WFCs as a function of both temperature (25°C or 37°C), the presence of sodium lauryl sulfate (0% or 3%) in 10 mM phosphate buffer, pH 6.0. to retain sink conditions, and the absence of the wax-backing layer (mono-layer film). For the studies, WFCs (3/8 inch diameter) were submerged in 10 mL buffer in glass scintillation vials under controlled stirring conditions. Five hundred microliter (500 μ L) aliquots were removed over time for assay and replaced with fresh medium. The data reported are the mean $\pm$ standard deviation for three replicates (n=3).

Figure 18 shows standard curve for testosterone in rabbit serum using a competitive testosterone immunoassay. Testosterone in 10 mM phosphate buffer, pH 6.0 with 3% SLS was spiked separately into rabbit serum to produce final testosterone concentrations ranging from 0-16 ng/mL. The concentration of free testosterone in the spiked serum samples was inversely proportional to the O.D. at 450 nm. A Best-Fit analysis was performed using three separate standard curves (N=3) and n=3 replicates/sample. For reference, the results are compared to the kit standard curve prepared in buffer (dotted line).

Figure 19 shows serum testosterone concentration in anaesthetized New Zealand White rabbits after both intravenous injection ("I.V.", n=3 rabbits) and transmucosal delivery ("Buccal", n=3 rabbits) of testosterone (4 mg dose). For transmucosal delivery, the mucoadhesive layer of the WFCs was pre-wetted with 33 μ L of 10 mM phosphate buffer, pH 6.0. The data reported are the mean of 2-5 replicates per sample. The overall CV of replicate measurements for the intravenous and buccal groups were 13.5% and 13.9%, respectively. The data for the naïve rabbits (n=2 rabbits) are not shown, but were measured to be at the baseline. The insert shows the serum testosterone levels (ng/mL) for the individual rabbits treated with the WFCs. All WFCs were removed from the buccal pouch of rabbits at 3 hours (180 minutes).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to delivery systems suitable for the topical and mucosal delivery of molecules of interest. Specifically, the invention relates to; 1) compositions of pH-sensitive mucoadhesive film-forming gels, 2) uses of pH-sensitive mucoadhesive film-forming gels, 3) compositions of pH-sensitive mucoadhesive wax-film composites, and 4) uses of pH-sensitive mucoadhesive wax-film composites. A summary of the compositions of the invention is shown in Figure 1.

The pH-sensitive mucoadhesive film-forming gel is a pharmaceutical gel which when applied to the skin or mucosal surface forms a film, said gel comprising a solvent vehicle, at least one water-insoluble swellable mucoadhesive polymer, at least one pH-sensitive film-forming

polymer, and at least one molecule of interest, said film forming due to changes in pH and desolvation of the polymer, said film providing for the delivery of the molecule of interest to or through the application site.

The pH-sensitive mucoadhesive wax-film composite is a bi-layer pharmaceutical film less than 5 mm in diameter that when applied to the skin or mucosal surface adheres to the application for at least 1 hour. The wax-film composite is comprised of a pH-sensitive mucoadhesive layer and a water-insoluble wax layer.

Definitions and Preferred Embodiments

As used herein, certain terms may have the following defined meanings.

As used in the specifications and claims, the singular form *a*, *an* and *the* include plural references unless the context clearly dictates otherwise. For example, the term *a pharmaceutical* may refer to one or more pharmaceuticals for use in the presently disclosed formulations and methods.

The term *molecule of interest* is defined as any synthetic or naturally occurring substance including elements, radioactive elements, synthetic or natural small molecules, peptides, proteins, nucleic acids, or any combinations thereof.

The term *active pharmaceutical* means any synthetic or naturally occurring substance including elements, radioactive elements, synthetic or natural small molecules, peptides, proteins, nucleic acids, or any combinations thereof intended to be administered to a warm-blooded mammal to elicit a pharmacological response. The terms *pharmaceutical*, *active pharmaceutical*, and *drug* as used herein are identical in meaning and thus are used interchangeably. It is preferred that the active pharmaceutical be an anti-microbial, antiviral, anti-inflammatory, antiseptic, antihistamine, tranquilizer, sedative, anti-nausea, local anesthetic, disinfectant, keratolytic, analgesics including anti-migraine, anti-fungal, sweetener, flavoring agent, diagnostic agent, peptide, protein, antigen, monoclonal antibody, polyclonal antibody, nucleic acid, or combinations thereof. More preferably, the active pharmaceutical is amlexanox, triclosan, lidocaine, dyclonine, benzocaine, a benzodiazepine, hirudin, antisense oligonucleotide, or plasmid DNA, or any derivative or combinations thereof.

Amlexanox: The chemical name of Amlexanox is 2-amino-7-(1-methylethyl)-5-oxo-5H-[1]benzopyrano-[2,3-b]-pyridine-3-carboxylic acid. Amlexanox is the active ingredient in the commercial product, Aphthasol, which accelerates the healing of aphthous ulcers in the mouth by an unknown mechanism (Binnie *et al.* 1997; Khandwala *et al.* 1997a; Khandwala *et al.* 1997b). The Aphthasol product is a 5% paste. In cell culture experiments, Amlexanox has been

shown to inhibit the formation or release of inflammatory mediators such as histamine and leukotrienes from mast cells, neutrophils, and mononuclear cells (Urisu *et al.* 1990). Further, in animals, Amlexanox has anti-allergic and anti-inflammatory properties and has been shown to inhibit both immediate and delayed hypersensitivity reactions (Saijo *et al.* 1985; Saijo *et al.* 1986). Amlexanox has very poor solubility in ethanol (0.58 mg/mL) and water (0.005 mg/mL). However, the solubility of Amlexanox increases with pH.

Triclosan: The chemical name of Triclosan is 2,4,4'-Trichloro 2'-hydroxy-diphenyl-ether 5-chloro 2-(2,4-dichlorophenoxy)-phenol. Triclosan is a broad-spectrum antibacterial that has activity against a wide range of gram-positive and gram-negative bacteria (Zuckerbraun *et al.* 1998). Triclosan has been shown to inhibit bacterial fatty acid synthesis at the enoyl-acyl carrier protein reductase (FabI) step (Heath *et al.* 1999). It has gained wide-spread use as antibacterial agent that is used in toothpastes, kitchen utensils and toys. Triclosan has also been formulated in mouthwashes (Mandel 1994) and liposomes (Jones *et al.* 1993). Triclosan has a pKa of 7.9 and is insoluble in water, except in alkaline solutions where it is readily soluble (Loftsson *et al.* 1999). However, triclosan is soluble in ethanol and polyethylene glycol 400 and many other organic solvents.

Hirudin: Hirudin is the most potent and specific known inhibitor of thrombin ($K_i = 0.2$ pM) (Markwardt 1989; Markwardt 1991a; Markwardt 1991b; Fenton *et al.* 1991). Hirudin was originally isolated from medicinal leeches (*Hirudo medicinalis*), but is now available in large quantities due to recombinant techniques (Riehl-Bellon *et al.* 1989). Unlike heparin, hirudin does not require endogenous cofactors to function and does not have associated bleeding complications. Hirudin is a relatively small 65 amino acid peptide (Mw 7000) with remarkable stability over a broad range of pH (pH 3-10), temperature (i.e., up to 60°C), and solvent conditions (Chang 1991; Eric and Caroline 1991). The main structure of hirudin is characterized by an apolar disulfide knot with a very anionic (negatively-charged) tail. The apolar knot of hirudin masks the catalytic site of thrombin by interacting with its apolar site (i.e., via hydrophobic interaction). The anionic tail of hirudin (18 amino acids) interacts with the anion-binding exosite of thrombin. It is the combination of the apolar binding and the anion-binding that accounts for the very high affinity of hirudin and thrombin. The presence of the apolar knot and anionic tail on hirudin results in both hydrophobic and hydrophilic surface interactions. For example, in solution, hirudin forms micellar complexes (e.g., tetramers) with itself. Further, the apolar region of hirudin is known to interact with cell membranes while the anionic tail remains extracellular (Fenton 1989). It is likely that the anionic portion of hirudin is electrostatically repelled from the surface of cell membranes. Novartis Pharma obtained marketing approval in

the European Union in 1998 for REVASC (desirudin; recombinant hirudin) for the prevention of deep-vein thrombosis following knee and hip replacement surgery. In clinical trials, patients receiving hirudin by subcutaneous injection twice a day for 8 days had a nearly 30% lower overall rate of deep-vein thrombosis than those who received low molecular weight heparin (Eriksson *et al.* 1997). Novartis is also performing clinical trials (Phase III) for the use of hirudin in acute coronary syndrome. Several other indications for hirudin are being pursued such as stasis-induced venous thrombosis, diffuse microthrombosis, and hemodialysis (Markwardt 1989). Most of the potential applications of hirudin require blood levels between 80 to 3500 ng/mL (Markwardt 1989, Markwardt 1991). A potential problem with the use of injected forms of hirudin is that there are no effective antidotes for hirudin overdosing. If upon frequent injection, the wrong dose is given, there are no methods to turn the activity of hirudin off. For this reason alone, hirudin is a good candidate for transmucosal delivery via the buccal tissue. It is envisioned that the pH-sensitive mucoadhesive film-forming gel or wax-film composite containing hirudin, can easily be applied to the buccal tissue in the mouth and provide a means to safely control the dosing and dose scheduling of hirudin.

Benzodiazepines: Benzodiazepines are among the most frequently prescribed drugs in the Western world. All benzodiazepine agonists exert similar pharmacodynamic actions (e.g., activate central GABAergic neuroinhibition, thereby inducing anxiolysis, sedation/hypnosis, anticonvulsion and muscle relaxation. In turn, benzodiazepines are used for the treatment of seizures and insomnia, preoperative or procedural sedation, and for the treatment of nausea and anxiety. Most if not all of the benzodiazepine bases have little or no solubility in water and are very lipophilic (Hjortkjaer *et al.* 1999, Bechgaard *et al.* 1997). As a result, a great deal of research has gone into developing efficacious dosage forms of these drugs including; *i*) synthesizing water-soluble derivatives such as their hydrochloride salts, *ii*) developing injectable formulations of the benzodiazepine bases that do not result in precipitation of the drugs, and *iii*) exploring alternative routes of administration including intranasal, rectal, and buccal. Previous work has shown that intranasal doses of the benzodiazepine bases dissolved in organic solvents lead to pharmacological responses with suitable pharmacokinetic profiles. However, the repeated use of organic solvents such as ethanol, polyethylene glycol, and propylene glycol for intranasal administration is not desirable. Alternatively, benzodiazepines have been given by buccal administration with very favorable results (Schwagmeier *et al.* 1998; Scott *et al.* 1999). For example, Schwagmeier *et al.* showed that the maximum plasma concentration of midazolam was 55.9 ng/mL at 30 minutes following buccal administration of 5 mg midazolam with a mean bioavailability of 74.5%. Scott *et al.* reported that the buccal administration of 10 mg

midazolam per 2 mL stopped 75% (30/40) seizures in children within an average time of 6 minutes after administration. Thus, buccal administration of benzodiazepines may be an attractive alternative to injection or intranasal administration. It is envisioned that the said pH-sensitive mucoadhesive film-forming gel or wax-film composite containing a benzodiazepine can easily be applied to the buccal tissue in the mouth and provide a means to safely and reproducibly deliver doses of the benzodiazepines for the treatment of seizures and insomnia, preoperative or procedural sedation, and for the treatment of nausea and anxiety. Benzodiazepines suitable for delivery are, but not limited to, alprazolam, camazepam, clobazam, clonazepam, desmethyldiazepam, diazepam, flunitrazepam, flurazepam, halazepam, lorazepam, lormethazepam, midazolam, nitrazepam, oxazepam, pinazepam, prazepam, triazolam, and derivatives and combinations thereof.

The term *pharmaceutically acceptable* means that the substance is generally regarded to be safe when used in such a manner that is widely acceptable by those skilled in the art. For reference of acceptable materials, refer to THE UNITED STATES PHARMACOPEIA.

The term *pH-sensitive* is defined as a substance that is affected by changes in pH so that the substance changes conformation, charge, solubility, or combinations thereof. A *pH-sensitive polymer* is defined as polymer that is affected by changes in pH so that the polymer changes conformation, charge, solubility, or combinations thereof. A *pH-sensitive film-forming polymer* is defined as polymer that is affected by changes in pH so that the polymer changes conformation, charge, solubility, or combinations thereof resulting in the precipitation, desolvation, or settling of the polymer into a film. For the purposes of this invention, it is preferred that the pH-sensitive film-forming polymers have some solubility in aqueous-based vehicles above pH 5 but limited or no solubility below pH 5. It is more preferred that the pH-sensitive film-forming polymers have some solubility in aqueous-based vehicles above pH 6 but limited or no solubility below pH 6. It is most preferred that the pH-sensitive film-forming polymers have some solubility in aqueous-based vehicles above pH 7 but limited no solubility below pH 7. Examples of pH-sensitive film-forming polymers that meet these criteria are, but not limited to, Eudragits® and cellulose acetate phthalate polymers, or derivatives thereof.

Eudragits® are synthetic cationic and anionic polymers of methacrylic acid and methacrylic acid esters in varying ratios. The polymers are generally regarded as safe and are included in the FDA Inactive Ingredients Guide (for oral capsules and tablets). The presence of positive charge (provided by the dimethylaminoethylmethacrylates), neutral charge (provided by the methacrylic acid esters) and negative charge (provided by the methacrylic acid) residues provides for interesting and useful solubility characteristics as a function of pH. In general, the

cationic Eudragits are soluble at lower pHs while the anionic Eudragits are soluble at higher pHs. Neutral Eudragits usually lack aqueous solubility at any pH. For the purposes of this invention, any Eudragit polymer or derivative thereof is preferred. Even more preferred is any anionic Eudragit or derivative thereof. Most preferred is Eudragit L100-55, Eudragit L100, and Eudragit S100 or derivatives thereof. Depending on the type of Eudragit polymer selected and its solubility as function of pH, films having different properties can be prepared. Eudragit gels have been reported for the sustained-release of drugs via rectal administration (Goto *et al.* 1991; Kawata *et al.* 1991; Kim *et al.* 1992a; Kim *et al.* 1992b; Kim *et al.* 1992c; Umejima *et al.* 1993). Eudragit has also been used in buccal patches (Wong *et al.* 1999). Wong *et al.* described the use of Eudragit patches with the water-soluble cellulose-based polymers and swellable Carbopol polymers to deliver a model drug. However, for these studies Wong *et al.* utilized Eudragit NE40D, a neutral poly(ethylacrylate methylmethacrylate) polymer that is insoluble at all pHs (non-pH-sensitive). Eudragit has also been used to film-coat tablets for pH-dependent colon targeting after oral delivery (Khan *et al.* 1999). Cationic Eudragit E, which is soluble at low pH, has also been proposed for the pH-controlled drug release from mini-tablets for the treatment of inflammatory bowel disease (Leopold and Eikeler, 1998). No references or other prior art could be found that describe the use of mucoadhesive gels comprised of anionic polymers, that when applied to the skin or mucosal surfaces, form films due to effect of pH on the solubility of the anionic polymers.

Solubility refers to the extent to which a solute is dissolved in a solvent. Solubility can be described in terms such as described in REMINGTON'S PHARMACEUTICAL SCIENCES 18th edition, 1990, ranging from very soluble (less than 1 part of solvent per 1 part of solute) to insoluble (more than 10,000 parts of solvent for 1 part of solute). The term *water-insoluble* refers to a substance or solute where more than 10,000 parts of water are needed to dissolve 1 part of solute. However, it is contemplated that certain advantages can be obtained with layers that are generally water-insoluble. The term *generally water-insoluble* refers to a substance (e.g., layer, gel, film and/or composite) or solute where more than 10,000 parts of water are needed to dissolve 10 parts of solute; preferably more than 20,000 parts of water are needed to dissolve 10 parts of solute. A water-insoluble swellable substance refers to a substance or solute that is water-insoluble but absorbs water and swells to form a colloidal dispersion. A *colloidal dispersion*, as defined by REMINGTON'S "consists of at least two discrete phases", namely a solid phase and a liquid phase. The term *desolvation* refers to phenomena whereby a solvent such as water diffuses from a swelled substance leaving primarily one solid phase comprised of the substance. A *gel* is defined as a semisolid consisting of particles interpenetrated by a liquid.

A *gel solution* is defined as a gel which has a molecule of interest dissolved in solution in the liquid phase. A *gel suspension* is defined as a gel which has a molecule of interest suspended in the liquid phase.

A *wax* refers to any water-insoluble substance composed of hydrocarbons, alcohols, fatty acids, and esters that are solids at temperatures below 40°C, but liquids at temperatures of above 40°C. Suitable waxes are, but not limited to the following, DENTSPLY® Utility Wax, beeswax, emulsifying wax, microcrystalline wax, carnauba wax, paraffin wax, white wax, yellow wax, or other suitable pharmaceutical wax. It is preferred that the wax has a melting temperature between 40°C and 100°C, more preferred that the wax has a melting temperature between 40°C and 80°C, and most preferred that the wax has a melting temperature between 40 °C and 65 °C.

A *wax-film composite* refers to a bi-layer film comprised of a pH-sensitive mucoadhesive layer and a water-insoluble wax layer. A molecule of interest may be loaded in and released from either the said pH-sensitive mucoadhesive layer or said water-insoluble wax layer. It is preferred that the molecule of interest is present in the wax-film composite at a concentration of 0.001% to 20% by weight, more preferably at a concentration of 0.001% to 10% by weight, and most preferably at a concentration of 0.001% to 5% by weight. It is preferred that the pH-sensitive mucoadhesive layer is present at a concentration in the wax-film composite of 20% to 90% by weight, more preferably at 30% to 80% by weight, and most preferably at 40% to 70% by weight. Preferably, the water-insoluble wax layer is present at a concentration in the wax-film composite of 10% to 80% by weight, more preferably at 20% to 70% by weight, and most preferably at 30% to 60% by weight. For bonding the two layers of the bi-layer wax-film composite, it is preferred that the water-insoluble wax layer contain at least one water-soluble or water-swellaable agents such as, but not limited to, tragacanth, polyvinyl pyrrolidone, polyvinyl alcohol, cross-linked polyacrylic acid, polyethylene glycol, a cellulose-based polymer or derivative thereof, a cross-linked polyacrylic acid polymer or derivative thereof, or other suitable pharmaceutical polymers that are water-soluble or water-swellaable. Preferably, the water-soluble or water-swellaable agents are present in the wax layer at a concentration of 0.05% to 10% by weight, more preferably at 1% to 8% by weight, and most preferably at 1% to 5% by weight. It is envisioned that the wax-film composite may be applied to any readily accessible topical site including, but not limited to the following; any skin surface, rectal, vaginal, nasal cavity, any location in the mouth, or other accessible topical or mucosal surfaces. A dry wax-film composite will readily adhere to the surfaces described when the surfaces are wet with saliva or other bodily fluids. However, for application to dry skin, it is envisioned that the dry wax-film composite could be easily wetted using tap water or another appropriate vehicle to

promote subsequent adherence of the film to the skin. It is preferred that the wax-film composite remain adhered to the application site for at least 1 hour, more preferably for at least 75 minutes, and most preferably for at least 90 minutes.

The term *mucoadhesive* refers to a substance that sticks to or adheres to the skin or mucosal surfaces by forces that are measurable and by any number of mechanisms such as, but not limited to the following: hydrogen-bonding, ionic interaction, hydrophobic interaction, van der Waals interaction, or combinations thereof.

Mucosal delivery is defined as the application of a formulation or delivery system containing one or more active pharmaceuticals to a mucosal site for the purpose of eliciting a pharmacological response at the site of application or alternatively, for the active pharmaceutical to be absorbed through the mucosal membrane into the systemic circulation. *Mucosal sites* applicable to the application of a formulation or delivery system are, but not limited to the following, vaginal, mouth, rectal, intranasal, and the eye.

Transmucosal Delivery: Peptides, proteins, and other larger molecules are becoming increasingly more important as scientists search for new drugs to treat serious human diseases. Most often, these new larger molecules have to be injected since bioavailability by other routes is low. However, developing injectable large molecule drugs is expensive and patient compliance is often low. Researchers in the field of drug delivery have sought for decades for methods to dose larger molecules like peptides and proteins orally. However these drugs are prone to rapid and extensive degradation due to the low pH of the stomach as well as to peptidases and proteolytic enzymes throughout the gastrointestinal tract. Alternative routes have been sought such as transmucosal delivery via the lung, nose, and buccal tissue. The foreseen advantages of transmucosal delivery are; *i*) a faster onset of activity (i.e., achieve higher drug blood levels faster), *ii*) the avoidance of the first hepatic pass metabolism and the related increase of bioavailability, and *iii*) the avoidance of side effects related to GI absorption (Rathbone *et al.* 1996). However, the barriers for effective transmucosal delivery (i.e., buccal delivery) are similar to those for oral delivery. For example, the oral mucosal tissue is also characterized by a mucous layer that lines the epithelia. The mucous layer provides a substantial physical barrier as well as a chemical barrier due to the presence of a multitude of peptidases and proteolytic enzymes. In theory, delivery systems designed to adhere to this mucous layer (i.e., mucoadhesives) can serve to protect peptides from rapid and/or extensive degradation (Bodde *et al.* 1990; Duchene *et al.* 1988; Harris and Robinson 1990). The mechanism of mucoadhesion is complicated but is known to involve hydrogen bonding between the components of the delivery systems (i.e., polymers) and sialic acid residues of mucin (0.5-2.0% glycoprotein), the primary

substance of mucous. The viscosity of the mucous layer is largely determined by the type and amount of the glycoproteins present. Usually, the mucous layer in the oral mucosa is about 500-600 μm thick and has a pH of 5.8-7.1. The combination of the mucous layer and the epithelial layer provides a substantial barrier for significant drug absorption. This is especially true for peptide drugs that are prone to a high degree of proteolytic degradation in the mucous layer. Further, if the peptide drugs are anionic, the negatively-charged epithelial membrane below the mucous provides a repulsive electrical "fence" of sorts.

In general, the oral transmucosal bioavailability of peptides is low (<5%), but can be increased with the use of penetration enhancers designed to disrupt the mucosal and epithelial membranes (Sayani *et al.* 1993; Bhatt and Johnston, 1996; Bayley *et al.* 1995; Bhatt and Johnston, 1997). The most often used types of penetration enhancers are surfactants (i.e., sodium lauryl sulfate), bile salts (i.e., sodium deoxycholate), and fatty acids (i.e., oleic acid). Although these penetration enhancers typically increase the absorption of peptides, the safety and long term use of these agents is of concern. Further, these penetration enhancers, by design, do not function to specifically protect peptides from proteolytic degradation in the mucosal membrane. Alternatively, a plausible strategy is to use more pharmaceutically acceptable excipients to physically protect peptides from rapid and extensive proteolytic degradation, rather than to rely on chemical means to disrupt biological membranes. For example, to enhance the transmucosal delivery of negatively-charged peptides, the peptides may be complexed with pharmaceutically acceptable excipients that have positive charges such as, but not limited to, chitosan, polylysine, or protamine. It is thought that complexation will serve two functions; *i*) decrease electrostatic repulsion of the peptide drugs with the negatively-charged epithelial membrane, and *ii*) decrease the extent of degradation of the peptide drugs by proteolytic enzymes in the mucosal membrane.

A *complex* refers to the physical interaction between two substances whereby a definite stoichiometry exists. Complexation can occur by any number of mechanisms, including, but not limited to: hydrogen-bonding, ionic interaction, hydrophobic interaction, van der Waals interaction, or combinations thereof. It is envisioned that molecules of interest formulated in said delivery systems may be complexed with various pharmaceutically acceptable agents to enhance their efficacy by any number of mechanisms described above, or those still unknown.

Chitosan: Chitosan is a biodegradable polysaccharide composed of two subunits, D-glucosamine and N-acetyl-D-glucosamine linked together by $\beta(1,4)$ glycosidic bonds. When dissolved in solution, the amino groups present in the glucosamine subunits (pKa 6.5) have a positive charge (Henriksen *et al.* 1997; Schipper *et al.* 1996). Chitosan is known to be a

mucoadhesive, and interacts ionically with the negatively charged sialic acid residues in mucin (Lehr *et al.* 1992). Chitosan has been used as a component of mucoadhesive systems (Takeuchi *et al.* 1996), as well as a controlled release and drug carrier systems (Berthold *et al.* 1993; Shiraishi *et al.* 1993). Chitosan has also been found to increase the nasal absorption of insulin and calcitonin (Illum *et al.* 1994; Aspden *et al.* 1996), although the mechanism by which this occurs is not clear. Finally, chitosan has been reported to form complexes with many negatively-charged molecules including indomethacin (Imai *et al.* 1991), sodium hyaluronate (Takayama *et al.* 1990), and pectin and acacia (Meshali *et al.* 1993). One problem that may be encountered with the use of chitosan (100 kDa) is the poor solubility of 100 kDa chitosan at neutral pH. This lack of solubility may hinder its ability to form complexes with hirudin or our ability to study the complex. A solution to this problem may be to obtain lower molecular weight chitosans (i.e., 10 kDa or 50 kDa). However, lower molecular weight chitosans are not commercially available. In our previous work with chitosan (MacLaughlin *et al.* 1998), we obtained lower molecular weight chitosans using a modified depolymerization technique first described by Peniston *et al.* (1975). This depolymerization process is a relatively simple deamination reaction where chitosan is chemically treated with sodium nitrite in 6% acetic acid at 25°C for 1 hour. The amount of sodium nitrite used in the reaction determines the resulting chitosan molecular weight since the reaction is stoichiometric. For example, reacting 1 g of 100 kDa chitosan (Seacure 143) with 2% and 7% sodium nitrite results in final chitosan molecular weights of 50 kDa and 10 kDa, respectively. The depolymerized chitosan polymers are purified and ready to use. The molecular weight of each depolymerized chitosan polymer is determined by gel chromatography through a Beckman Ultraspherogel SEC2000 column with a 1 mL/min flow-rate of eluent 0.1M acetic acid/sodium acetate buffer containing 0.05M sodium chloride as the mobile phase.

Mucosal Vaccines: It is thought that the most effective vaccines for important viral and bacterial pathogens will require mucosal immunity since this is the site at which these pathogens infect the body (McGhee *et al.* 1992). However, all marketed vaccines except one (oral polio vaccine) are now administered by injectable routes and are ineffective at inducing mucosal immunity. A new promising flu vaccine (FlumistTM from Aviron) is currently being tested in phase III clinical trials. FlumistTM is administered as a nasal spray. Since the nasal associated lymphoid tissue (NALT) is part of the mucosal network, this vaccine is the first of potentially many new mucosal vaccines. Other mucosal routes are actively being pursued by researchers including the vaginal route and the mucosal regions of the gut (i.e., Peyer's patches). However, the one mucosal route that has been largely overlooked by researchers is the oral mucosal region of the mouth. This may be due to the lack of suitable delivery systems to retain and protect

antigen in the mouth for an extended period of time. We hypothesize that the oral mucosa may be an ideal site for vaccination for the following reasons: *i*) the oral mucosa is accessible, *ii*) administration to the oral mucosa is needleless, and *iii*) the oral mucosa is a preferred site for antigen presentation. Vaccination without the use of needles would provide a distinct advantage in terms of both cost and safety over conventional vaccines that must be given with needles. The oral mucosa is theoretically a preferred site for antigen presentation since in the normal course of a day our bodies are exposed to hundreds of antigens during eating and other hand-to-mouth activities. In most cases, the oral mucosal tissue, which comprises a high density of Langerhans cells, and T-lymphocytes as well as the mucosal associated lymphoid tissue (MALT), is our first line of defense against both viral and bacterial pathogens. The percentage of Langerhan cells in the oral mucosa, about 4% of the total cells, is comparable to the percentage of Langerhan cells found in the skin epidermis. Langerhan cells are immature dendritic cells that are thought to be the most potent antigen presenting cell type. However, the oral mucosa has about a 40-fold increase in the number of T-lymphocytes as compared to the skin. The presence of Langerhan cells and T-lymphocytes along with MALT tissue (i.e., tonsils, salivary glands, Waldeyer's Ring, and the pharyngeal lymphoid tissue) makes the oral mucosa a theoretically potent immunization site. For example, unlike vaccination by the skin, intravenous, subcutaneous, and muscle routes, vaccination via the oral mucosa route may result in both cellular and mucosal (humoral) immune responses. Finally, vaccination by the oral mucosa route to target the MALT remains a relatively untested strategy as compared to previous efforts to target the nasal-associated lymphoid tissues (NALT) or the gut-associated lymphoid tissue (GALT). One explanation for the lack of published results on vaccination by the oral mucosa route is the lack of suitable pharmaceutical formulations to retain and stabilize antigens at the site for a suitable length of time.

Oral Mucosal Delivery and Vaccination: Many reports in the literature have described the use of polymeric delivery systems to deliver drugs locally in the mouth or transmucosally into the systemic circulation. See Leung *et al.* (1991) for a review on delivery systems for the oral mucosa. However, to our knowledge no group has reported on the use of such delivery systems to deliver antigens, specifically DNA plasmids expressing antigens, in the oral mucosa. Two recent published studies have investigated the application of antigens alone (with no delivery systems) to the oral mucosa route for immunization. Etchart *et al.* (1997) showed that the use of a DNA plasmid encoding measles virus hemoagglutinin (HA) injected into the buccal mucosa of mice induced HA-specific cytotoxic-T-lymphocyte (CTL) responses. The CTL responses to expressed HA after DNA plasmid was applied to the buccal mucosa were much stronger than those responses after oral or intrajejunal immunization suggesting the feasibility of

the oral mucosa as a route of immunization. Also, Casetta and Negretti (1998) demonstrated that antigenic mixtures of several bacteria could induce specific salivary IgA after rubbing the mixtures on the rat gingival mucosa. These publications demonstrate the potential utility of the oral mucosa as a route of immunization for both genetic vaccines (i.e., DNA plasmids expressing antigens) and subunit (protein antigen) approaches.

Genetic Vaccines: After the concept of genetic immunization was first demonstrated by Johnston's group in 1992 (Tang *et al.*, 1992), numerous studies have reported the potential prophylactic and therapeutic use of genetic vaccines for combating various infectious diseases (Taubes, 1997; Ulmer *et al.*, 1996; Robinson and Torres, 1997; Liu *et al.*, 1995). Vaccines of this composition appear to be both efficacious in the short term and able to elicit a prolonged anamnestic response, which is capable of preventing or resolving infection when challenged up to one year after vaccination (Yankauckas *et al.*, 1993). Genetic vaccines elicit a broader immune response than do subunit vaccines. They induce both cellular and humoral responses that are reminiscent of attenuated and whole-killed viral vaccines. Further, genetic vaccines can be prepared with relative ease of synthesis and production. Expression plasmids can be generated quickly once the antigen's coding sequence is known and small- and large-scale DNA purification methods are well established. Genetic vaccines also avoid some of the safety concerns of conventional vaccines in that there is no chance of disease due to co-purification of contaminating virus or reversion of the attenuated strain in the patient.

Vaginal Delivery: Many types of dosage forms have been proposed for the vaginal delivery of drugs including gels, creams, suppositories, films, foams, and tablets. In designing a dosage form intended for vaginal application, one must consider multiple parameters including, but not limited to, *i*) the physico-chemical properties of the drug (i.e., solubility, stability, etc.), *ii*) the intended duration of action or activity, *iii*) the context in which the dosage form is used, and *iv*) patient preferences. For example, clinical studies have shown that a majority of women prefer a gel to a cream formulation and both of these over the other dosage forms listed above (Hardy *et al.* 1998a). Women also prefer a dosage form that has no odor or a pleasant mild odor and also one that has no color (Hardy *et al.* 1998b). The gel formulations will be able to be applied manually or by use of a simple applicator. Importantly for use a microbicidal agents, the applied gel formulations will be able to cover a large area of the mucosal tissue, adhere to the tissue, and potentially provide prolonged pharmacological activity of the molecule of interest.

A plethora of different mucoadhesive gel formulations have been described in the literature and several are now marketed products. Typically, the mucoadhesive component of the formulation is a biocompatible polymer, such as polyacrylic acid that is cross-linked with an

acceptable agent to create an insoluble gel. The use of an insoluble gel is desirable since it remains in contact with the mucosal tissue for much longer periods of time. Cross-linked polyacrylic acid polymers, such as Noveon and Carbomer, have been shown to stay attached to the mucosal lining in the vagina for up to three to five days (March and Nakamura, 1993). Further, gels containing Noveon and/or Carbomer have been used as vaginal lubricants so it is envisioned that the described gels may be used during sexual intercourse. Noveon and Carbomer-based polymers are weak acids and contain many negatively-charged carboxyl-groups. The multiple negative charges on these polymers promote hydrogen-bonding between the polymers and the negatively charged mucin, the main glycoprotein that allows for the attachment of mucus to the epithelial lining of the vaginal wall (Park and Robinson, 1985). Noveon and Carbomer-based polymers have been shown to have maximum hydrogen-bonding in the pH range of 4.0 to 6.0. This is ideal for use in the vagina which has a normal pH value of about 4.5 (Stevens-Simon et al., 1994; Garcia-Closas, et al., 1999). It is envisioned that gels comprised of pH-sensitive polymers and water-insoluble mucoadhesive polymers such as Noveon and Carbomer may provide superior delivery of molecules of interest to the vagina since the lower pH of the vagina will cause the pH-sensitive polymer to form a long lasting film to retain and/or deliver the molecule of interest in a more efficacious manner.

The following Examples are described to illustrate the different aspects of the invention. These Examples should not be used in any way to limit the scope of the invention.

EXAMPLES

Materials: Recombinant hirudin and bovine α -thrombin are from Sigma Chemicals (St. Louis, MO). Chromozym-TH is from Boehringer Mannheim. Chitosan Seacure 143 (85-90% deacetylated) is from Natural Biopolymer Inc. (Raymond, WA). All Eudragit polymers were obtained from Rohm America, Inc. (Piscataway, NJ). Noveon and Carbomers were obtained from BF Goodrich (Cleveland, Ohio). Glycerin, polyethylene glycol 400, isopropyl myristate, ethanol, sodium hydroxide, and propylene glycol were all of USP/NF grade and were purchased from Spectrum Quality Products, Inc. (New Brunswick, NJ). PicoGreen dsDNA Quantitation Kit was purchased from Molecular Probes, Inc. (Eugene, OR). DENTSPLY® Utility Wax was obtained from DENTSPLY International (York, PA).

EXAMPLE 1

A placebo pH-sensitive mucoadhesive film-forming gel was made as follows. Water (44.1 % w/w) was added to a 250 mL stainless steel beaker and stirring was begun at 200 rpm using a Caframo Stirrer. Noveon (0.5% w/w) and Carbomer 971 (0.8% w/w) were added very

slowly to the stirring water until the solution was clear and viscous with no visible solid material in solution. Glycerin (50.4% w/w) was then added to the polymers in water. Eudragit L100 (2.0% w/w) was added and the solution and the viscous solution became slightly milky in color and less viscous. 18% sodium hydroxide (2.2% w/w) was then added and the whitish gel became viscous. The pH of the placebo gel was measured by taking 1 mL of the gel and dispersing it into 5 mL water and measuring the pH after 1 hour. The pH of the gel was 6.3 ± 0.02 ($n = 3$). When the placebo gel was spread onto the skin of a human volunteer's hand; it produced a clear film. Placebo gel stored under controlled conditions at 25°C/60% Relative Humidity for 1 week and 1 month had pH of 6.2 ± 0.07 ($n = 3$) and 6.2 ± 0.06 ($n = 3$), respectively. Placebo gel stored under controlled conditions at 40°C/75% Relative Humidity for 1 week and 1 month had pH of 6.1 ± 0.03 ($n = 3$) and 6.1 ± 0.09 ($n = 3$), respectively. These results demonstrated that the placebo gel was stable when stored under the conditions tested.

EXAMPLE 2

A 5% triclosan pH-sensitive mucoadhesive film-forming gel suspension was made as follows. Water (39.0% w/w) was added to a 250 mL stainless steel beaker and stirring was begun at 200 rpm using a Caframo Stirrer. Noveon (0.5% w/w) and Carbomer 971 (0.65% w/w) were added very slowly to the stirring water until the solution was clear and viscous with no visible solid material in solution. Glycerin (50.6 g) was then added to the polymers in water. Eudragit L100 (2.0% w/w) was added and the solution and the viscous solution became slightly milky in color and less viscous. 18% sodium hydroxide (2.4% w/w) was then added and the whitish gel became viscous. Triclosan (5.0% w/w) was then added to produce a homogenous whitish gel suspension. The pH of the 5% triclosan gel was measured by taking 1 mL of the gel and dispersing it into 5 mL water and measuring the pH after 1 hour. The pH of the gel was 6.5 ± 0.1 ($n = 3$). When the 5% triclosan gel was spread onto the skin of a human volunteer's hand, it produced a clear film. The 5% triclosan gel was stored under controlled conditions at 25°C/60% Relative Humidity for 1 week and 1 month had pH of 6.5 ± 0.1 ($n = 3$) and 6.4 ± 0.1 ($n = 3$), respectively. 5% triclosan gel stored under controlled conditions at 40°C/75% Relative Humidity for 1 week and 1 month had pH of 6.3 ± 0.02 ($n = 3$) and 6.2 ± 0.1 ($n = 3$), respectively. These results demonstrated that the 5% triclosan gel was stable when stored under the conditions tested.

EXAMPLE 3

A 1% triclosan pH-sensitive mucoadhesive film-forming gel solution was made as follows. Propylene glycol (37.6% w/w) was added to a 250 mL stainless steel beaker and

stirring was begun at 200 rpm using a Caframo Stirrer. Triclosan (1.0% w/w) was added to the stirring propylene glycol to produce a clear semi-viscous solution. Eudragit L100 (0.2% w/w) was then added to the solution. Polyethylene glycol 400 (30.8% w/w) was then added followed by 3 mM phosphate buffer, pH 8 (28.9% w/w). To the clear semi-viscous solution, Noveon (1.0% w/w) and Carbomer 971 (0.5% w/w) were added very slowly. After additional mixing for 2 hours, the final 1% triclosan gel was a very slightly opaque gel solution. When the 1% triclosan gel solution was spread onto the skin of a human volunteer's hand; it produced a clear film.

EXAMPLE 4

A placebo pH-sensitive mucoadhesive film-forming gel was made as follows. Water (94.2% w/w) was added to a 250 mL stainless steel beaker and stirring was begun at 200 rpm using a Caframo Stirrer. Noveon (1.0% w/w) and Carbomer 971 (0.5% w/w) were added very slowly to the stirring water until the solution was clear and viscous with no visible solid material in solution. Eudragit L100 (2.0% w/w) was added and the solution and the viscous solution became slightly milky in color and less viscous. 18% sodium hydroxide (1.2% w/w) was then added and the whitish gel became viscous. Propylene glycol (0.5% w/w) and polyethylene glycol 400 (0.6% w/w) were added to the viscous gel. The final placebo formulation was a white viscous gel. However, when the placebo gel was spread onto the skin of a human volunteer's hand, it produced a clear film.

EXAMPLE 5

A 5% amlexanox pH-sensitive mucoadhesive film-forming gel suspension was made as follows. Water (63.5% w/w) was added to a 250 mL glass jar and stirring was begun at 1100 rpm using a Caframo Stirrer. Carbomer 971 (0.5% w/w) was added to the stirring water and mixed until fully hydrated. Next, Noveon (1.0% w/w) was added to the clear viscous solution and mixed until fully hydrated. Glycerin (1.0% w/w) was then added followed by ethanol (21.0% w/w). To the clear viscous gel, Eudragit L100 (7.0% w/w) was added to produce a whitish gel. Amlexanox (5.0% w/w) was then added and stirring was continued to result in a homogenous suspension of Amlexanox in the viscous gel. Finally, 18% sodium hydroxide (1.0% w/w) was added to adjust the pH. The final formulation was a whitish gel suspension. When the 5% Amlexanox gel suspension was applied to the buccal region of a human volunteer's mouth, a whitish thin film formed within 20 seconds.

EXAMPLE 6

A 1.5% amlexanox pH-sensitive mucoadhesive film-forming gel solution was made as follows. A 50 mM glycine/sodium hydroxide buffer, pH 9.0 (75% w/w), 95% ethanol (7.4% w/w), and glycerin (1.0% w/w) were added to a 250 mL glass jar and stirring was begun at 200 rpm using a Caframo Stirrer. Noveon (1.0% w/w) was then added to the solution and mixed at 800 rpm until fully hydrated. Carbomer 971 (1.0% w/w) was then added to the viscous gel and mixed at 800 rpm until fully hydrated. To the clear viscous gel, Eudragit L100 (0.1% w/w) was added to produce a whitish gel. A volume of approximately 7 mL 2N sodium hydroxide was added slowly to raise the pH to 8.8. At this pH, the formulation was a clear and viscous gel. Amlexanox was then added slowly in 0.25 g increments causing the pH of the whitish gel to drop to between pH 7.0 and 7.5. After each addition of Amlexanox, approximately 0.75 mL to 1.0 mL of 2N sodium hydroxide was needed to raise the pH to 8.8 at which time the gel became clear. The final concentration of Amlexanox was 1.5% w/w and the total amount of 2N sodium hydroxide added was 12 mL (13% w/w). The final formulation was a clear and viscous gel. When the 1.5% Amlexanox gel solution was spread onto the skin of a human volunteer's hand, it produced a clear film.

EXAMPLE 7

Preparation of initial wax-film composites using different waxes. A gel of Noveon (2%) and Eudragit S100 (0.5%) in ethanol was casted on a plastic circular hollow ring (diameter = 2.357 inches) fixed on Mylar film and dried in a 55°C oven to form homogenous mucoadhesive films having a diameter of 2.357 inches. Several ¼ inch disks were cut using a punch. The disks were dipped into the following melted wax compositions to form a homogeneous coating on one side of the pH-sensitive mucoadhesive films: beeswax, emulsifying wax, and DENTSPLY® Utility Wax. The results showed that the dried beeswax and emulsifying wax layers peeled away from the mucoadhesive layer over 30 minutes. The DENTSPLY® Utility Wax exhibited a very slight tendency to peel away but was mostly adhered. In an effort to mold the two layers of the wax-film composites more efficiently, a water-swellable polymer, tragacanth, was dispersed into melted DENTSPLY® Utility Wax at a concentration of either 1% w/w, 7% w/w, and 10% w/w. Mucoadhesive disks were then dipped into the new melted wax compositions to form a homogeneous coating on one side of the mucoadhesive films. The results showed that the two layers, mucoadhesive layer and wax layer with 1% tragacanth, were permanently molded together when stored at ambient conditions. Wax-film composites made with either 7% w/w or 10% w/w tragacanth in the wax layer caused the wax to be too permeable to water.

EXAMPLE 8

Six different pH-sensitive mucoadhesive wax-film composites were made using six different Noveon and Eudragit S100 compositions in ethanol as shown in Table 1 below. A specific volume of each gel was added to a plastic circular hollow ring (diameter = 2.357 inches) fixed on Mylar film to result in 450-480 mg total polymer in the ring.

Table 1: Preparation of Wax-Film Composites Having Different Ratios of Noveon/Eudragit S100 in the pH-Sensitive Mucoadhesive Layer

Gel #	Noveon (%) in gel	Eudragit (%) in gel	Volume of Gel Added to Ring (mL)	Noveon (mg) in Dried Film	Eudragit (mg) in Dried Film	Ratio of Noveon (N)/Eudragit (S) in pH-sensitive muco-adhesive Wax-film composite
1	1	2	15	150	300	0.5N:1S
2	2	2	12	240	240	2N:2S
3	2	1	15	300	150	2N:1S
4	1	0.5	30	300	150	2N:1S*
5	2	0.67	12	240	113.9	3N:1S
6	2	0.5	18.5	370	92.5	4N:1S

The ethanol from each Gel 1-6 was evaporated in a 55°C oven to form homogenous films (Films 1-6) of having a diameter of 2.357 inches. The dry Films 1-6 still on the Mylar film were dipped in melted DENTSPLY® Utility Wax (containing 1% w/w tragacanth polymer) at 55°C resulting in a homogenous coating of a wax layer on one side of the pH-sensitive mucoadhesive wax-film composites 1-6. Three-¼ inch disks each of the wax-film composites 1-6 for were made using a ¼ inch punch. Exactly 6 µL of a 40 mM KH₂PO₄/NaOH Buffer, pH 6 was added to the mucoadhesive side of the ¼ inch wax-film composites 1-6 (n = 3) and the disks were fixed to glass microscope cover-slips (to simulate the negatively-charged mucosal surface). The disks were allowed to dry for 4 hours at room temperature and then each cover-slip containing adhered disk was placed in a bath of 40 mM KH₂PO₄/NaOH Buffer, pH 6 at 37°C (unstirred). The time at which each disk dislodged from the cover-slip was recorded and the results of the study are plotted, in part, in Figure 9. The data demonstrated that when the amount of total polymer per ¼ inch disk was held constant, the weight ratio of each Noveon and Eudragit S100 polymers used in the pH-sensitive mucoadhesive layer significantly affected the adhesive time on the glass.

EXAMPLE 9

Five different pH-sensitive mucoadhesive wax-film composites were made using the same ethanol-based gel containing 2% Noveon and 0.67% Eudragit S100. The purpose of this

experiment was to test the effect of having different amounts of total polymer in the mucoadhesive layer on the total adherence time of wax-film composites to glass. A specific volume of the gel was added to a plastic circular hollow ring (diameter = 2.375 inches) fixed on Mylar film to result in different amounts of total polymer as shown in Table 2 below.

Table 2: Preparation of Wax-Film Composites Having Different Total Polymer Contents (Noveon/Eudragit S100) in the pH-Sensitive Mucoadhesive Layer

Sample	Noveon (%) in gel	Eudragit (%) in gel	Volume of Gel Added to Ring (mL)	Total Amount of Polymer in pH-sensitive mucoadhesive Wax-film composite (mg)
1	2	0.67	4	1.2
2	2	0.67	11	3.3
3	2	0.67	19	5.6
4	2	0.67	31	9.2
5	2	0.67	38	11.2

The ethanol from each Samples 1-5 was evaporated in a 55°C oven to form homogenous films (Films 1-5) having a diameter of 2.375 inches. The dry Films 1-5 still on the Mylar film were dipped in melted DENTSPLY® Utility Wax (containing 1% w/w tragacanth polymer) at 55°C resulting in a homogenous coating of the pH-sensitive mucoadhesive wax-film composites 1-5. Three-¼ inch disks each of the wax-film composites 1-5 for were made using a ¼ inch paper punch. Exactly 6 µL of a 40 mM KH₂PO₄/NaOH Buffer, pH 6 was added to the mucoadhesive side of the ¼ inch wax-film composites 1-6 (n = 3) and the disks were fixed to glass microscope cover-slips (to simulate the negatively-charged mucosal surface). The disks were allowed to dry for 4 hours at room temperature and then each cover-slip containing adhered disk was placed in a bath of 40 mM KH₂PO₄/NaOH Buffer, pH 6 at 37°C (stirred at 100 rpms). The time at which each disk dislodged from the cover-slip was recorded and the results of the study are plotted in Figure 2. The data demonstrated that when the amount of total polymer per ¼ inch disk was increased, there was no effect on the adhesive time on the glass. This result was most likely due to the fact that both the glass and the wax layer are impermeable to the buffer. It is expected that if the wax-film composites as described in this Example were placed on a permeable substrate such as a membrane or the buccal tissue, differences in the adhesion time would be observed.

EXAMPLE 10

Pre-loading and post-loading of plasmid DNA into pH-sensitive mucoadhesive wax-film composites was carried out. Plasmid DNA (CMV-luciferase plasmid) was loaded into the wax-film composites during the manufacturing process or post-loaded onto pre-made composites. An ethanol-based gel containing 3% Noveon and 1% Eudragit S-100 was prepared. For pre-loaded composites, 12.6 g of the gel was diluted with 6.3 g ethanol and 400 μ L of plasmid DNA (920 μ g) was added. For post-loaded composites, 12.6 g of the gel was diluted with 6.3 g ethanol. Each gel was added to a plastic circular hollow ring (diameter = 2.375 inches) fixed on Mylar film. The ethanol from each gel was evaporated in a 55°C oven to form homogenous films (Pre-loaded and Post-Loaded) having a diameter of 2.375 inches. The dry Pre-loaded and Post-Loaded Films, still on the Mylar film, were dipped in melted DENTSPLY® Utility Wax (containing 1% w/w tragacanth polymer) at 55°C resulting in a homogenous coating of the pH-sensitive mucoadhesive wax-film composites. Three ¼ inch disks each of the wax-film composites were made using a ¼ inch paper punch. Pre-loaded wax-film composites contained 10 μ g plasmid DNA per ¼ inch disks. To Post-loaded wax-film composites, exactly 4.3 μ L of plasmid DNA (10 μ g) was added to the mucoadhesive layer and allowed to dry for 4 hours.

Adhesion time: To dry wax-film composites containing plasmid DNA (pre-loaded and post-loaded) exactly 6 μ L of a 40 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$ Buffer, pH 6 was added to the mucoadhesive side of the ¼ inch wax-film composites ($n = 3$ for both Pre-loaded and Post-loaded) and the disks were fixed to the inside of a 300 mL glass beaker (to simulate the negatively-charged mucosal surface). The disks were allowed to dry for 4 hours at room temperature and then submerged in 40 mM $\text{KH}_2\text{PO}_4/\text{NaOH}$ Buffer, pH 6 at 37°C (stirred at 100 rpms). The time at which each disk dislodged from the glass was recorded and the results of the study are plotted in Figure 12. The data demonstrated that pre-loading or post-loading plasmid DNA had no effect on the adhesion times of the wax-film composites to glass.

Quantitation of Plasmid DNA: Plasmid DNA in solution was quantified using the PicoGreen dsDNA Quantitation Kit. In brief, Phage Lamda DNA was diluted with TE (Tris-EDTA) buffer (pH=7.5) to obtain a DNA concentration of 0, 2, 20, 100, and 1000 ng/mL. These solutions were further diluted twice with PicoGreen containing TE buffer. One mL of the solution was measured by the fluorescence spectrophotometer (Hitachi Instrument, Inc., Fairfield, OH) to obtain a standard plasmid DNA calibration curve as shown in Figure 3. This curve was used for calibration of the concentration of unformulated plasmid DNA and plasmid DNA released from wax-film composites.

In-Vitro Release of Plasmid DNA from Wax-Film Composites: Five ¼ inch wax-film composites containing of plasmid DNA (5 µg) (pre-loaded and post-loaded) were submerged separately into 1 mL 10 mM PBS buffer, pH 7.4 at 37°C. At various times, exactly 100 µL solution was aliquoted for DNA quantitation using the PicoGreen DNA Quantitation Kit. Exactly 100 µL fresh PBS was added to replace the removed volume at each time point. As shown in Figure 10, the release rate of plasmid DNA pre-loaded into wax-film composites was low as only about 4% of the total amount of plasmid DNA loaded into the wax-film composites during the manufacturing process was released in 11 hours. This result may be explained by the time needed for a large molecule such as plasmid DNA to diffuse through the mucoadhesive layer of Noveon and Eudragit S100. In comparison, the release rate of plasmid DNA post-loaded onto wax-film composites was much more rapid, as approximately 60% of the plasmid DNA was released within 1 hour (see Figure 11). These results demonstrate that plasmid DNA may be either pre-loaded or post-loaded on wax-film composites and released at different rates as necessary. A wax-film composites containing either pre-loaded or post-loaded plasmid DNA expressing a relevant peptide or protein antigen can be placed in the mouth of a warm-blooded mammal to elicit a measurable and protective immune response.

EXAMPLE 11

Hirudin was both pre-loaded and post-loaded into wax-film composites in a similar manner as described in Example 10. The release rate of hirudin (0.5 mg) from ¼ inch wax-film composites was measured using both a HPLC assay and a chromogenic substrate assay.

Chromogenic substrate assay for hirudin: This anti-amidolytic assay was first reported by Spannagl *et al.* (1991). It can be used to both quantitate hirudin in solution and determine its retained stability/functionality (i.e., ability to inhibit thrombin-catalyzed digestion of the synthetic substrate). Samples of hirudin (500 uL) were mixed with 400 uL of reagent mixture consisting of 67 mM Tris-HCl buffer, pH 8.0, containing 133 mM NaCl and 0.1% Polyethylene glycol (Mw 8000) and 1 nM of bovine α -thrombin. Hirudin and thrombin were allowed to form a complex at room temperature for 2 minutes. After 2 minutes, 100 uL of the 200 uM synthetic substrate (Tos-Gly-Pro-Arg-pNA; Chromozym-TH from Boehringer-Mannheim) was added and the digestion of the substrate pNA (para-nitroaniline) was monitored at 405 nm for 3 minutes. As shown in Figure 4A, the reactions proceeded linearly for 3 minutes. The addition of different amounts of hirudin in the reaction resulted in different slopes. A slope of ~0 signifies complete inhibition of thrombin-catalyzed digestion of the substrate. Complete inhibition theoretically occurs when the mole ratio of hirudin to thrombin is 1. A calibration curve for released hirudin

(see Figure 4B) in solution was made by plotting the difference between the thrombin base value absorbance (thrombin in the presence of substrate only) and the hirudin sample values versus the concentration of hirudin standards from Figure 4A.

HPLC assay for hirudin: Three standard curves for hirudin were generated using a C18 Nucleosil® 5 μm analytical column (4.6 x 250 mm). The mobile phase consisted of water:acetonitrile:trifluoroacetic acid (59.95:40:0.05 w/w/w). The flow rate was 0.6 mL/min. Each 50 μL sample of each standard curve was injected three times and the average AUC was plotted as shown in Figure 5.

The data suggested that wax-film composites containing either pre-loaded or post-loaded hirudin can be placed in the mouth of a warm-blooded animal to result in the absorption of hirudin into the systemic circulation in an amount suitable to elicit a pharmacological response (i.e., effect blood-clotting time).

EXAMPLE 12

Hirudin/chitosan complexes were prepared by adding chitosan (from 13 μg to 125 μg) to hirudin (0.5 mg) in water so that the total volume was 1 mL. The particle size of the complexes was measured using a Coulter N4 Plus Sub-Micron Particle Sizer using 90° laser light scattering for 120 seconds. The results indicate that the complex size was effected by the weight ratio of hirudin and chitosan in solution with a hirudin/chitosan ratio of 4:1 w/w resulting in the smallest complex size of approximately 100 nm (Figure 6). Hirudin/chitosan complexes were also prepared by adding chitosan (from 2.5 μg to 25 μg) to hirudin (0.1 mg) in water so that the total volume was 1 mL. The zeta potential of the complexes was measured using a Malvern Zetasizer 2000. The zeta potential results in millivolt (mV) indicated that as the weight ratio of hirudin to chitosan increased, the overall charge of the complex decreased (Figure 7). This result was expected since hirudin is negatively-charged and chitosan is positively-charged. The particle size stability of selected complexes stored at 4°C size was also determined. Four complexes having hirudin/chitosan weight ratios of 0.1, 0.15, 0.2, and 0.25 w/w were particle sized on Day 1, 2, 6, and 7 and the results are shown in Figure 8. For the Day 7 sample analysis, exactly 100 μL of hirudin/chitosan complexes in water was diluted in 900 μL ethanol to determine the stability of the complexes in ethanol. The results showed that selected complexes were very stable when stored at 4°C and when diluted into ethanol. These results suggest that selected hirudin/chitosan complexes could be incorporated into the wax-film composites as described in previous Examples and remain stable. Further, the results suggest that hirudin could be released from the wax-film composites and retain its ability to inhibit thrombin. Lastly, the results

indicated that wax-film composites containing either pre-loaded or post-loaded hirudin, complexed with chitosan, could be placed in the mouth of a warm-blooded animal to result in the absorption of hirudin into the systemic circulation in an amount suitable to elicit a pharmacological response (i.e., effect blood-clotting time).

Study of Bilayer Films for Mucosal (Genetic) Immunization via the Buccal Route in Rabbits

The oral buccal mucosa may be an ideal site for mucosal immunization allowing for needleless administration of cost-effective vaccines. A novel mucoadhesive bilayer film was developed to test the feasibility of this route of immunization in rabbits. Bilayer films were developed using different ratios of Noveon and Eudragit S-100 as the mucoadhesive layer, and a pharmaceutical wax as the impermeable backing layer. Optimal 3/8 inch films were post-loaded with 100 μ g plasmid DNA (CMV- β -gal) or β -gal protein $\pm$ 100 μ g cholera toxin as an adjuvant. The in-vitro release rates and stability of all post-loaded antigens was determined. The films were applied to the buccal pouch of rabbits on day 0, 7, and 14 and the humoral and splenocyte proliferative immune responses to β -gal were determined through day 28 and compared to those responses after conventional subcutaneous injection of adjuvanted protein. The weight ratio of Noveon and Eudragit S-100 had a significant effect on adhesion time of bilayer films. Postloaded plasmid DNA and β -gal remained stable after being released from bilayer films (release rates of ~65-80% in 2 hours for both). Buccal immunization using novel bilayer films (109 $\pm$ 6 μ m thickness) containing plasmid DNA led to comparable antigen-specific IgG titer to that of subcutaneous protein injection. All rabbits immunized via the buccal route, but none by the subcutaneous route with protein antigen, demonstrated splenocyte proliferative immune responses. The feasibility of buccal (genetic) immunization was demonstrated with these novel bilayer films.

INTRODUCTION

It is thought that the most effective vaccines for important viral and bacterial pathogens will require mucosal immunity since these pathogens infect the body primarily at mucosal sites (Cripps *et al.*, 2001; McGhee and Kiyono, 1992). Moreover, the feasibility of immunization at mucosal sites such as intestinal (Roy *et al.*, 1999), nasal (Imaoka *et al.*, 1998), and vaginal (Shen *et al.*, 2001) tissues has been demonstrated. However, all marketed vaccines except one (oral polio vaccine) are now administered by systemic routes and are ineffective at inducing mucosal immunity. A new promising intranasal flu vaccine (FlumistTM) is currently awaiting final regulatory approval. However, the one mucosal route that has been largely overlooked by

researchers is the oral mucosal region of the mouth. This may be due to the lack of suitable delivery systems to retain and protect antigen in the mouth for an extended period of time.

The oral mucosa may be an ideal site for vaccination for the following reasons: i) the oral mucosa is accessible, ii) administration to the oral mucosa is needleless, iii) the oral mucosa is a preferred site for antigen presentation, and iv) oral mucosa immunization is a largely untested vaccination strategy. The buccal mucosa is covered by a network of dendritic cells analogous to skin Langerhans's cells (LCs), which are the most potent professional antigen presenting cells (APC) (Ahlfors and Czerkinsky, 1991). LCs represent the major APC in human buccal mucosal epithelium, as demonstrated by the presence of Birbeck granule and the expression of CD1a and HLA-DR (Barret *et al.*, 1996). In addition, a high density of T lymphocytes (40-fold greater than in the skin) and mucosal associated lymphoid tissue (MALT), such as tonsils, salivary glands, Waldeyer's Ring, and pharyngeal lymphoid tissue, are present in the buccal mucosa (McGhee *et al.*, 1999). Thus, immunization via the buccal mucosa may provide for both cellular and mucosal (humoral) immune responses. For example, Etchart *et al.* (1997) reported that a single immunization with recombinant DNA injected transepithelially in the buccal mucosa of mice induced a measles virus haemagglutinin (HA)-specific class I-restricted cytotoxic lymphocytes (CTL) responses in the spleens (Etchart *et al.*, 1997). Lundholm *et al.* (1999) found using jet injection immunization that pDNA administered in the oral cheek of mice elicited very high IgA mucosal responses specific to HIV-1 proteins (gp160, p24, or TAT) (Lundholm *et al.*, 1999). Also, the immune response was more Th1 biased evidenced by the high ratio of IgG2a to IgG1 (Lundholm *et al.*, 1999). More recently, Wang *et al.* (2001) reported that gene gun-mediated oral mucosal transfer of interleukin 12 (IL-12) cDNA coupled with an irradiated melanoma vaccine in a hamster model led to successful treatment of oral melanoma and distant skin lesion (Wang *et al.* 2001). The feasibility to deliver protein antigen via the buccal mucosa was also demonstrated by Etchart *et al.* (2001). Etchart *et al.* showed that a single buccal immunization with measles virus nucleoprotein (NP), by either topical application onto or intradermal injection in the buccal mucosa, induced in-vivo priming of protective class I-restricted specific CD8+ CTL (Etchart *et al.* 2001). As a whole, these previous studies demonstrate that immunization via the buccal mucosa is feasible. However, more pharmaceutically acceptable and cost-effective delivery systems are still needed to fully elucidate the potential of this immunization method.

Conceivably, an effective buccal delivery system will provide for easy administration thereby increasing patient compliance. Several different types of buccal delivery systems, such as sprays, solutions, mono- or multi-layer adhesive films, buccal tablets, and lollipops, have been

developed (Hoogstraate and Wertz, 1998). In addition to factors relating to safety and cost, ideal delivery systems for buccal immunization should provide for prolonged exposure (i.e., 2-4 hours) of the antigen (protein or plasmid DNA) to the mucosal tissue while at the same time ensuring the retention of its immunogenicity.

The overall goal of the present study was to develop a mucoadhesive bilayer film containing a model protein antigen (β -galactosidase) or plasmid DNA expressing β -galactosidase, and to test the feasibility of buccal immunization in rabbits. The bilayer film was comprised of a thin wax layer bonded to a mucoadhesive layer containing Noveon AA-1, a cross-linked mucoadhesive polyacrylate polymer, and Eudragit S-100, an anionic pH-sensitive co-polymer of polymethacrylic acid-co-methyl methacrylate. The use of plasmid DNA as a potential genetic vaccine was emphasized in the present studies since it is well known that such vaccines are able to induce both humoral and cellular immune responses in animals (Gurunathan *et al.*, 2000).

MATERIALS AND METHODS

Materials

Polycarbophil (Noveon AA1) was a gift from BF Goodrich (Charlotte, NC). Eudragit® S-100 was a gift from Rohm America, Inc. (Piscataway, NJ). Dentsply® Utility Wax was purchased from DENTSPLY International Inc. (York, PA). Ethanol, β -galactosidase, Tragacanth, and 2-Mercaptoethanol were from Spectrum Laboratory Products, Inc. (New Brunswick, NJ). Plasmid DNA containing a CMV promoter and β -galactosidase reporter gene (CMV- β -gal) was gift from Valentis, Inc. (The Woodlands, TX). O-Nitrophenyl- β -D-galactopyranoside (ONPG) and a MicroBCA protein quantification kit were purchased from Pierce (Rockford, IL). PicoGreen DNA Quantification Kit was purchased from Molecular Probes (Eugene, OR). Glass microscope cover slips were purchased from Fisher Scientific Inc. Cholera Toxin (CT, #101D) was purchased from List Biological Laboratories, Inc. (Campbell, CA).

Preparation of mucoadhesive bilayer films

Five different ethanol-based gels were prepared comprised of the following final Noveon/Eudragit ratios (% w/w): 1:2, 2:2, 2:1, 3:1, and 4:1. These gels and subsequent films were designated by the final ratio of Noveon (N) and Eudragit (S) as 0.5N:1S, 1N:1S, 2N:1S, 3N:1S, and 4N:1S, respectively. Briefly, the required amount of Noveon was dispersed into absolute ethanol. The dispersion was then vigorously stirred (Caframo Mixer BDC1850, Ontario, Canada) until a homogenous viscous opaque gel was formed. Then, the required amount of

Eudragit S-100 was slowly added into the Noveon gel while stirring. High-speed stirring (1,000 rpm) was employed until the Eudragit was dispersed in a homogenous gel. After the addition of ethanol to the required weight, the viscous gel was slowly stirred overnight in a closed container. The gel was then sonicated for 30-60 min in an Ultrasonic Cleaner (Branson Ultrasonic Co., Danbury, CT) to remove air bubbles prior to film casting. The required weight of gel was poured into a plastic hollow ring (diameter = 6.2 cm; area = 30.175 cm²) glued onto Mylar film. The total weight of polymers (Noveon and Eudragit) in the casted gels was held constant by pouring a corresponding volume of gel that would result in final 3/8-inch films containing 10 ± 0.5 mg of total polymer, which was confirmed for all five ethanol-based gels. The casted gels were then left at room temperature overnight to form the mucoadhesive layer and to ensure evaporation of ethanol. The complete removal of ethanol was verified by confirming that the dried film reached a uniform weight. The mucoadhesive films (diameter = 6.2 cm) were coated with melted DENTSPLY[®] Utility Wax containing 1% (w/w) tragacanth by carefully dipping one side of the mucoadhesive film into the melted wax at 88°C for 1-2 seconds. The thin wax coating on the films was allowed to cool to dryness at ambient condition. The thin wax coating hardened within 5 seconds and cooled to room temperature within 30 seconds. This process resulted in a homogenous and permanent thin wax coating on the mucoadhesive layer. Circular bilayer films (3/8 inch diameter) were punched from the larger films using Arch punches (C.S. Osborne and Co., Harrison, NJ) and stored in ambient conditions away from light.

Effect of Noveon/Eudragit ratio on the adhesion time of the bilayer films to glass

The adhesion time of bilayer films was determined using glass as a simple model of the negatively-charged buccal mucosa. The mucoadhesive side of the films (n=5-9) were pre-wetted with 2 μ L 10 mM phosphate buffered saline (PBS, pH 6.0) and attached to a glass microscope cover slip with slight pressure. The films were left at room temperature to dry for at least 2.5 h. Subsequently, the glass cover slips with attached bilayer films were then submerged vertically into PBS buffer, pH 6.0 (n=5 films per 200 mL PBS) at 37°C maintained in a rotating waterbath at 100 rpm. The detachment time of the films from the glass was recorded and reported as the adhesion time. Statistical analysis was completed using a two-sample t-test, assuming equal variances. A p value ≤ 0.05 was considered to be significant.

Loading of plasmid DNA or β -galactosidase on/in the mucoadhesive bilayer film

Plasmid DNA (CMV- β -gal) was loaded on/in the mucoadhesive layer of the bilayer film using either a preloading or postloading method. For preloading, a required volume pDNA was added into the ethanol-based Noveon/Eudragit gels and stirred for 30 min prior to casting. The

volume of pDNA added was controlled so that a resulting 3/8-inch bilayer film contained 10 μg of pDNA. For post-loading, 3/8-inch bilayer films were made as described above, and then a required volume of pDNA was added to the mucoadhesive layer. Briefly, 5 μL of 0.2 M NaOH was first applied to the mucoadhesive side of the film. The NaOH was required to ensure that plasmid DNA was not affected by the low pH environment of the mucoadhesive layer. Without neutralization with NaOH, the pH of the PBS buffer under the conditions used in the method decreased to between pH 4-5 within two hours (data not shown). After the film was dried, 10 μg of pDNA (4.4 μL of 2.3 $\mu\text{g}/\mu\text{L}$ in 10 mM PBS buffer, pH 7.4) was applied onto the mucoadhesive layer of the bilayer film. The films were then left at room temperature for 2 h to dry. To ascertain whether preloading or postloading of pDNA in/on the bilayer films affected the adhesion time of the bilayer films, glass adhesion studies were performed as described above.

For postloading β -galactosidase protein, five hundred micrograms (500 μg) of β -galactosidase dissolved into 10 mM PBS buffer, pH 7.4 was applied to the mucoadhesive layer of the bilayer films and then left at room temperature for 2 h to dry. Preloading of β -galactosidase in the bilayer films was not investigated.

Release of pDNA or β -galactosidase from bilayer films

The release of preloaded or postloaded pDNA from bilayer films was investigated by submerging 3/8-inch bilayer films ($n=5$) into 1 mL of 10 mM PBS buffer (pH 7.4) in a glass scintillation vial. The vial was kept at 37°C in a C76 Water Bath Shaker rotating at 100 rpm. One hundred μL of solution was withdrawn at specified time for pDNA quantification using the PicoGreen DNA Quantification Kit (Molecular Probes). One hundred microliters (100 μL) of fresh 10 mM PBS buffer (pH 7.4) at 37°C was added to maintain a constant total volume of 1 mL. Placebo films without pDNA loading were used as negative control. The stability of the pDNA released from postloaded bilayer films was measured using gel electrophoresis with 1% SeaKem Gold agarose gel (BioWhittaker Molecular Applications, Rockland, ME) in 1X TAE buffer (pH 7.4).

The release of β -galactosidase from bilayer films was determined in a similar manner as described above. The concentration of released β -galactosidase protein was measured using a MicroBCA protein quantification kit (Pierce). The enzymatic activity of released β -galactosidase was also measured to evaluate the stability of the protein using a protocol from Edge BioSystems (Gaithersburg, MD) with modification. Briefly, 30 μL of release solution was placed into a 7-mL glass vial. Two hundred μL of Z buffer [60 mM Na_2HPO_4 , 40 mM NaH_2PO_4 , 10 mM KCl, and 1 mM MgSO_4 , (pH 7.0) with 50 mM of β -Mercaptoethanol added freshly before use] was added

into the sample and incubated at 37°C for 5 min. Seventy μ L of ONPG was then added. After mixing, the sample was monitored until it developed a faint yellow color. The reaction was stopped by adding 0.5 mL of Na₂CO₃ (1 M). The OD420 was measured using an ELX 800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT). A standard curve between β -galactosidase concentration and OD420 was constructed to convert the OD420 from release samples to enzyme activity.

Rabbit immunization studies

Female New Zealand White rabbits (2.5-3.0 kg) from Myrtle's Rabbitry (Thompson's Station, TN) were used in the present studies. NIH guidelines for the care and use of laboratory animals were observed. All animal studies were started after an acclimation period of one week. Rabbits were anesthetized prior to dosing and blood collection by intramuscular injection of Ketamine HCl (40 mg/kg) and Xylazine (5 mg/kg). All rabbits remained anesthetized for at least 2 h. In the first preliminary study, rabbits (n=2) were immunized with either pDNA (100 μ g) or β -galactosidase protein (100 μ g), both adjuvanted with 100 μ g of Cholera Toxin (CT), via the buccal mucosa by applying 3/8-inch postloaded bilayer films on day 0, 7, and 14. As positive control, 2 rabbits were also subcutaneously injected with β -galactosidase (100 μ g) adjuvanted with CT (100 μ g). Naïve rabbits received no treatment other than anesthesia. In a second rabbit study, four rabbits were immunized with pDNA (100 μ g) adjuvanted with CT (100 μ g) via the buccal mucosa by applying the pDNA postloaded films. Three rabbits (n=3) were immunized with β -galactosidase (100 μ g) adjuvanted with 'Alum' (100 μ g) by subcutaneous injection on the back.

In both studies, rabbit blood was withdrawn via the ear vein on day 0, 7, 14, 21, and 28. Sera were separated and stored as previously described (Cui and Mumper, 2001). β -galactosidase-specific total IgG titer in the sera was determined by ELISA (Cui and Mumper, 2001). In addition, in the second study, rabbit spleens were collected at day 28 and splenocytes were prepared as previously described (Cui and Mumper, 2001). A splenocyte proliferation assay was completed as described elsewhere (Cui and Mumper, 2001) except that 1.6×10^5 cells/well were co-incubated with 0 or 10 μ g/well β -galactosidase for 4 days prior to assay using the CellTiter 96[®] Aqueous Non-radioactive Cell Proliferation Assay Kit (Promega). Proliferation results were reported as the percent increase in the splenocyte number four days after stimulation. The proliferation index was calculated as the ratio of splenocyte number with and without stimulation after 4 days. Statistical analysis was completed with a two-sample t-test assuming unequal variances. A result of $p < 0.05$ was considered statistically significant.

RESULTS

Effect of Noveon/Eudragit ratio on the adhesion time of the bilayer films to glass

Fig. 9 shows the glass adhesion time of the 3/8-inch bilayer films ($109 \pm 6 \mu\text{m}$ thickness) having different ratios of Noveon and Eudragit (0.5N:1S, 1N:1S, 2N:1S, 3N:1S, and 4N:1S). Increasing the proportion of Noveon to Eudragit from 0.5:1 to 3:1 (w/w) significantly enhanced the adhesion time of the bilayer film from an average of only a few minutes to up to 175 minutes. Bilayer films comprised of a Noveon/Eudragit ratio of 3:1 (w/w) appeared to be optimal, and the adhesion times were statistically greater than all of other bilayer films ($p < 0.05$). Therefore, bilayer films comprised of a Noveon/Eudragit ratio of 3:1 (w/w) was used for all further studies.

Release of plasmid DNA and β -galactosidase from bilayer films

As shown in Fig. 10, the release rate of plasmid DNA preloaded in bilayer films was very slow and incomplete. However, as shown in Fig. 11, the release rate of pDNA postloaded on bilayer films was greater and more extensive. Over 60% of the postloaded pDNA was released in 2 hours, in contrast to only about 2% from the preloaded films. Gel electrophoresis showed that the pDNA released from the postloaded films was mostly in supercoiled form (data not shown). Further, as shown in Fig 12 postloading pDNA had no significant effect on the adhesion time of the bilayer films as compared to both placebo and preloaded bilayer films ($p = 0.43$ by ANOVA). Based on the desirable release profile and the stability of pDNA, the postloading process for pDNA was used to prepare bilayer films for subsequent rabbit studies.

β -galactosidase was successfully postloaded on the mucoadhesive layer of the bilayer films and the loading did not affect the cosmetic properties of the films. Fig. 13 shows that about 80% of the postloaded β -galactosidase was released within 2 hours. In addition, the released β -galactosidase retained its enzymatic activity as determined by its ability to hydrolyze ONPG to *o*-nitrophenol and D-galactose.

Immune responses in rabbits

The total β -galactosidase-specific IgG titer profile in sera of immunized rabbits for the first rabbit immunization study is shown in Figure 14. Since only two rabbits were used for each group, statistical analyses were not completed. However, the results did demonstrate that both β -galactosidase protein and pDNA (CMV- β -gal) induced antigen-specific IgG when applied to the buccal mucosa of the rabbits via the mucoadhesive bilayer films. In addition, the level of the IgG titers in rabbits immunized via the buccal route for at least one rabbit in each group was

comparable to that of rabbits immunized by subcutaneous injection of adjuvanted protein. Genetic immunization using plasmid DNA resulted in a delay of the onset of detectable titer until day 21. All treated rabbits demonstrated positive cholera toxin specific IgG titers in sera (results not shown). Further, all immunized rabbits demonstrated positive, but low, IgG titer in nasal swabs. Rabbits immunized by the buccal route with bilayer films containing plasmid DNA resulted in nasal IgG titer that were generally greater than those after immunization with protein either by the buccal route or by subcutaneous injection. For example, the increase in O.D. in the ELISA for rabbits immunized by the buccal route with plasmid DNA were generally 4-10 fold greater than naïve rabbits over the 28 day study, whereas rabbits immunized with protein by either route were only 1-3 fold greater than naïve rabbits.

In a second rabbit immunization study, the number of rabbits in each group were increased to n=3-4. The total β -galactosidase-specific IgG titer profile in sera of immunized rabbits is shown in Fig. 15. At day 7, a low level of IgG was detected in one of the four rabbits immunized via the buccal route. The overall IgG titer a day 7 was significantly lower than that of rabbits immunized by subcutaneous injection ($p = 0.04$). However, by day 14, all four rabbits immunized by the buccal route responded with titers that were comparable to those after subcutaneous injection. By day 28, the titer for the rabbits immunized by the buccal route were statistically greater than those rabbits immunized by subcutaneous injection ($p = 0.03$). As shown in Table 3, all rabbits immunized via the buccal route with bilayer films containing 100 μ g plasmid DNA, but none by the subcutaneous route with 100 μ g protein adjuvanted with Alum, demonstrated splenocyte proliferative immune responses.

Table 3. Proliferation of isolated rabbit splenocytes after *in-vitro* stimulation.

Rabbit No.	Naïve			Bilayer film with Postloaded pDNA				β -Gal protein via subcutaneous injection		
	1	2	3	4	5	6	7	8	9	10
% increase in cell Number	<0	<0	<0	168	277	52	148	<0	<0	<0
Proliferation Index	<1	<1	<1	2.68	3.77	1.52	2.48	<1	<1	<1

Referring to Table 3, rabbits were immunized as in Fig. 15 with either pDNA (100 μ g) adjuvanted with CT (100 μ g) via buccal mucosa or β -galactosidase protein (100 μ g) adjuvanted

with 'Alum' (100 µg) subcutaneously on day 0, 7, and 14. On day 28, rabbits were sacrificed and their spleens were collected. Splenocytes (1.6×10^5) (n=2) were seeded into 48-well plates and incubated together with β -galactosidase (0 or 10 µg/well) for 4 days at 37°C with 5 % CO₂. Cell proliferation was quantified with a CellTiter 96[®] Aqueous Non-radioactive Cell Proliferation Assay Kit (Promega). Reported was the average of two numbers for each sample.

Finally, although the two rabbit studies were completed at different times, the results suggested that the titers after buccal immunization using bilayer films containing 100 µg pDNA were comparable. Further, the results also demonstrated that for rabbits immunized by subcutaneous injection of protein, both Alum and CT adjuvanted the immune response to a similar extent.

DISCUSSION

Vaccination without the use of needles would provide a distinct advantage in terms of both cost and safety over conventional vaccines that must be given with needles. In addition, needleless vaccination would make the prospects of widespread vaccination more practical and cost-effective. Although the intestinal and nasal mucosa routes have been intensively explored to delivery (genetic) vaccines, the oral mucosa route remains a largely untested vaccination strategy (Roy *et al.*, 1999; Imaoka *et al.*, 1998). This may be due to the lack of suitable delivery systems to retain and protect antigen in the mouth for an extended period of time.

The oral buccal mucosa has all the necessary cells (high density of Langerhans' cells and T lymphocytes) and the mucosal associated lymphoid tissues for the development of immune responses (Ahlfors and Czerkinsky, 1991; Barret *et al.*, 1996; McGhee *et al.*, 1999). Further, several recent publications have confirmed that it is feasible to elicit both humoral and cellular immune responses by applying (genetic) vaccines to the oral buccal mucosa using either transepithelial needle injection, needle-free jet injection, or gene gun-mediated injection (Etchart *et al.*, 1997; Lundholm *et al.*, 1999; Wang *et al.*, 2001; Etchart *et al.*, 2001). However, these reports also underscore the need for improved, cost-effective, and more pharmaceutically acceptable and patient friendly delivery systems.

The rabbit model was chosen in this present study since the rabbit is the only laboratory rodent that has non-keratinized mucosal lining similar to human tissue (Harris and Robinson, 1992). However, one drawback associated with using rabbits for immunization studies is that unlike other rodent models such as the mouse and rat, most of the necessary antibodies and cytokine ELISA kits are not commercially available. Thus, for this present study, we could not

readily assess IgA titer or Th1 versus Th2-biased immune responses via the detection of cytokines such as IL-2, IFN- γ , and IL-4, etc.

To determine the feasibility of buccal (genetic) immunization in rabbits, novel mucoadhesive bilayer films were developed. The mucoadhesive layer of the bilayer film is comprised of Noveon and Eudragit S-100. Noveon, or polycarbophil, is a homopolymer of acrylic acid cross-linked with divinyl glycol. Noveon has USP designation and is generally regarded as safe. It has been extensively formulated in a variety of drug delivery systems and is commonly used as a bioadhesive (The U.S. Pharmacopeia; Natl Formulary, 1995). The high molecular weight polycarbophils readily swell in water, providing a large adhesive surface for maximum contact with the mucosal tissue. The exact mechanism of mucoadhesion of these polymers to the buccal tissue is unknown, but is thought to involve extensive hydrogen bonding between the carboxylic acid moieties of the polymers to the sialic acid residues of mucin, the primary substance of the viscous mucous layer on epithelial cells (The U.S. Pharmacopeia; Natl Formulary, 1995). The viscosity of the mucous layer is largely determined by the type and amount of the glycoproteins present in the mucin. Usually, the mucous layer in the oral mucosa is about 500-600 μm thick and has a pH of 5.8-7.1. Eudragit S-100 is a poly(methacrylic acid, methyl methacrylate) 1:2 polymer. Polymethacrylates are primarily used as film-coating agents for oral capsule and tablet formulations (Wade and Weller, 1994). Although Eudragit has some mucoadhesive properties, we mainly took advantage of its film-forming properties by combining it with the Noveon to provide some rigidity to the mucoadhesive layer. Eudragit S-100 is a pH-sensitive polymer that has solubility in water above pH 7, but is insoluble below pH 7. This insolubility below pH 7 is ideal for use in the oral mucosa to promote longer adhesion time of the bilayer films due to retardation of hydration and swelling of the polycarbophil polymer. Thus, the optimal combinations of Noveon and Eudragit in the mucoadhesive layer provided for a bilayer film with excellent and prolonged adhesive properties. The presence of the wax-backing layer greatly enhanced the adhesion time of the mucoadhesive layer by retarding the diffusion of (simulated) saliva (data not reported). Further, the presence of the wax-backing layer presumably helps to retard the diffusion of plasmid DNA and β -galactosidase into the mouth.

The results of this present study demonstrated that two large biomolecules, β -galactosidase (256 kDa) and plasmid DNA could be postloaded on optimal bilayer films, and that the majority of the postloaded material was released within 2 hours. Further, the stability of the released biomolecules was retained as measured by retained enzymatic activity of the protein, and the retention of supercoiled topology for plasmid DNA. The application of these

agents to premade and quality controlled bilayer films may have several advantages including cost. For example, in contrast to the preloading process, the postloading process creates no waste of the protein or plasmid DNA since they are added directly to the delivery system to be applied.

Taken together, the two rabbit immunization studies demonstrated that buccal (genetic) immunization is feasible. In addition, the results showed that the buccal immunization was quite reproducible in that all rabbits responded eliciting titer that was equivalent, if not greater, than those rabbits immunized by conventional subcutaneous injection of adjuvanted protein. Moreover, it was demonstrated that all rabbits immunized by the buccal route with bilayer films containing plasmid DNA showed positive splenocyte proliferative responses, which may be indicative of cellular-based immunity. As expected, no proliferative immune responses were observed with conventional subunit protein immunization, which is well known to produce more humoral-based immune responses. Cholera toxin (CT) was included into the bilayer film since it is known to be a strong mucosal adjuvant (Holmgren *et al.*, 1993). The use of 100 µg CT as an adjuvant by either the buccal or subcutaneous routes did not cause any discernable toxic effects in the rabbits. Additional buccal immunization studies are planned to determine the importance of cholera toxin, or other adjuvants such as Alum or cationic lipids, in terms of the breadth and depth of the immune responses.

These results suggest that immunization with mucoadhesive bilayer films containing plasmid DNA via the buccal mucosa may elicit systemic humoral and cellular-based immune responses. Although, mucosal IgA was not determined in these studies, it is believed based on the presence of nasal IgG and previous literature reports, that it is likely that IgA antibodies are also produced after buccal immunization. Methods to quantitatively determine IgA antibodies in rabbits are being actively pursued in our laboratories. The elicitation of systemic humoral, cellular, and mucosal responses via buccal immunization may warrant additional testing with important pathogens.

Study of Transmucosal Delivery of Testosterone in Rabbits using Novel Bi-Layer Mucoadhesive Wax-Film Composite Disks

Testosterone exhibits very low oral bioavailability due to low aqueous solubility and extensive first pass metabolism. The purpose of this study was to develop a novel bi-layer mucoadhesive wax-film composite (WFC), and to test the relative bioavailability of testosterone via the buccal route in rabbits. The release rate of testosterone from optimal WFCs (3/8 inch diameter) per unit surface area was $5.6 \mu\text{g cm}^2 \text{ mL}^{-1} \text{ min}^{-1}$ and was zero-order. Bi-layer WFCs

(average weight of 14 ± 2.6 mg and thickness of 186 ± 34 microns) containing 4 mg testosterone were applied to the buccal pouch of anesthetized New Zealand White Rabbits. Rabbits (n=3) injected intravenously had $C_{\max}$ and AUC values of 1200 ± 46 ng/mL, and $48,227 \pm 12,995$ ng*min/mL, respectively. Rabbits (n=3) dosed via the buccal pouch had $C_{\max}$, $T_{\max}$, and AUC values of 127 ± 13 ng/mL, 200 ± 35 min, and $24,221 \pm 1543$ ng*min/mL. The relative bioavailability for rabbits treated with the WFC was $50.2 \pm 3.2\%$ with a CV of 6.4%. It was concluded that these bi-layer mucoadhesive WFCs disks could deliver physiologically relevant amounts of insoluble drugs such as testosterone across the buccal mucosa.

INTRODUCTION

Buccal delivery for the transmucosal absorption of drugs into the systemic circulation offers a number of advantages over oral delivery, especially for those drugs that have poor oral bioavailability and/or those drugs that suffer from extensive first pass metabolism in the liver (Hoogstraate and Wertz, 1998; Junginger *et al.*, 1999). Conceivably, buccal delivery systems provide for easy administration thereby increasing patient compliance. Many different types of buccal delivery systems have been developed including sprays, solutions, erodable or non-erodable multi-layer adhesive films, adhesive tablets, and lollipops (Hoogstraate and Wertz, 1998; Voorspoels *et al.*, 1996; Benes *et al.*, 1997; Alur *et al.*, 1999; Li *et al.*, 1997; Schechter *et al.*, 1995; Schwagmeier *et al.*, 1998). Several peptides have been delivered via the buccal route including thyrotropin-releasing hormone, insulin, octreotide, leuprolide, and oxytocin among others (Hoogstraate and Wertz, 1998; Junginger *et al.*, 1999; Li *et al.*, 1997; Schurr *et al.*, 1985; Heiber *et al.*, 1994). In most cases the relative bioavailability of these peptides by the buccal route was quite low (i.e., 0.1-5.0%), but was significantly increased by the inclusion of chemical penetration enhancers. In contrast, recent studies in humans have shown that the relative bioavailability of small lipophilic drugs by the buccal route (such as buprenorphine, testosterone, atipamezole, fentanyl, butorphanol, melatonin, nifedipine among others) are relatively high (i.e., 20-80%) and routinely comparable to nasal administration (Hoogstraate and Wertz, 1998; Junginger *et al.*, 1999; Voorspoels *et al.*, 1996; Benes *et al.*, 1997; Alur *et al.*, 1999; Schechter *et al.*, 1995; Schwagmeier *et al.*, 1998).

The barriers for effective buccal delivery are similar to those for oral delivery including a substantial physical barriers (mucous layer that lines a multi-layered epithelium) and chemical barriers due to the presence of a multitude of peptidases and proteolytic enzymes (Hoogstraate and Wertz, 1998; Junginger *et al.*, 1999). These barriers contribute to the relatively low permeability of the buccal mucosa. However, the buccal mucosa is well vascularized. Rapid

and extensive drug absorption is possible, especially for those drugs that are small, lipophilic, and remain stable to enzymatic degradation. Further, unlike formulations intended for injection or nasal administration, buccal delivery systems do not have to be sterile which greatly reduces manufacturing costs. Chemical penetration (absorption) enhancers such as surfactants, bile salts, and fatty acids have been used to markedly increase the bioavailability of drugs via the buccal route. However, there are no approved products on the market with designated penetration enhancers so the inclusion of these chemicals in buccal delivery systems may be undesirable (Hoogstraate and Wertz, 1998).

Cui and Mumper (2001) have recently described a novel bi-layer mucoadhesive wax-film composite (WFC) comprised of a pH-sensitive mucoadhesive layer and a pharmaceutical wax as the impermeable backing layer (Cui and Mumper, 2001). The mucoadhesive layer consisted of Noveon[®] AA-1, a cross-linked polyacrylate polymer, and Eudragit S-100, the pH-sensitive anionic polymer polymethacrylic acid-co-methyl methacrylate. The pharmaceutical wax is DENTSPLY[®] Utility Wax. Cui and Mumper reported that bi-layer mucoadhesive WFC, containing either plasmid DNA or subunit protein antigen, applied to the buccal pouch in rabbits produced enhanced antigen-specific antibody and proliferative immune responses to expressed antigen or subunit protein antigen over those responses observed after conventional subcutaneous injection of adjuvanted protein antigen (Cui and Mumper, 2001). It was also demonstrated that the adhesion time of these WFCs to both glass (simple *in-vitro* model) and the rabbit buccal pouch could be controlled by modifying the relative ratio of these two excipients, the thickness of the disk, and the extent of wax-backing layer. These novel WFCs potentially provide advantages over other reported buccal delivery systems mentioned above due to their, *i*) relative low cost, *ii*) ease of production, *iii*) controllable mucoadhesion time from minutes to hours, and *iv*) biocompatibility and bioerodability.

The overall objective of these studies was to further develop the bi-layer mucoadhesive wax-film composites for the transmucosal delivery of testosterone, and to determine the relative bioavailability after buccal administration to that of intravenous administration in rabbits. To this end, factors influencing the adhesion of the WFCs both *in-vitro* and *in-vivo* as well as the rate of release of testosterone under the conditions of temperature, media, and drug loading were also investigated.

MATERIALS AND METHODS

Materials

Polycarbophil (Noveon[®] AA-1, USP) was obtained from BF Goodrich (Cleveland, Ohio). Polymethacrylic acid-co-methyl methacrylate (Eudragit S-100) was a generous gift of Röhm America Inc. (Piscataway, New Jersey). Testosterone and potassium phosphate dibasic were purchased from Aldrich Chemicals (Milwaukee, WI). DENTSPLY[®] Utility Wax was obtained from DENTSPLY International (York, PA). Sodium hydroxide NF, ethanol (95%) USP, and sodium lauryl sulfate NF, were purchased from Spectrum Laboratory Products (Gardena, California). Testosterone ELISA kits were acquired from IBL Immuno-Biological Laboratories (Hamburg, Germany). Rabbit serum (cat. 16120-102) was purchased from Invitrogen Corporation (Carlsbad, California).

Preparation of wax-film composites containing testosterone

Placebo wax-film composites (WFCs) were made as previously described by Cui and Mumper (2001). Briefly, ethanol-based gels were first prepared by mechanical mixing for up to three hours using a Caframo Mixer. The final clear mucoadhesive gel consisted of Noveon AA-1 (3% w/w) and Eudragit S-100 (1% w/w) in ethanol. For testosterone wax-film composites, the appropriate concentration of testosterone was first dissolved in the mucoadhesive gel. The mucoadhesive gel solutions were sonicated for 10-15 min to remove air bubbles. Next, 7 mL of each ethanol-based gel was cast in a plastic circular hollow ring (diameter = 6.2 cm; total area = 30.175 cm²) fixed on Mylar film. The gels were dried overnight under various conditions (i.e., 0°C, 5°C, 25°C, and 55°C) to produce optimal mucoadhesive mono-layer films. The complete removal of ethanol was confirmed by verifying that each dried mono-layer film reached a constant weight. The mono-layer films (diameter = 6.2 cm) were coated with melted DENTSPLY[®] Utility Wax by carefully dipping one side of the mucoadhesive film into the melted wax at 88°C for 1-2 seconds. The thin wax coating on the films was allowed to cool to dryness at ambient condition. The thin wax coating hardened within 5 seconds and cooled to room temperature within 30 seconds. This process resulted in a homogenous and permanent thin wax coating on the mucoadhesive layer. Circular WFC disks (3/8 inch diameter) were punched from the films using Arch punches (C.S. Osborne and Co., Harrison, NJ) and stored in ambient conditions away from light.

Physical characterization of the wax-film composites (WFCs)

Placebo and testosterone mono-layered 3/8 inch mucoadhesive films (no wax-backing layer) and 3/8 inch bi-layer WFCs were weighed on a calibrated Mettler AC100 Balance (n=10

each). The thickness of both the mono-layered mucoadhesive films and the bi-layer WFCs (n=10 each) was determined using an Electronic Digital Micrometer Model 030025 EMD (0-25 mm, resolution of 0.001 mm).

Content Uniformity of the wax-film composites (WFCs)

The intra-disk and inter-disk content uniformity of WFCs punched from three larger films was determined using a Beckman Diode-Array Spectrophotometer (Model DU75000) at a wavelength of 240 nm. To determine the intra-disk variability in content uniformity, ten 3/8 inch WFCs were punched from all regions of the larger film (perimeter, body, and center). Testosterone WFCs were suspended in 15 mL ethanol and mixed for 2 hr using a magnetic stirrer. Samples were filtered using a 0.2 μ m filter and diluted with ethanol. Testosterone concentrations were calculated using a standard curve (linear range = 2-16 μ g/mL corresponding to an absorbance range of ~0.05 to 0.8 absorbance units; $R^2 = 0.9933$).

Adhesion time of wax-film composites to a glass matrix

The adhesion time of both placebo WFCs and testosterone WFCs (4 mg) was determined using glass as a simple model of the negatively-charged buccal mucosa. The adhesion time of WFCs (n=5/group) was determined as a function of both temperature (25°C or 37°C) and the presence of sodium lauryl sulfate (0% or 3%) in 10 mM phosphate buffer, pH 6.0. WFCs were wetted with 25 μ L of water and adhered to the inside of a 50 mL dry glass beaker. Ten (10) mL of the release media was added to the 50 mL beaker with adhered WFCs, and the beakers were rotated at 120 rpm using a water bath at the temperature indicated. The adhesion times for the WFCs were visually determined and recorded. Statistical analyses were completed using a two-sample t-test assuming unequal variances wherein p-values <0.05 were considered statistically significant.

***In-vitro* release studies of testosterone**

The *in-vitro* release profiles of testosterone from various WFCs in 10 mM potassium phosphate buffer, pH 6.0 were determined as a function of surfactant concentration (0% or 3% sodium lauryl sulfate), temperature (25°C or 37°C), and the presence or absence of the wax-backing layer. For these studies, WFCs (3/8 inch diameter) were submerged in covered glass scintillation vials containing 10 mL of buffer under controlled stirring conditions. At 5, 10, 15, 20, 30, 45, 60, 90, and 120 minutes, five hundred microliter (500 μ L) aliquots were removed and assayed for testosterone using a Beckman Diode-Array Spectrophotometer (Model DU75000) at a wavelength of 240 nm. Released testosterone concentrations were calculated using a standard

curve (linear range = 2-16 $\mu\text{g/mL}$ corresponding to an absorbance range of ~ 0.05 to 0.8 absorbance units; $R^2 = 0.9933$). At each time point, five hundred microliters (500 μL) of fresh media were replaced. Cumulative % testosterone released was calculated by the ratio of testosterone released in the entire volume divided by the total amount of testosterone loaded in the WFC. These values were corrected for the amount of testosterone removed at each time point. The data reported are the mean $\pm$ standard deviation for three replicates ($n=3$) for each condition tested.

In-vivo studies in rabbits

Female New Zealand White rabbits (2.5 kg to 3.0 kg) from Myrtle's Rabbitry (Thompson's Station, Tennessee) were used for the studies. NIH guidelines for the care and use of laboratory animals were observed. After an acclimation period of one week, rabbits were weighed and anesthetized by intramuscular injection of Ketamine HCl (40 mg/kg) and Xylazine (5 mg/kg). All rabbits remained anesthetized for at least 2 hours, but not more than 3 hours. For intravenous injection via the ear-vein ($n=3$ rabbits), the hair on the ear was shaved with clippers and swabbed with alcohol. Five hundred microliters (500 μL = 4 mg testosterone) of a sterile solution of testosterone in propylene glycol/ethanol (60:40 v/v) were injected via a 26-gauge needle in the ear vein over a period of approximately 30 seconds. Care was taken so as to verify that an accurate and complete injection was made. For buccal application, testosterone WFCs containing 4 mg testosterone were applied directly to the buccal pouch of the rabbits ($n=3$ rabbits). For ease of application to the anesthetized rabbits, the mucoadhesive layer of each WFC was pre-wetted with 33 μL of 10 mM phosphate buffer, pH 6.0. Naïve rabbits ($n=2$) were also used as controls for the experiment. For two additional rabbits, the WFCs were pre-wetted with 33 μL of 10 mM phosphate buffer, pH 6.0 containing 3% sodium lauryl sulfate. All placebo and testosterone WFCs were removed at 3 hours and analyzed for residual testosterone. At various times after either intravenous injection or buccal application, 1.0 mL of blood was removed via ear-vein using a 26-gauge needle. The blood was transferred into Vacutainer Brand Blood Collection Tubes with gel for serum separation (Becton Dickinson and Company, Franklin Lakes, NJ). The serum was separated by centrifugation and stored frozen at -20°C . Testosterone concentrations in the serum ($n = 2-5$ replicates/sample) were detected and quantified using a commercially available competitive immunoassay for testosterone (see below). Pharmacokinetic parameters such as C_{max} , T_{max} , AUC, and elimination half-life were determined using WinNonlin[®] Professional Version 3.1 from Pharsight Corporation (Mountain View, CA) employing a noncompartmental modeling (NCA Model 200) and the Linear

Trapezoidal Method. When applicable, statistical analyses were completed using a two-sample t-test assuming unequal variances wherein p-values <0.05 were considered statistically significant.

Detection of testosterone in rabbit serum

A competitive immunoassay for testosterone was used to detect and quantify the concentration of testosterone in rabbit serum. The immunoassay kit contained all necessary components such as standards, buffers, substrate, washes, and plates. The assay has a reported sensitivity of 0.1 ng/mL and comes with testosterone standards in the range of 0.2-16 ng/mL. The kits were used as directed by the manufacturer. The principle of the assay is based on the competitive binding of free testosterone and a fixed concentration of alkaline phosphatase-labeled testosterone to a mouse monoclonal antibody. After washing steps, the substrate tetramethylbenzidine (TMB) was added and the concentration of bound alkaline phosphatase-labeled testosterone was measured. The concentration of free testosterone in serum was inversely proportional to the optical density (OD) at 450 nm using a Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VM). For qualification of the assay for these studies, rabbit serum (from Invitrogen Corp.) was spiked in triplicate with final concentrations of testosterone in 10 mM phosphate buffer, pH 6.0 with 3% SLS ranging from 0-16 ng/mL. The competitive testosterone assay was then completed in triplicate and compared to the normal standard curve performed in buffer. Best-fit analysis was performed using GraphPad Prism® (Version 3) from GraphPad Software, Inc. (San Diego, CA). A new standard curve of testosterone spiked in rabbit serum was completed for each new 96-well plate used.

RESULTS

Preparation and physical characterization of wax-film composites containing testosterone

Initial testosterone pre-loading experiments focused on the incorporation of testosterone in the mucoadhesive gel solution prior to casting films in plastic circular hollow rings (diameter = 6.2 cm; total area = 30.175 cm²). Testosterone was very soluble in the ethanol-based gel containing 3% Noveon (w/w) and 1% Eudragit S-100 (w/w). Mucoadhesive gel solutions comprised of Noveon (1.5 g) and Eudragit S-100 (0.5 g) with and without testosterone (1.803 g) were homogeneous and slightly opaque gels after preparation. Various methods were investigated to dry the mucoadhesive gel solutions containing testosterone. Casted gels dried overnight at 0°C, 5°C, and 25°C resulted in films containing large sections of precipitated testosterone. In order to better elucidate this problem, mucoadhesive gel solutions containing

different amounts of testosterone (i.e., 100%, 75%, 50%, 25%, and 0% corresponding to 4 mg, 3 mg, 2 mg, 1 mg, and 0 mg, testosterone per disk, respectively) were cast in order to ascertain the effect of testosterone loading on the presence of the observed precipitate. Visual observations of all dried films at 0°C, 5°C, and 25°C showed a direct correlation between testosterone loading and the degree of precipitation. Likewise, post-loading a concentrated solution of testosterone dissolved in ethanol on placebo films, and dried overnight at 0°C, 5°C, and 25°C, also resulted in a similar precipitate. In contrast, it was observed that drying casted mucoadhesive gel solutions overnight at 55°C produced mono-layered mucoadhesive films that were transparent and elastic. No testosterone precipitates were visible even when viewed under a microscope with 100X magnification.

The physical properties of mono-layered mucoadhesive films and wax-film composites for both placebo and testosterone (4 mg) are listed in Table 4. On average, WFCs with testosterone (4 mg) weighed 14 mg (RSD = 18.4%) and had an overall thickness of 186 μm (RSD = 18.4%). For both the placebo and testosterone WFC, Noveon AA-1 and Eudragit S-100 in the mucoadhesive layer comprised about 60% of the total weight of the WFCs. For testosterone WFCs, testosterone (4 mg) comprised about 40% of the total weight of the mucoadhesive layer and about 28% of the total weight of the WFCs.

The intra-disk content uniformity of testosterone (4 mg) in WFCs (3/8 inch disks, n=10) punched from ten locations from the larger films casted in plastic circular hollow rings (diameter = 6.2 cm; total area = 30.175 cm^2) was 4.2 ± 0.8 mg (range = 4.1-4.4 mg; RSD = 1.8%). The inter-disk content uniformity of WFCs punched from three larger films was 4.2 ± 0.08 mg (n=10; RSD = 1.8%), 4.2 ± 0.05 mg (n=3; RSD = 1.1%), and 4.2 ± 0.06 mg (n=3; RSD = 1.4%).

The adhesion time of placebo and testosterone WFCs to glass was measured as a function of both temperature and sodium lauryl sulfate (SLS) concentration in the buffer. In general, disks adhered to the glass matrix on average from approximately 2 to 2.5 hours (Figure 16). There was no statistical difference ($p>0.05$) between the adhesion time of placebo WFCs and testosterone WFCs at either 25°C versus 37°C or in 0% SLS versus 3% SLS as determined using a two-sample t-test assuming unequal variances.

***In-vitro* release studies of testosterone**

As shown in Figure 17, there was a marked effect of sodium lauryl sulfate (SLS) concentration in the release media on both the overall release profile and extent of release of testosterone from WFCs containing testosterone. For WFCs containing 4 mg testosterone per disk in buffer containing 3% SLS, the average cumulative amount of testosterone released after

120 minutes was $67.5\% \pm 0.3$ (RSD = 0.4%). In contrast, the cumulative amount of testosterone released in buffer containing no SLS was significantly lower at $38.2 \pm 1.3\%$ after 120 minutes ($p = 0.0005$). Temperature was also found to influence the overall release profile and extent of release of testosterone from WFCs as shown in Figure 17. The extent of testosterone release from WFCs in buffer containing 3% SLS at 37°C was nearly complete at 120 minutes ($93.5\% \pm 7.9\%$). Further, the release rate of testosterone from WFCs under these conditions was zero-order with k being equal to $4 \mu\text{g mL}^{-1} \text{min}^{-1}$ corresponding to a release rate of $1\% \text{min}^{-1}$. For a WFC having a diameter of $3/8$ inch and a total surface area equal to 0.712 cm^2 , the release rate of testosterone from the disk was calculated as $5.6 \mu\text{g cm}^2 \text{mL}^{-1} \text{min}^{-1}$. A comparison of the release rate of testosterone from either mono-layer mucoadhesive films or bi-layer WFCs was also determined in order to determine the effect, if any, of the thin wax-backing layer on the diffusion of testosterone out of the mucoadhesive layer. As shown in Figure 17, the release rate of testosterone from mono-layer mucoadhesive films was found to be zero-order after an initial lag period of about 20 minutes.

Detection of testosterone in rabbit serum

For assay qualification, three independent standard curves of spiked commercially available rabbit serum were generated and assayed using the competitive testosterone ELISA. The mean and standard deviations of the O.D. readings of the three measurements at each testosterone concentration are plotted in Figure 18. For comparison, the standard curve of testosterone in buffer (using the standards provided in the kit) is shown. The assayed samples at each testosterone concentration showed good reproducibility especially at testosterone concentrations equal to or greater than 1 ng/mL (CV ranged from 3.5% to 13.2%, with an average CV of 8.1%). The deviation of the standard curve of testosterone in spiked rabbit serum from that of the standard curve of testosterone provided in the kit was expected based on information provided in the competitive ELISA kit. Based on these assay results, the working range of testosterone concentrations for the *in-vivo* rabbit study was $1\text{-}16 \text{ ng/mL}$. Serum samples collected from rabbits were diluted with 10 mM phosphate buffer, pH 6.0 in order to provide testosterone concentrations within this working range. A new standard curve of testosterone spiked in rabbit serum was completed for each new 96-well plate used.

***In-vivo* studies in rabbits**

All placebo ($n=2$) and testosterone WFCs ($n=5$) removed after 3 hours from the buccal pouch of the rabbits were observed to have remained intact with the mucoadhesive layer and

wax-backing layer still bonded to each other. For all rabbits treated with WFCs, there was no discernable redness or irritation on the treated buccal tissue at 3 hours.

Mean serum testosterone levels after both intravenous and buccal application of WFCs containing 4 mg of testosterone are shown for individual rabbits in Figure 19. The mean serum testosterone levels are for $n=2-5$ replicates/sample. The overall CV of replicate measurements for the intravenous and buccal groups were 13.5% and 13.9%, respectively. The range and median CVs of replicate measurements for each serum sample assayed for the intravenous group ($n=3$ rabbits) was 3.0 - 41% and 11.1%, respectively. The range and median CVs of replicate measurements for each serum sample assayed for the buccal group ($n=5$ rabbits) was 2.5 - 40.8% and 11.7%, respectively. Collectively, these data provided sufficient confidence that the competitive ELISA for testosterone was reliable and reproducible.

Pharmacokinetic parameters such as C_{max} , T_{max} , and AUC for each individual rabbit are reported in Table 5. The average relative bioavailability after buccal application of the testosterone WFCs was $50.2 \pm 3.2\%$ ($n=3$) with a coefficient of variation of 6.4%. WFCs removed at 3 hours and analyzed for residual testosterone in the disks showed WFCs had 0.5 mg, 0.2 mg, and 0.9 mg of retained testosterone in the disks at the time of removal. Thus, mass balance analysis demonstrated that from 77.5-95% of the 4 mg loaded testosterone was released within 180 minutes in the rabbit buccal pouch.

The average C_{max} for the rabbits treated with the WFC was 127 ± 13 ng/mL, which was 9.4-fold lower than the C_{max} for rabbits injected intravenously with testosterone. The average T_{max} for the rabbits treated with the WFCs was 200 ± 35 min. The elimination half-lives of testosterone after intravenous injection ($n=3$) and buccal application ($n=3$) were 42 ± 13 min and 32 ± 2 min, respectively. The differences in the elimination half-lives were found to be statistically insignificant ($p>0.05$) using a two-sample t-test assuming unequal variances.

The relative bioavailability after buccal application of the testosterone WFCs pre-wetted with 1 mg sodium lauryl sulfate was 45.7% and 47.5% for the two rabbits. These values were very comparable to the relative bioavailability of testosterone from WFCs pre-wetted with buffer alone ($p>0.05$). Thus, these results suggest no apparent benefit in pre-wetting WFCs with a known penetration enhancer such as SLS. However, it is acknowledged that this group consisted of only two rabbits. WFCs pre-wetted with 1 mg SLS had 1.6 mg and 1.1 mg of retained testosterone in the disks at the time of removal.

DISCUSSION

Androgen deficiency, also known as hypogonadism, occurs in about 1 in 1000 newborn males (Hamerton *et al.*, 1975; Plymate, 1994; Hellstrom, 1999). There are currently several marketed products of testosterone indicated for androgen replacement therapy including intramuscular oil-based depot injections, transdermal systems, oral tablets, and sublingual tablets (Voorspoels *et al.*, 1996; Dobs *et al.*, 1998; Winters, 1999; Slater *et al.*, 2001; Kim *et al.*, 1995; Cutter, 2001). The oral bioavailability of testosterone has been reported to be from 1-6% due to extensive first pass metabolism and low aqueous solubility (Voorspoels *et al.*, 1996; Cutter, 2001). The solubility of testosterone in water at 37°C is only 46.3 µg/mL (Voorspoels *et al.*, 1996). There are currently three marketed transdermal products for delivering from 2.5 mg to 6 mg testosterone. However, these patches are as large as 44 cm² and have been reported to cause skin irritation in as high as 60% of patients due to the inclusion of penetration enhancers (Jordan, 1997; Jordan and Atkinson, 1998; Dobs *et al.*, 1999).

Due to these limiting factors, buccal delivery of testosterone may be a plausible approach. Several groups have reported on the buccal delivery of testosterone with various results. Voorspoel *et al.* reported that the bioavailability of testosterone (60 mg) using a bioadhesive buccal tablet in dogs was $14 \pm 7\%$ providing testosterone plasma levels at above 3 ng/mL for 15 to 24 hours (Voorspoels *et al.*, 1996). The relatively low bioavailability of testosterone using the buccal tablet was explained by the lack of an impermeable backing layer on the tablet, which caused a significant amount of the total dose to be swallowed. Dobs *et al.* reported that transmucosal delivery of testosterone (10 mg) in a buccal tablet produced peak serum testosterone levels within 30 minutes in hypogonadal males with a mean serum concentration of 270 ± 15 ng/mL (Dobs *et al.*, 1998). Testosterone levels were found to return to baseline within 4-6 hours resulting in area under the curves that were far less than that using other commercially available testosterone products.

The purpose of these studies was to further develop a novel bi-layer mucoadhesive wax-film composite (WFC) comprised of a pH-sensitive mucoadhesive layer and a pharmaceutical wax as the impermeable backing layer (Cui and Mumper, 2001), and to test the relative bioavailability of testosterone via the buccal route in rabbits. The rabbit model was chosen since the rabbit is the only laboratory rodent that has non-keratinized mucosal lining similar to human tissue (Alur *et al.*, 1999; Li *et al.*, 1997; Oh and Ritschel, 1990; Siegel *et al.*, 1981; Siegel and Gordon, 1986; Dowty *et al.*, 1992; Alur *et al.*, 1999). The goal of this present study was to demonstrate sufficiently high relative bioavailability of testosterone, ideally without the need for a commonly employed penetration enhancer such as sodium lauryl sulfate (SLS). SLS has been

reported to increase the transmucosal delivery of a number of drugs by the buccal route when included in various dosage forms (Hoogstraate and Wertz, 1998; Junginger *et al.*, 1999). However, SLS has also been reported to cause marked irritation to the buccal epithelium (Siegel *et al.*, 1981; Siegel and Gordon, 1986; Griffiths *et al.*, 1996; Nicander *et al.*, 1997).

It was demonstrated that bi-layer placebo or testosterone WFCs could be manufactured with excellent uniformity in both weight and thickness. Further, optimal bi-layer WFCs were found to adhere to either glass or the buccal pouch of rabbits for an extended period of time (i.e., 2.5 to 4 hours). Although the *in-vitro* glass adhesion model is a very simple model, good correlation was found between the adhesion time of various WFCs to glass and the adhesion time to the buccal pouch of rabbits (Cui and Mumper, 2001). Important correlates to adhesion time were found to be the ratio of Noveon/Eudragit S-100, and the presence and thickness of the wax-backing layer. In general, increasing percentage of Noveon, a mucoadhesive polymer, increased the adhesion time. However, too much Noveon created a mucoadhesive layer that swelled too rapidly and extensively. The presence of Eudragit S-100, a pH-sensitive polymer with some mucoadhesive properties, provided rigidity and allowed the mucoadhesive layer to adhere longer. The presence of the wax-backing layer greatly enhanced the adhesion time of the mucoadhesive layer by retarding the diffusion of (simulated) saliva. Further, the presence of the wax-backing layer retarded the diffusion of drug into the mouth.

The *in-vitro* release studies with testosterone WFCs demonstrated that temperature and the presence of sodium lauryl sulfate in the buffer (to promote sink conditions) greatly affected both the release rate and extent of release of testosterone from the WFCs. Ideally, under *in-vitro* conditions that most closely simulated physiological conditions (37°C, sink conditions), the release rate of testosterone was found to be zero-order. Further, all of the testosterone was released under these conditions within two hours, which may be important for compliance reasons when dosing humans. In contrast, other buccal delivery systems have been shown to release drug very slowly up to 10-24 hours post-application Voorspoels *et al.*, 1996; Benes *et al.*, 1997; Li *et al.*, 1997).

In the present study, the relative bioavailability of testosterone (4 mg) via the buccal pouch in rabbits (n=3) treated with the WFC was $50.2 \pm 3.2\%$ (n=3) with a coefficient of variation of only 6.3%. These results are noteworthy for several reasons. First, the overall relative bioavailability was 3.5-fold greater than those values previously reported using bioadhesive buccal tablets in dogs. Possible reasons for this could be explained by species differences, by the inclusion of an impermeable wax-backing layer in our WFC that impeded drug loss to the mouth, and/or by the application of a more optimal buccal delivery system.

Second, the CV of only 6.4% in the present study was markedly lower than those of other reported studies. For example, Alur *et al.* showed that the CVs of chlorpheniramine maleate after buccal application of mucoadhesive tablets to rabbits ranged from 13-29% (Alur *et al.*, 1999). Further, Li *et al.* showed that the CV of oxytocin after buccal application to rabbits using a mucoadhesive buccal patch was 21% (Li *et al.*, 1997). Finally, Benes *et al.* demonstrated that the CV of melatonin after buccal application of the bi-layer CydotTM system to humans was 29%. (Benes *et al.*, 1997). Third, the relative bioavailability of testosterone in this present study could be achieved in anaesthetized rabbits without the use of a known penetration enhancer such as SLS. Presumably, overall blood flow to the buccal tissue is reduced in animals under anesthesia.

CONCLUSIONS

Mucoadhesive bi-layer wax-film composites (WFCs) were developed containing 4 mg of pre-loaded testosterone. The release rate of testosterone from optimal WFCs was zero-order and was shown to be dependent on both temperature and surfactant concentration in the buffer media. The relative bioavailability of testosterone via the buccal route in rabbits of about 50% was 3.5-fold higher than previously reported for testosterone and far less variable. Further, the inclusion of a penetration enhancer was not needed. It was concluded that these bi-layer mucoadhesive WFC disks could deliver physiologically relevant amounts of testosterone across the buccal mucosa in rabbits and that this system may be amenable to a number of other poorly-soluble drugs and/or those exhibiting extensive first-pass metabolism.

Table 4: Physical Properties of Mono-Layered Films and Wax-Film Composites (3/8 inch diameter disks)

Sample (n = 10)	Weight (mg)	Thickness (μm)
Placebo		
Mono-layered film	5.5 ± 0.7	51 ± 4
Wax-film composite	9.7 ± 1.2	109 ± 6
Testosterone (4 mg)		
Mono-layered film	10.1 ± 1.0	120 ± 14
Wax-film composite	14.0 ± 2.6	186 ± 34

Table 5: Pharmacokinetic Parameters for Testosterone (4 mg) Administered to Rabbits by Either Intravenous Injection or Buccal Wax-Film Composite (WFC) Application

Treatment	$C_{\max}$ (ng/mL)		$AUC_{(0-\infty)}$ (ng*min/mL)
<i>Intravenous</i>			
Rabbit #1	1150		62,077
Rabbit #2	1240		36,302
Rabbit #3	1209		46,301
	1200 ± 46		$48,227 \pm 12,995$ $CV = 26.9\%$
<i>Buccal WFC</i>			
Rabbit #1	129	240	24,095
Rabbit #2	139	185	22,745
Rabbit #3	114	176	25,823
	127 ± 13	200 ± 35	$24,221 \pm 1543$ $CV = 6.4\%$
<i>Buccal WFC with sodium lauryl sulfate (1 mg)</i>			
Rabbit #4	139	174	22,040
Rabbit #5	159	119	22,893

The pharmacokinetic parameters were determined using WinNonlin® Professional Version 3.1 from Pharsight Corporation (Mountain View, CA) using a noncompartmental modeling (NCA Model 200) and the Linear Trapezoidal Method. Serum testosterone concentrations were determined using a commercially available competitive ELISA kit as described in the Materials and Methods section (n = 2-5 replicates/sample).

LITERATURE CITED

Ahlfors and Czerkinsky, "Contact sensitivity in the murine oral mucosa. I. An experimental model of delayed-type hypersensitivity reactions at mucosal surfaces," *Clin. Exp. Immunol.*, 86:449-456, 1991.

Alur, Beal, Pather, Mitra, Johnston, "Evaluation of a novel, natural oligosaccharide gum as a sustained-release and mucoadhesive component of calcitonin buccal tablets," *J. Pharm. Sci.*, 88:1313-1319, 1999.

Alur, Pather, Mitra, Johnston, "Transmucosal sustained-delivery of chlorpheniramine maleate in rabbits using a novel, natural mucoadhesive gum as an excipient in buccal tablets," *Int. J. Pharm.*, 188:1-10, 1999.

Aspden, Adler, Davis, Skaugrud, Illum, "Chitosan as a nasal delivery system: evaluation of the effect of chitosan on mucociliary clearance rate in the frog palate model," *Int. J. Pharm.*, 122:69-78, 1995.

Barret, Cruchley, Williams, "Oral mucosal Langerhans cells," *Crit. Rev. Oral Biol. Med.*, 7:36, 1996.

Bayley, Temple, Clay, Steward, Lowther, "The transmucosal absorption of recombinant human interferon-alpha B/D hybrid in the rat and rabbit," *J. Pharm. Pharmacol.*, 47:721-724, 1995.

Bechgaard, Gizurason, Hjorkjaer, "Solubilization of various benzodiazepines for intranasal administration, a pilot study," *Pharm. Dev. Tech.*, 2:293-296, 1997.

Benes, Claustrat, Horriere, Geoffriau, Konsil, Parrott, DeGrande, McQuinn, Ayres, "Transmucosal, oral controlled-release, and transdermal drug administration in human subjects: a crossover study with melatonin," *J. Pharm. Sci.*, 86:1115-1119, 1997.

Berthold, Cremer, Kreuter, "Preparation and characterization of chitosan microspheres as drug carriers for prednisolone sodium phosphate as a model for anti-inflammatory drugs," *J. Cont. Rel.*, 39:17-25, 1996.

Bhatt and Johnston, "In vitro release and permeation of oxytocin from a mucoadhesive buccal patch," *Pharm. Dev. Technol.*, 1:357-364, 1996.

Bhatt and Johnston, "Transmucosal delivery of oxytocin to rabbits using a mucoadhesive buccal patch," *Pharm. Dev. Technol.*, 2:265-274, 1997.

Binnie, Curro, Khandwala, Van Inwegen, "Amlexanox oral paste: a novel treatment that accelerates the healing of aphthous ulcers," *Compend Contin Educ Dent.*, 18:1116-1118, 1997.

Bodde, de Vries, Junginger, "Mucoadhesive polymers for the buccal delivery of peptides, structure-adhesiveness relationships," *J. Cont. Rel.*, 13:225-231, 1990.

Casetta and Negretti, "Salivary immune responses after gingival immunization with tetravaccinal and bivaccinal formulations of inactivated micro-organisms," *Dev. Biol. Stand.*, 92:317-321, 1998.

Chang, "Stability of hirudin, a thrombin-specific inhibitor," *J. Biol. Chem.*, 266:10839-10843, 1991.

Cripps, Kyd, Foxwell, "Vaccines and mucosal immunization," *Vaccine*, 19:2513-2515, 2001.

Cui and Mumper, "Bi-layer disks for mucosal (genetic) immunization via the buccal route in rabbits," *AAPS PharmSci.*, 3(3)Suppl, 2001.

Cui and Mumper, "Chitosan-based nanoparticles for topical genetic immunization," *J. Control. Rel.*, 75:409-419, 2001.

Cui and Mumper, "Enhanced humoral and proliferative immune responses after topical application of nanoengineered genetic vaccines," *J. Control. Rel.*, Submitted, 2001.

Cutter, "Compounded percutaneous testosterone gel: use and effects in hypogonadal men," *J. Am. Board Fam. Pract.*, 14:22-32, 2001.

Dobs, Hoover, Chen, Allen "Pharmacokinetic characteristics, efficacy, and safety of buccal testosterone in hypogonadal males: a pilot study," *J. Clin. Endoc. Metab.*, 83:33-39, 1998.

Dobs, Meikle, Arver, Sanders, Caramelli, Mazer, "Pharmacokinetics, efficacy, and safety of a permeation-enhanced testosterone transdermal system in comparison with bi-weekly injections of testosterone enanthate for the treatment of hypogonadal men," *J. Clin. Endocrinol. Metab.*, 84:3469-3478, 1999.

Dowty, Knuth, Robinson, "Enzyme characterization studies on the rate-limiting barrier in rabbit buccal mucosa," *Int. J. Pharm.*, 88:293-302, 1992.

Duchene, Touchard, Peppas, "Pharmaceutical and medical aspects of bioadhesive systems for drug administration," *Drug Devol. Ind. Pharm.*, 14:283-318, 1988.

Eric and Caroline, "Influence of storage conditions on the activity of recombinant hirudin," *Thromb Res.*, 61:87-89, 1991.

Eriksson, Ekman, Lindbratt, Baur, Bach, Torholm, Kalebo, Close, "Prevention of thromboembolism with use of recombinant hirudin. Results of a double-blind, multicenter trial comparing the efficacy of desirudin (Revasc) with that of unfractionated heparin in patients having a total hip replacement," *J Bone Joint Surg Am.*, 79:326-33, 1997.

Esslinger, Haas, Maurer, Lassmann, Dubbers, Muller-Peltzer, "Pharmacodynamic and safety results of PEG-hirudin in healthy volunteers," *Thromb. Haemost.*, 77:911-919, 1997.

Etchart, Buckland, Liu, Wild, Kaiserlian, "Class I-restricted CTL induction by mucosal immunization with naked DNA encoding measles virus haemagglutinin," *J. Gen. Virol.*, 78:1577-1580, 1997.

Etchart, Desmoulins, Chemin *et al.*, "Dendritic cells recruitment and in vivo priming of CD8+ CTL induced by a single topical or transepithelial immunization *via* the buccal mucosa with measles virus nucleoprotein," *J. Immunol.*, 164:384-391, 2001.

Fenton, "Thrombin interactions with hirudin," *Sem. Thromb. Hemost.*, 15:265-268, 1989.

Fenton, Villanueva, Ofosu, Maraganore, "Thrombin inhibition by hirudin: how hirudin inhibits thrombin," *Haemostasis*, 21:27-31, 1991.

Garcia-Closas, Herrero, Bratti, Hildesheim, Sherman, Morera, Schiffman, "Epidemiologic determinants of vaginal pH," *Am. J. Obstet. Gynecol.*, 180:1060-1066, 1999.

Goto, Kawata, Suzuki, Kim, Ito, "Preparation and evaluation of Eudragit gels. I: Eudragit organogels containing drugs as rectal sustained-release preparations," *J Pharm Sci.*, 80:958-961, 1991.

Griffiths, Dabelsteen, Voorhees, "Topical retinoic acid changes the epidermal cell surface glycosylation pattern toward of a mucosal epithelium," *Br. J. Dermatol.*, 134:431-436, 1996.

Gurunathan, Klinman, Seder, "DNA vaccines: Immunology, application, and optimization," *Annu. Rev. Immunol.*, 18:927-974, 2000.

Hamerton, Canning, Ray, Smith, "A cytogenetic survey of 14,069 newborn infants," *Clin Genet.*, 8:223-243, 1975.

Hardy, de Padu, Osis, Jimenez, Zaneveld, "Women's preferences for vaginal antimicrobial contraceptives. IV. Attributes of a formulation that would protect from STD/AIDS," *Contraception*, 58:251-255, 1998b.

Hardy, Jimenez, de Padue, Zaneveld, "Women's preferences for vaginal antimicrobial contraceptives. III. Choice of a formulation, applicator, and packaging," *Contraception*, 58:245-249, 1998a.

Harris and Robinson, "Bioadhesive polymers in peptide and drug delivery," *Biomaterials*, 11:652-558, 1990.

Harris and Robinson, "Drug delivery via the mucous membranes of the oral cavity," *J. Pharm. Sci.*, 81:1-10, 1992.

Heath, Rubin, Holland, Zhang, Snow, Rock, "Mechanism of triclosan inhibition of bacterial fatty acid synthesis," *J Biol Chem.*, 274:11110-11114, 1999.

Heiber, Ebert, Dave, Smith, Kim, Mix, "In vivo buccal deliver of calcitonin," *J. Controlled Release*, 28:269-270, 1994.

Hellstrom, "Testosterone replacement therapy," Digital Urology Journal. website: <http://www.duj.com/Article/Hellstrom2/Hellstrom2.html>, 1999.

Henriksen, Vagen, Sande, Smistad, Karlsen, "Interactions between liposomes and chitosan II: effect of selected parameters on aggregation and leakage," *Int. J. Pharm.*, 146:193-204, 1997.

Hjortkjaer, Bechgaard, Gizurarson, Suzdak, McDonald, Greenough, Single- and repeated-dose local toxicity in the nasal cavity of rabbits after intranasal administration of different glycols for formulations containing benzodiazepines," *J. Pharm. Pharmacol.*, 51:377-383, 1999.

Holmgren, Lycke, Czerhinsky, "Cholera toxin and cholera toxin B-subunit as oral-mucosal adjuvant in antigen vector systems," *Vaccine*, 11:1179-1184, 1993.

Hoogstraate and Wertz, "Drug delivery via the buccal mucosa," *Pharm. Sci. Tech. Today*, 1:309-316, 1998.

Illum, Farraj, Davis, "Chitosan as a novel nasal delivery system for peptide drugs," *Pharm. Res.*, 11:1186-1189, 1994.

Imai, Shiraishi, Saito, Otagiri, "Interaction of indomethacin with low molecular weight chitosan, and improvements of some pharmaceutical properties of indomethacin by low molecular weight chitosans," *Int. J. Pharm.*, 67:11-20, 1991.

Imaoka *et al.*, "Nasal immunization of nonhuman primates with simian immunodeficiency virus p550gag and cholera toxin adjuvant induced Th1/Th2 help for virus specific immune responses in reproductive tissue," *J. Immunol.* 161:5952-8, 1998.

Jones, Francis, Hutchinson, Handley, Lyle, "Targeting and delivery of bactericide to adsorbed oral bacteria by use of proteoliposomes," *Biochim Biophys Acta.*, 1147:251-261, 1993.

Jordan and Atkinson, "Comparison of the skin irritation potential of two testosterone transdermal systems: An investigational system and a marketed product," *Clin. Ther.*, 20:80-87, 1998.

Jordan, "Allergy and topical irritation associated with transdermal testosterone administration: A comparison of scrotal and nonscrotal transdermal systems," *Am. J. Contact Derm.*, 8:103-113, 1997.

Junginger, Hoogstraate, Coos, "Recent advances in buccal delivery and absorption – *in vitro* and *in vivo* studies," *J. Controlled Release*, 62:149-159, 1999.

Kawata, Suzuki, Kim, Ito, Kurita, Miyagoe, Goto, "Preparation and evaluation of Eudragit gels. II: In vitro release of salicylic acid, sodium salicylate, and ketoprofen from Eudragit L and S organogels," *J Pharm Sci.*, 80:1072-1074, 1991.

Khan, Prebeg, Kurjakovic, "A pH-dependent colon targeted oral drug delivery system using methacrylic acid copolymers. I. Manipulation of drug release using Eudragit L100-55 and Eudragit S100 combinations," *J Controlled Release*, 58:215-22, 1999.

Khandwala, Van Inwegen, Alfano, "5% amlexanox oral paste, a new treatment for recurrent minor aphthous ulcers: I. Clinical demonstration of acceleration of healing and resolution of pain," *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.*, 83:222-230, 1997a.

Khandwala, Van Inwegen, Charney, Alfano, "5% amlexanox oral paste, a new treatment for recurrent minor aphthous ulcers: II. Pharmacokinetics and demonstration of clinical safety," *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.*, 83:231-238, 1997b.

Kim, Ito, Kawata, Uchida, T., Goto, S. Preparation and evaluation of Eudragit gels. IV: Rectal gel preparations for sustained release and avoidance of first-pass metabolism of propentofylline. *J Pharm Sci.* 81:904-907, 1992b.

Kim, Kawata, Uchida, Goto, "Preparation and evaluation of Eudragit gels. III: Rectal gel preparations for sustained release of pentoxifylline," *J Pharm Sci.*, 81:537-40, 1992.

Kim, Snipes, Hodgen, Anderson, "Pharmacokinetics of a single dose of buccal testosterone," *Contraception*, 52:313-316, 1995

Kim, Umejima, Ito, Uchida, Goto, "Preparation and evaluation of Eudragit gels. V. Rectal gel preparations for sustained release and avoidance of first-pass metabolism of lidocaine," *Chem Pharm Bull.*, 40:2800-2804, 1992a.

Lehr, Bouwstra, Schacht, Junginger, "In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers," *Int. J. Pharm.*, 78:43-48, 1992.

Leopold and Eikeler, "Eudragit E as a coating material for the pH-controlled drug release in the topical treatment of inflammatory bowel disease (IBD)," *J. Drug Targeting*, 6:85-94, 1998.

Leung, Nagai, Machida, "Mucoadhesive dosage forms for peptide and protein drug delivery," In: *Peptide and Protein Drug Delivery*, Lee (Ed), Marcel Dekker, Inc. New York, pp. 741-767, 1991.

Li, Bhatt, Johnston, "Transmucosal delivery of oxytocin to rabbits using a mucoadhesive buccal patch," *Pharm. Dev. Tech.*, 2:265-274, 1997.

Liu, Hilleman, Kurth, *DNA Vaccines: A New Era in Vaccinology*. Vol. 772. *Ann. N.Y. Acad. Sci.* New York, NY, 1995.

Loftsson, Leeves, Bjornsdottir, Duffy, Masson, "Effect of cyclodextrins and polymers on triclosan availability and substantivity in toothpastes *in vivo*," *J Pharm Sci.*, 88:1254-1258, 1999.

Lundholm, Asahura, Hinkula, Lucht, Wahren, "Induction of mucosal IgA by a novel jet delivery technique for HIV-1 DNA," *Vaccine*, 17:2036-2042, 1999.

MacLaughlin, Mumper, Wang, Tagliaferri, Rolland, "Chitosan and depolymerized chitosan oligomers as condensing carriers for in-vivo plasmid delivery," *J. Controlled Rel.*, 56:259-272, 1998.

Mandel, "Antimicrobial mouthrinses: overview and update," *J Am Dent Assoc.*, 125:2S-10S, 1994.

March and Nakamura, "Evaluation of the duration of effect of a bioadhesive vaginal moisturizing gel on vaginal pH, 7th International Congress on the Menopause, Replens Symposium. Stockholm, Sweden, June 20-22, 1993.

Markwardt, "Development of hirudin as an antithrombotic agent," *Sem. Thromb. Hemost.*, 15:269-282, 1989.

Markwardt, "Hirudin and derivatives as anticoagulant agents," *Thromb. Haemost.*, 66:141-152, 1991a.

Markwardt, "Past, present and future of hirudin," *Haemostatis*, 21:11-26, 1991b.

Martin's Physical Pharmacy: Physical Chemical Principles in the Pharmaceutical Sciences,. Martin, Swarbrick, Cammarata (Eds.), Third Edition. Lea and Febiger, Philadelphia, 1983.

McGhee and Kiyono, "Mucosal immunity to vaccines: Current concepts for vaccine development and immune response analysis," In: *Genetically engineered vaccines*, Ciardi (Ed.), Plenum Press, New York, N.Y. pp. 3-12, 1992a.

McGhee, Lamn, Strober, "Mucosal immune responses," In: *Mucosal immunolog*, Orga, Mestechky, Lamn, Stober, Bienenstock, McGhee (Eds.), 2nd ed. San Diego: Academic Press, 1999.

Meshali and Gabr, "Effect of interpolymer complex formation of chitosan with pectin or acacia on the release behavior of chlorpromazine HCl," *Int. J. Pharm.*, 89:177-181, 1993.

Nicander, Rozell, Rundquist, Ollmar, "Electrical impedance. A method to evaluate subtle changes of the human oral mucosa," *Eur. J. Oral Sci.*, 105:576-582, 1997.

Oh and Ritschel, "Biopharmaceutic aspects of buccal absorption of insulin," *Meth. Find. Exp. Clin. Pharmacol.*, 12:205-212, 1990.

Park and Robinson, "Physico-chemical properties of water insoluble polymers important to mucin/epithelial adhesion," *J. Cont. Rel.*, 2:47-57, 1985.

Peniston and Johnson, "Process for depolymerization of chitosan," U. S. Pat. No. 3,922,260, 1975.

Plymate, "Hypogonadism," *Endoc. Metab. Clin. N. Am.*, 23:749-772, 1994.

Rathbone, Ponchel, Ghazali, In: *Oral Mucosal Drug Delivery*, Rathbone (Ed), Marcel Dekker Inc. New York, 1996.

Remington's Pharmaceutical Sciences. 18th Edition. A.R. Gennaro, ed. Mack Publishing Company, Easton, Pennsylvania, 1990.

Riehl-Bellon, Carvalho, Acker, Van Dorsselaer, Marquet, Loison, Lemoine, Brown, Courtney, Roitsch, "Purification and biochemical characterization of recombinant hirudin produced by *Saccharomyces cerevisiae*," *Biochemistry*, 28:2941-2949, 1989.

Robinson and Torres, "C.A.T. DNA Vaccines," *Sem. Immun.*, 9:271-283, 1997.

Roy, Mao, Huang, Leong, "Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy," *Nat. Med.*, 5:387-391, 1999.

Saijo, Kuriki, Ashida, Makino, Maki, "Inhibition by amoxanox (AA-673) of the immunologically, leukotriene D4- or platelet-activating factor-stimulated bronchoconstriction in guinea pigs and rats," *Int Arch Allergy Appl Immunol.*, 77:315-321, 1985.

Saijo, Makino, Tamura, Kuriki, Ashida, Terao, Maki, "The antiallergic agent amoxanox suppresses SRS-A generation by inhibiting lipoxygenase," *Int Arch Allergy Appl Immunol.*, 79:231-237, 1986.

Sayani, Chun, Chien, "Transmucosal delivery of leucine enkephalin: stabilization in rabbit enzyme extracts and enhancement of permeation through mucosae," *J. Pharm. Sci.*, 82:1179-1185, 1993.

Schechter, Weisman, Rosenblum, Bernstein, Conard, "The use of oral transmucosal fentanyl citrate for painful procedures in children," *Pediatrics*, 95:335-339, 1995.

Schipper, Varum, Artursson, "Chitosans as absorption enhancers for poorly absorbable drugs. I: influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells," *Pharm. Res.*, 13:1686-1692, 1996.

Schurr, Knoll, Ziegler, Anders, Merkle, "Comparative study of intravenous, nasal, oral, and buccal TRH administration among healthy subjects," *J. Endocrinol. Invest.*, 8:41-44, 1985.

Schwagmeier, Alincic, Striebel, "Midazolam pharmacokinetics following intravenous and buccal administration," *Br. J. Clin. Pharmacol.*, 46:203-206, 1998.

Scott, Besag, Neville, "Buccal midazolam and rectal diazepam for treatment of prolonged seizures in childhood and adolescence: a randomized trial," *The Lancet.*, 353:623-626, 1999.

Shen, Goldberg, Saltzman, "Local DNA delivery polymer disks stimulate mucosal immunity in the vagina," The 28th International Symposium on Controlled Release of Bioactive materials, San Diego, CA, June 23-27, 2001.

Shiraishi, Imai, Otagiri, "Controlled release of indomethacin by chitosan-polyelectrolyte complex: optimization and in vivo/in vitro evaluation," *J. Cont. Rel.*, 25:217-225, 1993.

Siegel and Gordon, "Surfactant-induced alterations of permeability of rabbit oral mucosa *in vitro*," *Exp. Mol. Path.*, 44:132-137, 1986.

Siegel, Izutsu, Watson, "Mechanisms of non-electrolyte penetration across dog and rabbit oral mucosa *in vitro*," *Arch. Oral Biol.*, 26:357-361, 1981.

Slater, Souter, Zhang, Guan, Stanczyk, Mishell, "Pharmacokinetics of testosterone after percutaneous gel or buccal administration," *Fertil Steril*, 76:32-37, 2001.

Spannagl, Bichler, Lill, Schramm, "A fast photometric assay for the determination of hirudin," *Haemostasis*, 21:36-40, 1991.

Stevens-Simon, Jamison, McGregor, Douglas, "Racial variation in vaginal pH among healthy sexually active adolescents," *Sex. Transm. Dis.*, 21:168-172, 1994.

Takayama, Hirata, Machida, Masada, Sannan, Nagai, "Effect of interpolymer complex formation on bioadhesive property and drug release phenomenon of compressed tablet consisting of chitosan and sodium hyaluronate," *Chem. Pharm. Bull.*, 38:1993-1997, 1990.

Takeuchi, Yamamoto, Niwa, Hino, Kawashima, "Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes," *Pharm. Res.*, 13:896-901, 1996.

Tang, DeVit, Johnston, "Genetic immunization is a simple method for eliciting an immune response," *Nature*, 356:152-154, 1992.

Taubes, "Salvation in a snippet of DNA," *Science*, 278:1711-1714, 1997.

The United States Pharmacopeia (23)/The National Formulary (18), United States Pharmacopeial Convention, Inc. Rockville, MD, 1995.

Ulmer, Donnelly, Liu, "Toward the development of DNA vaccines," *Curr. Opin. Immun.*, 8:531-536, 1996.

Umejima, Kim, Ito, Uchida, Goto, "Preparation and evaluation of Eudragit gels. VI: In vivo evaluation of Eudragit rectal hydrogel and xerogel containing salicylamide," *J Pharm Sci.*, 82:195-9, 1993.

Urisu, Iimi, Kondo, Horiba, Masuda, Tsuruta, Yazaki, Torii, "Inhibitory action amlexanox on interleukin-3-induced enhancement of histamine releasability of human leukocytes," *Arerugi.*, 39(10):1448-1454, 1990.

Voorspoels Remon, Eechaute, De Sy, "Buccal absorption of testosterone and its esters using a bioadhesive tablet in dogs," 13:1228-1232, 1996.

Wade and Weller, "Handbook of pharmaceutical excipients," (2nd ed.) American Pharmaceutical Association, Washington, and The Pharmaceutical Press, London, 1994.

Wang *et al.*, "Gene gun-mediated oral mucosal transfer of interleukin 12 cDNA coupled with an irradiated melanoma vaccine in a hamster model: Successful treatment of oral melanoma and distant skin lesion," *Cancer Gene Ther.*, 8:705-712, 2001.

Winters, "Current status of testosterone replacement therapy in men," *Arch. Fam. Med.*, 8:257-263, 1999.

Wong, Yuen, Peh, "Formulation and evaluation of controlled release Eudragit buccal patches," *Int. J. Pharmaceutics.*, 178:11-22, 1999.

Yankauckas, Morrow, Parker, Abai, Rhodes, Dwarki, Gromkowski, "Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing NP gene," *DNA Cell. Biol.*, 12:771-776, 1993.

Zuckerbraun, Babich, May, Sinensky, "Triclosan: cytotoxicity, mode of action, and induction of apoptosis in human gingival cells *in vitro*," *Eur J Oral Sci.*, 1998 106:628-636, 1998.

CLAIMS

What is claimed is:

1. A pH-sensitive mucoadhesive generally water-insoluble layer, comprising at least one generally water-insoluble swellable mucoadhesive polymer wherein the water-insoluble swellable mucoadhesive polymer is a cross-linked polyacrylic acid, and at least one molecule of interest,

characterized in that the pH-sensitive mucoadhesive generally water-insoluble layer includes at least one pH-sensitive film forming polymer and is formed due to changes in pH and/or desolvation of polymers.

2. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the at least one generally water-insoluble swellable mucoadhesive polymer includes at least one water-insoluble swellable mucoadhesive polymer.

3. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the generally water-insoluble swellable mucoadhesive polymer includes polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol.

4. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the generally water-insoluble swellable mucoadhesive polymer includes Noveon or Carbomer.

5. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the generally water-insoluble swellable mucoadhesive polymer is present at a concentration of from 0.1 % to 20% by weight.

6. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the pH-sensitive film forming polymer includes a copolymer of methacrylic acid and acrylic or methacrylic ester.

7. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the pH-sensitive film forming polymer is present at a concentration of from 0.05% to 10% by weight.

8. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the pH-sensitive film forming polymer includes a Eudragit polymer, or a chemical derivative thereof.

9. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest comprises an active pharmaceutical.

10. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 9, wherein the active pharmaceutical includes at least one member selected from the group consisting of an antimicrobial, an antiviral, an antiinflammatory, an antiseptic, an antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent and a diagnostic agent.

11. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest includes amlexanox.

12. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest includes triclosan.

13. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest includes hirudin.

14. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest includes DNA.

15. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest includes lidocaine, benzocaine, or dyclonine.

16. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the molecule of interest includes at least one benzodiazepine drug or derivative thereof.

17. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the at least one molecule of interest includes an antigen.

18. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the at least one molecule of interest includes DNA expressing a protein.

19. The pH-sensitive mucoadhesive generally water-insoluble layer of claim 1, wherein the at least one molecule of interest includes a hormone.

20. A method of making a pH-sensitive mucoadhesive generally water-insoluble layer, comprising combining a solvent vehicle, at least one generally water-insoluble swellable mucoadhesive polymer wherein the water-insoluble swellable mucoadhesive polymer is a cross-linked polyacrylic acid, and at least one molecule of interest,

characterized in that combining includes adding at least one pH-sensitive film forming polymer and making includes forming the pH-sensitive mucoadhesive water-insoluble layer due to changes in pH and/or desolvation of polymers.

21. The method of claim 20, wherein the at least one generally water-insoluble swellable mucoadhesive polymer includes at least one water-insoluble swellable mucoadhesive polymer.

22. The method of claim 20, wherein the solvent vehicle is comprised of at least 25 to 100 parts water or buffered water with 0 to 75 parts of ethanol, propylene glycol, glycerin, polyethylene glycol, or combinations thereof.

23. The method of claim 20, wherein the at least one molecule of interest includes an antigen.

24. The method of claim 20, wherein the at least one molecule of interest includes DNA expressing a protein.

25. The method of claim 20, wherein the at least one molecule of interest includes a hormone.

26. A product made by the method of claim 20.

27. A pharmaceutical gel composition comprising:
a solvent vehicle comprising at least 25 to 100 parts water or buffered water with 0 to 75 parts of ethanol, propylene, glycol, glycerin, polyethylene glycol, or combinations thereof, where the solvent vehicle is 100 parts by weight total,
at least one water-insoluble swellable mucoadhesive polymer, wherein the water-insoluble swellable mucoadhesive polymer is a cross-linked polyacrylic acid,
at least one pH-sensitive anionic copolymer of methacrylic acid and methacrylic acid esters which form a film when applied to skin or a mucosal surface, and
at least one molecule of interest.
28. The gel of claim 27, wherein the water-insoluble swellable mucoadhesive polymer includes polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol.
29. The gel of claim 27, wherein the water-insoluble swellable mucoadhesive polymer includes Noveon or Carbomer.
30. The gel of claim 27, wherein the water-insoluble swellable mucoadhesive polymer is present at a concentration of from 0.1% to 20% by weight.
31. The gel of claim 27, wherein the pH-sensitive polymer is present at a concentration of from 0.05% to 10% by weight.
32. The gel of claim 27, wherein the pH-sensitive polymer includes a Eudragit polymer, or a chemical derivative thereof.
33. The gel of claim 27, wherein the molecule of interest comprises an active pharmaceutical.
34. The gel of claim 33, wherein the active pharmaceutical includes at least one member selected from the group consisting of an antimicrobial, an antiviral, an antiinflammatory, an antiseptic, an antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent and a diagnostic agent.

35. The gel of claim 27, wherein the molecule of interest includes amlexanox.
36. The gel of claim 27, wherein the molecule of interest includes triclosan.
37. The gel of claim 27, wherein the molecule of interest includes hirudin.
38. The gel of claim 27, wherein the molecule of interest includes plasmid DNA.
39. The gel of claim 27, wherein the molecule of interest includes lidocaine, benzocaine, or dyclonine.
40. The gel of claim 27, wherein the molecule of interest includes at least one benzodiazepine drug or derivative thereof.
41. A pharmaceutical gel which when applied to the skin or mucosal surface forms a film, said gel comprising a solvent vehicle, at least one water-insoluble swellable mucoadhesive polymer, at least one pH-sensitive film-forming polymer, and at least one molecule of interest, wherein said film is formed due to changes in pH and desolvation of the polymer, and wherein said film provides for the delivery of the molecule of interest to or through the application site.
42. The gel of claim 41, wherein the solvent vehicle is comprised of at least 25 to 100 parts water with 0 to 75 parts of ethanol, propylene glycol, glycerin, polyethylene glycol, or combinations thereof.
43. The gel of claim 41, wherein the water-insoluble swellable mucoadhesive polymer includes polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol.
44. The gel of claim 41, wherein the water-insoluble swellable mucoadhesive polymer includes Noveon or Carbomer.
45. The gel of claim 41, wherein the water-insoluble swellable

mucoadhesive polymer is present at a concentration of from 0.1% to 20% by weight.

46. The gel of claim 41, wherein the pH-sensitive polymer includes a copolymer of methacrylic acid and acrylic or methacrylic ester.

47. The gel of claim 41, wherein the pH-sensitive polymer is present at a concentration of from 0.05% to 10% by weight.

48. The gel of claim 41, wherein the pH-sensitive polymer includes a Eudragit polymer, or chemical derivative thereof.

49. The gel of claim 41, wherein the molecule of interest comprises an active pharmaceutical.

50. The gel of claim 49, wherein the active pharmaceutical includes at least one member selected from the group consisting of an antimicrobial, an antiviral, an antiinflammatory, an antiseptic, an antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent and a diagnostic agent.

51. The gel of claim 41, wherein the molecule of interest includes amlexanox.

52. The gel of claim 41, wherein the molecule of interest includes triclosan.

53. The gel of claim 41, wherein the molecule of interest includes a peptide or protein.

54. The gel of claim 41, wherein the molecule of interest includes hirudin.

55. The gel of claim 41, wherein the molecule of interest includes plasmid DNA.

56. The gel of claim 41, wherein the molecule of interest includes lidocaine, benzocaine, or dyclonine.

57. The gel of claim 41, wherein the molecule of interest includes at least one benzodiazepine drug or derivative thereof.

58. The gel of claim 41, wherein the application site is the skin, mouth, vagina, nose, nasal cavity, or other accessible mucosal site.

59. A wax-film composite having a total thickness of less than 5 mm, comprised of a pH-sensitive mucoadhesive layer that is free of any natural polymers and free of any neutral polymers and a water-insoluble wax layer:

wherein the pH-sensitive mucoadhesive layer is comprised of at least one water-insoluble swellable anionic mucoadhesive polymer, at least one anionic pH-sensitive film-forming polymer, and at least one molecule of interest.

60. The wax-film composite of claim 59, wherein the pH-sensitive mucoadhesive layer is present at a concentration of 20% to 90% by weight, and the water-insoluble wax layer is present at a concentration of 10% to 80% by weight.

61. The wax-film composite of claim 59, wherein the water-insoluble swellable anionic mucoadhesive polymer includes polyacrylic acid cross-linked with polyalkenyl ether or divinyl glycol.

62. The wax-film composite of claim 59, wherein the water-insoluble swellable anionic mucoadhesive polymer includes Noveon or Carbomer.

63. The wax-film composite of claim 59, wherein the water-insoluble swellable mucoadhesive polymer is present in the pH-sensitive mucoadhesive layer at a concentration from 0.1% to 20% by weight.

64. The wax-film composite of claim 59, wherein the pH-sensitive polymer present in the pH-sensitive mucoadhesive layer includes a copolymer of methacrylic acid and acrylic or methacrylic ester.

65. The wax-film composite of claim 59, wherein the pH-sensitive polymer is present in the pH-sensitive mucoadhesive layer at a concentration of from 0.05% to 10% by

weight.

66. The wax-film composite of claim 59, wherein the pH-sensitive polymer present in the pH-sensitive mucoadhesive layer includes a Eudragit polymer, or chemical derivative thereof.

67. The wax-film composite of claim 61, wherein the water-insoluble wax layer comprises at least one water-insoluble pharmaceutical wax having a melting point between 40°C and 100°C and at least one water-soluble or water-swellaable polymer.

68. The water-insoluble pharmaceutical wax of claim 67, wherein said wax includes DENTSPLY® Utility Wax, beeswax, emulsifying wax, microcrystalline wax, carnauba wax, paraffin wax, white wax, yellow wax, or other suitable pharmaceutical wax.

69. The water-soluble or swellaable polymer of claim 67, wherein said polymer is present in the insoluble wax layer at a concentration of 0.05 to 10% by weight.

70. The water-soluble or swellaable polymer of claim 67 where said water-soluble or water-swellaable polymer includes tragacanth, polyvinyl pyrrolidone, polyvinyl alcohol, cross-linked polyacrylic acid, polyethylen glycol, a cellulose polymer derivative, or other suitable pharmaceutical polymer that is water-soluble or water-swellaable.

71. The wax-film composite of claim 59, wherein the molecule of interest is contained in and released from either the pH-sensitive mucoadhesive layer or the water-insoluble wax layer.

72. The wax-film composite of claim 59, wherein the molecule of interest comprises an active pharmaceutical compound.

73. The wax-film composite of claim 72, wherein the active pharmaceutical compound includes at least one member selected from the group consisting of an antimicrobial, an antiviral, an antiinflammatory, an antiseptic, an antihistamine, a local anesthetic, a disinfectant, a keratolytic, an analgesic, an anti-migraine, an anti-fungal, a sweetener, a flavoring agent and a diagnostic agent.

74. The wax-film composite of claim 59, wherein the molecule of interest includes amlexanox.

75. The wax-film composite of claim 59, wherein the molecule of interest includes triclosan.

76. The wax-film composite of claim 59, wherein the molecule of interest includes lidocaine, benzocaine, or dyclonine.

77. The wax-film composite of claim 59, wherein the molecule of interest includes a peptide or protein.

78. The wax-film composite of claim 59, wherein the molecule of interest includes at least one benzodiazepine drug or derivative thereof.

79. The wax-film composite of claim 59, wherein the molecule of interest includes hirudin or hirudin complexed with a substance of opposite charge.

80. The wax-film composite of claim 79, wherein said substance of opposite charge includes chitosan or protamine.

81. The wax-film composite of claim 59, wherein the molecule of interest includes plasmid DNA or plasmid DNA complexed with a substance of opposite charge.

82. The wax-film composite of claim 81, wherein the substance of opposite charge includes at least one member selected from the group consisting of chitosan, protamine and a cationic lipid.

83. The wax-film composite of claim 59, wherein the application site is the skin, mouth, vagina, nasal cavity, or other accessible mucosal site.

84. The wax-film composite of claim 59, wherein the wax-film composite adheres to the application site for at least 1 hour.

85. A method of making the pharmaceutical gel composition according to claim 27, comprising:

- (i) adding a mucoadhesive polymer to a stirring water to form a solution that is clear and viscous,
- (ii) adding the pH-sensitive film-forming polymer to the solution formed in step (i) and measuring the pH of the solution,
- (iii) adding the molecule of interest to the solution of step (ii), and
- (iv) forming the final gel composition.

86. A method of making the wax film composite according to claim 59, comprising:

- (i) forming a homogeneous mucoadhesive film by fusing a mucoadhesive polymer and a pH-sensitive film-forming polymer,
- (ii) homogeneously coating one side of the mucoadhesive film of step (i) with a melted wax composition, and
- (iii) drying the wax composition to form the wax-film composite, wherein the molecule of interest is added in either step (i) or (ii), or the molecule of interest is added to the mucoadhesive side after the wax-film composite is formed.

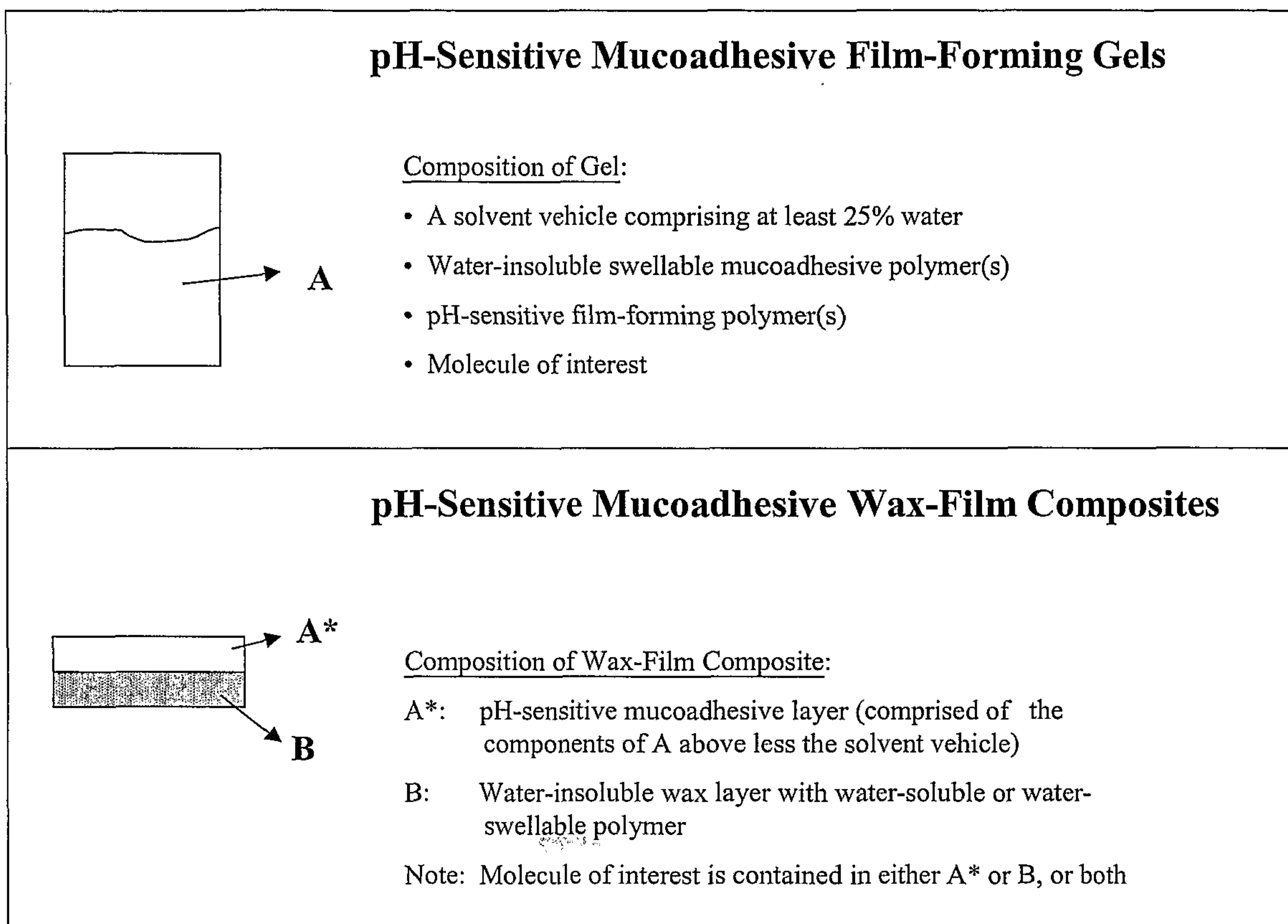
Figure 1

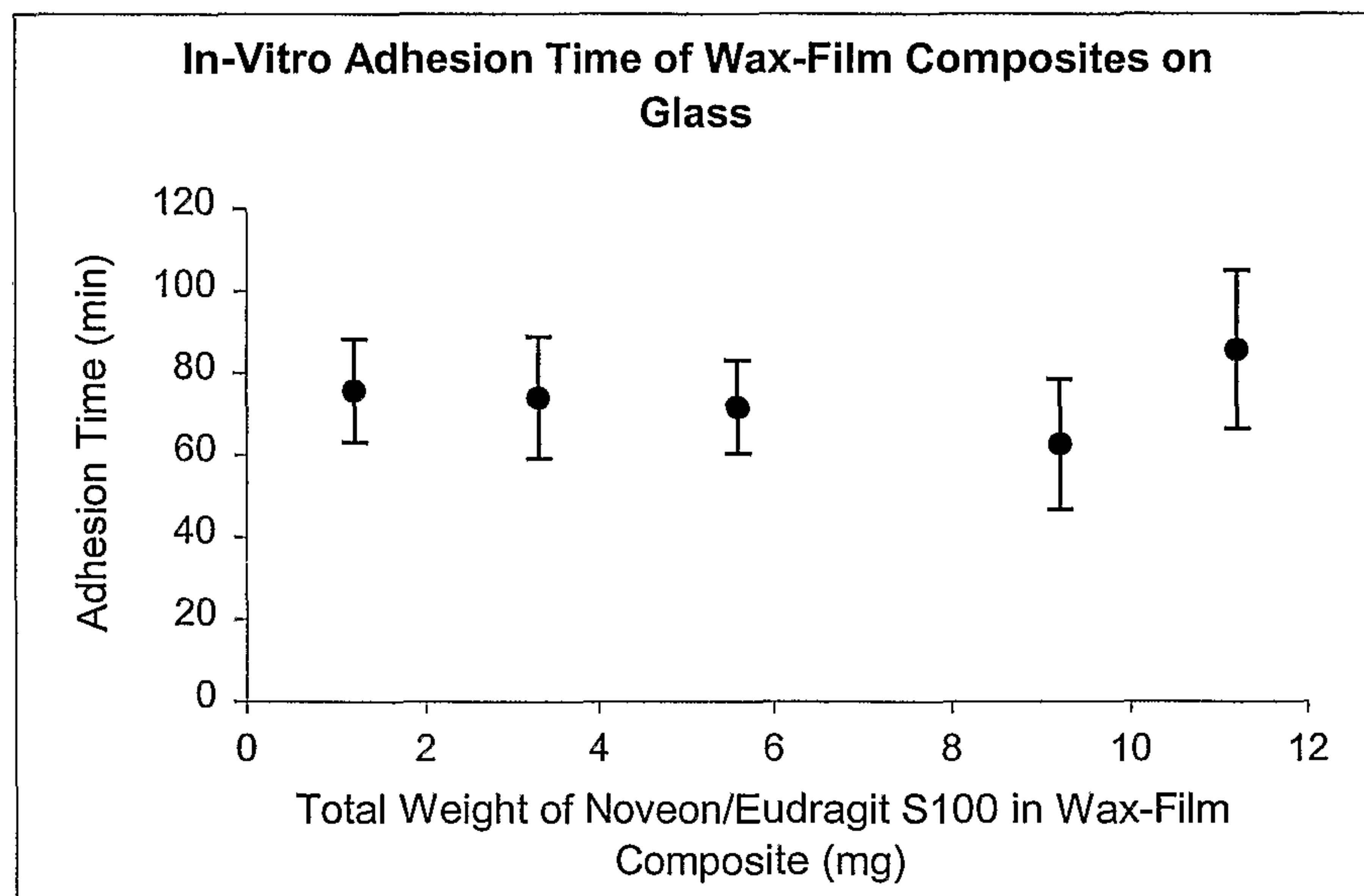
Figure 2

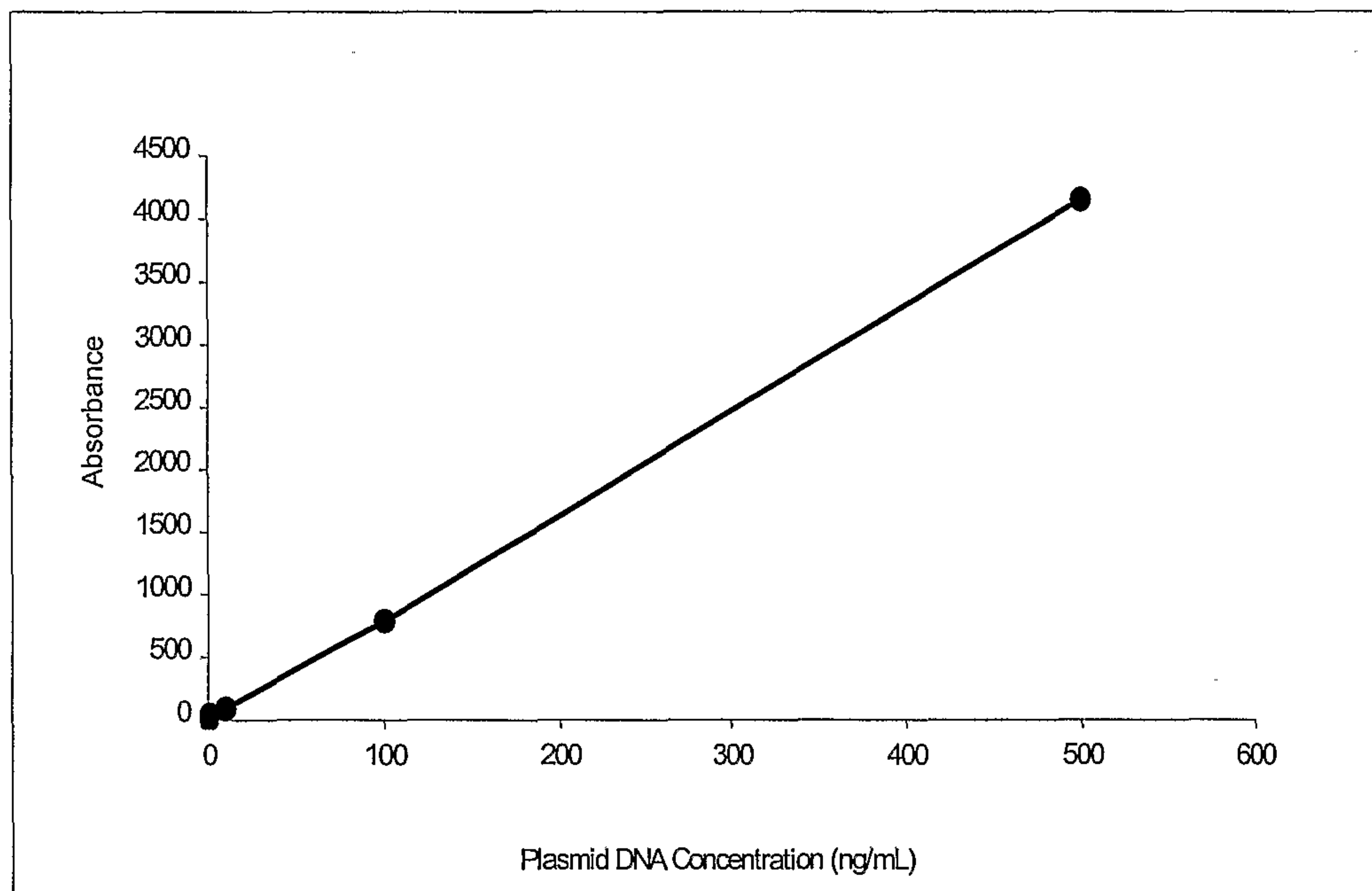
Figure 3

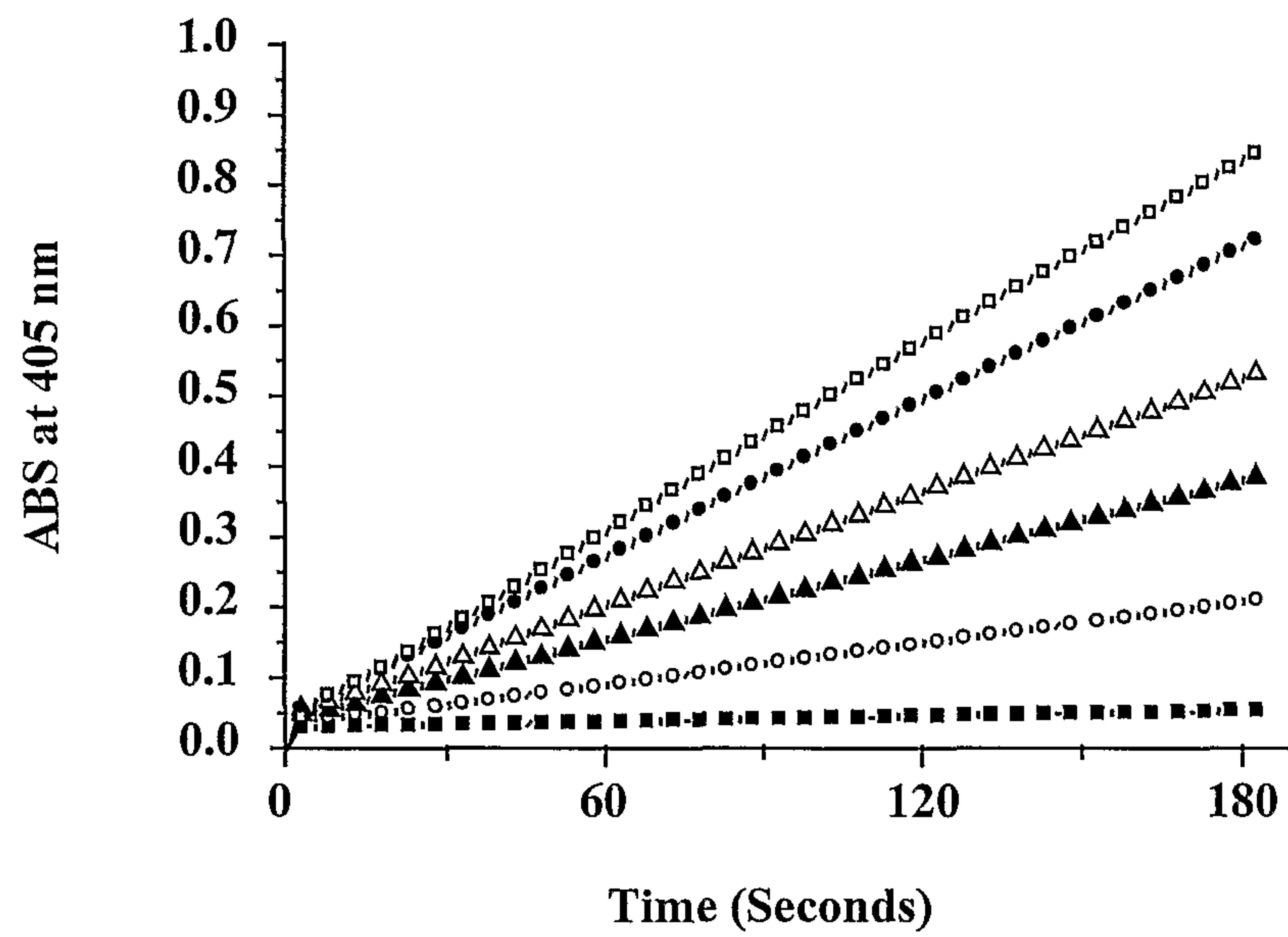
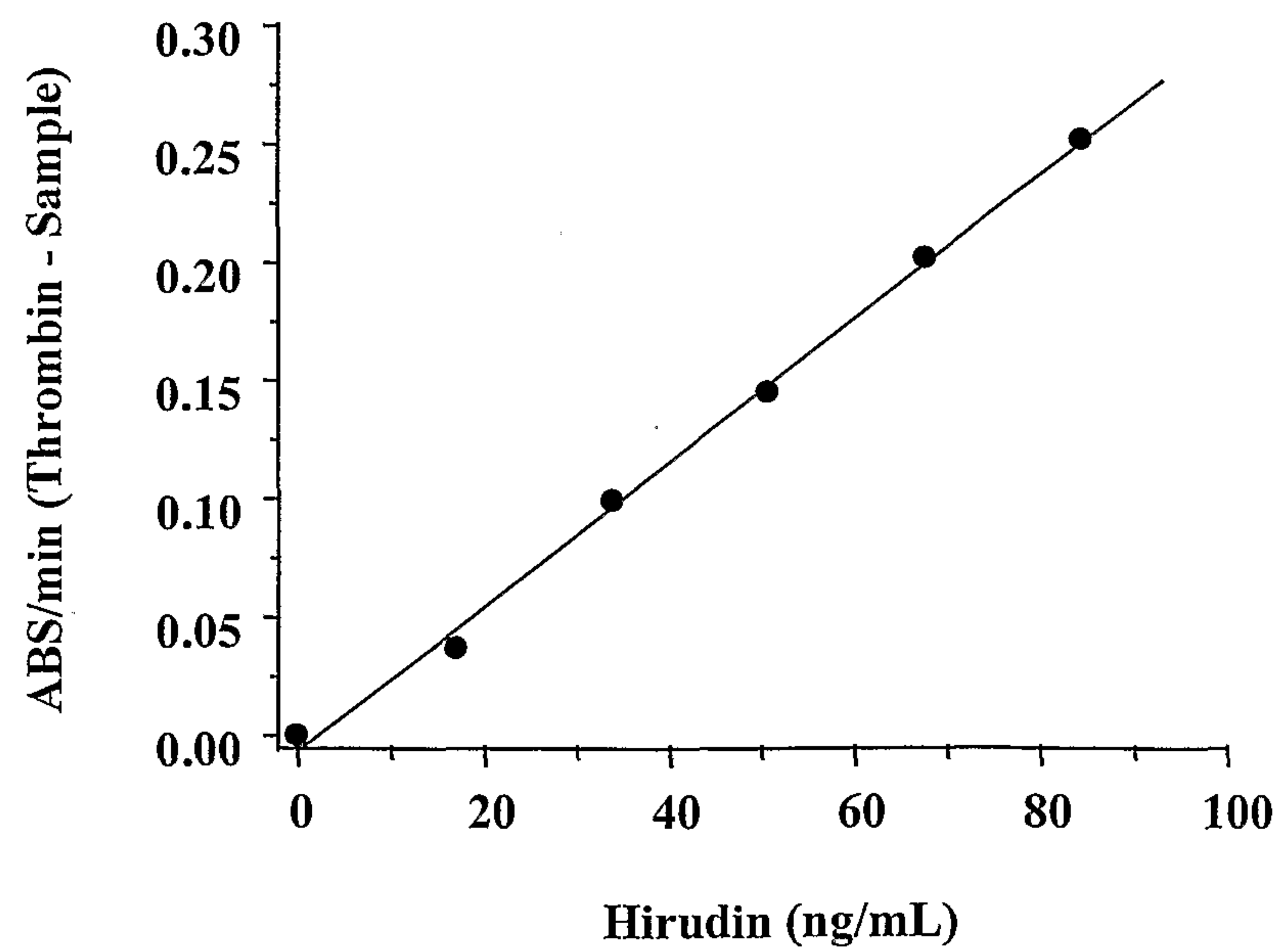
Figure 4**A****B**

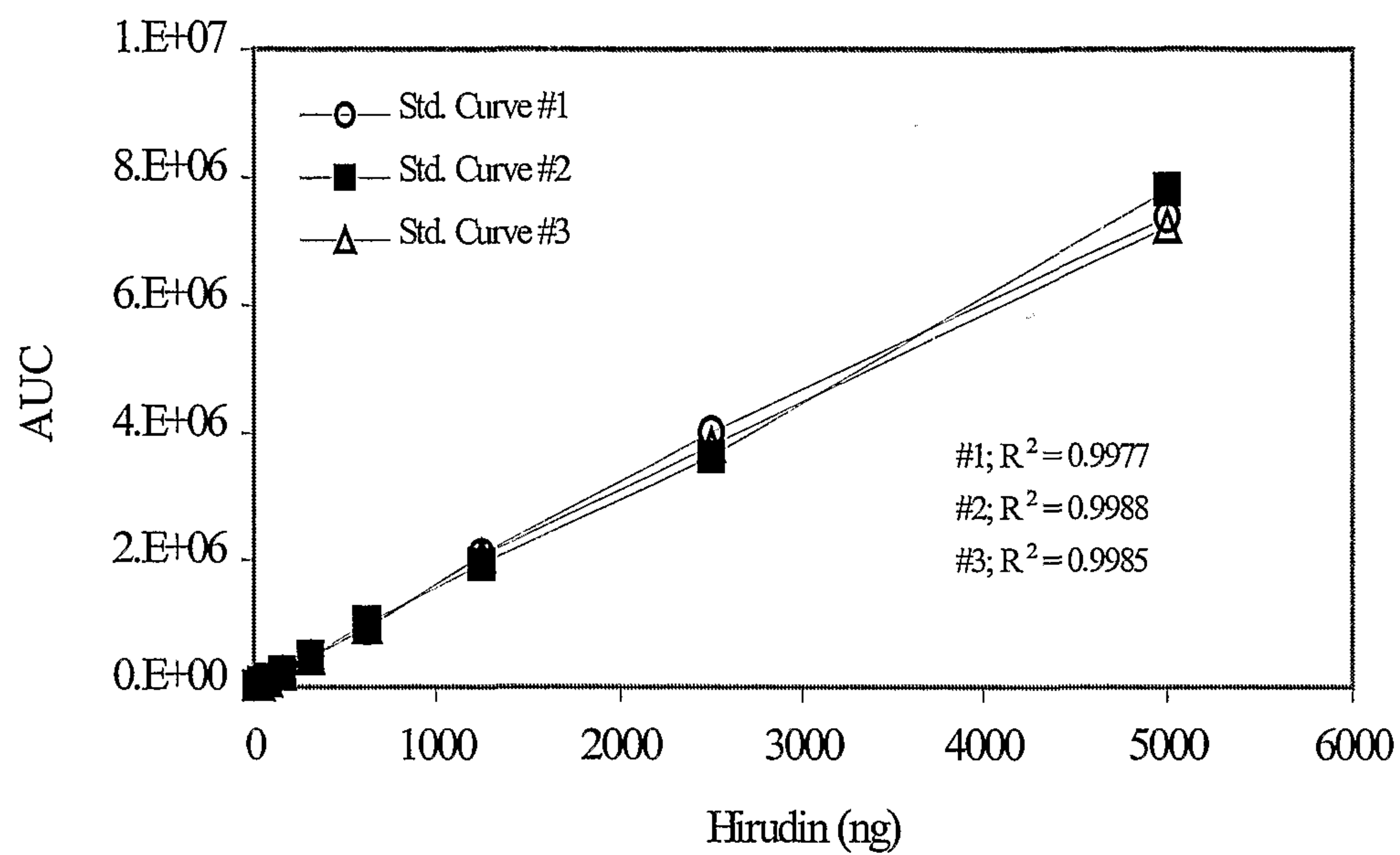
Figure 5

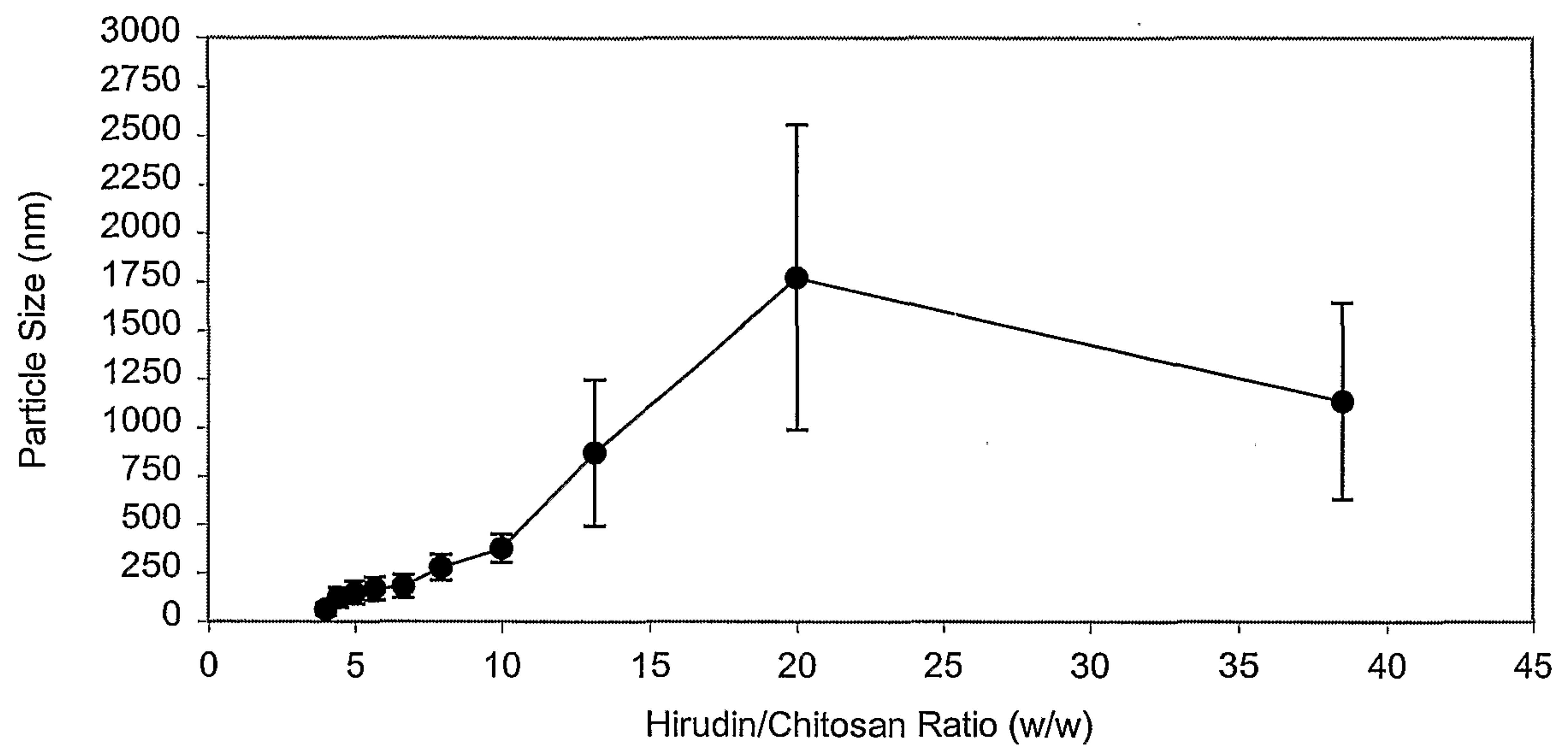
Figure 6

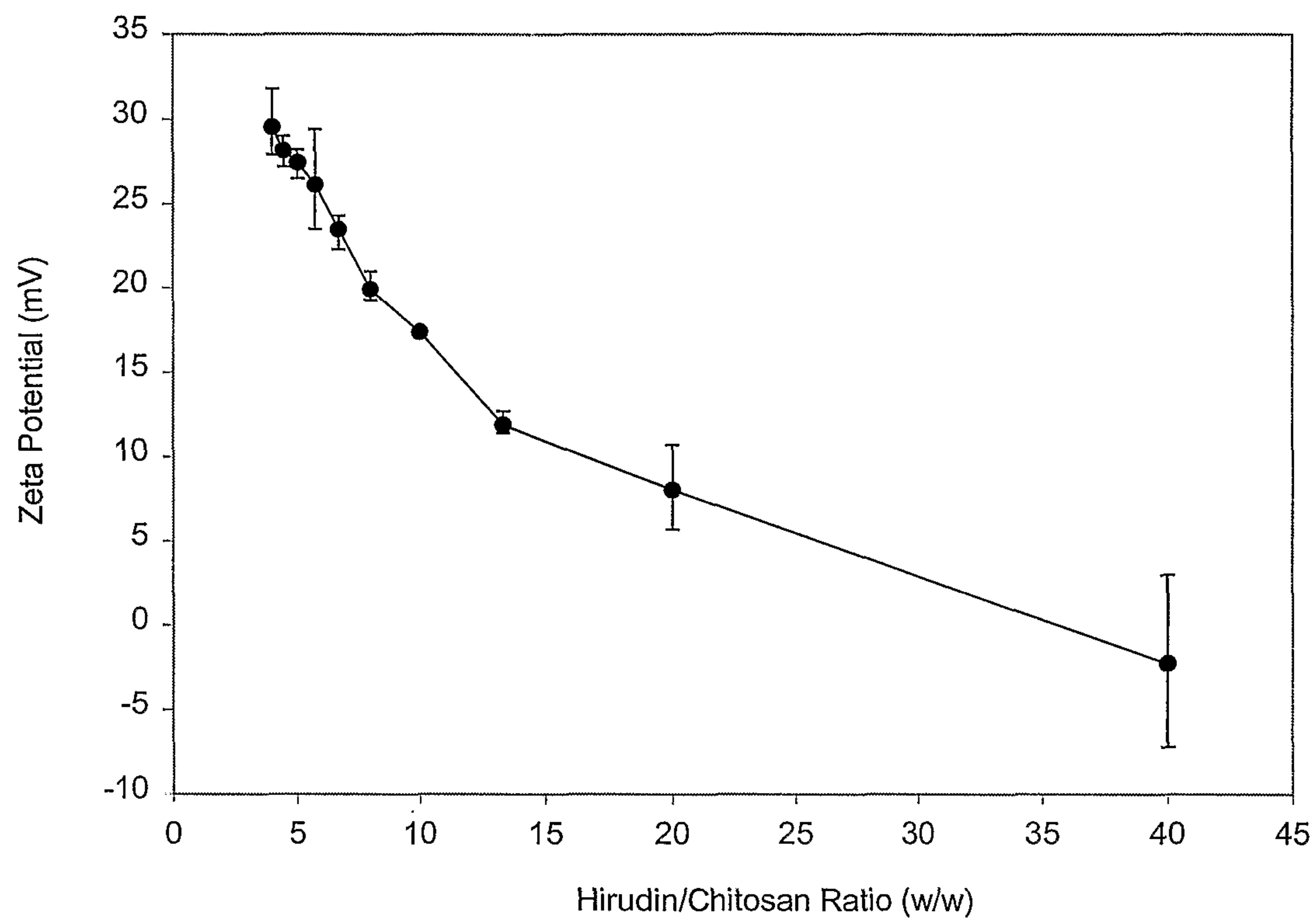
Figure 7

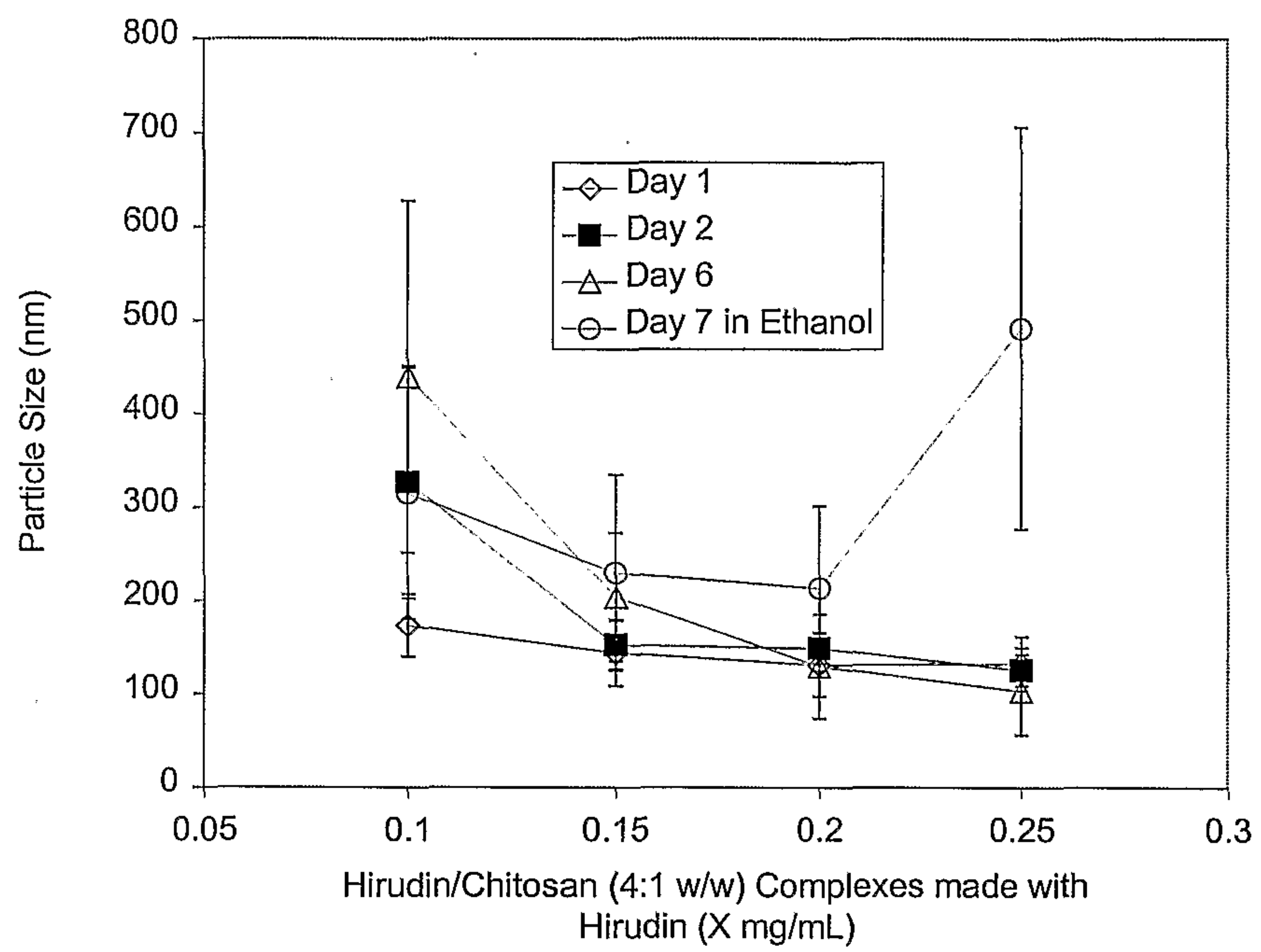
Figure 8

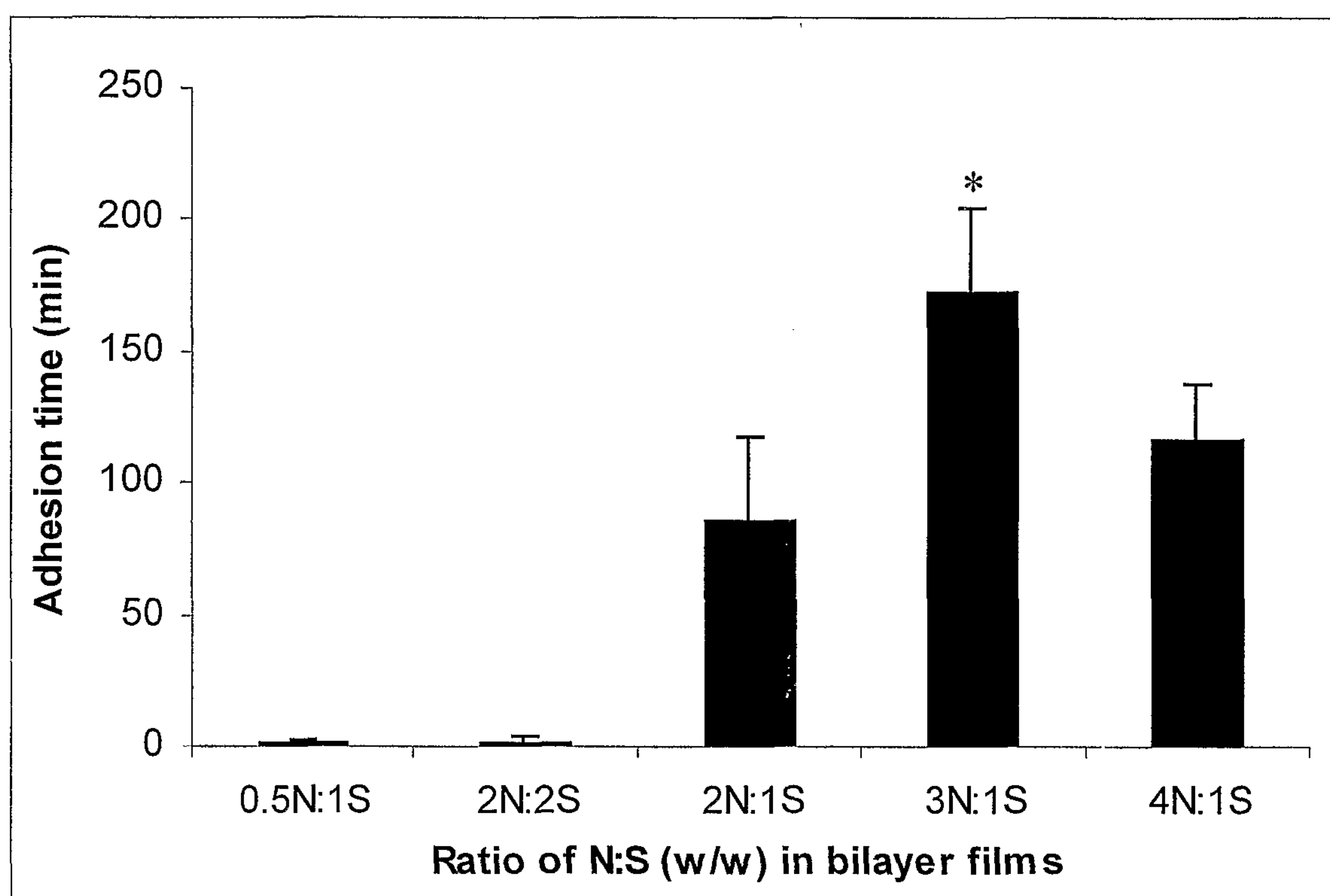
Figure 9

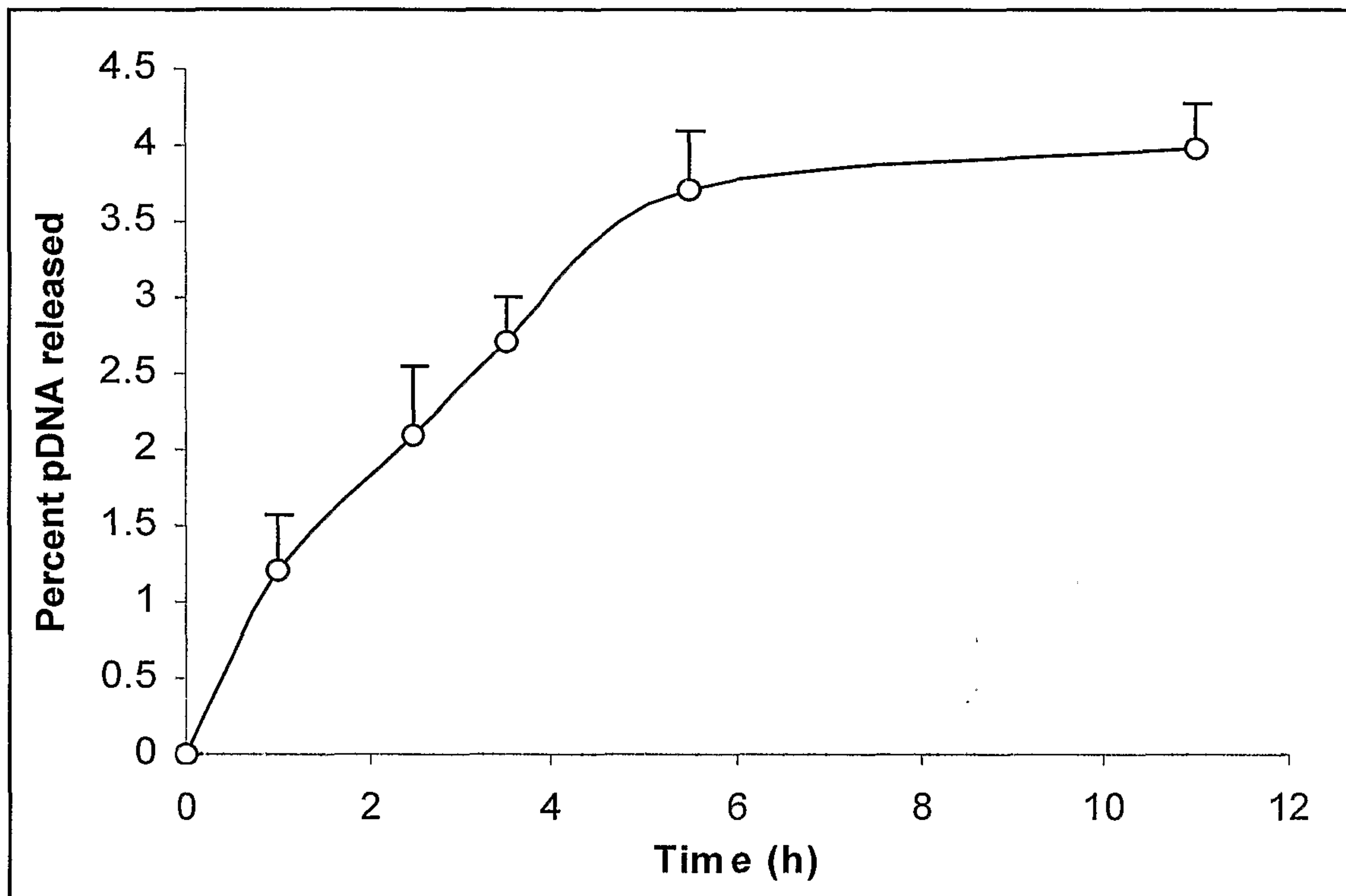
Figure 10

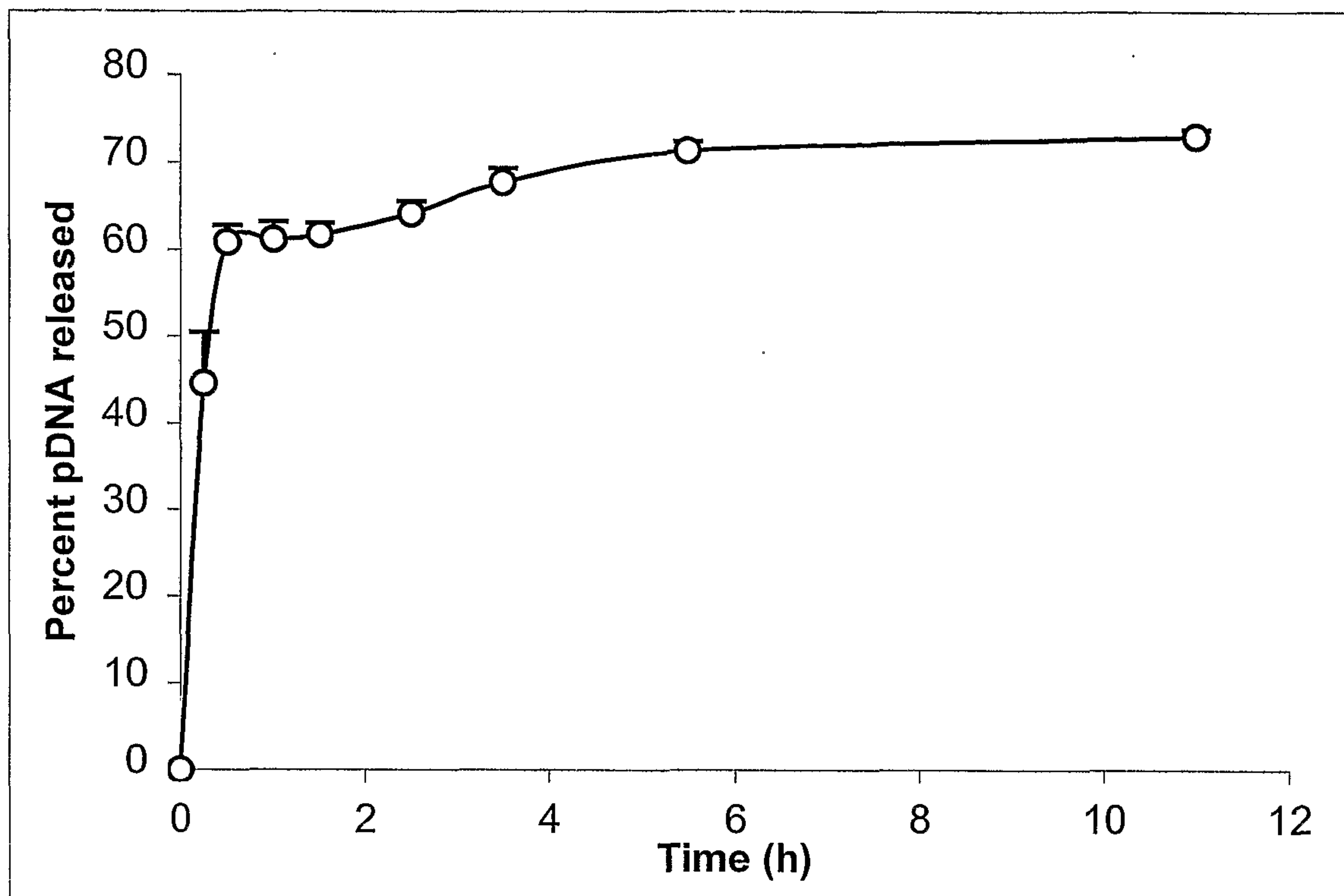
Figure 11

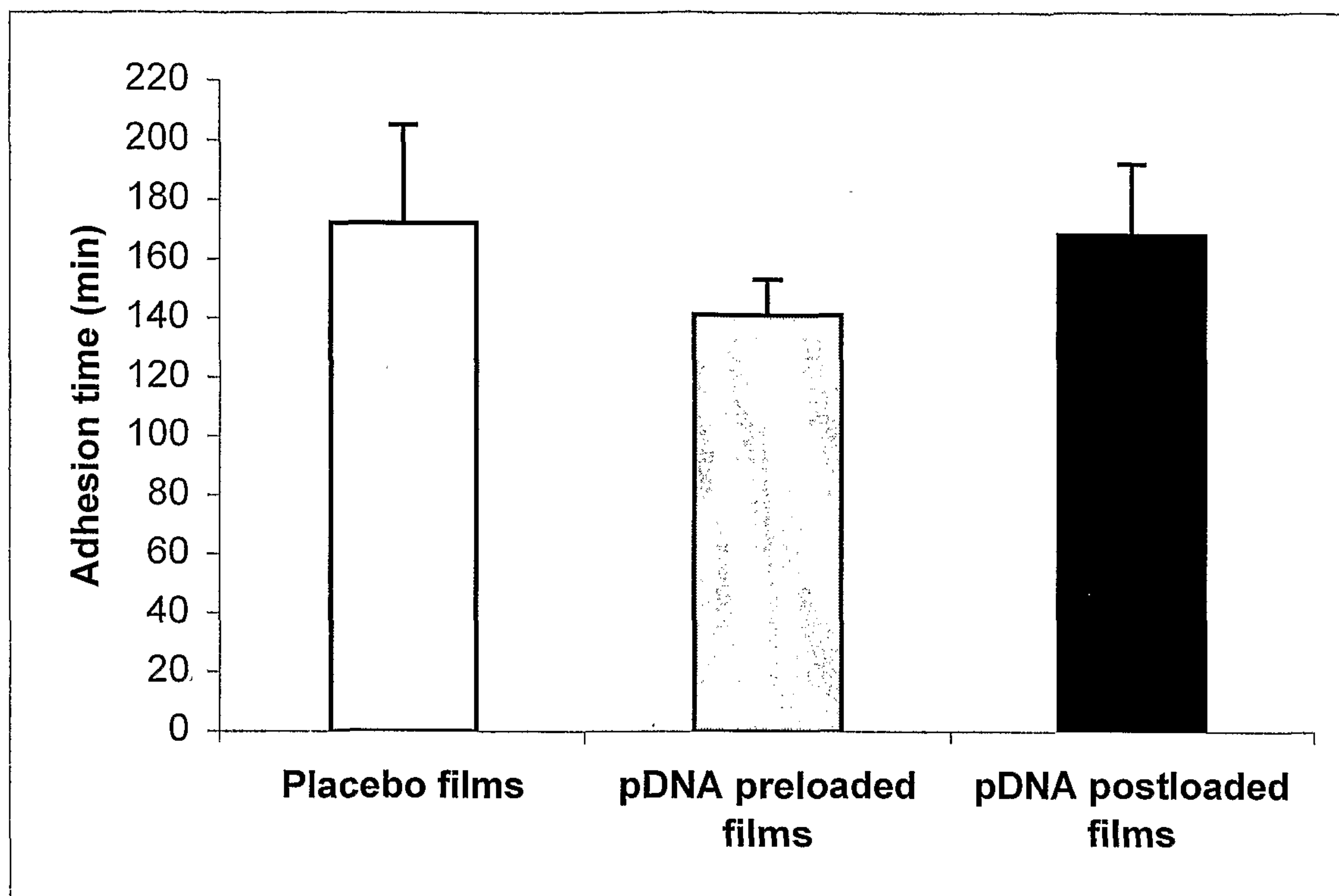
Figure 12

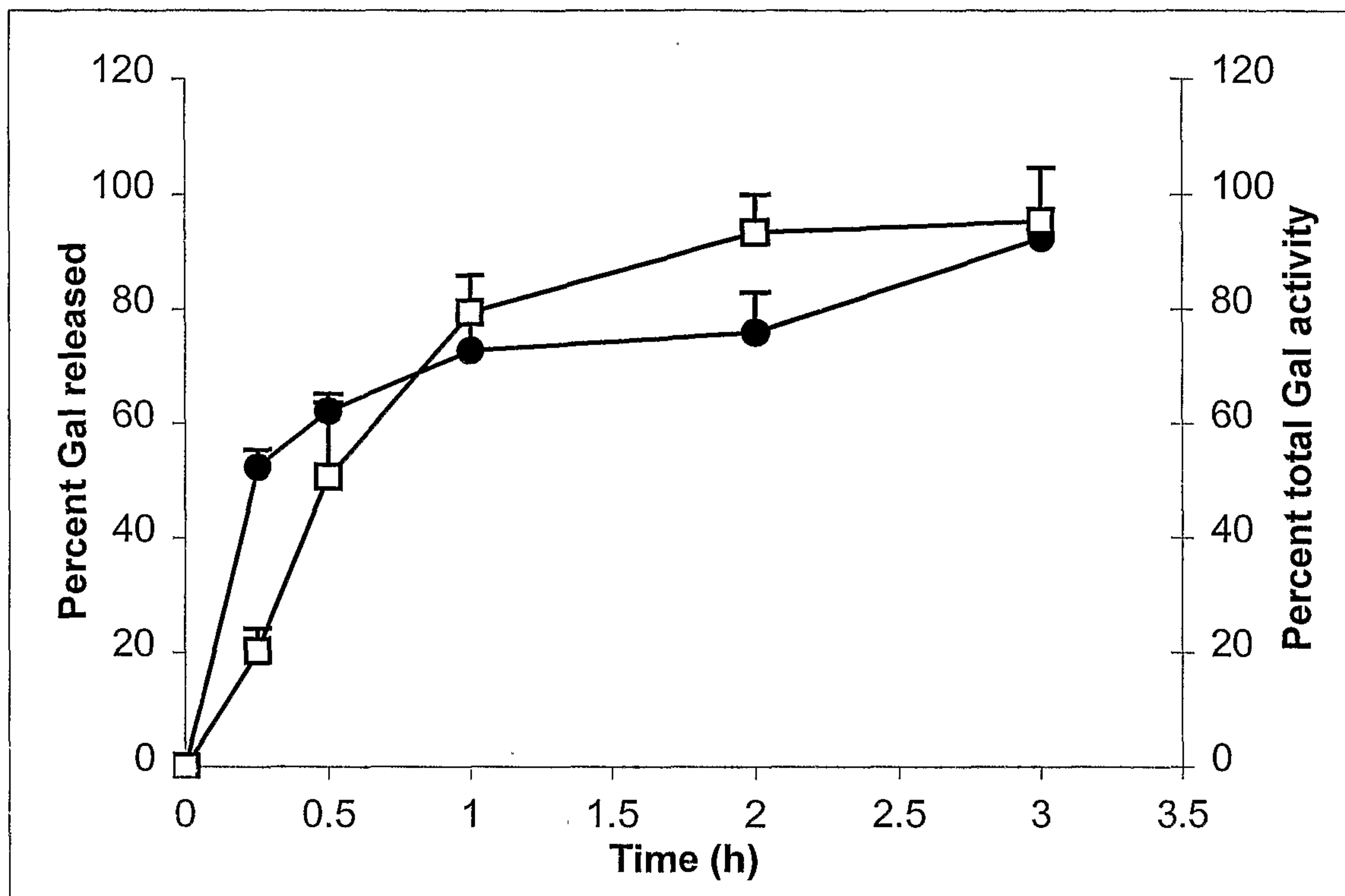
Figure 13

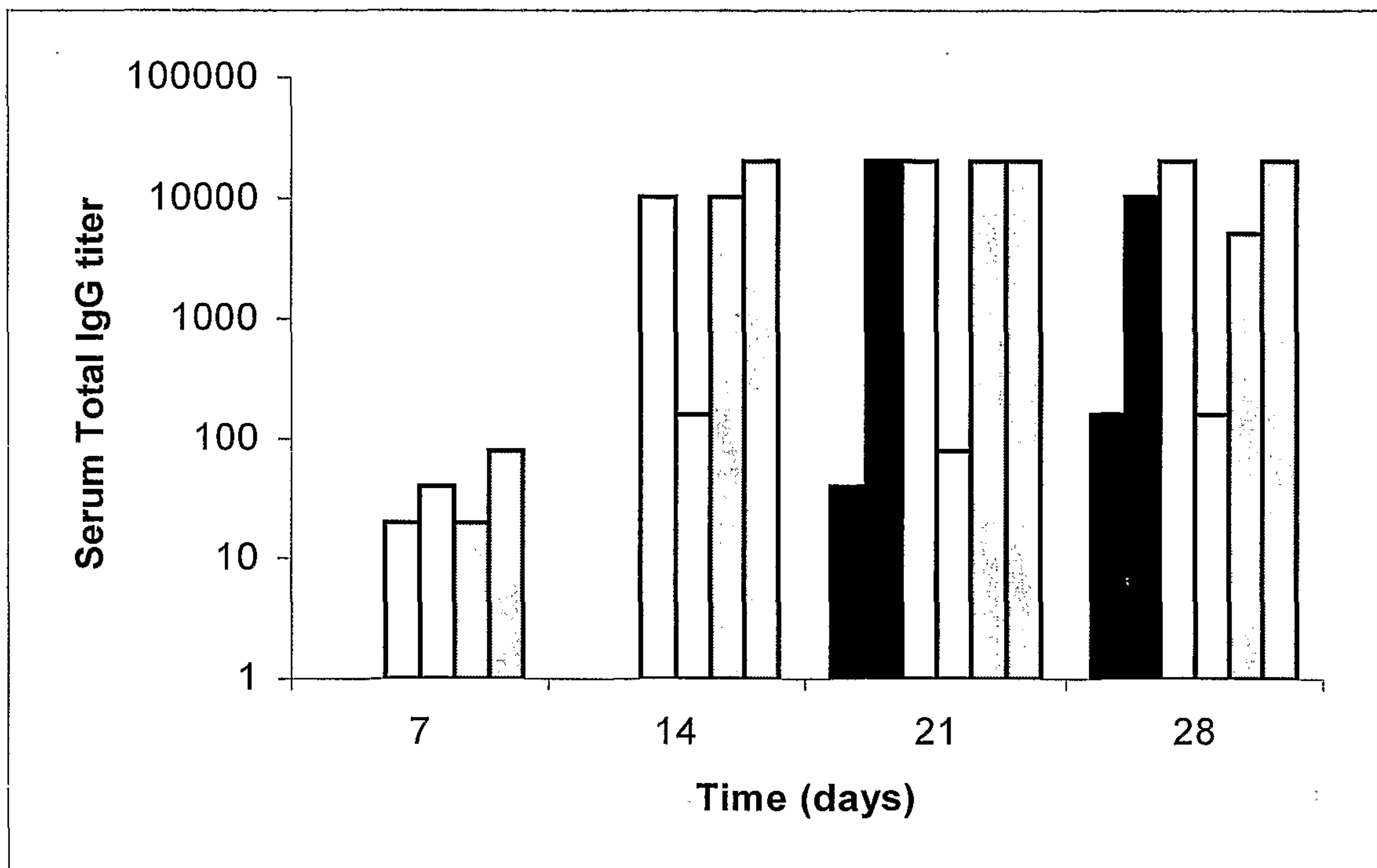
Figure 14

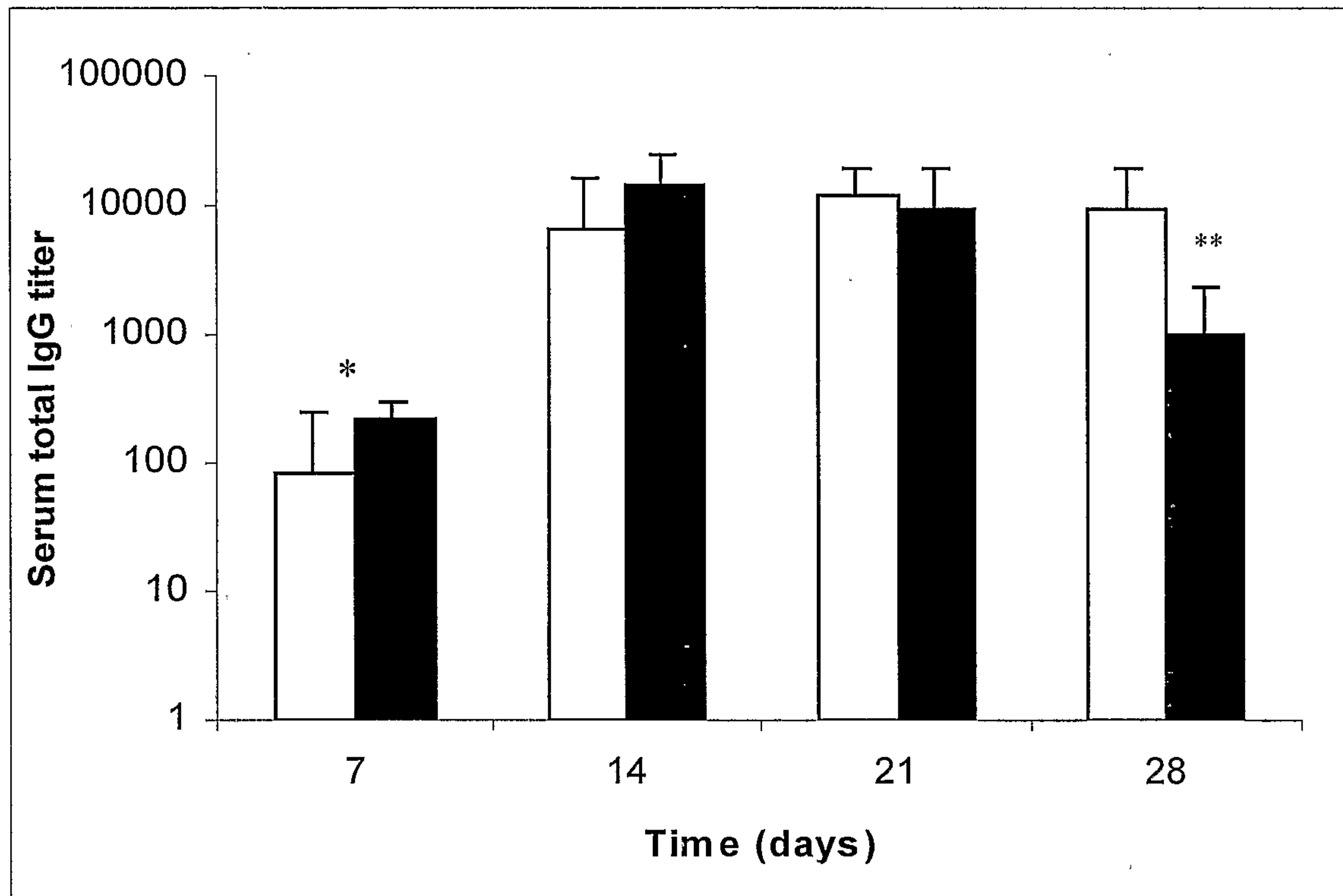
Figure 15

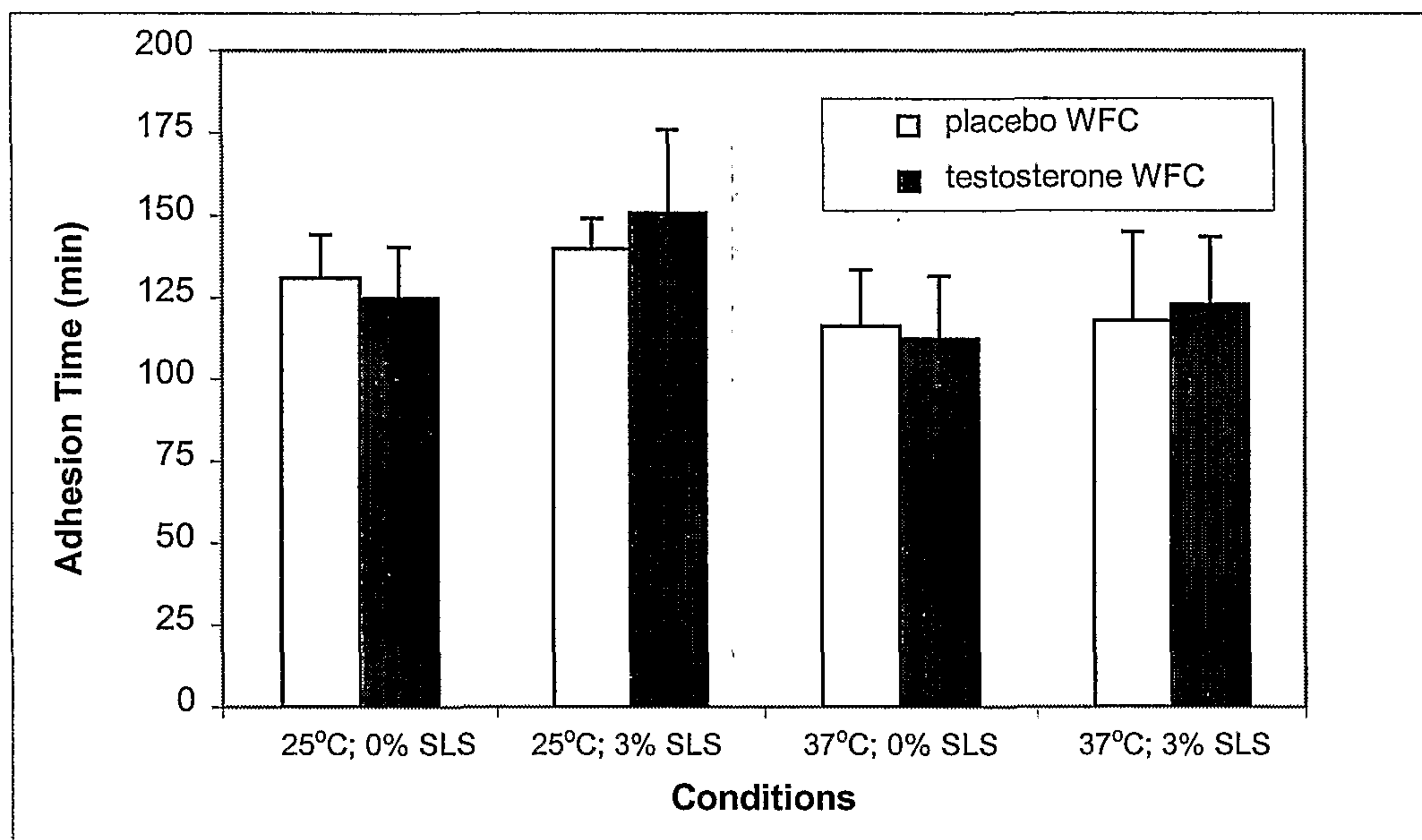
Figure 16

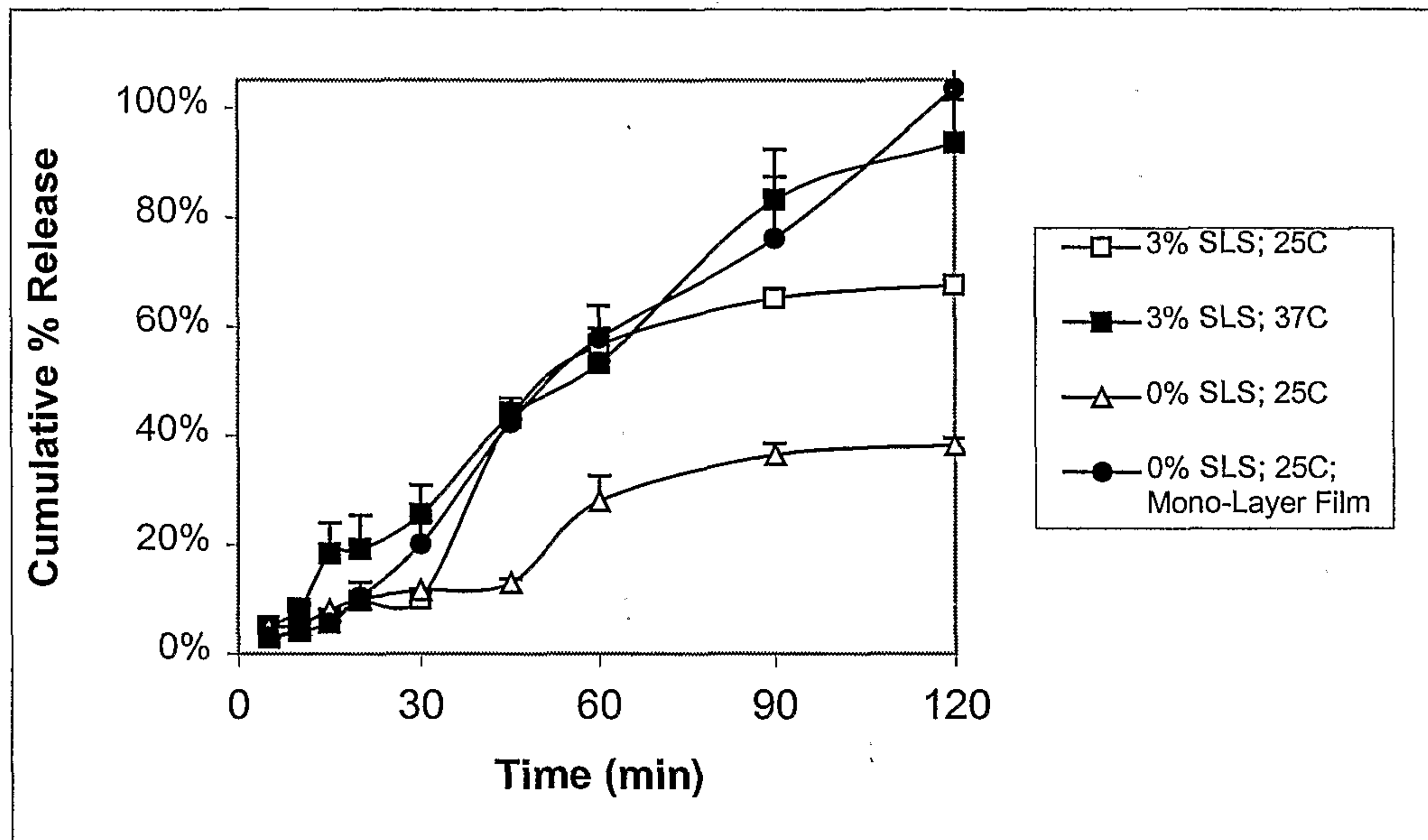
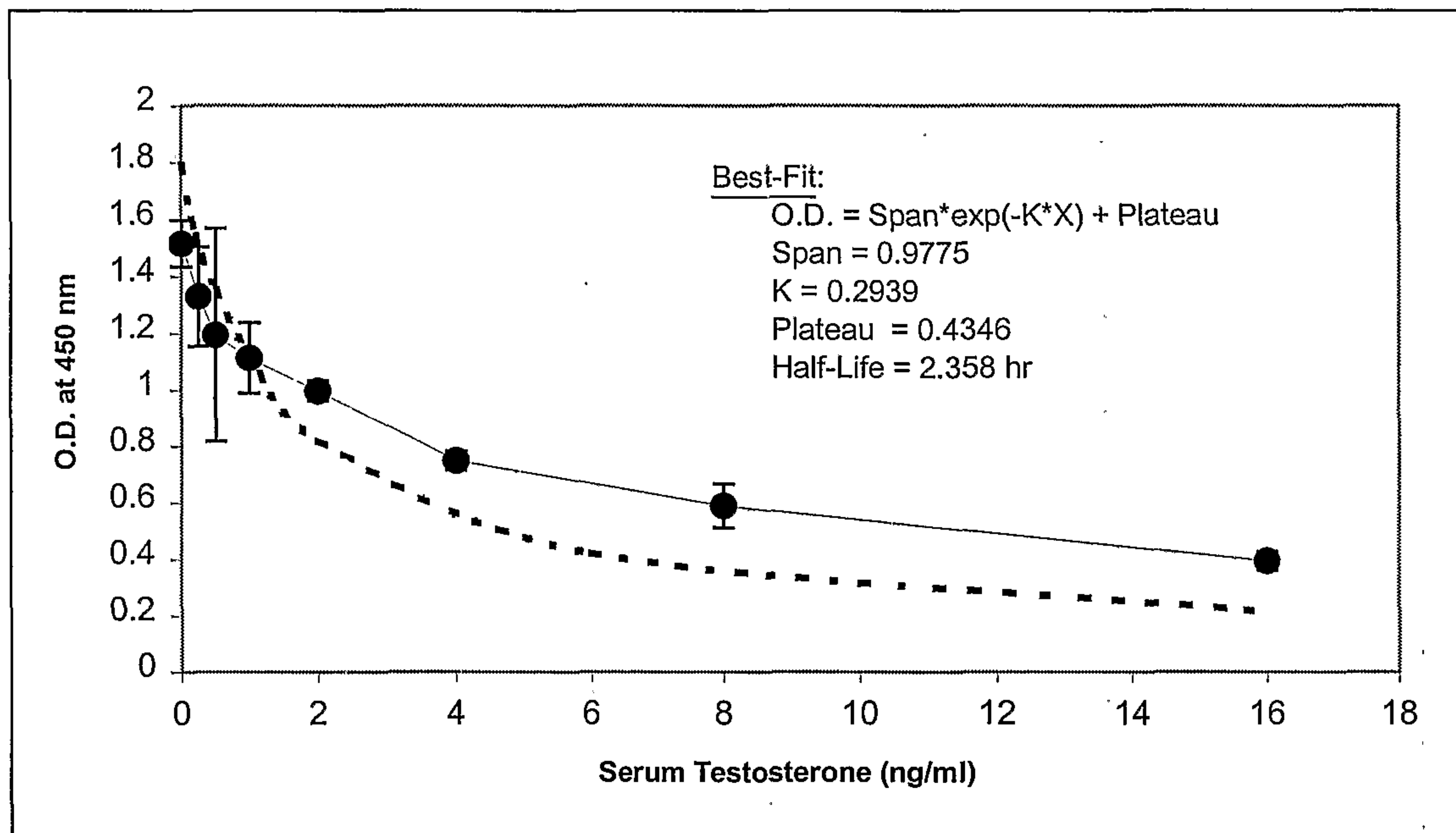
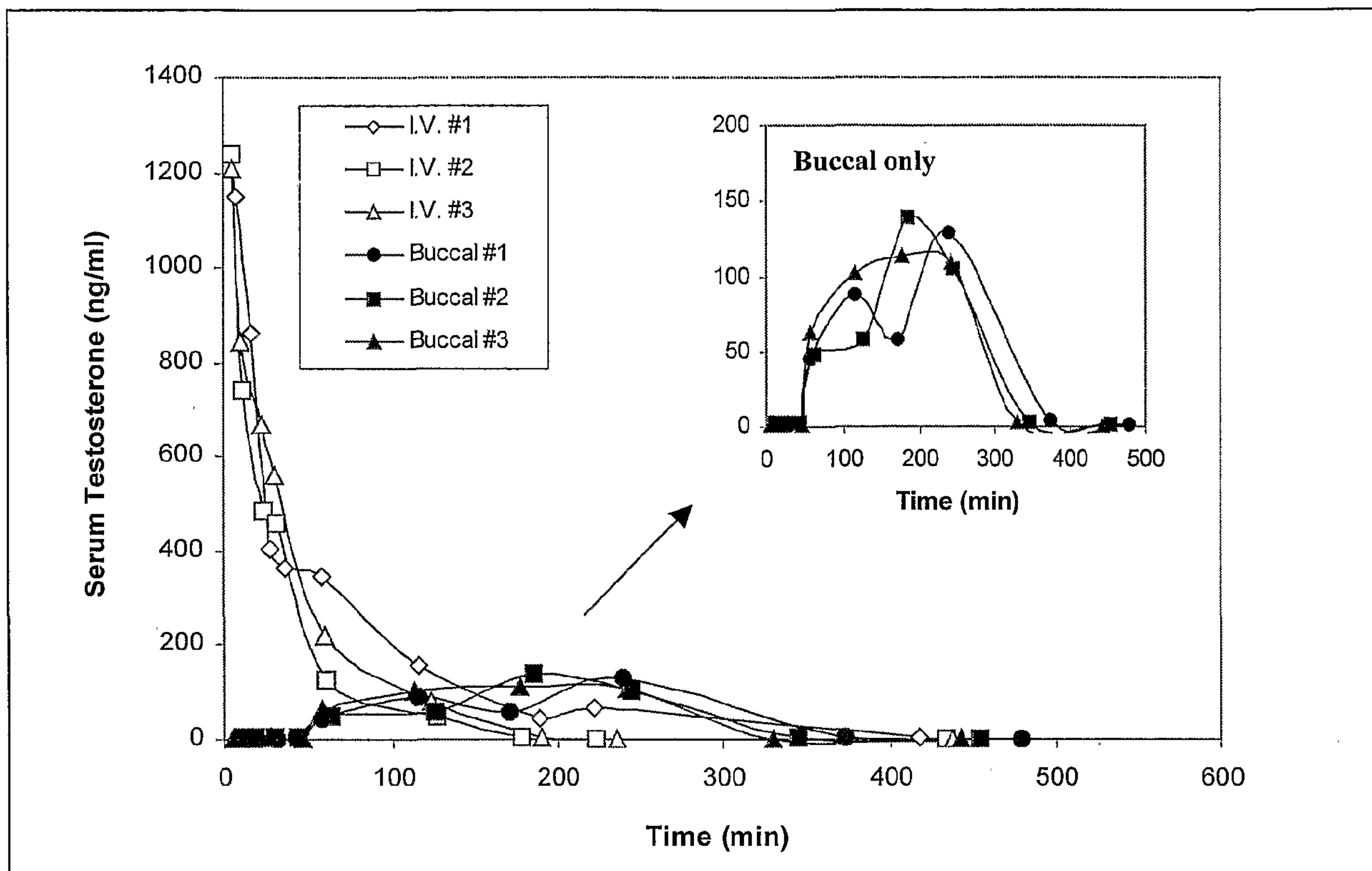
Figure 17

Figure 18



**Figure 19**