JPP Journal of Pharmacy and Pharmacology

Royal Pharmaceutical Society of Great Britain

JPP Journal of Pharmacy and Pharmacology

Short Communication

JPP 2010, 62: 1189–1194

Received January 15, 2010
Accepted May 11, 2010
DOI 10.1111/j.2042-7158.2010.01153.x
ISSN 0022-3573

In-vitro permeation of bevacizumab through human sclera: effect of iontophoresis application

Silvia Pescina[a], Giulio Ferrari[b], Paolo Govoni[c], Claudio Macaluso[b], Cristina Padula[a], Patrizia Santi[a] and Sara Nicoli[a]

[a]Department of Pharmacy, [b]Department of Ophthalmology and [c]Department of Experimental Medicine, University of Parma, Italy

Abstract

Objectives Bevacizumab (Avastin) is a recombinant humanized monoclonal antibody used in ophthalmology (off-label) for the treatment of neovascularization in diseases such as diabetic retinopathy and age-related macular degeneration (wet form). Bevacizumab is currently administrated by repeated intravitreal injection, which can cause severe complications; a non-invasive delivery route is therefore desirable. The passive permeation of bevacizumab through isolated human sclera was evaluated and the iontophoretic technique was explored as a method to enhance its transscleral transport *in vitro*.

Methods Bevacizumab was fluorescently labelled using fluorescein isothiocyanate (FITC). Permeation experiments were conducted for 2 h in Franz-type diffusion cells using human sclera as the barrier. The donor compartment contained FITC-bevacizumab (2.5 mg/ml) in phosphate-buffered saline at pH 7.4. In the iontophoretic experiments, a current intensity of 2.3 mA (current density 3.8 mA/cm^2) was applied. The permeation samples were analysed with a fluorescence detector (excitation and emission wavelengths were 490 and 520 nm, respectively). The stability of FITC-bevacizumab conjugate was checked by thin layer chromatography.

Key findings The main finding of this work is that anodal iontophoresis can significantly enhance bevacizumab transport through isolated human sclera (enhancement factor 7.5), even though the drug is essentially uncharged. Due to the relatively constant characteristics of antibodies, these results can probably be extended to other molecules of the same family.

Conclusions Preliminary results indicate that anodal iontophoresis could be a promising strategy to non-invasively deliver bevacizumab through the sclera. The presence in the eye of other barriers, both static and dynamic, necessitates further evaluation of the technique on more complex ex-vivo and in-vivo models.

Keywords bevacizumab; fluorescein isothiocyanate; iontophoresis; transscleral permeation

Introduction

Therapeutic and diagnostic antibodies have become a very fast growing area and the study of their non-invasive administration is a timely topic.[1] Bevacizumab (MW 149 KDa) is a recombinant humanized IgG1 monoclonal antibody that inhibits angiogenesis by binding with high affinity to human vascular endothelial growth factor.[2] Bevacizumab is active against different types of cancer and, recently, it has been successfully used in ophthalmology, although off-label, for the treatment of diseases such as diabetic retinopathy, age-related macular degeneration (wet form), neovascular glaucoma and several other conditions characterized by neovascularization.[3] In the treatment of diseases of the posterior segment of the eye, bevacizumab is currently administered by repeated intravitreal injection, which can be the cause of severe complications; a non-invasive delivery route is therefore highly desirable. The relatively high permeability of the sclera to macromolecules[4] could encourage the transscleral administration of bevacizumab, but the passive flux is probably too low to reach therapeutically active concentrations in the back of the eye.[5,6] Drug transport across the sclera can be increased by means of enhancing techniques such as microneedles, alone or in combination with enzymatic treatment,[7,8] or iontophoresis, that is the application of low-intensity electrical current.[9]

Correspondence: Sara Nicoli, Department of Pharmacy, University of Parma, Viale Usberti 27/A, 43100 Parma, Italy.
E-mail: sara.nicoli@unipr.it

Iontophoresis has been extensively studied for the transdermal administration of drugs, but the knowledge acquired from this route cannot be directly applied to the transscleral route due to the different characteristics of the two barriers. In fact, the low permeability of the skin restricts passive administration to molecules with a molecular weight lower than 1000 Da[9] (although high molecular weight compounds can be administered using enhancers)[10] and the high electric resistance limits current density to 0.5 mA/cm^2. In the case of the sclera, the high permeability allows molecules up to 150 kDa to be delivered[4] and its porous structure and high water content[11] permits current intensities of 3–4 mA (corresponding to current density of 5.8–7.7 mA/cm^2).[12]

The purpose of this work was to evaluate the permeability of bevacizumab across isolated human sclera *in vitro* and to explore the effect of iontophoresis on the transscleral permeation of this antibody. Since the outermost barrier to drug transport to the back of the eye is represented by the sclera, this tissue was used as a screening barrier for studying bevacizumab passive and current-assisted transport.

Materials and Methods

Materials

Avastin (Roche Pharma, Reinach, Switzerland) was used as the bevacizumab source. Composition: bevacizumab 25 mg/ml; α,α-trehalose dihydrate 60 mg/ml; polysorbate 20 0.4 mg/ml; sodium phosphate monobasic monohydrate 5.8 mg/ml; sodium phosphate dibasic anhydrous 1.2 mg/ml (pH = 6.2).[2]

Tissue preparation

Human corneoscleral rims discarded following harvesting of the corneal button (Regional Cornea Bank, Bologna, Italy) were frozen in liquid nitrogen and used within 15 days of explant. The freezing procedure does not modify scleral histology[13] and, according to Olsen *et al*.,[14] should not influence scleral permeability. The mean thickness of the scleral samples used for permeation experiments was measured with a digital caliper (Absolute Digimatic 547-401, resolution 0.001 mm; Mitutoyo, Milan, Italy,) and found to be 0.60 ± 0.07 mm.

Bevacizumab derivatization

Fluorescein isothiocyanate (FITC) conjugation occurs through the free amino groups of the antibody forming stable thiourea bonds.[15] FITC solution (250 μl, 1 or 4 mg/ml) in 0.1 M carbonate/bicarbonate buffer (pH 9)[15] was added to 1 ml of bevacizumab solution (5 or 10 mg/ml) in the same buffer. The mixture was incubated at 20 ± 2°C for 2 h with or without gentle stirring, protected from light. Then, FITC-labelled bevacizumab was purified from free FITC on a size exclusion Sephadex G-25M column (Sigma-Aldrich, St Louis, MO, US): the reaction mixture was applied to the top and the column was eluted with 10 ml of phosphate-buffered saline (PBS; 0.19 g/L KH_2PO_4; 5.98 g/L Na_2HPO_4 $12H_2O$; NaCl 8.8 g/L; pH 7.4). Fractions of 1 ml were collected: the conjugate, due to its high molecular weight, eluted in fractions 3–4. Unbound FITC was retained on the column and eluted only after extensive (40–50 ml) PBS washing. The presence of free FITC in the conjugate sample was checked by thin layer chromatography (TLC).

The concentration of bevacizumab was determined by measuring the absorbance of the solution at 280 nm (A_{280}), taking into account the extinction coefficient of the conjugate ($E_{280}^{0.1\%} = 1.4$, assuming it was not altered by conjugation)[16] and the absorbance of FITC at 280 nm ($0.35 \times A_{495}$):[17]

$$\text{Bevacizumab}_{(\text{mg/ml})} = \left(A_{280} - \left(0.35 \times A_{495}\right)\right) / E_{280}^{0.1\%}$$

FITC concentration was calculated from the absorption at 495 nm (A_{495}) and the extinction coefficient of bound FITC ($E_{495}^{0.1\%} = 195$):[18]

$$\text{FITC}_{(\text{mg/ml})} = A_{495} / E_{495}^{0.1\%}$$

The FITC to bevacizumab molar ratio obtained after conjugation was calculated as:

$$\begin{aligned}\text{Molar ratio} &= \left(\text{MW}_{\text{bevacizumab}} / \text{MW}_{\text{FITC}}\right) \times \\ &\quad \left(\text{FITC}_{(\text{mg/ml})} / \text{bevacizumab}_{(\text{mg/ml})}\right) \\ &= (149\,000/389) \times \text{FITC}_{(\text{mg/ml})} / \text{bevacizumab}_{(\text{mg/ml})}\end{aligned}$$

The presence of free FITC in the conjugate sample was checked by TLC.

FITC-bevacizumab quantification

A fluorimetric method was used for the quantification of FITC-bevacizumab. Standard solutions were prepared by dilution of FITC-bevacizumab samples of known concentration with PBS (pH 7.4) and analysed with a fluorimeter (series 200a; Perkin Elmer, Norwalk, CT, USA), the excitation and emission wavelengths being 490 and 520 nm, respectively. The linearity was checked in the interval 38–910 ng/ml ($y = 0.1131x$; $R^2 = 0.9925$), the limit of quantification was 38 ng/ml, the relative standard deviation was between 1.3 and 13.3%, and the relative standard error was between 2.5 and 13.1, depending on the concentration.

Blank permeation experiments were conducted both in passive and iontophoretic conditions to verify the specificity of the method used. The fluorimetric analysis of the samples collected excluded the presence of interference from the sclera.

Thin layer chromatography

In order to test the presence of free FITC in the conjugate as well as the stability of the FITC-bevacizumab conjugate after current application, a TLC method was set up. Different amounts of FITC (1–25 ng) were spotted on a silica gel plate (Silica gel 60 F_{254}; Merck, Darmstadt, Germany) using 1-μl calibrated pipettes (Sigma-Aldrich). The plate was run using a mixture of methanol and chloroform (1 : 1). With this mixture, free FITC migrated with an Rf value (migration of compound / migration of solvent front) of 0.85, while the FITC-bevacizumab conjugate remained at the deposition site. After air-drying, the plate was examined under a UV lamp at

365 nm. The limit of quantification of free-FITC was 2.5 ng. Then, standard solutions of FITC-bevacizumab conjugate were analysed before and after current application (i = 2.3 mA; 2 h). These solutions (containing 15 μg of FITC-bevacizumab conjugate, corresponding to approximately 25 ng of FITC) were deposed and run together with four lanes of FITC standard solutions at different concentrations and the presence of free FITC was checked.

Dynamic light scattering analysis: size and zeta potential

Solutions of bevacizumab and FITC-bevacizumab in PBS pH 7.4 were analysed with a 90Plus/BI-MAS apparatus (Brookhaven Instruments Corporation, Holtsville, NY, US) and a Nanoseries Zetasizer, ZS, (Malvern Instruments, Malvern, UK).

The size analysis was performed at 25°C, 658 nm, 90° (fluid refractive index: 1.33, viscosity 0.89 cP). Each measure lasted 2 min and was repeated 6 times.

Permeation experiments

Permeation experiments were performed in Franz-type diffusion cells (0.6 cm^2); human sclera was mounted with the conjunctival side facing the donor compartment and, to reduce the edge damage, the minimum force necessary to keep the cell sealed was applied. The receptor compartment contained 4 ml of PBS pH 7.4 at physiological temperature (37°C) while the donor compartment contained 200 μl of FITC-labelled bevacizumab (2.67 mg/ml) in PBS. In the iontophoretic experiments, both anodal and cathodal, silver-silver chloride electrodes were used. The drug donor solution was separated from the electrodes by salt bridges (agar 2% p/v in 1 M KCl). The current (i = 2.3 mA; d = 3.8 mA/cm^2) was applied through a constant current generator. At predetermined time intervals, the receptor solution was sampled and analysed. The duration of the experiment was 2 h because preliminary tests indicated that steady state is reached within this time.

Transscleral flux (μg/cm^2 per h) was calculated as the slope of the regression line at steady-state, while the permeability coefficient (cm/s) was calculated by dividing flux per drug donor concentration C_D (μg/ml). The enhancement factor was calculated as $P_{iontophoresis} / P_{passive}$.

Passive and anodal permeation experiments were performed for 30 min, to evaluate drug distribution inside the tissue after a shorter application time. The sclera was then frozen in liquid nitrogen, embedded and sectioned (8 μm; Reichert-Jung Frigocut 2700, Nussloch, Germany). The sectioning was performed from the choroidal (receiver) to the conjunctival (donor) side of the sclera and the blade was cleaned before each cut to avoid contamination from the scleral surface. Images were taken using an optical microscope (Nikon Eclipse 80i, Nikon Instruments, Calenzano, Italy) equipped with a 465–495 bandpass filter and a camera (Nikon Digital Sight DS-2Mv, Nikon Instruments, Calenzano, Italy).

Statistical analysis

Permeation experiments were replicated 3–6 times. All results are expressed as mean ± SD. Statistical analysis of the effect of anodal and cathodal iontophoresis on bevacizumab flux was performed using the Kruskal-Wallis test.

Table 1 Dynamic light scattering results

	Size (nm)	Polydispersity index	Zeta potential (mV)
Bevacizumab	15.1 ± 0.1	0.19	–3.32 ± 0.87
Bevacizumab after iontophoresis	14.9 ± 0.3	0.18	–4.67 ± 1.14
FITC-bevacizumab	19.6 ± 0.9	0.22	–

Results

The conditions for derivatization of bevacizumab with FITC were found to be critical, because aggregation occurred. A molar ratio of bevacizumab to FITC of 1 : 20 and the avoidance of stirring were found to be the optimal conditions to obtain a clear solution containing 2.67 ± 0.14 mg/ml of bevacizumab as conjugate, with a FITC/bevacizumab ratio of 0.64 ± 0.16. TLC analysis showed that the separation step after conjugation was efficient since no free-FITC was present. With the same technique, the stability of the conjugate upon iontophoresis application was verified: no detachment of FITC was pointed out.

Because the main degradation pathways of bevacizumab are aggregation and formation of charge-related variants,[2] both the antibody size[19,20] and zeta potential were determined before and after current application. Bevacizumab size was 15.1 ± 0.1 nm (polydispersity index = 0.19) and zeta potential was very close to zero (–3.32 ± 0.87 mV). Both size and charge were not influenced by iontophoresis application (Table 1). A modest size increase was observed in bevacizumab conjugate. Unfortunately, it was not possible to determine the zeta potential of the conjugate (the solution was too diluted), but the low conjugation ratio of FITC to bevacizumab (0.64) suggests that it should not vary.

FITC-bevacizumab transscleral permeation was tested passively and in the presence of cathodal and anodal iontophoresis for 2 h. The permeation profiles (Figure 1) show the ability of anodal iontophoresis to enhance drug permeation. Flux, permeability coefficient and enhancement factor are reported in Table 2.

Permeation experiments in passive and anodal conditions were also performed for 30 min only; vertical sections of the sclera were then analysed with a fluorescence microscope. The results confirm the enhancing effect of anodal current (Figure 2).

Discussion

In order to quantify bevacizumab, a derivatization procedure with FITC was set up. With this method it is not possible to verify the biological activity of the antibody, however, due to the very low metabolic activity of the sclera,[11] it can be reasonably assumed that bevacizumab was not degraded during transscleral transport.

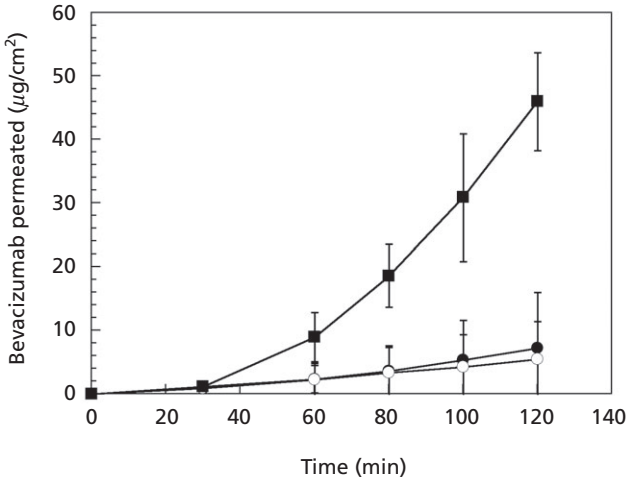

Figure 1 Transscleral permeation profiles of bevacizumab. Permeation profiles of bevacizumab (2.67 mg/ml) following anodal iontophoresis (■), passive permeation (●) and cathodal iontophoresis (○). Mean ± SD ($n \geq 3$).

Table 2 Transscleral permeation parameters of fluorescein isothiocyanate-bevacizumab

	Flux (μg/cm^2 per h)	Permeability coefficient (cm/s) $\times 10^6$	Enhancement factor
Passive	4.92 ± 6.73	0.53 ± 0.73	–
Cathodal	3.17 ± 3.38	0.34 ± 0.36	0.7 ± 0.6
Anodal	37.01 ± 9.37*	4.02 ± 1.02*	7.5 ± 1.9

Data are mean ± SD. *$P < 0.01$, significantly different compared with the passive value.

At first FITC-bevacizumab permeation was tested passively: despite its big size, the drug was able to passively cross human sclera *in vitro* giving rise to a flux of 4.92 ± 6.73 μg/cm^2 per h (Table 2). The permeability coefficient (0.53 ± 0.73 × 10^{-6} cm/s) was lower than that obtained with a model IgG through rabbit sclera (4.61 ± 2.17 × 10^{-6} cm/s),[4] in agreement with the different thickness. Iontophoresis was then tested. The conditions chosen (i = 2.3 mA, d = 3.8 mA/cm^2) were significantly lower than the ocular tolerance values reported on human volunteers[12] to avoid tissue damaging effects. When cathodal iontophoresis was applied, bevacizumab flux did not change compared with passive permeation (see Table 2), while anodal iontophoresis produced a dramatic increment of bevacizumab permeation, well evident after approximately 1 h. The presence of such a lag-time in transscleral transport has already been described when iontophoresis was applied to high molecular weight neutral dextrans.[21]

Bevacizumab transscleral flux after anodal iontophoresis application was 37.01 ± 9.37 μg/cm^2 per h: the enhancement factor obtained in this condition was 7.5 ± 1.9 (6.4 ± 2.2 if calculated on the cumulative amount of bevacizumab permeated after 2 h).

Generally speaking, the mechanisms involved in iontophoresis are an increase of passive permeability due to current application, electroosmosis and electrorepulsion. Current application could in principle modify scleral permeability. However, Li *et al*.[22] found no difference in the permeability of rabbit sclera *in vitro* after 20 min of application of a very high current density (10 mA/cm^2). Moreover, experiments performed in our laboratory on porcine sclera demonstrated that the application of current for 2 h (anodal, 1.75 mA/cm^2) did not modify scleral permeability toward a 40-KDa dextran (unpublished data). The lack of enhancement obtained with cathodal iontophoresis in this work supports the absence of scleral damage following current application.

Since bevacizumab is substantially neutral (the zeta potential is slightly negative and very close to zero) and assuming

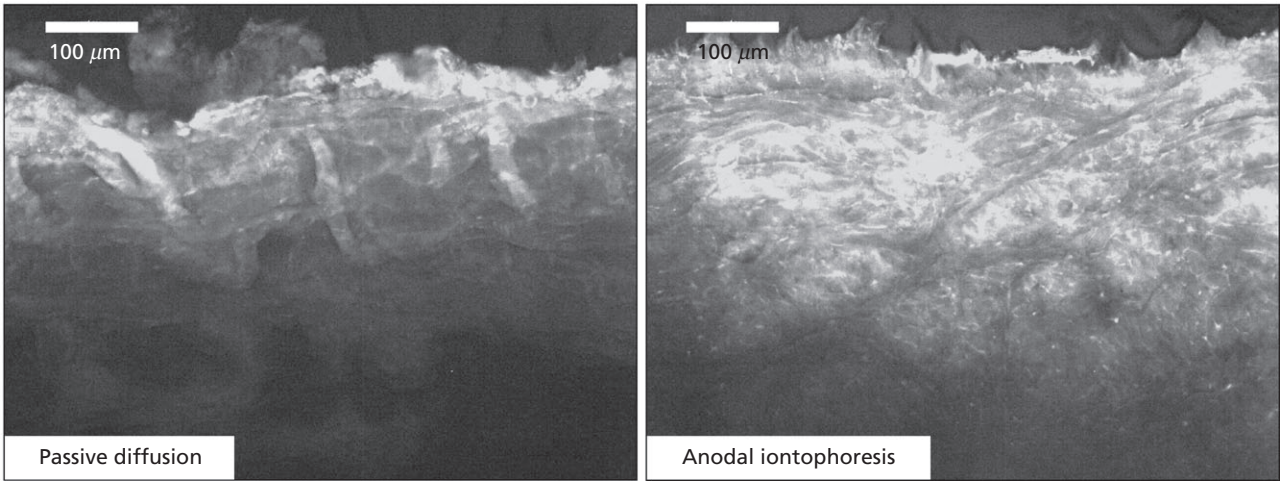

Figure 2 Fluorescence microscopy. Sections of human sclera were treated for 30 min with fluorescein isothiocyanate-bevacizumab (2.67 mg/ml) in passive and iontophoretic (anodal, 2.3 mA) conditions.

that passive permeability is not altered by iontophoresis, the only mechanism responsible for the enhancement is electroosmosis, a convective solvent flow caused by the application of electric current on a charged membrane.[23] Human sclera has an isoelectric point of approximately 3,[21] meaning that at physiological pH it is negatively charged (probably due to the presence of sulfate and uronic acid groups in glicosaminoglycans). As a consequence, the electroosmotic flow is in the anode to cathode direction, as was previously demonstrated using neutral dextrans as markers.[21]

Apparently, the amount of bevacizumab permeated is low when compared with the most used clinical protocol (intravitreal injection of 1.25 mg of bevacizumab monthly*)*; nevertheless intravitreal injection is intended to deliver a depot dose for 1 month or more. Due to its non-invasivity and to the future availability of devices, now in development,[24,25] iontophoretic administration could be applied more frequently and hence a lower dose could be sufficient. Additionally, recent data on human volunteers suggest that bevacizumab can be active at doses as low as 12.5 μg.[26]

The results obtained are encouraging, however, a long time was necessary for iontophorsesis to achieve a significant enhancement in drug transport across the tissue (1 h). To have more information about the kinetics of the process, permeation experiments were also conducted for 30 min and vertical sections of the sclera were then analysed with a fluorescence microscope. After passive diffusion, bevacizumab remained mainly in the more external part of the sclera, filling the superficial spaces between collagen fibres[11,13] (Figure 2). In the presence of anodal iontophoresis (Figure 2), a deeper penetration was evident, confirming the effectiveness of the technique after 30 min.

It is necessary to consider that the sclera is not the only barrier involved. In fact, the topically applied drug has to overcome other barriers both static (choroid, Bruch's membrane, retinal pigmented epithelium) and dynamic (choroidal circulation, retinal pigmented epithelium)[27,28] to reach the posterior segment of the eye. For this reason, it will be necessary to use more complex ex-vivo and in-vivo models to evaluate the effectiveness of this enhancing technique.

Conclusions

The effect of iontophoresis on the transport of an antibody through the sclera was studied. The main finding was that anodal iontophoresis is able to significantly enhance bevacizumab transport through human sclera. Since bevacizumab zeta potential (a measure of the superficial charge) is close to zero and the current applied does not alter the passive permeability of the tissue, the mechanism involved is probably electroosmosis. Due to the relatively constant characteristics of antibodies, these results can probably be extended to other molecules of the same family. Limitations should also be mentioned: the time necessary to obtain a significant enhancement was quite long and therefore optimization of the technique is necessary. Moreover, the presence, besides the sclera, of other barriers both static and dynamic requires further evaluation of the technique on more complex ex-vivo and in-vivo models.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Acknowledgements

The authors want to thank Dr Stefano Barbieri (Department of Pharmacy, University of Parma) and Dr Barbara Stella (Department of Drug Science and Technology, University of Turin) for the dynamic light scattering analysis.

References

1. Daugherty AL, Mrsny RJ. Formulation and delivery issues for monoclonal antibody therapeutics. *Adv Drug Deliv Rev* 2006; 58: 686–706.
2. FDA (2004). Avastin (bevacizumab). US Food and Drug Administration: Silver Spring, MD. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/STN-125085_Avastin.cfm (accessed 28 June 2010).
3. Sharif NA, Klimko P. Ophtalmic agents. In: Triggle D, Taylor J, eds. *Comprehensive Medicinal Chemistry II*, vol 6. Oxford: Elsevier, 2006: 297–320.
4. Ambati J *et al*. Diffusion of high molecular weight compounds through sclera. *Invest Ophthalmol Vis Sci* 2000; 41: 1181–1185.
5. Jarvinen K *et al*. Ocular absorption following topical delivery. *Adv Drug Deliv Rev* 1995; 16: 3–19.
6. Urtti A. Challenges and obstacles of ocular pharmacokinetics and drug delivery. *Adv Drug Deliv Rev* 2006; 58: 1131–1135.
7. Jiang J *et al*. Intrascleral drug delivery to the eye using hollow microneedles. *Pharm Res* 2009; 26: 395–403.
8. Jiang J *et al*. Coated microneedles for drug delivery to the eye. *Invest Ophthalmol Vis Sci* 2007; 48: 4038–4043.
9. Kalia YN *et al*. Iontophoretic drug delivery. *Adv Drug Deliv Rev* 2004; 56: 619–658.
10. Li YZ *et al*. Trypsin as a novel potential absorption enhancer for improving the transdermal delivery of macromolecules. *J Pharm Pharmacol* 2009; 61: 1005–1012.
11. Watson PG, Young RD. Scleral structure, organisation and disease. A review. *Exp Eye Res* 2004; 78: 609–623.
12. Parkinson TM *et al*. Tolerance of ocular iontophoresis in healthy volunteers. *J Ocul Pharmacol Ther* 2003; 19: 145–151.
13. Nicoli S *et al*. Porcine sclera as a model of human sclera for in vitro transport experiments: histology, SEM, and comparative permeability. *Mol Vis* 2009; 15: 259–266.
14. Olsen TW *et al*. Human scleral permeability. Effects of age, cryotherapy, transscleral diode laser, and surgical thinning. *Invest Ophthalmol Vis Sci* 1995; 36: 1893–1903.
15. Brinkley M. A brief survey of methods for preparing protein conjugates with dyes, haptens, and cross-linking reagents. *Bioconjug Chem* 1992; 3: 2–13.
16. The TH, Feltkamp TE. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. *Immunology* 1970; 18: 865–873.
17. Wood BT *et al*. Fluorescent antibody staining. 3. Preparation of fluorescein-isothiocyanate-labeled antibodies. *J Immunol* 1965; 95: 225–229.

18. McKinney RM *et al*. A simple method for determining the labeling efficiency of fluorescein isothiocyanate products. *Anal Biochem* 1966; 14: 421–428.
19. Mahler HC *et al*. Induction and analysis of aggregates in a liquid IgG1-antibody formulation. *Eur J Pharm Biopharm* 2005; 59: 407–417.
20. Nobbmann U *et al*. Dynamic light scattering as a relative tool for assessing the molecular integrity and stability of monoclonal antibodies. *Biotechnol Genet Eng Rev* 2007; 24: 117–128.
21. Nicoli S *et al*. In vitro transscleral iontophoresis of high molecular weight neutral compounds. *Eur J Pharm Sci* 2009; 36: 486–492.
22. Li SK *et al*. Influence of asymmetric donor-receiver ion concentration upon transscleral iontophoretic transport. *J Pharm Sci* 2005; 94: 847–860.
23. Pikal MJ. The role of electroosmotic flow in transdermal iontophoresis. *Adv Drug Deliv Rev* 2001; 46: 281–305.
24. Eljarrat-Binstock E, Domb AJ. Iontophoresis: a non-invasive ocular drug delivery. *J Control Release* 2006; 110: 479–489.
25. Eyegate Pharma. www.eyegatepharma.com (accessed 10 March 2010).
26. Avery RL *et al*. Intravitreal bevacizumab (Avastin) in the treatment of proliferative diabetic retinopathy. *Ophthalmology* 2006; 113: 1695: e1–e15.
27. Kim SH *et al*. Transport barriers in transscleral drug delivery for retinal diseases. *Ophthalmic Res* 2007; 39: 244–254.
28. Robinson MR *et al*. A rabbit model for assessing the ocular barriers to the transscleral delivery of triamcinolone acetonide. *Exp Eye Res* 2006; 82: 479–487.