

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

REGENERON PHARMACEUTICALS, INC.

Petitioner,

v.

NOVARTIS PHARMA AG,
NOVARTIS TECHNOLOGY LLC,
NOVARTIS PHARMACEUTICALS CORPORATION,
Patent Owners

Patent Number: 9,220,631

DECLARATION OF JAMES L. MULLINS. Ph.D.

TABLE OF CONTENTS

	Page
I. Introduction.....	1
II. Qualifications and Compensation.....	1
III. Background.....	3
IV. Documents	7
A. Document 1. Bruno Reuter and Claudia Petersen. “Die Silikonisierung von Spritzen: Trends, Methoden, Analyseverfahren,” <i>TechnoPharm</i> 2, Nr. 4 (2012): 238-244. (“Reuter”)	7
1. Authentication	7
2. Public Accessibility	8
3. Conclusion	10
B. Document 2. Arno Fries. “Drug Delivery of Sensitive Biopharmaceuticals with Prefilled Syringes.” <i>Drug Delivery Technology</i> . Volume 9. Number 5 (May 2009): 22-27. (“Fries”).....	10
1. Authentication.....	10
2. Public Accessibility	11
3. Conclusion	12
C. Document 3. James A. Dixon, et al., “VEGF Trap-Eye for the Treatment of Neovascular Age-related Macular Degeneration.” <i>Expert Opinion on Investigational Drugs</i> . Volume 18. Number 10 (October 2009): 1573-1580. (“Dixon”).....	13
1. Authentication.....	13
2. Public Accessibility	14
3. Conclusion	15
V. Declaration.....	17

I. Introduction

I, James L. Mullins, hereby declare under penalty of perjury:

1. In this declaration, I opine on the authenticity of certain documents referenced herein and explain when and how these documents were disseminated or otherwise made available to the extent that persons interested and ordinarily skilled in the subject matter or art, exercising reasonable diligence, could have located the document. I am being paid my usual hourly fee of \$250/hour. My compensation in no way depends on the content of my testimony or the outcome of the proceeding.

II. Qualifications and Compensation

2. I am the Founder and Owner of the firm Prior Art Documentation Librarian Services, LLC at 106 Berrow, Williamsburg, VA 23188. Attached as Exhibit 1060 is a true and correct copy of my Curriculum Vitae describing my background and experience. Further information about my firm, Prior Art Documentation Librarian Services, LLC (PADLS), is available at www.priorartdoelib.com.

3. I am presently Dean Emeritus of Libraries and Esther Ellis Norton Professor Emeritus, Purdue University, 2018 – present. I was previously employed as follows:

(a) Dean of Libraries and Professor & Esther Ellis Norton Professor, Purdue University, West Lafayette, IN, 2004-2017;

(b) Assistant/Associate Director for Administration, Massachusetts Institute of Technology (MIT), Cambridge, MA, 2000-2004;

(c) University Librarian and Director, Falvey Memorial Library, Villanova University, Villanova, PA, 1996-2000;

(d) Director of Library Services, Indiana University South Bend, South Bend, IN, 1978-1996. Part-time instructor, School of Library and Information Science, Indiana University, Bloomington, IN, 1979-1996;

(e) Associate Law Librarian, and associated titles, Indiana University School of Law, Bloomington, IN, 1974-1978;

(f) Catalog Librarian, Assistant Professor, Georgia Southern College (now University), Statesboro, GA, 1973-1974.

4. Over the course of my career as a librarian, instructor of library science, author of scholarly publications, and presenter at national and international conferences, I have had experience with catalog records and online library management systems built around Machine-Readable Cataloging (MARC) standards.

5. During more than forty-four years as an academic librarian and scholar, I have been an active researcher. In my years as a librarian, I have facilitated the research of faculty colleagues, either directly or through the provision of and access to the requisite print and/or digital materials and services at the universities I worked.

I have kept current on the professional library science literature and served on the editorial board of the most prominent library journal, *College and Research Libraries*. This followed service as the chair of the Research Committee of the Association of College and Research Libraries (ACRL), a division of the American Library Association (ALA). As an academic library administrator, I have had responsibility to ensure that students were educated to identify, locate, assess and integrate information garnered from library resources.

III. Background

6. *Authentication.* I understand that an item is considered authentic if there is sufficient evidence to support a finding that the item is what it is claimed to be. I am also informed that authenticity can be established based on the contents of the documents themselves, such as the appearance, content, substance, internal patterns, or other distinctive characteristics of the item, taken together with all of the circumstances.

7. *Public Accessibility.* I understand that a given reference is publicly accessible upon a satisfactory showing that such a document has been disseminated or otherwise made available to the extent that persons interested and ordinarily skilled in the subject matter or art exercising reasonable diligence, can locate it. I have also been informed by counsel that materials available in a library constitute

printed publications if they are cataloged and indexed according to general library practices that make the references available to members of the interested public.

8. *Materials considered.* For purposes of this declaration I have reviewed the documents and appendices referenced herein. These materials are records created in the ordinary course of business by publishers, libraries, indexing services, and others. I am familiar with the process for creating many of these records, and I know these records are created by people with knowledge of the information in the record. Further, these records are created with the expectation that researchers and other members of the public will use them. All materials cited in this declaration and its appendices are of a type that professionals in my field would reasonably rely upon and refer to in forming their opinions.

9. *WorldCat.* WorldCat is the world's largest public online catalog, maintained by the Online Computer Library Center, Inc., or OCLC, and built with the records created by the thousands of libraries that are members of OCLC. WorldCat is easily accessible on the internet to all who wish to search it; there are no restrictions to be a member of a particular community, etc.

10. *OPAC.* Online public access catalog, or "OPAC," refers to a catalog of a library or a group of libraries. OPACs, generally, and both OPACs mentioned in this declaration, are easily accessible on the internet to all who wish to search them

and there are no generally restrictions on who can search an OPAC and/or access its records.

11. *Periodical publications.* A library typically creates a catalog record for a periodical publication when the library receives its first issue. When the institution receives subsequent issues/volumes of the periodical, the issues/volumes are generally checked in (often using a date stamp), added to the institution's holding records, and made available very soon thereafter – normally within a few days of receipt or (at most) within a few weeks of receipt.

12. *Indexing.* A common way of searching for a publication is to look for relevant information in an index of periodical and other publications. Having found relevant material, the researcher will then normally obtain it online, look for it in libraries, or purchase it from the publisher, a bookstore, a document delivery service, or other provider. Sometimes, the date of a document's public accessibility will involve both indexing and library date information. Date information for indexing entries is, however, often unavailable. This is especially true for online indices. Online indexing services commonly provide bibliographic information, abstracts, and full-text copies of the indexed publications, along with a list of the documents cited in the indexed publication. These services also often provide lists of publications that cite a given document. A citation of a document is evidence that

the document was publicly available and in use by researchers no later than the publication date of the citing document.

13. *Persons of ordinary skill in the art.* I understand that a “person of ordinary skill in the art” (“POSITA”) is a hypothetical person who is presumed to have been familiar with the relevant field and its literature at the time of the inventions. This hypothetical person is also a person of ordinary creativity, capable of understanding the scientific principles applicable to the pertinent field.

14. I am told by counsel that a POSITA in this subject matter or art would either (a) have had at least an advanced degree (Dipl.Ing, M.S., or Ph.D.), with research experience in mechanical engineering, biomedical engineering, materials science, chemistry, or a related field or at least 2-3 years of professional experience in one or more of those fields and would have had experience with (i) the design of prefilled syringes; and (ii) sterilization of drug delivery devices, including those containing sterilization sensitive therapeutics, wherein such sterilization experience would include experience with microbiology; and with respect to certain claims in the challenged patent that deal with administering a drug, a POSITA would be an ophthalmologist with some experience administering VEGF-antagonist drugs to patients via the intravitreal route.

15. Such a person would have been engaged in academic research, learning through study and practice in the field and possibly through formal instruction the

bibliographic resources relevant to his or her research. In and prior to 2012, such a person would have had access to a vast array of print resources in, for example, mechanical engineering, biomedical engineering, materials science, chemistry, or medicine, as well as to a set of online resources.

IV. Documents

A. Document 1. Bruno Reuter and Claudia Petersen. “Die Silikonisierung von Spritzen: Trends, Methoden, Analyseverfahren,” *TechnoPharm* 2, Nr. 4 (2012): 238-244. (“Reuter”)

1. Authentication

16. Appendix 1A is a true and accurate copy from the issue of *TechnoPharm*, 2, Nnr.4 (2012) pages 238-244. Appendix 1A includes a cover, with ownership stamp of Württembergische Landesbibliothek in Stuttgart, table of contents, and the Reuter article pages 238-244. This scan was provided to me at my request by the Wisconsin TechSearch (WTS),¹ from the Württembergische Landesbibliothek in Stuttgart, Germany. I understand that is not the practice of German libraries to date stamp the receipt of an issue of a journal.

17. Appendix 1B is a true and accurate copy from the issue of *TechnoPharm* 2, Nr. 4 (2012) by Bruno Reuter and Claudia Petersen, downloaded by me from the TechnoPharm website:

¹ WTS (<https://wts.wisc.edu/>) is a service from the Libraries department at the University Wisconsin-Madison, which provides article delivery and certain librarian research services on request.

https://www.ecv.de/download/download/Zeitschriften/TechnoPharm/volltext/tp-2012-04-0238-reuter-silikonisierung-web_d.pdf.

18. Appendix 1A and Appendix 1B are in a condition that appear in all respects to be authentic. The sequence from pages 238-244 is not missing intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Appendix 1A was located and recovered from a library, the Württembergische Landesbibliothek. Appendix 1B was downloaded from the TechnoPharm website, which is where a person looking for authentic copies would expect to find them. Therefore, I conclude that Appendix 1A and Appendix 1B are authentic copies of the Reuter article.

2. Public Accessibility

19. Appendix 1C is a view of the WorldCat record for *TechnoPharm* making science work. WorldCat provides an international access point to locate TechnoPharm by title and by subject: Pharmazeutische Technologie. Listed among the eleven international libraries holding TechnoPharm is Württembergische Landesbibliothek in Stuttgart, Germany.

20. Appendix 1D is a letter from the Württembergische Landesbibliothek. In order to verify that the print version was processed and publicly accessible to a researcher at the Württembergische Landesbibliothek, counsel obtained a letter from

Birgit Hacker, Head of Journals Department, Württembergische Landesbibliothek.

This letter dated July 25, 2018 states:

I hereby confirm, that the printed issue of the journal
TechnoPharm : making science work / APV,
Arbeitsgemeinschaft für Pharmazeutische
Verfahrenstechnik e.V.
Aulendorf : ECV, Edito-Cantor-Verlag für Medizin und
Naturwissenschaften
Volume 2.2012, no. 4
has been received in our library in the month of August
2012.
Since the 14th of August, it has always been open to the
public.
It is part of a bound volume and is lendable with the shelf
number Za 15974-2.2012,4-6.

21. Appendix 1E is the OPAC (public catalog) record of Württembergische Landesbibliothek for TechnoPharm. Below the middle of the record on the left-hand side, under Band [Volume] are the holdings of the Württembergische Landesbibliothek. Among the holdings is: 2. 2012 (2012), Heft. [Issue] 4-6.

22. After verifying by a search on the internet that Birgit Hacker is with the Württembergische Landesbibliothek and from my years of experience in academic libraries, I can state that this letter is consistent with ones sent to verify the receipt and availability of library materials. I see no reason to suspect that this is not an

authentic and reliable statement on the receipt and availability of *Techno Pharm.* 2, nr. 4 (2012) and its availability as of August 14, 2012.

3. Conclusion

23. I conclude, based on finding Document 1, the Reuter article, in a library and on finding library catalog records and an online record for the Reuter article. Appendices 1D and 1E indicate it was locatable by a researcher in August 2012. Document 1, the Reuter article, would have been available to an interested person no later than August 14, 2012.

B. Document 2. Arno Fries. “Drug Delivery of Sensitive Biopharmaceuticals with Prefilled Syringes.” *Drug Delivery Technology*. Volume 9. Number 5 (May 2009): 22-27. (“Fries”)

1. Authentication

24. Appendix 2A is a true and accurate copy from the issue of *Drug Delivery Technology*, volume 9, number 5, May 2009, pages 22 - 27. Appendix 2A includes cover, with ownership and date stamp of National Library of Medicine (NLM) May 18, 2009 (upper left hand corner of cover); the front material relating to the publication of the journal, *Drug Delivery Technology* and the Fries article pages 22-27. This scan was provided to me at my request by the Wisconsin TechSearch (WTS) from the National Library of Medicine (NLM).

25. Appendix 2B is a download from the proprietary website of *Drug Development & Delivery* (formerly “Drug Delivery Technology” through 2010):

<http://drug-dev.com/wp-content/uploads/2018/05/May-2009.pdf>. It provided me access to the digital copy of the Fries article.

26. Appendices 2A and 2B are in a condition that creates no suspicion about their authenticity. Specifically, the cover, the front material, the table of contents, and the sequence from page 22-27 is not missing intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Appendix 2B was found within the custody of a library – National Library of Medicine - a place where, if authentic, it would likely be found. Appendix 2B was located and downloaded by me from the proprietary website of the journal, where, if authentic, it would be found. Therefore, with the documentation provided above, I conclude that Document 2, the Fries article, is authentic.

2. Public Accessibility

27. Appendix 2A has the check-in date (on label on upper left hand corner of the cover) by the National Library of Medicine of May 18, 2009. Based on my knowledge and experience of academic libraries, I understand that it would take at most a week for such a periodical to be accessible on the library's shelf, the Fries article would have been publicly accessible no later than May 25, 2009.

28. Appendix 2C is a download from WorldCat that indicates that *Drug Delivery Technology*, as of September 21, 2018, is held by 59 libraries among these

is the National Library of Medicine. The WorldCat record indicates it would have been accessible by title, *Drug Delivery Technology*, and by subjects: Drug delivery systems – periodicals; Drug delivery systems; and Biological transport.

29. Appendix 2D is a download from the National Library of Medicine OPAC (online catalog). The NLM OPAC record indicates that *Drug Delivery Technology* began publication in 2001 and ceased with volume 10, no. 9 (Nov. – Dec. 2010). The available volumes held by NLM are shown as v.2 (2002) – 10 (2010), inclusive of volume 9, number 5, the issue in which the Fries article was published.

30. Appendix 2E is an article by D. Brett Ludwig, et al., titled “Flow cytometry: A Promising Technique for the Study of Silicone Oil-induced Particulate Formation in Protein Formulations,” published in *Analytical Biochemistry*, volume 410 (2011): 191-199. On page 199, in References, is number 11, the Fries article.

3. Conclusion

31. I conclude, based on finding Document 2 in a library and on finding library catalog records, indicating it was locatable and accessible to the public, that Document 2, the Fries article, is an authentic document and that Appendix 2A and Appendix 2B are authentic copies of Document 2 and that Appendix 2C and Appendix 2D are accurate reflections of the acquisition and cataloging of Document 2 and its accessibility to a researcher. Further evidence of its public accessibility is

it being listed as a reference in an article (Appendix 2E) published in 2011. It is, therefore, my conclusion that Document 2, the Fries article, is an authentic document and was publicly accessible no later than May 25, 2009.

C. Document 3. James A. Dixon, et al., “VEGF Trap-Eye for the Treatment of Neovascular Age-related Macular Degeneration.” *Expert Opinion on Investigational Drugs*. Volume 18. Number 10 (October 2009): 1573-1580. (“Dixon”)

1. Authentication

32. Appendix 3A is a true and accurate copy, requested by me from the Wisconsin TechSearch (WTS), of the article by James A. Dixon, et al., titled “VEGF Trap-Eye for the Treatment of Neovascular Age-related Macular Degeneration” published in *Expert Opinion on Investigational Drugs*, volume 18, no. 10 (October 2009) pages 1573-1580.

33. Appendix 3A includes cover of the October 2009 issue with National Library of Medicine (NLM) label (upper left hand corner of cover), indicating the issue was checked in by the NLM staff on October 20, 2009; table of contents; publication information, and the Dixon article, pages 1573-1580.

34. Appendix 3B is a download from the Taylor and Francis website, that provides the bibliographic description of the Dixon article and also indicates it was published online 20 August 2009. Appendix 3B was downloaded by me on September 21, 2018 at the Taylor and Francis website: <https://www.tandfonline.com/doi/abs/10.1517/13543780903201684>.

35. Appendix 3A is in a condition that creates no suspicion about its authenticity. Specifically, the cover, the front material, the table of contents, and the sequence from page 1573 to 1580 is not missing intermediate pages, the text on each page appears to flow seamlessly from one page to the next, and there are no visible alterations to the document. Appendix 3A was found within the custody of a library – the National Library of Medicine - a place where, if authentic, it would likely be found.

36. Appendix 3B documents that the Dixon article was published online by Taylor and Francis in its publication *Expert Opinion on Investigational Drugs* on August 20, 2009.

37. With the evidence identified above, I have determined that Document 3, the Dixon article, is an authentic document.

2. Public Accessibility

38. Appendix 3C is the view of the WorldCat record for the journal *Expert Opinion on Investigational Drugs*. It illustrates the availability to the public of the journal. One-hundred eighty-one libraries worldwide hold *Expert Opinion on Investigational Drugs* (in all formats and editions) as of September 2018, among these it shows the National Library of Medicine as owning the journal. The ability to locate this record is by title and by subject: Drugs – Periodicals – Design; and Drugs.

39. Appendix 3D is a download from the National Library of Medicine OPAC (online catalog). The NLM OPAC record indicates that *Expert Opinion on Investigational Drugs* began publication in 1994 in London by Ashley Publications Ltd. The OPAC record indicates that the National Library of Medicine started receiving the journal with Vol. 3, no. 2, (Feb. 1994) – and has continued to subscribe to the present. In the availability portion of the OPAC record it indicates volumes held by NLM are shown as v.3: no.2 (1994)-v.3: no. 4 (1994), v.3: no. 6 (1994) – v.22: no. 4 (2013), inclusive of volume 18, number 10, October 2009, the issue in which the Dixon article was published.

40. Appendix 3E is an article by Dong Hyun Jo, et al., titled “How to Overcome Retinal Neuropathy: The Fight Against Angiogenesis-related Blindness,” published in *Archives of Pharmacal Research*, volume 33, number 10. (2010) pages 1557-1565. On page 1563, in References, in alphabetical order, is the citation to the Dixon article.

3. Conclusion

41. I conclude, based on finding Document 3 in a library and on finding library catalog records, indicating it was locatable and accessible to the public, that Document 3, the Dixon article, is an authentic document and that Appendix 3A and Appendix 3B are authentic copies of Document 3 and that Appendix 3C and Appendix 3D are accurate reflections of the acquisition and cataloging of Document

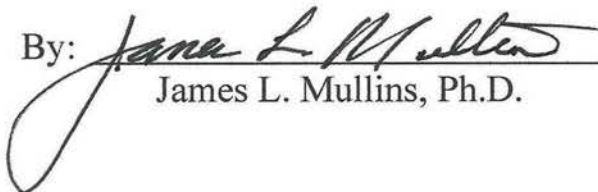
3 and its accessibility to a researcher. Further evidence of its public accessibility is it being listed as a reference in an article (Appendix 3E) published in 2010. It is, therefore, my conclusion that Document 3, the Dixon article, is an authentic document and was publicly accessible no later than end of October 2009 in print and online no later than August 20, 2009.

V. Declaration

42. I reserve the right to supplement this declaration in the future to respond to any arguments that Patent Owner or its expert(s) or counsel may raise, and to take into account new information as it becomes available to me.

43. I declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: July 14, 2020

By: 
James L. Mullins, Ph.D.

Appendix 1A

Techno Pharm

MAKING SCIENCE WORK

APV – Arbeitsgemeinschaft für
Pharmazeutische Verfahrenstechnik e.V.



04 · 2012

Silikonisierung von Spritzen

Trends, Analysen, Methoden

Fälschungssicherer DataMatrix-Code

Weiterhin eine Vision oder bald Standard?

Semiautomatisches aseptisches Füllen

Hochaktive Substanzen in Fertigspritzen und Flaschen einbringen

NIR- Beispiele zeigen das Potenzial

Online-Prozesskontrolle mit der Nahinfrarot-Spektroskopie

Partikelgrößenanalytik für Nanopartikel

Kombinierte Messmethoden erbringen die besten Ergebnisse

ACHEMA 2012

Volle Auftragsbücher für Pharma-Zulieferer

www.ecv.de

2a 15976

26,3

TERMINE 236

FOKUS VERPACKUNG

Reuter, Bruno; Petersen, Claudia 238
 Die Silikonisierung von Spritzen
 Trends, Methoden, Analyseverfahren

Wesch, Martin 246
 Elektronische Kennzeichnung für
 Pharmaverpackungen

Völcker, Thomas 250
 Serialisierung und digitale
 Authentifizierung von Arzneimitteln
 Der fälschungssichere DataMatrix-Code – eine
 Vision?

PROZESSTECHNIK

Caesar, Thomas 254
 Leistungsprüfung von Luftfiltern: Normen
 und Richtlinien für das 21. Jahrhundert

Bässler, Hans-Jürgen; Lehmann, Frank 260
 Semiautomatisches aseptisches Füllen
 Aktive pharmazeutische Substanzen in Fertig-
 spritzen und Flaschen unter Isolatorbedingungen
 im Technikumsmaßstab füllen

Bienert, Roland; Grimm, Christian;
 Höhse, Marek 266
 Ein prozessanalytisches Resümee
 Online-Prozesskontrolle mit der Nahinfrarot-
 Spektroskopie

AUTOMATION

Reis, C.; Brode, T.; Laske, C.; Traube, A.;
 Carlidge, D.; Hayes, T.; Behr, L. 274
 Neues Produktionsverfahren
 Test an synthetischen Wundauflagen mit
 mikroverkapseltem Antibiotikum

ANALYTIK

Keck, Cornelia M. 279
 Partikelgrößenanalytik für Nanopartikel
 Ein Kinderspiel oder doch eine verflixte Kiste?

MESSE

ACHEMA 2012 – Eine Retrospektive 288

PRODUKTE 302

IMPRESSUM 304

APVnews

Nachrichten und Mitteilungen von der Arbeitsgemeinschaft für
 Pharmazeutische Verfahrenstechnik e.V.
 (Ausgabe 04/12, nach S. 304)

Beiratsgremium

Prof. Dr. Jürgen Hannemann, Biberach · Dr. Udo Janske, Darmstadt · Prof. Dr. Gerd Kutz, Detmold · Heinz Kudernatsch, Nürnberg · Hans Ulrich Peterreit, Darmstadt · Dr. Elke Sternberger-Rützel, Freiberg a.N. · Dr. Mike Schäfers, Eschweiler · Prof. Dr. Hartwig Steckel, Kiel · Dr. Frank Stieneker, Mainz · Roland Szymoniak, Frankfurt am Main · Dr. Jochen Thies, Warendorf · Dipl. Ing. Frank Wilde, Stuttgart · Prof. Dr. Ing. Dominik Rabus, Denkendorf · Dipl. Ing. Frank Lehmann, Allschwil (Schweiz)

Die Silikonisierung von Spritzen

Trends, Methoden, Analyseverfahren

Bruno Reuter, Claudia Petersen • Gerresheimer Bünde GmbH, Bünde

Korrespondenz: Claudia Petersen, Gerresheimer Bünde GmbH, 32257 Bünde, Erich-Martens-Str. 26-32;
e-mail: c.petersen@gerresheimer.com

Zusammenfassung

Ready-to-Fill, d. h. sterile vorfüllbare Glasspritzen, werden durch den Primärpackmittelhersteller gewaschen, silikonisiert, verpackt und sterilisiert. Diese Spritzensysteme können somit ohne weitere Behandlungsschritte durch den Pharmazeuten zur Abfüllung eingesetzt werden. Heutzutage werden hauptsächlich vorfüllbare Glasspritzensysteme in Ready-to-Fill-Qualität gefertigt und dieser Trend setzt sich auch in der Zukunft fort. Bei der Fertigung von sterilen vorfüllbaren Glasspritzen ist die optimale Silikonisierung des Spritzenkörpers von großer Bedeutung. Das Zusammenspiel von Glaskörper- und Kolbenstopfensilikonisierung ist ausschlaggebend für die Funktionalität des gesamten Systems. Sowohl eine unzureichende als auch eine übermäßige Silikonisierung können hierbei Probleme hervorrufen. Für Glasspritzen kann durch den Einsatz moderner Technologien eine hohe Gleichmäßigkeit der Silikonölverteilung bei verringerten Mengen erreicht werden. Eine weitere Möglichkeit zur Minimierung der freien Silikonölmenge in einer Spritze ist das thermische Fixieren des Silikonöls auf der Glasoberfläche, die sogenannte Einbrennsilikonisierung. Silikonölfreie oder silikonölarmer vorfüllbare Spritzensysteme auf Kunststoffbasis sind eine relativ neue Option. In Entwicklung befinden sich zudem Gleitfähigkeitsbeschichtungen für Spritzen, welche nicht auf Silikonöl basieren.

Einleitung

Für die Verpackung von Injektabilia werden fast ausschließlich Primärverpackungen verwendet, die aus einem Glasbehälter (Karpulen, Spritzen, Fläschchen) und einem elastomeren Verschluss bestehen. Eine Ausnahme bilden hier die Ampullen.

Elastomere haben die Eigenschaft, von Natur aus leicht klebrig zu sein. Alle Elastomerverschlüsse (Kolbenstopfen, Injektions- oder Lyophilisationsstopfen) werden daher silikonisiert. Durch die Silikonisierung wird vermieden, dass die Stopfen aneinander kleben und die Verarbeitung der Artikel auf den Abfülllinien wird vereinfacht. Zum Beispiel werden beim Einsetzen der Stopfen die Mon-

tagekräfte minimiert. Die Silikonisierung ist somit unerlässlich für deren Prozessfähigkeit.

Glasbehälter werden ebenfalls silikonisiert. Bei Fläschchen und Ampullen ist dies seltener der Fall. Hier dient die Silikonisierung als Barrierebeschichtung zwischen Glas und Arzneimittelformulierung. Die Adsorption von Formulierungsbestandteilen an der Glasoberfläche wird unterbunden. Die hydrophobe Deaktivierung der Oberflächen führt zudem zu einer besseren Entleerbarkeit der Behälter.

Bei vorfüllbaren Spritzen und Karpulen dient die Silikonisierung einem weiteren Zweck. Sie ist unerlässlich, um das Gleiten des Kolbens im Spritzen- oder Karpulenkörper zu ermög-

lichen. Eine alleinige Silikonisierung des Kolbenstopfens wäre hierfür nicht ausreichend.

Silikonöle sind für die beschriebenen Aufgaben gut geeignet. Sie sind weitgehend inert, hydrophob und viskoelastisch. Chemikalische und physikalische Anforderungen sind in den relevanten Monographien des amerikanischen (United States Pharmacopoeia, USP) und des europäischen (Pharmacopoea Europaea, Phar. Eur) Arzneibuches beschrieben [1, 2]. Im Abschnitt 3.1.8 der Phar. Eur. ist zudem definiert, dass für Silikonöle zur Verwendung als Gleitmittel die kinematische Viskosität zwischen 1 000 und 30 000 mm²/s liegen sollte [3]. Die Monographie zu Polydimethylsiloxanen (PDMS) in

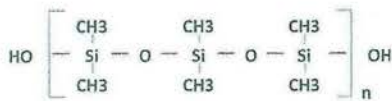


Abb. 1: Polydimethylsiloxan.

der USP [2] erlaubt hingegen die Verwendung von Silikonölen mit einer Viskosität von 20 bis 30 000 centistokes. Steigende Qualitätsanforderungen und neue biotechnologisch hergestellte Medikamente zeigen aber auch die Grenzen der Silikonisierungstechnik auf. Eine inhomogene Silikonisierung, wie sie vor allem bei einfachen Beschichtungstechniken und längeren Spritzenkörpern auftreten kann, führt eventuell zu mechanischen Problemen. Beispielhaft seien hier eine unvollständige Entleerung der Spritze in einem Autoinjektor oder hohe Gleitkräfte genannt.

Silikonöltropfen sind in abgefüllten Spritzen immer zu beobachten. Die Anzahl an Silikonöltropfen in Lösung nimmt mit steigender Silikonölmenge zu. Visuell erkennbare Tropfen können als kosmetischer Fehler betrachtet werden. Für Silikonölpartikel im subvisuellen Bereich wird diskutiert, ob sie ein Auslöser für die Aggregation von Proteinen sein können [4].

Vor diesem Hintergrund zeigt sich daher ein deutlicher Trend zu optimierten oder alternativen Beschichtungstechniken. Beispiele hierfür sind eine möglichst gleichmäßige Beschichtung mit reduzierter Silikonölmenge oder eine Minimierung der freien Silikonölmenge durch das sogenannte Einbrennverfahren. In diesem Kontext sind zuverlässige Analysetechnologien unabdingbar, mit denen die Qualität der Beschichtung qualitativ und quantitativ überprüft werden kann. Darüber hinaus befinden sich auch alternative Beschichtungsverfahren in der Entwicklung.

Silikonöle und ihre Eigenschaften

Silikonöle werden schon seit einem halben Jahrhundert in zahlreichen

pharmazeutischen Anwendungen eingesetzt zum Beispiel als Hilfsstoffe in der Pharmaproduktion (Schmiermittel) und als inerte pharmazeutische Grundmasse (z. B. Weichkapselwände) [5]. Für die Silikonisierung werden in der Regel Trimethylsiloxy-endgeblockte Polydimethylsiloxane (PDMS, Dimethicone) in verschiedenen Viskositäten eingesetzt (Abb. 1).

Das am häufigsten genutzte Silikonöl zur Silikonisierung von Primärpackmitteln ist das DOW CORNING® 360 Medical Fluid mit einer Viskosität von 1 000 cSt.

Die Herstellung von PDMS erfolgt über die Reduktion von Quarzsand zu metallischem Silizium. Im nächsten Schritt wird das Silizium durch eine Müller-Rochow-Synthese mit Methylchlorid zu Methylchlorsilanen weiterverarbeitet. Hierbei entsteht ein Gemisch unterschiedlicher Silane, bei dem Dimethyldichlorsilan (CH_3)₂ SiCl₂ mit 75 % – 90 % überwiegt. Nach einer destillativen Abtrennung wird das Dimethyldichlorsilan durch Hydrolyse oder Methanolyse zu Silanolen umgesetzt, die zu niedermolekularen Ketten und Zyklen kondensieren. In einer sauer (kationisch) oder alkalisch (anionisch) katalysierten Polymerisation werden Polydimethylsiloxane mit Hydroxy-Funktionen erzeugt, die abschließend durch Zugabe von Trimethylchlorsilan Trimethylsiloxy-Endgruppen erhalten. Aus den entstehenden polydispersen Polymeren werden die kurzkettigen Moleküle durch Verdampfen entfernt, um einsetzfähiges PDMS zu erhalten.

Prägend für das PDMS-Molekül ist die Si-O-Bindung. Sie ist mit einer Bindungsenergie von 108 kcal/mol deutlich stabiler als beispielsweise die C-O-Bindung (83 kcal/mol) oder die C-C-Bindung (85 kcal/mol). PDMS ist dementsprechend unempfindlich gegenüber thermischen Belastungen, UV-Strahlung oder Oxidationsmitteln. Erst oberhalb von 130 °C kommt es zu Reaktionen wie Oxidation, Polymerisierung oder Depolymerisierung. Für das Molekül ist

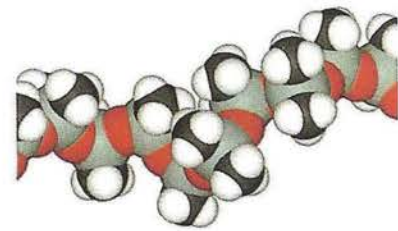


Abb. 2: Räumliche Darstellung Polydimethylsiloxan.

darüber hinaus ein flacher Bindungswinkel typisch (Si-O-Si 130 °C), der eine geringe Rotationsenergie zur Folge hat und es besonders flexibel macht (Abb. 2). Eine hohe Bindungslänge (1,63 Å Si-O im Vergleich zu 1,43 Å für C-O) macht das Molekül vergleichsweise gasdurchlässig [6].

Das spiralförmige (und daher gut komprimierbare) Molekül ist von CH₃-Gruppen umhüllt, welche das chemische und mechanische Verhalten von PDMS bestimmen. Die Methylgruppen der Moleküle entwickeln nur sehr geringe Wechselwirkungen untereinander. Dies sorgt selbst bei hohen Molekulargewichten für eine niedrige Viskosität, die das Verteilen von PDMS auf Oberflächen erleichtert und die Substanz zu einem sehr effektiven Gleitmittel macht. Zudem ist PDMS weitgehend inert, Reaktionen mit Glas, Metallen, Kunststoffen oder Körpergewebe finden nur in sehr geringem Umfang statt. Die CH₃-Gruppen machen PDMS stark hydrophob. Es ist daher in Wasser unlöslich, in nichtpolaren Lösungsmitteln dagegen löslich [6].

Silikonisierte Spritzen

Wie schon dargestellt, funktioniert das System „Spritze“ nur, wenn die Glaskörper- und Kolbenstopfensilikonisierung homogen und richtig aufeinander abgestimmt sind. Bei Hohnadelspritzen ist außerdem eine Silikonisierung derselben unerlässlich, um ein Kleben der Haut an der Nadel zu vermeiden und damit eine schmerzarme Injektion zu ermöglichen. Für die Innensilikonisierung des Glaskörpers wird bei der soge-

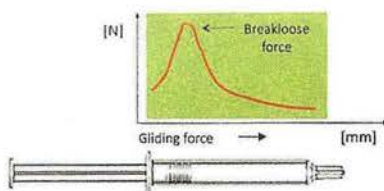


Abb. 3: Exemplarisches Kräfteprofil einer vorfüllbaren Spritze.

nannten Ölig-Silikonisierung DOW CORNING® 360 mit einer Viskosität von 1000 cSt verwendet. Für die Einbrennsilikonisierung wird oftmals die Silikonölemulsion DOW CORNING® 365 eingesetzt. Die Nadelsilikonisierung erfolgt im Verlauf der ready-to-fill Prozessierung mit einer Wischtechnik. Hierfür wird DOW CORNING® 360 mit einer Viskosität von 12.500 cSt verwendet. Alternativ hierzu ist auch eine thermische Fixierung von Silikonöl während der Nadelmontage möglich.

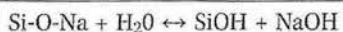
Ziel der Silikonisierung des Spritzenkörpers ist eine über die gesamte Spritzenlänge möglichst gleichförmige Gleitbeschichtung, welche im Zusammenspiel mit dem Kolbentopfen zu möglichst niedrigen Losbrech- und Gleitkräften führt (Abb. 3).

Eine zu geringe Silikonisierung des Spritzenkörpers, vor allem aber das Vorhandensein von unsilikonisierten Bereichen, stellt die Funktionalität der Spritze in Frage, sogenannter Slip-Stick-Effekt. Die erforderlichen Kräfte beim Injektionsvorgang können zu hoch sein oder ein vollständiges Versagen des Systems zur Folge haben. Da Untersilikonisierung und Lücken besonders häufig im vorderen Bereich der Spritze (Luerkonus-/Kanülenseite) auftreten, muss die vollständige Entleerung der Spritze in Frage gestellt werden. Insbesondere bei Autoinjektoren kann dies auf Grund der geschlossenen Systeme unentdeckt bleiben und damit ist die ausreichende Dosierung des Medikaments nicht sichergestellt werden.

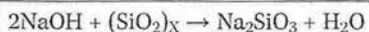
Die naheliegendste Lösung ist es, eine homogene Silikonisierung durch Erhöhung der Silikonölmenge zu er-

reichen. Eine Erhöhung der Silikonölmenge ist aber wie schon gesagt mit einer Erhöhung der Anzahl von Silikonölparkeln in der Lösung verbunden. Vor allem bei protein-basierten Medikamenten können unerwünschte Wechselwirkungen mit derartigen Silikonölparkeln nicht ausgeschlossen werden. Subvisuelle Silikonölparkel stehen in dem Verdacht, eine Proteinaggregatbildung zu begünstigen, welche Immunreaktionen verstärken können und die Verträglichkeit des Medikaments verschlechtern. Der zugrunde liegende Mechanismus ist noch nicht vollständig geklärt. Es wird diskutiert, ob diese Aggregatbildung durch zusätzliche Bewegung, wie z. B. durch Schütteln, beeinflusst wird [7]. In Versuchen wurde außerdem festgestellt, dass ab einer Silikonölmenge von mehr als 1mg/Spritze, das zusätzliche Silikonöl nicht zu einer weiteren Reduktion der Gleitkraft führt.

Die Innensilikonisierung von Glasspritzen hat noch einen weiteren Vorteil. Sie verhindert die Interaktion der Arzneimittellösung mit der Glasoberfläche und damit eventuell verbundene Probleme wie Wirkstoffverlust durch Adsorption oder pH-Wertverschiebung durch Alkali-Leaching. Vorfüllbare Glasspritzen werden nur aus hochwertigem Borosilikatglas Typ I gefertigt. Doch auch hier werden Natriumionen bei längerer Lagerung aus der Glasoberfläche in wässrige Lösungen ausgewaschen und können in ungepufferten Systemen eine problematische Erhöhung des pH-Werts bewirken. Durch eine saure Umgebung wird dieser Vorgang begünstigt.



In basischen Umgebungen findet dagegen ein Ätzprozess statt.



Wässrige Lösungen mit hohem pH-Wert können daher nicht über längere Zeit in Behältern aus Borosi-

likatglas gelagert werden, sondern müssen lyophilisiert und vor dem Gebrauch rekonstituiert werden. Insbesondere das Anätzen der Oberfläche kann im Extremfall zu Delamination der Glasoberfläche führen. Die hydrophobe Deaktivierung der Behälter durch Silikonisierung hat sich als eine Maßnahme zum Schutz der Glasoberfläche bewährt.

Optimierte Silikonisierung

Aus den dargestellten Gründen ergibt sich, dass es hierbei das Ziel sein muss, eine möglichst homogene Beschichtung mit möglichst geringen Silikonölmengen herzustellen. Hierzu ist es zunächst erforderlich, die minimale Silikonölmenge zu ermitteln, bei der die spezifischen Qualitätsanforderungen der jeweiligen Anwendung zuverlässig erfüllt werden. Standardmäßig erfolgt die Spritzensilikonisierung im Ready-to-Fill- Prozess nach dem Waschen und Trocknen. Feststehende Düsen, welche unterhalb des Spritzenkörpers an der Fingerauflagensseite positioniert sind, versprühen das Silikonöl auf der Spritzenkörperinnenseite. Bei längeren Spritzen kann es zu einer ungleichmäßigen Verteilung des Silikonöls kommen, wobei die Silikonölkonzentration zum Spritzenende (Luerkonus-/Kanülenseite) abnimmt. Durch den Einsatz von Tauchdüsen kann die Gleichmäßigkeit der Beschichtung über die gesamte Länge des Spritzenkörpers deutlich verbessert werden. Die Düsen tauchen hierzu in die Spritze ein und applizieren das Silikonöl (feinst zerstäubt) während des Bewegungsablaufes. Nahezu lineare, eng gebündelte Verläufe der Gleitkräfte im Kraft/Weg-Diagramm (Abb. 4) sind das Resultat.

In Studien mit 1 ml long Spritzen zeigte sich darüber hinaus ein deutliches Reduktionspotenzial bzgl. der benötigten Silikonölmenge. Die Menge an Silikonöl/Spritze konnte im Versuch um 40 % reduziert werden ohne Qualitätseinbußen in Bezug auf die funktionellen Eigenschaften.

ecv

Wissenschaft und Technik in der Anwendung



Computervalidierung im GxP-regulierten Umfeld

ISBN 978-3-87193-412-4

- € 64,00
- Neuauflage 2010, 208 Seiten



Gute Hygiene Praxis

ISBN 978-3-87193-379-0

- € 64,00
- 2. überarbeitete Auflage 2008, 248 Seiten

GMP- / FDA-Compliance in der Biotechnologie

ISBN 978-3-87193-329-5

- € 64,00
- 1. Auflage 2006, 192 Seiten

Die Qualified Person

ISBN 978-3-87193-362-2

- € 64,00
- 1. Auflage 2007, 144 Seiten

GMP- / FDA-Anforderungen an die Qualitätssicherung

ISBN 978-3-87193-380-6

- € 64,00
- 1. Auflage 2009, 214 Seiten

Fertigspritzen

ISBN 978-3-87193-393-6

- € 64,00
- 1. Auflage 2009, 176 Seiten

GMP- / FDA-gerechte Validierung

ISBN 978-3-87193-376-9

- € 64,00
- 2. überarbeitete Auflage 2010, 248 Seiten



GMP-Inspektionen und -Audits

ISBN 978-3-87193-381-3

- € 86,00
- 2. überarbeitete Auflage 2010, 176 Seiten



Herausgegeben von **CONCEPT HEDELBERG**

Zielgruppen

- Pharmaunternehmen
- Zulieferindustrie
- Behörden / Überwachungsämter
- Hochschulen / Universitäten
- Planungs- / Beratungsunternehmen

Bestellung:

Tel. +49 (0)7525-940 148, Fax: +49 (0)7525-940 147
eMail: vertrieb@ecv.de
Onlineshop, Leseproben und Inhaltsverzeichnisse
www.ecv.de

ECV - Gutes Konzept Verlag

PLETT **DER TURBO** HIE

BEWEGUNG MAXIMAL

BEI GROSSENAUFRÄGEN

Blistermaschine B 1660. Innovative Maschinenteknik für rasante Auflagen.

- Hochleistung für feste Produktformen bei Großaufträgen
- Bis zu 750 Blister pro Minute
- Mit Platten- oder Walzensiegelung
- 1-, 2- oder 3-bahnige Arbeitsweise
- Direkte Blisterübergabe an den Kartonierer

Uhlmann

Uhlmann Pac-Systeme GmbH & Co. KG
Uhlmannstrasse 14-18
88471 Laupheim, Germany
www.uhlmann.de

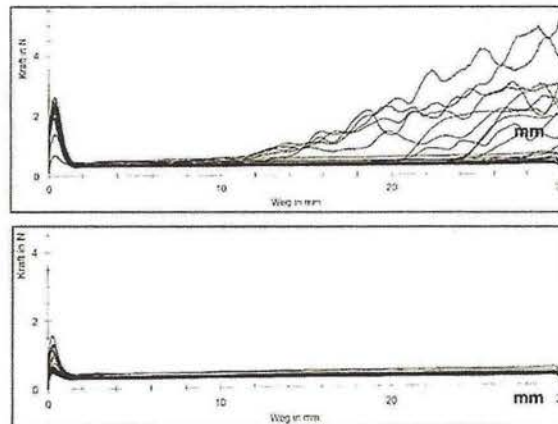
A member of
Excellence United
www.excellence-united.com

ten des Systems in Kauf nehmen zu müssen (Abb. 5). In der Praxis sollten bei der Ermittlung der optimalen Silikonöldosierung neben dem jeweiligen Spritzenvolumen auch der Kolbenstopfentyp (beschichtet, unbeschichtet), die Methode des Kolbenstopfensetzens (Setzrohr/Vakuum) sowie die Anforderungen während der Applizierung (Injektionsysteme) berücksichtigt werden. Kolbenstopfen verschiedener Lieferanten unterscheiden sich nicht nur in Bezug auf die verwendete Gummisorte und Design. Es werden zudem Silikonöle mit unterschiedlichen Viskositäten verwendet. Auch die Silikonisierungsverfahren unterscheiden sich deutlich voneinander. Diese Variabilität kann einen größeren Einfluss auf die funktionellen Eigenschaften eines Spritzensystems haben, als die Spritzensilikonisierung unterschiedlicher Lieferanten, wie von Eu et al [8] gezeigt.

Einbrennsilikonisierung

Eine weitere entscheidende Weiterentwicklung in der Silikonisierungstechnik ist das häufig auch als Härten bezeichnete Einbrennen. Silikonöl wird in Form einer Emulsion aufgebracht und in einem speziellen Ofen mit einem bestimmten Temperatur-/Zeitprogramm auf der Glasoberfläche fixiert.

Beim Einbrennen bilden sich sowohl Wasserstoffbrücken als auch kovalente Bindungen zwischen den der Glasoberfläche und den Polydimethylsiloxanketten. Die Bindung ist hierbei so fest, dass ein Teil des Silikonöls nicht mehr durch Lösungsmittel entfernt werden kann und eine dauerhafte, hydrophobe Schicht entsteht (Abb. 6). Zudem erhöht sich das durchschnittliche Molekulargewicht durch Polymerisierung und das Verdampfen kurzkettiger Polymere. Die entstehende, extrem dünne Silikonschicht, im Zusammenhang mit der geringen Silikonölmenge innerhalb der Emulsion, minimiert das freie Silikon in der Spritze und sorgt dennoch für die



$m = 0.8 \text{ mg}$, $v = 300 \text{ mm/min}$, leere 1ml long LC Spritzen

Abb. 4: Vergleich Kräfteprofil Tauchdüse gegen feststehende Düse.

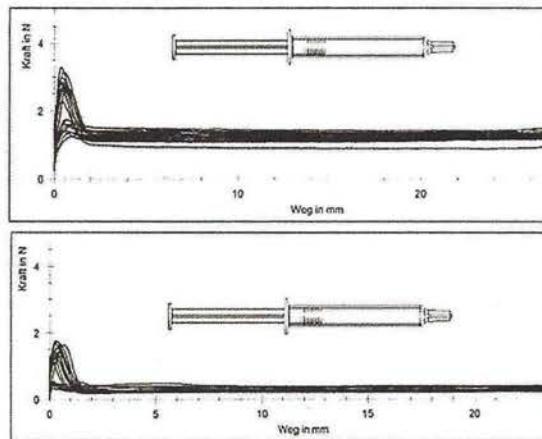


Abb. 5: Kräfteprofil nach optimierter Silikonisierung.

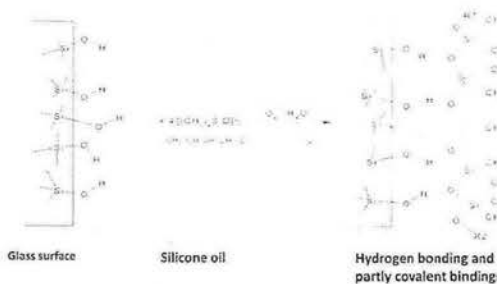


Abb. 6: Einbrennsilikonisierung.

gewünschte Qualität des Finishs. Es wird eine Schichtdicke von 15 – 50 nm gemessen. Im Vergleich dazu beträgt die Schichtdicke bei öliger Silikonisierung im Mittel 500 – 1000 nm.

■ Feststehende Düse
 - F_{av} Losbrechkraft = 2.1 N
 - F_{av} Gleitkraft = 2.4 N

■ Tauchdüse
 - F_{av} Losbrechkraft = 1.7 N
 - F_{av} Gleitkraft = 0.5 N

Standard 1ml long Spritze*
 Feststehende Düse
 $m = 0.8 \text{ mg}$
 $v = 100 \text{ mm/min}$
 $BF_{mean} = 2.5 \text{ N}$
 $EF_{mean} = 1.7 \text{ N}$

Optimierte Silikonisierung

Standard 1ml long Spritze*
 Tauchdüse
 $m = 0.5 \text{ mg}$
 $v = 100 \text{ mm/min}$
 $BF_{mean} = 1.7 \text{ N}$
 $EF_{mean} = 0.5 \text{ N}$
 * Leere Spritze

Durch eine Einbrennsilikonisierung wird die messbare Menge an freiem Silikonöl auf ca. 10 % des normalen Wertes reduziert. Es folgt hieraus, dass weniger subvisuelle und visuelle Silikonölpartikel in der Lösung zu finden sind. Dieses Silikonisierungsverfahren empfiehlt sich somit zum Einsatz mit empfindlichen Proteinformulierungen. Sie ist auch vorteilhaft bei der Verwendung für ophthalmologische Zubereitungen, welche sehr hohe Anforderungen im Hinblick auf die

erlaubte Partikelbelastung erfüllen müssen.

Ein weiterer Pluspunkt ist die Stabilität der mechanischen Eigenschaften der abgefüllten Spritze über die Lagerdauer hinweg. Die Rippen eines Kolbenstopfens drücken sich bei längerer Lagerung in die Silikonschicht einer ölig silikonisierten Spritze ein und es kommt zu einem direktem Glas/Gummi-Kontakt. Da Elastomere immer leicht klebrig sind, erhöhen sich dadurch die Losbrechkraften über die Lagerdauer. Bei einer eingebrannten Beschichtung hingegen kann man dieses Phänomen nicht in dem Ausmaß beobachten (Abb. 7). Die Losbrechkraft bleibt über die Lagerzeit nahezu konstant.

Analysetechniken

Die Optimierung des Silikonisierungsprozesses setzt zuverlässige qualitative und quantitative Analysemethoden voraus. Online-Methoden für eine hundertprozentige Kontrolle der Silikonisierung während der Produktion stehen nach dem derzeitigen Stand der Technik nicht zur Verfügung. Die Prozesskontrolle erfolgt daher an Stichproben, wobei mehrere zerstörende und zerstörungsfreie Methoden zum Einsatz kommen.

Beim Glasstaub-Test wird die Silikonisierung durch das Bestäuben mit feinsten Glaspartikeln sichtbar gemacht (Abb. 8). Das destruktive Verfahren ist einfach, aber zeitaufwän-

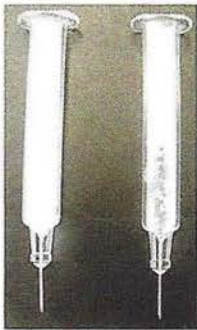


Abb. 8: Glasstaub-Test: links - Spritze mit Tauchdüse silikonisiert, rechts - Spritze mit feststehender Düse silikonisiert.

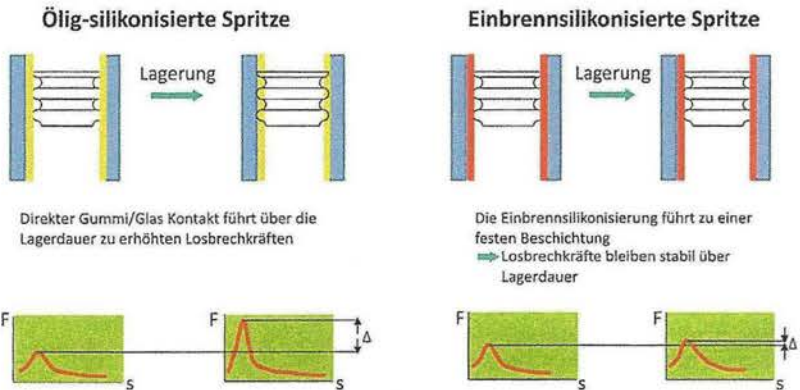


Abb. 7: Vergleich ölig- und einbrennsilikonisierte Spritze.

dig. Problematisch sind zudem die subjektive Beurteilung der Silikonisierungsqualität sowie die Abhängigkeit der Ergebnisse von Temperatur und Luftfeuchtigkeit.

Durch die Messung der Gleitkräfte kann die Gleichmäßigkeit der Silikonisierung indirekt bestimmt werden (Abb. 9). Das Verfah-



Abb. 9: Gleitkraftmessung.

ren ist ebenfalls destruktiv. Problematisch ist bei diesem Verfahren, dass die Ergebnisse durch die Platzierung des Kolbenstopfens beeinflusst werden und keine Standardisierung für die Extrusionsgeschwindigkeit besteht. Für leere Spritzensysteme wird oft mit einer Vorschub-Geschwindigkeit von 100 mm/min gearbeitet; bei gefüllten mit bis zu 380 mm/min.

Relativ schnelle, quantitative und zerstörungsfreie Ergebnisse

können auf reflexometrischem Weg gewonnen werden. Der Layer Explorer UT (Abb. 10) der Firma rapID zum Beispiel tastet den Spritzenkörper zeilenweise ab und misst dabei Schichtdicken von 15 nm bis zu einigen Tausend nm bei einer Genauigkeit von 5 nm (Abb. 10.1). Für die Untersuchung einer 40 mm langen Spritze wird ca. 1 min benötigt.

Ein weiterer zerstörungsfreier Ansatz basiert auf bildverarbeitenden Verfahren wie das der Firma ZebraScience (Abb. 11). Bei dieser Analyseverfahren wird die gesamte Innenseite des Spritzenkörpers erfasst und auf die charakteristischen Oberflächenstrukturen der Silikonisierung hin untersucht. Hierbei werden ausreichend silikonisierte Bereiche und eventuelle Lücken sichtbar gemacht (Abb. 11.1). Das Verfahren

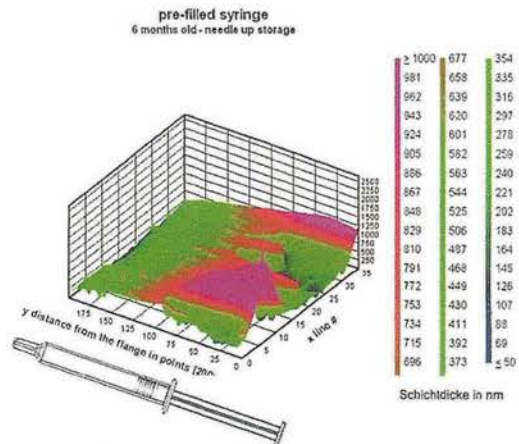


Abb. 10: Silikonölschichtdickenmessung mit dem Layer Explorer RapID (Quelle: Gerresheimer Buende).

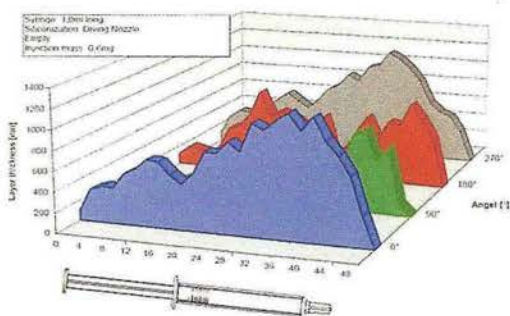


Abb. 10.1: Silikonölschichtdickenmessung mit dem Layer Explorer (Quelle: Gerresheimer Buende).



Abb. 11: ZebraScience Visualisierung Silikonisierung (Quelle: Gerresheimer Buende).

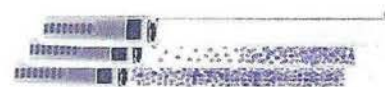


Abb. 11.1: Visualisierung der Spritzenkörpersilikonisierung (Zebra Science).

liefert schnelle qualitative Ergebnisse und kann für leere und gefüllte Spritzen eingesetzt werden. Leere Spritzen sollten jedoch nur unmittelbar nach dem Silikonisieren gemessen werden. Schon nach einer halben Stunde ergibt sich durch die Verteilung des Silikonöls ein völlig anderes Bild, dessen richtige Interpretation sehr viel Erfahrung benötigt.

Auch diese Methode ist derzeit leider nicht schnell genug, um eine

100 %-On-Line-Kontrolle während des Wasch- und Silikonisierungsprozesses zu ermöglichen.

Ausblick

Beim Finishing von Glasspritzen geht der Trend zu silikonölradierten Systemen oder Einbrennsilikonisierung. Verbesserte Analysetechniken und ein verbessertes Verständnis der involvierten Phänomene unterstützen einen optimierten Silikonöleinsatz.

Neue Fragestellungen ergeben sich durch die Nutzung innovativer Materialien oder Beschichtungen. Vor dem Hintergrund immer komplexer werdender

Devices und der weiterhin kontinuierlichen Zunahme an Biopharmazeutika mit ihren spezifischen Anforderungen, werden auch neue Materialoptionen für Primärverpackungen zunehmend interessant. So können Fläschchen- und Spritzeninnenflächen beispielsweise im Plasmaverfahren mit reinem SiO_2 beschichtet werden, um Interaktionen mit Medikamenten zu minimieren. Darüber hinaus gewinnen bei vorgefüllten Spritzen und Fläschchen Kunststoffsysteme auf Basis von zyklischen Olefinen (COP/COC) an Bedeutung. COP-Spritzen wie z. B. ClearJect TasPack™ von Taisei Kako Co. Ltd bieten die Transparenz von Glas. Darüber hinaus ist ihre Bruchsicherheit höher, der nutzbare pH-Bereich größer und es findet kein Auswaschen von Metallionen statt.

Besonders wichtig für die Verpackung biotechnologisch hergestellter Arzneimittel ist auch die hervorragende Dosiergenauigkeit. Auch bei COP-Spritzen ist eine Silikonisierung meist unerlässlich.

Einen völlig neuen Ansatz bieten dagegen silikonölfreie Systeme. Die Gleitfähigkeit der Fluorpolymerbeschichtung speziell entwickelter Kolbenstopfen wird genutzt, um die Silikonisierung von Plastikspritzen komplett überflüssig zu machen. Innovativen Medikamenten und Injektionssystemen stehen also ebenso innovative Ideen aus der Entwicklung für Primärpackmittelsysteme gegenüber.

Fachliteratur

- [1] United States Pharmacopoeia 35 NF 30. Dimethicone, The United States Pharmacopoeial Convention Inc, Rockville, USA, 2011
- [2] Pharmacopoea Europaea. 7. Ausgabe. Dimeticon. Deutscher Apotheker Verlag, Stuttgart, Deutschland, 2011, s. 2788
- [3] Pharmacopoea Europaea. 7. Ausgabe. 3.1.8 Siliconöl zur Verwendung als Gleitmittel, Deutscher Apotheker Verlag, Stuttgart, Deutschland, 2011, s. 486
- [4] Jones LS, Kaufmann A, Middaugh CR. Silicone Oil induced aggregation of proteins. *J Pharm Sci* 2005; 94(4):918-927
- [5] Colas A, Siang J, Ulman K. Silicone in Pharmaceutical Applications Part 2: Silicone Excipients. Dow Corning Corporation, Midland, USA, 2001
- [6] Colas A. Silicone in Pharmaceutical Applications. Dow Corning Corporation, Midland, USA, 2001
- [7] Thirumangalathu R, Krishnan S, Speed Ricci M, Brems DN, Randolph TW, Carpenter JF. Silicone Oil- and agitation-induced aggregation of a monoclonal antibody in aqueous suspension. *J Pharm Sci* 2009; 98(9):3167-3181
- [8] Rathore N, Pranay P, Eu B. Variability in syringe components and its impact on functionality of delivery systems. *PDA J Pharm Sci and Tech* 2011; 65:468-480

Appendix 1B

Die Silikonisierung von Spritzen

Trends, Methoden, Analyseverfahren

Bruno Reuter, Claudia Petersen • Gerresheimer Bünde GmbH, Bünde

Korrespondenz: Claudia Petersen, Gerresheimer Bünde GmbH, 32257 Bünde, Erich-Martens-Str. 26-32;
e-mail: c.petersen@gerresheimer.com

Zusammenfassung

Ready-to-Fill, d. h. sterile vorfüllbare Glasspritzen, werden durch den Primärpackmittelhersteller gewaschen, silikonisiert, verpackt und sterilisiert. Diese Spritzensysteme können somit ohne weitere Behandlungsschritte durch den Pharmazeuten zur Abfüllung eingesetzt werden. Heutzutage werden hauptsächlich vorfüllbare Glasspritzensysteme in Ready-to-Fill-Qualität gefertigt und dieser Trend setzt sich auch in der Zukunft fort. Bei der Fertigung von sterilen vorfüllbaren Glasspritzen ist die optimale Silikonisierung des Spritzenkörpers von großer Bedeutung. Das Zusammenspiel von Glaskörper- und Kolbenstopfensilikonisierung ist ausschlaggebend für die Funktionalität des gesamten Systems. Sowohl eine unzureichende als auch eine übermäßige Silikonisierung können hierbei Probleme hervorrufen. Für Glasspritzen kann durch den Einsatz moderner Technologien eine hohe Gleichmäßigkeit der Silikonölverteilung bei verringerten Mengen erreicht werden. Eine weitere Möglichkeit zur Minimierung der freien Silikonölmenge in einer Spritze ist das thermische Fixieren des Silikonöls auf der Glasoberfläche, die sogenannte Einbrennsilikonisierung. Silikonölfreie oder silikonölarne vorfüllbare Spritzensysteme auf Kunststoffbasis sind eine relativ neue Option. In Entwicklung befinden sich zudem Gleitfähigkeitsbeschichtungen für Spritzen, welche nicht auf Silikonöl basieren.

Einleitung

Für die Verpackung von Injektabilia werden fast ausschließlich Primärverpackungen verwendet, die aus einem Glasbehälter (Karpulen, Spritzen, Fläschchen) und einem elastomeren Verschluss bestehen. Eine Ausnahme bilden hier die Ampullen.

Elastomere haben die Eigenschaft, von Natur aus leicht klebrig zu sein. Alle Elastomerverschlüsse (Kolbenstopfen, Injektions- oder Lyophilisationsstopfen) werden daher silikonisiert. Durch die Silikonisierung wird vermieden, dass die Stopfen aneinander kleben und die Verarbeitung der Artikel auf den Abfülllinien wird vereinfacht. Zum Beispiel werden beim Einsetzen der Stopfen die Mon-

tagekräfte minimiert. Die Silikonisierung ist somit unerlässlich für deren Prozessfähigkeit.

Glasbehälter werden ebenfalls silikonisiert. Bei Fläschchen und Ampullen ist dies seltener der Fall. Hier dient die Silikonisierung als Barrierebeschichtung zwischen Glas und Arzneimittelformulierung. Die Adsorption von Formulierungsbestandteilen an der Glasoberfläche wird unterbunden. Die hydrophobe Deaktivierung der Oberflächen führt zudem zu einer besseren Entleerbarkeit der Behälter.

Bei vorfüllbaren Spritzen und Karpulen dient die Silikonisierung einem weiteren Zweck. Sie ist unerlässlich, um das Gleiten des Kolbens im Spritzen- oder Karpulenkörper zu ermög-

lichen. Eine alleinige Silikonisierung des Kolbenstopfens wäre hierfür nicht ausreichend.

Silikonöle sind für die beschriebenen Aufgaben gut geeignet. Sie sind weitgehend inert, hydrophob und viskoelastisch. Chemikalische und physikalische Anforderungen sind in den relevanten Monographien des amerikanischen (United States Pharmacopoeia, USP) und des europäischen (Pharmacopoea Europaea, Phar. Eur) Arzneibuches beschrieben [1, 2]. Im Abschnitt 3.1.8 der Phar. Eur. ist zudem definiert, dass für Silikonöle zur Verwendung als Gleitmittel die kinematische Viskosität zwischen 1 000 und 30 000 mm²/s liegen sollte [3]. Die Monographie zu Polydimethylsiloxanen (PDMS) in

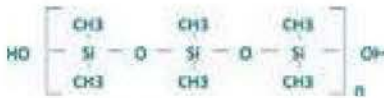


Abb. 1: Polydimethylsiloxan.

der USP [2] erlaubt hingegen die Verwendung von Silikonölen mit einer Viskosität von 20 bis 30 000 centistokes. Steigende Qualitätsanforderungen und neue biotechnologisch hergestellte Medikamente zeigen aber auch die Grenzen der Silikonisierungstechnik auf. Eine inhomogene Silikonisierung, wie sie vor allem bei einfachen Beschichtungstechniken und längeren Spritzenkörpern auftreten kann, führt eventuell zu mechanischen Problemen. Beispielhaft seien hier eine unvollständige Entleerung der Spritze in einem Autoinjektor oder hohe Gleitkräfte genannt.

Silikonöltropfen sind in abgefüllten Spritzen immer zu beobachten. Die Anzahl an Silikonöltropfen in Lösung nimmt mit steigender Silikonölmenge zu. Visuell erkennbare Tropfen können als kosmetischer Fehler betrachtet werden. Für Silikonölpartikel im subvisuellen Bereich wird diskutiert, ob sie ein Auslöser für die Aggregation von Proteinen sein können [4].

Vor diesem Hintergrund zeigt sich daher ein deutlicher Trend zu optimierten oder alternativen Beschichtungstechniken. Beispiele hierfür sind eine möglichst gleichmäßige Beschichtung mit reduzierter Silikonölmenge oder eine Minimierung der freien Silikonölmenge durch das sogenannte Einbrennverfahren. In diesem Kontext sind zuverlässige Analysetechnologien unabdingbar, mit denen die Qualität der Beschichtung qualitativ und quantitativ überprüft werden kann. Darüber hinaus befinden sich auch alternative Beschichtungsverfahren in der Entwicklung.

Silikonöle und ihre Eigenschaften

Silikonöle werden schon seit einem halben Jahrhundert in zahlreichen

pharmazeutischen Anwendungen eingesetzt zum Beispiel als Hilfsstoffe in der Pharmaproduktion (Schmiermittel) und als inerte pharmazeutische Grundmasse (z. B. Weichkapselwände) [5]. Für die Silikonisierung werden in der Regel Trimethylsiloxy-endgeblockte Polydimethylsiloxane (PDMS, Dimethicone) in verschiedenen Viskositäten eingesetzt (Abb. 1).

Das am häufigsten genutzte Silikonöl zur Silikonisierung von Primärpackmitteln ist das DOW CORNING[®] 360 Medical Fluid mit einer Viskosität von 1 000 cSt.

Die Herstellung von PDMS erfolgt über die Reduktion von Quarzsand zu metallischem Silizium. Im nächsten Schritt wird das Silizium durch eine Müller-Rochow-Synthese mit Methylchlorid zu Methylchlorosilanen weiterverarbeitet. Hierbei entsteht ein Gemisch unterschiedlicher Silane, bei dem Dimethyldichlorsilan ($\text{CH}_3)_2\text{SiCl}_2$ mit 75 % – 90 % überwiegt. Nach einer destillativen Abtrennung wird das Dimethyldichlorsilan durch Hydrolyse oder Methanolyse zu Silanolen umgesetzt, die zu niedermolekularen Ketten und Zyklen kondensieren. In einer sauer (kationisch) oder alkalisch (anionisch) katalysierten Polymerisation werden Polydimethylsiloxane mit Hydroxy-Funktionen erzeugt, die abschließend durch Zugabe von Trimethylchlorosilan Trimethylsiloxy-Endgruppen erhalten. Aus den entstehenden polydispersen Polymeren werden die kurzkettigen Moleküle durch Verdampfen entfernt, um einsetzbares PDMS zu erhalten.

Prägend für das PDMS-Molekül ist die Si-O-Bindung. Sie ist mit einer Bindungsenergie von 108 kcal/mol deutlich stabiler als beispielsweise die C-O-Bindung (83 kcal/mol) oder die C-C-Bindung (85 kcal/mol). PDMS ist dementsprechend unempfindlich gegenüber thermischen Belastungen, UV-Strahlung oder Oxidationsmitteln. Erst oberhalb von 130 °C kommt es zu Reaktionen wie Oxidation, Polymerisierung oder Depolymerisierung. Für das Molekül ist

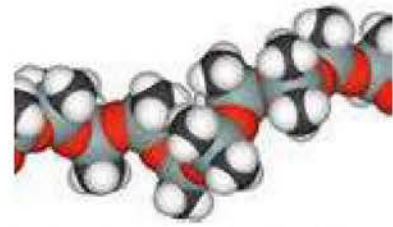


Abb. 2: Räumliche Darstellung Polydimethylsiloxan.

darüber hinaus ein flacher Bindungswinkel typisch (Si-O-Si 130 °C), der eine geringe Rotationsenergie zur Folge hat und es besonders flexibel macht (Abb. 2). Eine hohe Bindungslänge (1,63 Å Si-O im Vergleich zu 1,43 Å für C-O) macht das Molekül vergleichsweise gasdurchlässig [6].

Das spiralförmige (und daher gut komprimierbare) Molekül ist von CH_3 -Gruppen umhüllt, welche das chemische und mechanische Verhalten von PDMS bestimmen. Die Methylgruppen der Moleküle entwickeln nur sehr geringe Wechselwirkungen untereinander. Dies sorgt selbst bei hohen Molekulargewichten für eine niedrige Viskosität, die das Verteilen von PDMS auf Oberflächen erleichtert und die Substanz zu einem sehr effektiven Gleitmittel macht. Zudem ist PDMS weitgehend inert, Reaktionen mit Glas, Metallen, Kunststoffen oder Körpergeweben finden nur in sehr geringem Umfang statt. Die CH_3 -Gruppen machen PDMS stark hydrophob. Es ist daher in Wasser unlöslich, in nichtpolaren Lösungsmitteln dagegen löslich [6].

Silikonisierte Spritzen

Wie schon dargestellt, funktioniert das System „Spritze“ nur, wenn die Glaskörper- und Kolbenstopfensilikonisierung homogen und richtig aufeinander abgestimmt sind. Bei Hohnadelspritzen ist außerdem eine Silikonisierung derselben unerlässlich, um ein Kleben der Haut an der Nadel zu vermeiden und damit eine schmerzarme Injektion zu ermöglichen. Für die Innensilikonisierung des Glaskörpers wird bei der sog-

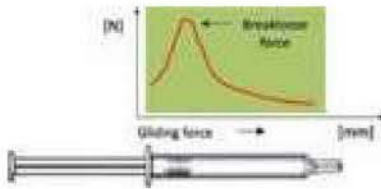


Abb. 3: Exemplarisches Kräfteprofil einer vorfüllbaren Spritze.

nannten Ölig-Silikonisierung DOW CORNING® 360 mit einer Viskosität von 1000 cSt verwendet. Für die Einbrennsilikonisierung wird oftmals die Silikonölemulsion DOW CORNING® 365 eingesetzt. Die Nadelsilikonisierung erfolgt im Verlauf der ready-to-fill Prozessierung mit einer Wischtechnik. Hierfür wird DOW CORNING® 360 mit einer Viskosität von 12500 cSt verwendet. Alternativ hierzu ist auch eine thermische Fixierung von Silikonöl während der Nadelmontage möglich.

Ziel der Silikonisierung des Spritzenkörpers ist eine über die gesamte Spritzenlänge möglichst gleichförmige Gleitbeschichtung, welche im Zusammenspiel mit dem Kolbentopfen zu möglichst niedrigen Losbrech- und Gleitkräften führt (Abb. 3).

Eine zu geringe Silikonisierung des Spritzenkörpers, vor allem aber das Vorhandensein von unsilikonisierten Bereichen, stellt die Funktionalität der Spritze in Frage, sogenannter Slip-Stick-Effekt. Die erforderlichen Kräfte beim Injektionsvorgang können zu hoch sein oder ein vollständiges Versagen des Systems zur Folge haben. Da Untersilikonisierung und Lücken besonders häufig im vorderen Bereich der Spritze (Luerkonus-/Kanülenseite) auftreten, muss die vollständige Entleerung der Spritze in Frage gestellt werden. Insbesondere bei Autoinjektoren kann dies auf Grund der geschlossenen Systeme unentdeckt bleiben und damit ist die ausreichende Dosierung des Medikaments nicht sichergestellt werden.

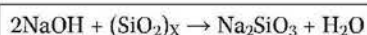
Die naheliegendste Lösung ist es, eine homogene Silikonisierung durch Erhöhung der Silikonölmenge zu er-

reichen. Eine Erhöhung der Silikonölmenge ist aber wie schon gesagt mit einer Erhöhung der Anzahl von Silikonölparkeln in der Lösung verbunden. Vor allem bei protein-basierten Medikamenten können unerwünschte Wechselwirkungen mit derartigen Silikonölparkeln nicht ausgeschlossen werden. Subvisuelle Silikonölparkel stehen in dem Verdacht, eine Proteinaggregatbildung zu begünstigen, welche Immunreaktionen verstärken können und die Verträglichkeit des Medikaments verschlechtern. Der zugrunde liegende Mechanismus ist noch nicht vollständig geklärt. Es wird diskutiert, ob diese Aggregatbildung durch zusätzliche Bewegung, wie z. B. durch Schütteln, beeinflusst wird [7]. In Versuchen wurde außerdem festgestellt, dass ab einer Silikonölmenge von mehr als 1mg/Spritze, das zusätzliche Silikonöl nicht zu einer weiteren Reduktion der Gleitkraft führt.

Die Innensilikonisierung von Glasspritzen hat noch einen weiteren Vorteil. Sie verhindert die Interaktion der Arzneimittellösung mit der Glasoberfläche und damit eventuell verbundene Probleme wie Wirkstoffverlust durch Adsorption oder pH-Wertverschiebung durch Alkali-Leaching. Vorfüllbare Glasspritzen werden nur aus hochwertigem Borosilikatglas Typ I gefertigt. Doch auch hier werden Natriumionen bei längerer Lagerung aus der Glasoberfläche in wässrige Lösungen ausgewaschen und können in ungepufferten Systemen eine problematische Erhöhung des pH-Werts bewirken. Durch eine saure Umgebung wird dieser Vorgang begünstigt.



In basischen Umgebungen findet dagegen ein Ätzprozess statt.



Wässrige Lösungen mit hohem pH-Wert können daher nicht über längere Zeit in Behältern aus Borosi-

likatglas gelagert werden, sondern müssen lyophilisiert und vor dem Gebrauch rekonstituiert werden. Insbesondere das Anätzen der Oberfläche kann im Extremfall zu Delamination der Glasoberfläche führen. Die hydrophobe Deaktivierung der Behälter durch Silikonisierung hat sich als eine Maßnahme zum Schutz der Glasoberfläche bewährt.

Optimierte Silikonisierung

Aus den dargestellten Gründen ergibt sich, dass es hierbei das Ziel sein muss, eine möglichst homogene Beschichtung mit möglichst geringen Silikonölmengen herzustellen. Hierzu ist es zunächst erforderlich, die minimale Silikonölmenge zu ermitteln, bei der die spezifischen Qualitätsanforderungen der jeweiligen Anwendung zuverlässig erfüllt werden. Standardmäßig erfolgt die Spritzensilikonisierung im Ready-to-Fill- Prozess nach dem Waschen und Trocknen. Feststehende Düsen, welche unterhalb des Spritzenkörpers an der Fingerauflageposition positioniert sind, versprühen das Silikonöl auf der Spritzenkörperinnenseite. Bei längeren Spritzen kann es zu einer ungleichmäßigen Verteilung des Silikonöls kommen, wobei die Silikonölkonzentration zum Spritzenende (Luerkonus-/Kanülenseite) abnimmt. Durch den Einsatz von Tauchdüsen kann die Gleichmäßigkeit der Beschichtung über die gesamte Länge des Spritzenkörpers deutlich verbessert werden. Die Düsen tauchen hierzu in die Spritze ein und applizieren das Silikonöl (feinst zerstäubt) während des Bewegungsablaufes. Nahezu lineare, eng gebündelte Verläufe der Gleitkräfte im Kraft/Weg-Diagramm (Abb. 4) sind das Resultat.

In Studien mit 1 ml long Spritzen zeigte sich darüber hinaus ein deutliches Reduktionspotenzial bzgl. der benötigten Silikonölmenge. Die Menge an Silikonöl/Spritze konnte im Versuch um 40 % reduziert werden ohne Qualitätseinbußen in Bezug auf die funktionellen Eigenschaf-

Nur für den privaten oder firmeninternen Gebrauch / For private or internal corporate use only

ecv

Wissenschaft und Technik in der Anwendung



Computervalidierung im GxP-regulierten Umfeld

ISBN 978-3-87193-412-4

- € 64,00
- Neuauflage 2010, 208 Seiten



Gute Hygiene Praxis

ISBN 978-3-87193-379-0

- € 64,00
- 2. überarbeitete Auflage 2008, 248 Seiten

GMP- / FDA-Compliance in der Biotechnologie

ISBN 978-3-87193-329-5

- € 64,00
- 1. Auflage 2006, 192 Seiten

Die Qualified Person

ISBN 978-3-87193-362-2

- € 64,00
- 1. Auflage 2007, 144 Seiten

GMP- / FDA-Anforderungen

an die Qualitätssicherung

ISBN 978-3-87193-380-6

- € 64,00
- 1. Auflage 2009, 214 Seiten

Fertigspritzen

ISBN 978-3-87193-393-6

- € 64,00
- 1. Auflage 2009, 176 Seiten

GMP- / FDA-gerechte Validierung

ISBN 978-3-87193-376-9

- € 64,00
- 2. überarbeitete Auflage 2010, 248 Seiten



GMP-Inspektionen und -Audits

ISBN 978-3-87193-381-3

- € 86,00
- 2. überarbeitete Auflage 2010, 176 Seiten



Herausgegeben von 

Zielgruppen

- Pharmaunternehmen
- Zulieferindustrie
- Behörden / Überwachungsämter
- Hochschulen / Universitäten
- Planungs- / Beratungsunternehmen

Bestellung:

Tel. +49 (0)7525-940 148, Fax: +49 (0)7525-940 147
eMail: vertrieb@ecv.de
Onlineshop, Leseproben und Inhaltsverzeichnisse
www.ecv.de

ECV · Editio Cantor Verlag



Blistermaschine B 1660. Innovative Maschinenteknik für rasante Auflagen.

- Hochleistung für feste Produktformen bei Großaufträgen
- Bis zu 750 Blister pro Minute
- Mit Platten- oder Walzensiegelung
- 1-, 2- oder 3-bahnige Arbeitsweise
- Direkte Blisterübergabe an den Kartonierer

 Uhlmann

Uhlmann Pac-Systeme GmbH & Co. KG
Uhlmannstrasse 14-18
88471 Laupheim, Germany
www.uhlmann.de

 A member of
Excellence United
www.excellence-united.com

Regeneron Exhibit 1033.034

ten des Systems in Kauf nehmen zu müssen (Abb. 5). In der Praxis sollten bei der Ermittlung der optimalen Silikonöldosierung neben dem jeweiligen Spritzenvolumen auch der Kolbenstoptyp (beschichtet, unbeschichtet), die Methode des Kolbenstoppens (Setzrohr/Vakuum) sowie die Anforderungen während der Applizierung (Injektionssysteme) berücksichtigt werden. Kolbenstopfen verschiedener Lieferanten unterscheiden sich nicht nur in Bezug auf die verwendete Gummisorte und Design. Es werden zudem Silikonöle mit unterschiedlichen Viskositäten verwendet. Auch die Silikonisierungsverfahren unterscheiden sich deutlich voneinander. Diese Variabilität kann einen größeren Einfluss auf die funktionellen Eigenschaften eines Spritzensystems haben, als die Spritzensilikonisierung unterschiedlicher Lieferanten, wie von Eu et al [8] gezeigt.

Einbrennsilikonisierung

Eine weitere entscheidende Weiterentwicklung in der Silikonisierungstechnik ist das häufig auch als Härten bezeichnete Einbrennen. Silikonöl wird in Form einer Emulsion aufgebracht und in einem speziellen Ofen mit einem bestimmten Temperatur-/Zeitprogramm auf der Glasoberfläche fixiert.

Beim Einbrennen bilden sich sowohl Wasserstoffbrücken als auch kovalente Bindungen zwischen den der Glasoberfläche und den Polydimethylsiloxanketten. Die Bindung ist hierbei so fest, dass ein Teil des Silikonöls nicht mehr durch Lösungsmittel entfernt werden kann und eine dauerhafte, hydrophobe Schicht entsteht (Abb. 6). Zudem erhöht sich das durchschnittliche Molekulargewicht durch Polymerisierung und das Verdampfen kurzkettiger Polymere. Die entstehende, extrem dünne Silikonschicht, im Zusammenhang mit der geringen Silikonölmenge innerhalb der Emulsion, minimiert das freie Silikon in der Spritze und sorgt dennoch für die

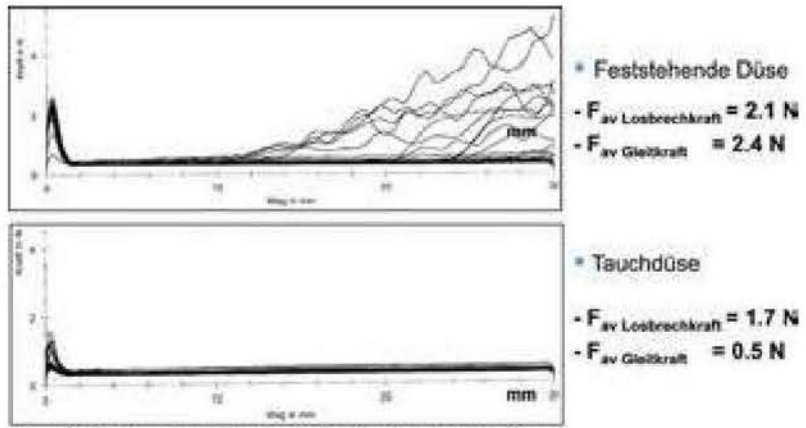


Abb. 4: Vergleich Kräfteprofil Tauchdüse gegen feststehende Düse.

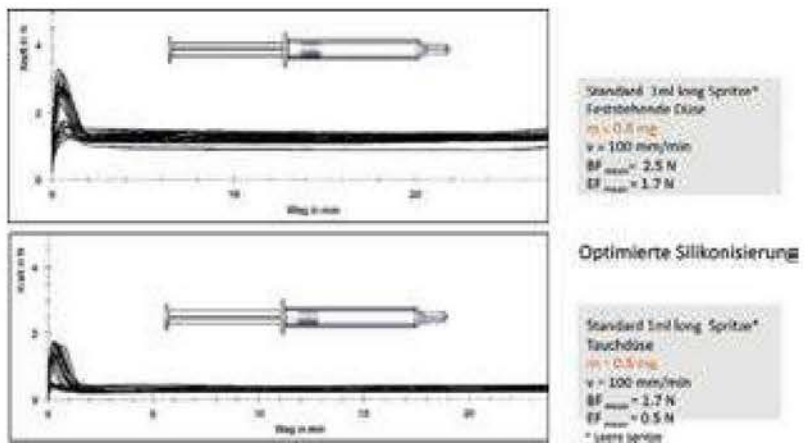


Abb. 5: Kräfteprofil nach optimierter Silikonisierung.

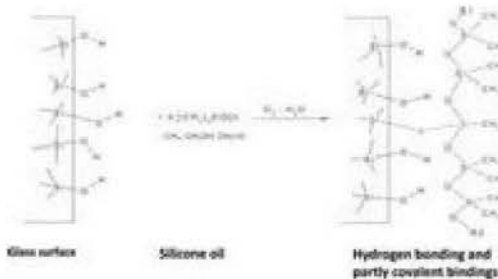


Abb. 6: Einbrennsilikonisierung.

gewünschte Qualität des Finishes. Es wird eine Schichtdicke von 15 - 50 nm gemessen. Im Vergleich dazu beträgt die Schichtdicke bei öliger Silikonisierung im Mittel 500 - 1000 nm.

Durch eine Einbrennsilikonisierung wird die messbare Menge an freiem Silikonöl auf ca. 10 % des normalen Wertes reduziert. Es folgt hieraus, dass weniger subvisuelle und visuelle Silikonölpartikel in der Lösung zu finden sind. Dieses Silikonisierungsverfahren empfiehlt sich somit zum Einsatz mit empfindlichen Proteinformulierungen. Sie ist auch vorteilhaft bei der Verwendung für ophthalmologische Zubereitungen, welche sehr hohe Anforderungen im Hinblick auf die

Nur für den privaten oder firmeninternen Gebrauch / For private or internal corporate use only

erlaubte Partikelbelastung erfüllen müssen.

Ein weiterer Pluspunkt ist die Stabilität der mechanischen Eigenschaften der abgefüllten Spritze über die Lagerdauer hinweg. Die Rippen eines Kolbenstopfens drücken sich bei längerer Lagerung in die Silikonschicht einer ölig silikonisierten Spritze ein und es kommt zu einem direktem Glas/Gummi-Kontakt. Da Elastomere immer leicht klebrig sind, erhöhen sich dadurch die Losbrechkräfte über die Lagerdauer. Bei einer eingebraunten Beschichtung hingegen kann man dieses Phänomen nicht in dem Ausmaß beobachten (Abb. 7). Die Losbrechkraft bleibt über die Lagerzeit nahezu konstant.

Analysetechniken

Die Optimierung des Silikonisierungsprozesses setzt zuverlässige qualitative und quantitative Analysemethoden voraus. Online-Methoden für eine hundertprozentige Kontrolle der Silikonisierung während der Produktion stehen nach dem derzeitigen Stand der Technik nicht zur Verfügung. Die Prozesskontrolle erfolgt daher an Stichproben, wobei mehrere zerstörende und zerstörungsfreie Methoden zum Einsatz kommen.

Beim Glasstaub-Test wird die Silikonisierung durch das Bestäuben mit feinsten Glaspartikeln sichtbar gemacht (Abb. 8). Das destruktive Verfahren ist einfach, aber zeitaufwändig.



Abb. 8: Glasstaub-Test: links – Spritze mit Tauchdüse silikonisiert, rechts- Spritze mit feststehender Düse silikonisiert.

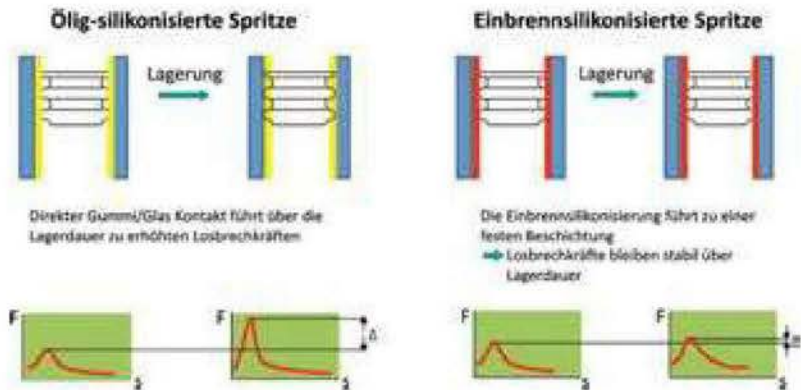


Abb. 7: Vergleich ölig- und einbrennsilikonisierte Spritze.

dig. Problematisch sind zudem die subjektive Beurteilung der Silikonisierungsqualität sowie die Abhängigkeit der Ergebnisse von Temperatur und Luftfeuchtigkeit.

Durch die Messung der Gleitkräfte kann die Gleichmäßigkeit der Silikonisierung indirekt bestimmt werden (Abb. 9). Das Verfah-



Abb. 9: Gleitkraftmessung.

ren ist ebenfalls destruktiv. Problematisch ist bei diesem Verfahren, dass die Ergebnisse durch die Platzierung des Kolbenstopfens beeinflusst werden und keine Standardisierung für die Extrusionsgeschwindigkeit besteht. Für leere Spritzen-systeme wird oft mit einer Vorschub-Geschwindigkeit von 100 mm/min gearbeitet; bei gefüllten mit bis zu 380 mm/min.

Relativ schnelle, quantitative und zerstörungsfreie Ergebnisse

können auf reflexometrischem Weg gewonnen werden. Der Layer Explorer UT (Abb. 10) der Firma rapID zum Beispiel tastet den Spritzenkörper zeilenweise ab und misst dabei Schichtdicken von 15 nm bis zu einigen Tausend nm bei einer Genauigkeit von 5 nm (Abb. 10.1). Für die Untersuchung einer 40 mm langen Spritze wird ca. 1 min benötigt.

Ein weiterer zerstörungsfreier Ansatz basiert auf bildverarbeitenden Verfahren wie das der Firma ZebraScience (Abb. 11). Bei dieser Analyse-methode wird die gesamte Innenseite des Spritzenkörpers erfasst und auf die charakteristischen Oberflächenstrukturen der Silikonisierung hin untersucht. Hierbei werden ausreichend silikonisierte Bereiche und eventuelle Lücken sichtbar gemacht (Abb. 11.1). Das Verfahren

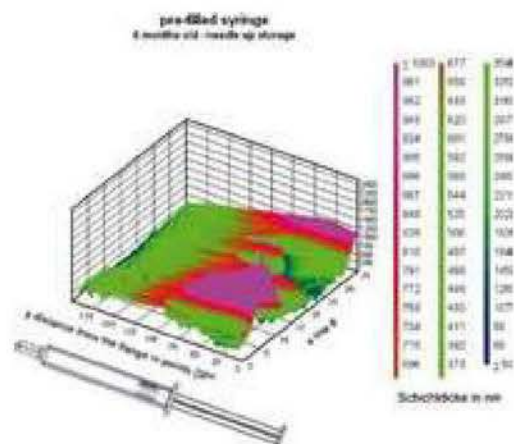


Abb. 10: Silikonölschichtdickenmessung mit dem Layer Explorer RapID (Quelle: Gerresheimer Buende).

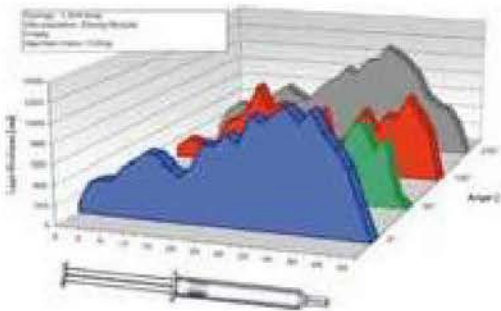


Abb. 10.1: Silikonölschichtdickenmessung mit dem Layer Explorer (Quelle: Gerresheimer Buende).

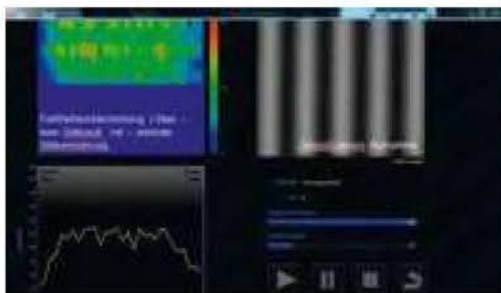


Abb. 11: ZebraScience Visualisierung Silikonisierung (Quelle: Gerresheimer Buende).



Abb. 11.1: Visualisierung der Spritzenkörpersilikonisierung (Zebra Science).

liefert schnelle qualitative Ergebnisse und kann für leere und gefüllte Spritzen eingesetzt werden. Leere Spritzen sollten jedoch nur unmittelbar nach dem Silikonisieren gemessen werden. Schon nach einer halben Stunde ergibt sich durch die Verteilung des Silikonöls ein völlig anderes Bild, dessen richtige Interpretation sehr viel Erfahrung benötigt.

Auch diese Methode ist derzeit leider nicht schnell genug, um eine

100 %-On-Line-Kontrolle während des Wasch- und Silikonisierungsprozesses zu ermöglichen.

Ausblick

Beim Finishing von Glasspritzen geht der Trend zu silikonölradierten Systemen oder Einbrennsilikonisierung. Verbesserte Analysetechniken und ein verbessertes Verständnis der involvierten Phänomene unterstützen einen optimierten Silikonöleinsatz.

Neue Fragestellungen ergeben sich durch die Nutzung innovativer Materialien oder Beschichtungen. Vor dem Hintergrund immer komplexer werdender

Devices und der weiterhin kontinuierlichen Zunahme an Biopharmazeutika mit ihren spezifischen Anforderungen, werden auch neue Materialoptionen für Primärverpackungen zunehmend interessant. So können Fläschchen- und Spritzeninnenflächen beispielsweise im Plasmaverfahren mit reinem SiO₂ beschichtet werden, um Interaktionen mit Medikamenten zu minimieren. Darüber hinaus gewinnen bei vorgefüllten Spritzen und Fläschchen Kunststoffsysteme auf Basis von zyklischen Olefinen (COP/COC) an Bedeutung. COP-Spritzen wie z. B. ClearJect TasPack™ von Taisei Kako Co. Ltd bieten die Transparenz von Glas. Darüber hinaus ist ihre Bruchsicherheit höher, der nutzbare pH-Bereich größer und es findet kein Auswaschen von Metallionen statt.

Besonders wichtig für die Verpackung biotechnologisch hergestellter Arzneimittel ist auch die hervorragende Dosiergenauigkeit. Auch bei COP-Spritzen ist eine Silikonisierung meist unerlässlich.

Einen völlig neuen Ansatz bieten dagegen silikonölfreie Systeme. Die Gleitfähigkeit der Fluorpolymerbeschichtung speziell entwickelter Kolbenstopfen wird genutzt, um die Silikonisierung von Plastikspritzen komplett überflüssig zu machen. Innovativen Medikamenten und Injektionssystemen stehen also ebenso innovative Ideen aus der Entwicklung für Primärpackmittelsysteme gegenüber.

Fachliteratur

- [1] United States Pharmacopoeia 35 NF 30. Dimethicone, The United States Pharmacopoeial Convention Inc, Rockville, USA, 2011
- [2] Pharmacopoea Europaea. 7. Ausgabe. Dimeticon, Deutscher Apotheker Verlag, Stuttgart, Deutschland, 2011, s. 2788
- [3] Pharmacopoea Europaea. 7. Ausgabe. 3.1.8 Silikonöl zur Verwendung als Gleitmittel, Deutscher Apotheker Verlag, Stuttgart, Deutschland, 2011, s. 486
- [4] Jones LS, Kaufmann A, Middaugh CR. Silicone Oil induced aggregation of proteins. J Pharm Sci 2005; 94(4):918-927
- [5] Colas A, Siang J, Ulman K. Silicone in Pharmaceutical Applications Part 2: Silicone Excipients. Dow Corning Corporation, Midland, USA, 2001
- [6] Colas A. Silicone in Pharmaceutical Applications. Dow Corning Corporation, Midland, USA, 2001
- [7] Thirumangalathu R, Krishnan S, Speed Ricci M, Brems DN, Randolph TW, Carpenter JF. Silicone Oil- and agitation-induced aggregation of a monoclonal antibody in aqueous suspension. J Pharm Sci 2009; 98(9):3167-3181
- [8] Rathore N, Pranay P, Eu B. Variability in syringe components and its impact on functionality of delivery systems. PDA J Pharm Sci and Tech 2011; 65:468-480



Befreit nachhaltig

BEKOKAT® übertrifft mit konstant ölfreier Druckluft höchste Anforderungen

BEKOKAT® setzt mit zukunftsweisender Katalysertechnik neue Standards in der Druckluftaufbereitung. Das Ergebnis: jederzeit konstant ölfreie Druckluft für höchste Prozesssicherheit. Dabei übertrifft BEKOKAT® sogar die Anforderungen der ISO 8573-1, Klasse 1, Ölgehalt. Außerdem ist er besonders umweltfreundlich und unabhängig von Umgebungstemperatur, Luftfeuchtigkeit oder Öleingangskonzentration.

Interessiert? Alles über BEKOKAT® erfahren Sie unter www.beko-technologies.de



BEKO TECHNOLOGIES GmbH
 Im Taubental 7
 D-41468 Neuss
 Fon +49 2131 988-1000
 Fax +49 2131 988-912
beko@beko.de
www.beko-technologies.de



Patientenschutz steht im Vordergrund

Die Fälschungsrichtlinie 2011/62/EU dient dem Schutz der Patienten vor gefälschten Arzneimitteln. Sind Sie bereit für deren Umsetzung?

Lassen Sie sich von unserem Know-how führen!

Besuchen Sie uns auf **der FachPack in Nürnberg: Halle 3, Stand 407**



www.domino-deutschland.de

Domino. Do more.

Kartonqualitäten von MMK für pharmazeutische Produkte



Printing 2D-Matrix-Codes

Codes auf Verpackungen müssen in weniger als einer halben Sekunde trocken und wischfest sein. Damit sind sie hervorragend geeignet, die neuen gesetzlichen Regelungen in Bezug auf 2D-Matrix-Codes zu erfüllen. Mit diesen Codes bietet sich für pharmazeutische Unternehmen die Möglichkeit, fälschungssichere Verpackungen und eine lückenlose Rückverfolgbarkeit zu gewährleisten. Zudem können zusätzliche Informationen für Marketingaktionen eingesetzt werden.

Codes, die auf der MMK Frischfaserqualität Exzellent Top 2D™ und den Recyclingqualitäten MCM 2D™ und Supra 2D™ mit optimiertem Strich gedruckt wurden, sind nach Prüfung gemäß der Norm ISO 15415 für diesen Einsatz bestens geeignet.

Exzellent Top™ ist im Grammaturbereich zwischen 230 und 450g/m², MCM™ zwischen 280 und 450g/m² und Supra™ zwischen 250 und 500g/m² verfügbar.

Mehr Info finden Sie bei www.mm-karton.com.



Regeneron Exhibit 1033.038

Appendix 1C



Search WorldCat

Search

[Advanced Search](#) [Find a Library](#)

[<< Return to Search Results](#)

[Cite/Export](#)

[Print](#)

[E-mail](#)

[Share](#)

[Permalink](#)

[Add to list](#)

[Add tags](#)

[Write a review](#)

Rate this item: 1 2 3 4 5

TechnoPharm making science work

Publisher: Aulendorf München ECV, Editio-Cantor-Verl. für Medizin und Naturwiss 2011-

Edition/Format: Journal, magazine : German

Rating: (not yet rated) 0 with reviews - [Be the first.](#)

Subjects [Pharmazeutische Technologie](#)

More like this [Similar Items](#)

Get a Copy

[Find a copy in the library](#)

Search this publication for other articles with the following words:

Find a copy online

Links to this item

[Volltext](#)

[Volltext](#)

Find a copy in the library

Enter your location:

Submit a complete postal address for best results.

Displaying libraries 1-6 out of 11 (Konrad-Adenauer-Straße 8, 70173 Stuttgart, Germany)

Show libraries holding [just this edition](#)

[<< First](#) [< Prev](#) 1 [2](#) [Next >](#) [Last >>](#)

Library	Held formats	Distance	
1. Württembergische Landesbibliothek Stuttgart, 70173 Germany	Journal / Magazine / Newspaper	< 1 mile MAP IT	Library info Add to favorites
2. Deutsche Nationalbibliothek Leipzig / Frankfurt am Main Frankfurt am Main, 60322 Germany	Journal / Magazine / Newspaper	96 miles MAP IT	Library info Add to favorites
3. Hochschule Anhalt, Hochschulbibliothek Köthen, 06366 Germany	Journal / Magazine / Newspaper	240 miles MAP IT	Library info Add to favorites
4. Bibliothek im Kurt-Schwitters-Forum Gemeinsame Bibliothek der Hochschule	Journal / Magazine / Newspaper	247 miles MAP IT	Library info Add to favorites

**Hannover und der Hochschule für Musik,
Theater und Medien Hannover**
Hannover, 30539 Germany

5. [Bibliothek der Hochschule Hannover](#)
BHSH, Hochschule Hannover
Hannover, D-30459 Germany



[Journal / Magazine / Newspaper](#)

249 miles
MAP IT

[Library info](#)
[Add to favorites](#)

6. [Universitätsbibliothek Braunschweig](#)
Braunschweig, 38106 Germany



[Journal / Magazine / Newspaper](#)

249 miles
MAP IT

[Library info](#)
[Add to favorites](#)

« First < Prev 1 2 Next > Last »

Details

Genre/Form: Zeitschrift

Material Type: Internet resource

Document Type: Journal / Magazine / Newspaper, Internet Resource

ISSN: 2191-8341

OCLC Number: 959030334

Notes: Volltext auch als Teil einer Datenbank verfügbar

Description: 1 Online-Ressource

Other Titles: Techno Pharm

Responsibility: APV, Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V

Reviews

User-contributed reviews

[Add a review](#) and share your thoughts with other readers. [Be the first.](#)

Tags

[Add tags](#) for "TechnoPharm making science work". [Be the first.](#)

Similar Items

Related Subjects: (1)

[Pharmazeutische Technologie](#)

Linked Data

Appendix 1D



WÜRTTEMBERGISCHE LANDESBIBLIOTHEK

Zeitschriftenstelle

email: zeitschriften@wlb-stuttgart.de

EINGEGANGEN

30. Juli 2018

WGM FRANKFURT

Württembergische Landesbibliothek Postfach 105441-D-70047 Stuttgart

Weil, Gotshal & Manges LLP
zu Händen Frau Ellen Bauche
Taunusanlage 1 (Skyper)
60329 FRANKFURT AM MAIN

Ihre Zeichen

Unsere Zeichen
EA/Zss ash/häc

☎ (0711) 212-4383
Fax (0711) 212-4391

Datum
25.07.2018

Dear Sir or Madam,

I hereby confirm, that the printed issue of the journal

TechnoPharm : making science work / APV, Arbeitsgemeinschaft für Pharmazeutische
Verfahrenstechnik e.V.

Aulendorf : ECV, Editio-Cantor-Verlag für Medizin und Naturwissenschaften

Volume 2.2012, no. 4

has been received in our library in the month of August 2012.

Since the 14th of August, it has always been open to the public.

It is part of a bound volume and is lendable with the shelf number Za 15974-2.2012,4-6.

Best regards


Birgit Häcker
Head of Journals Department

Appendix 1E



Württembergische
Landesbibliothek
Stuttgart

Komplette Titelangaben

Katalogangaben	
	Zitierlink
Medienart	 [Zeitschrift]
Titel	TechnoPharm : making science work / APV, Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik e.V.
Weitere Titel	Techno Pharm
Veröffentlichung	Aulendorf: ECV, Editio-Cantor-Verl. für Medizin und Naturwiss., 2011
Umfang / Format	30 cm
Verlauf	1.2011 -
Anmerkungen	Ersch. 6x jährl.
Sprache	Deutsch
ISSN	2191-8341 2191-8341
Nummer	11,A47 (Nationalbibliographienummer / WV-Nummer (NBN)) 959030334 (OCLC) 2596825-7 (ID-Nr. ZDB) 362772053 (SWB-Katalog Nr.)
Weitere Ausgabe	Erscheint auch als Online-Ausgabe: TechnoPharm
Hinweis auf	Ab 2011 Beil. zu: APV news : Nachrichten und Mitteilungen; official communications; eine Kooperation zwischen APV und ECV / Arbeitsgemeinschaft für Pharmazeutische Verfahrenstechnik
Schlagwortfolge	Pharmazeutische Technologie ; Zeitschrift
Bestand	1.2011 -; Bestandslücken: [N=2013,1-5;2014,2u.6] // Signatur: Za 15974
Band	1.2011 (2011) 2.2012 (2012), Heft 1-3 2.2012 (2012), Heft 4-6 3-4.2013-14 (2014) 5.2015 (2015) 6.2016 (2016)
Begleitmaterial	APV news
Heft(e)	8 (2018), Heft 3 8 (2018), Heft 2 8 (2018), Heft 1 7 (2017), Heft 6 7 (2017), Heft 5 7 (2017), Heft 4 7 (2017), Heft 3 7 (2017), Heft 2 7 (2017), Heft 1

Katalogangaben

6 (2016), Heft 6
6 (2016), Heft 5
6 (2016), Heft 4
6 (2016), Heft 3
6 (2016), Heft 2
6 (2016), Heft 1

Appendix 2A

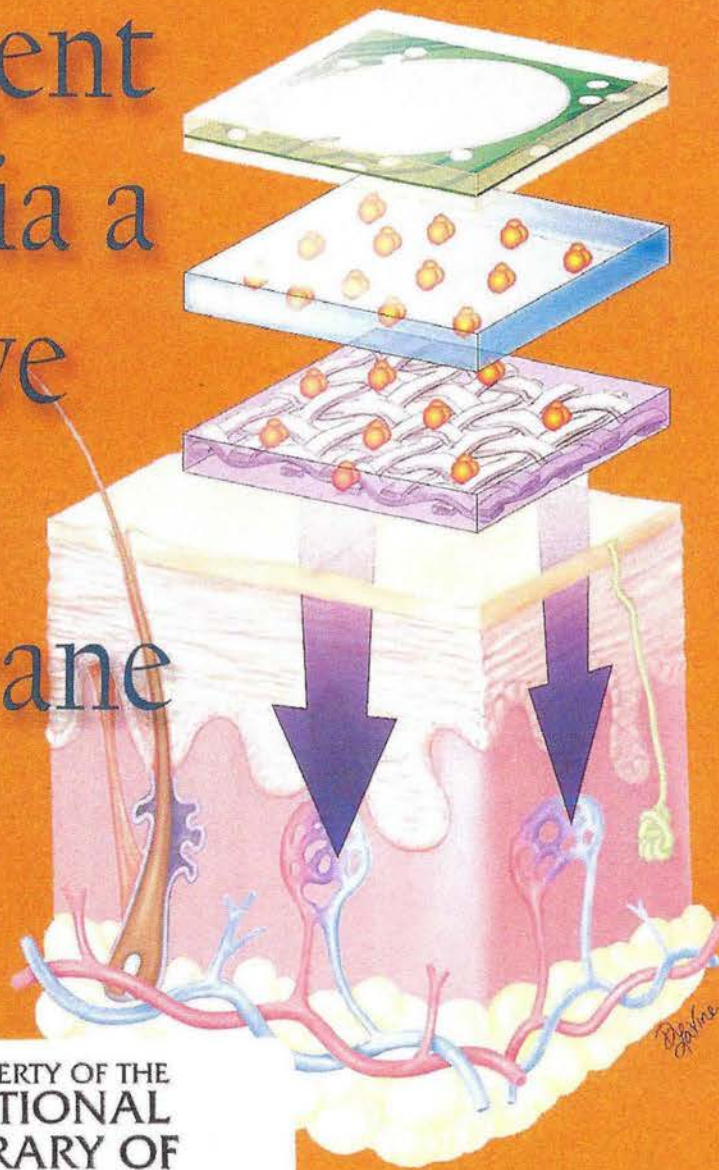
Drug delivery technology,
 v. 9 no. 5 (May 2009)
 General Collection
 W1 DFG149J
 2009-05-16 15:19:58

Drug Delivery[®] Technology

May 2009 Vol 9 No 5

www.drugdeliverytech.com

Intelligent TDD Via a Selective Barrier Membrane



PROPERTY OF THE
 NATIONAL
 LIBRARY OF
 MEDICINE

The science & business of drug development in specialty pharma, biotechnology, and drug delivery



**Arno Fries,
 PhD**
 Drug Delivery of
 Sensitive
 Biopharmaceuticals
 With Prefilled Syringes



**Emma A.
 Durand**
 Successful,
 Sustained-Release
 Using a Selective
 Charged Barrier
 Membrane



**Cherng-ju
 Kim, PhD**
 A New Process
 Producing
 Biodegradable
 Microspheres of
 Peptide Drugs:
 Suspension-Encapsulation

This material was copied
 at the NLM and may be
 Subject US Copyright Laws

IN THIS ISSUE



INTERVIEW WITH
 SYMYX TECHNOLOGIES'
 PRESIDENT

RICHARD BOHNER

Characterization Services 20

Derek G. Hennecke, MBA

Inhaled Formulations 32

M.W. Samaha, PhD

Biopharmaceutical Development 40

Dave Mead, PhD

Particle Size Distribution 44

Philo Morse, MS

FEATURING

SPECIALTY 
 Strategic For
 Business Development **PHARMA**

Protein/Peptide Manufacturing 66

Cindy H. Dubin

Winning Partnerships 69

Matt Siefert, MBA

Azopharma™
Product Development Group Inc.

The Total Product Development Company™

**Get the most out of your resources
with a single outsourcing partner!**

Azopharma Product Development Group offers bundled services from key sections of the drug development process including the Preclinical, CMC and Clinical phases that extend your financial resources by maximizing communication and minimizing downtime! Contact Azopharma to develop a comprehensive solution for your product development needs.

AZOPHARMA PRODUCT DEVELOPMENT GROUP OF COMPANIES:

Azopharma™
Contract Pharmaceutical Services

Integrated product development including synthesis, analysis, formulation and CTM manufacturing for all dosage forms

AVIVOCLIN™
Clinical Services

Human clinical pharmacology and monitoring services for Phase III clinical trials.

AniClin™
PRECLINICAL SERVICES

Preclinical services in support of early product development.

954-241-4340 • development@azopdogroup.com • www.azopdogroup.com/ADU

Drug Delivery Technology **FREE** SUBSCRIPTION OFFER

Keep abreast of the marketplace with the industry's only publication entirely devoted to drug delivery and development.

Go to www.drugdeliverytech.com
and click on Subscription Services

Once on the website you can also subscribe
to our eNewsletter!

For more information contact Ralph Vitaro at 973-299-1200
or e-mail him at rvitaro@drugdeliverytech.com

Drug Delivery

Technology

May 2009 Vol 9 No 5

PUBLISHER/PRESIDENT
Ralph Vitaro

EXECUTIVE EDITORIAL DIRECTOR
Dan Marino, MSc
dmarino@drugdeliverytech.com

CREATIVE DIRECTOR
Shalamar Q. Eagel

CONTROLLER
Debbie Carrillo

CONTRIBUTING EDITORS
Cindy H. Dubin
Debra Bingham
Jason McKinnie

TECHNICAL OPERATIONS
Mark Newland

EDITORIAL SUPPORT
Nicholas D. Vitaro

ADMINISTRATIVE SUPPORT
Kathleen Kenny

Corporate/Editorial Office
219 Changebridge Road, Montville, NJ 07045
Tel: (973)299-1200
Fax: (973) 299-7937
www.drugdeliverytech.com

Advertising Sales Offices

East & Midwest

Victoria Geis - Account Executive
Cheryl S. Stratos - Account Executive
103 Oronoco Street, Suite 200
Alexandria, VA 22314
Tel: (703) 212-7735
Fax: (703) 548-3733
E-mail: vgeis@drugdeliverytech.com
E-mail: cstratos@drugdeliverytech.com

West Coast

Warren De Graff
Western Regional Manager
818 5th Avenue, Suite 301
San Rafael, CA 94901
Tel: (415) 721-0644
Fax: (415) 721-0665
E-mail: wjdegtraff@drugdeliverytech.com

International

Ralph Vitaro
219 Changebridge Road
Montville, NJ 07045
Tel: (973) 299-1200
Fax: (973) 299-7937
E-mail: rvitaro@drugdeliverytech.com

Mailing List Rental

Candy Brecht
Tel: (703) 706-0383
Fax: (703) 549-6057
E-mail: cbrecht@mgilists.com

All editorial submissions are handled with reasonable care, but the publishers assume no responsibility for the safety of artwork, photographs, or manuscripts. Every precaution is taken to ensure accuracy, but publishers cannot accept responsibility for the accuracy of information supplied herein or for any opinion expressed. Drug Delivery Technology (ISSN 1944-818X) is published 10 times in 2009, January, February, March, April, May, June, July/August, September, October, and November/December by Drug Delivery Technology LLC, 219 Changebridge Road, Montville NJ 07045. Subscription rates: \$99.00 for 1 year in the United States, Canada, and Mexico. \$153.00 for 1 year outside the United States, Canada, and Mexico. All subscriptions are payable in US funds, drawn on US banks. Send payment to: Drug Delivery Technology LLC subscription Department, 219 Changebridge Road, Montville NJ 07045. Single copies (prepaid) \$15.00, US, Canada, and Mexico; \$24.00 in all other countries. Add \$5.00 per order for shipping and handling. Periodicals Postage Paid at Montville, NJ 07045-9998 and additional mailing offices. Postmaster: please send address changes to Drug Delivery Technology, 219 Changebridge Road, Montville NJ 07045. All rights reserved under the US International and Pan-American Copyright Conventions. All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including by photocopy, recording, or information storage and retrieval system, without written permission from the publisher. Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Drug Delivery Technology LLC for libraries and other users registered with the Copyright Clearance, 222 Rosewood Drive, Danvers, MA 01923; phone: (978) 750-8400, fax: (978) 750-4470.

This material was copied
at the NLM and may be

Regeneron Exhibit 1033.049

ADVANCED DELIVERY DEVICES

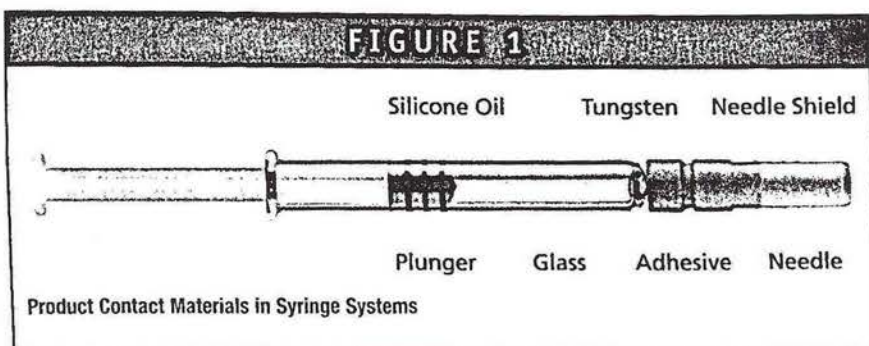
Drug Delivery of Sensitive Biopharmaceuticals With Prefilled Syringes

By: Arno Fries, PhD

Recombinant proteins, monoclonal antibodies, and other biopharmaceuticals offer medication for life-threatening diseases. However, these products consist of sensitive molecules. Among the causes for chemical and physical instability are leachables in container closure systems.^{1,5} Interactions of leached contaminants with therapeutic proteins can result in aggregation, particulate formation, and loss of native protein tertiary structures.^{6,7} Even small fractions of aggregated proteins might reduce biological activity and enhance immunogenicity.⁸ For these reasons, strategies to prevent aggregation pathways and monitor aggregate levels in biopharmaceutical formulations are important elements of product development.⁹

BIOMOLECULES RAISE THE BAR

Strength, efficacy, and safety of active molecules are closely related to their chemical and physical properties. Most biopharmaceuticals are more sensitive toward product contact materials from container closure systems than small molecules. The difference can be attributed to several reasons.^{3,10} Biomolecules contain, due to their large size, a high number of functional groups that are prone to react with other compounds. This opens a wide range of pathways for undesirable reactions with leachables. In addition, the stability of biopharmaceutical products hinges on the three-dimensional orientation of the molecules (eg, native folding



of proteins). Biopharmaceuticals are primarily administered as injectables, and liquid formulations increase the risk posed by leachables. Because these products often contain the active molecule in low concentrations, trace amounts of contaminants might interact with the whole quantity.

commercialized in vials as lyophilized formulations. This means the advantages of ready-to-use injection solutions in prefilled syringes are not leveraged.

THE PROCESS IS THE PRODUCT

Container closure compatibility is a regulatory requirement to protect the potency, efficacy, and safety of therapeutics. In glass-based syringe systems, a range of materials gets in immediate contact with active ingredients: silicone oil, tungsten, closure, plunger, glass, and (for staked needle syringes) adhesive and needle (Figure 1). The fact that closures are considered product contact materials is reflected by change control procedures in the biopharmaceutical industry. When the rubber formulation of an established needle shield is modified by the supplier, 93.3% of the companies run complete stability studies.²⁹

An evolving trend among biopharmaceutical companies is to enter closer partnerships with syringe suppliers and to scrutinize all aspects of their processes. The paradigm from biopharmaceutical

PREFILLED SYRINGES

Both for the ultimate end-users and biopharmaceutical companies, prefilled syringes offer advantages over traditional container systems.¹¹⁻¹⁵ Medical staff and patients prefer ready-to-use injection solutions in syringes because they are convenient and prevent medication errors. The industry is utilizing these benefits with life cycle strategies to gain competitive advantages and increase market shares.^{16,17} When molecules are expensive to manufacture, prefilled syringes increase revenues and earnings as they reduce product overfill compared to vials. Due to these benefits, the use of prefilled syringes grows at double-digit rates. The trend is predicted to continue over the coming years.^{18,19} However, for stability reasons, a number of biotherapeutics is

This material was copied at the NLM and may be

ADVANCED DELIVERY DEVICES

manufacturing, "the process is the product," is being transferred to the production of prefilled syringes.

The rationale behind this shift in attention is that all substances used during glass cutting, forming, printing, needle staking, washing, siliconization, assembly, packaging, and sterilization are potential contact materials with sensitive biomolecules.

Biopharmaceutical companies want to catalog these materials and understand how syringe suppliers control their processes.

The following outlines recent advances in the field of prefilled syringes. Strategies to mitigate stability risks for sensitive biopharmaceuticals are discussed. Special focus is placed on alkalinity, tungsten, and silicone oil as sources of incompatibilities.

pH RANGE

When sensitive products are applied in glass syringes, the pH value of the formulation needs to be considered.¹⁰ Elevated pH might trigger oxidation and hydrolysis of biopharmaceuticals.

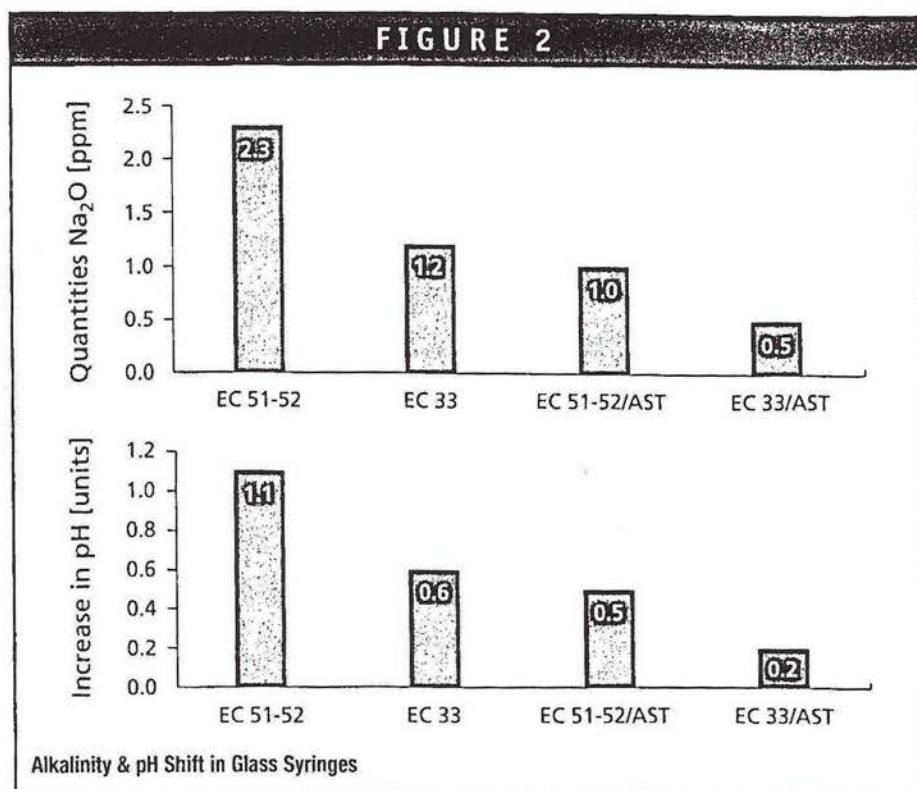
For the production of syringe barrels, glass tubing from Type I borosilicate glass according to USP, EP, and JP is used. Standard glass tubing has an extension coefficient of 51-52 and consists of 70% to 80% SiO₂, 15% B₂O₃/Al₂O₃, and up to 7% Na₂O. The role of sodium is to lower the forming temperatures of glass to 1,000°C to 1,200°C, a prerequisite for industrial converting processes. Glass is a well-characterized material, and Type I borosilicate has excellent hydrolytic resistance.²¹ However, the material is being heated during the syringe manufacturing cycle, and at temperatures above 800°C, sodium cations are migrating from inside the glass barrel to the surface. Each single syringe-forming step increases the quantity of sodium oxide on the glass surface by 15% to 30%.²² When an aqueous formulation is filled and stored in a syringe, sodium cations are being leached from the glass surface into solution. This causes in unbuffered solutions an increase in pH.

How can alkali ion leachables in glass syringes be reduced? The principal strategies are use of glass material with lower sodium content, treatment of the glass surface, and a combination of both. Figure 2 compares analytical results with 1-ml long Luer cone syringes manufactured with these methods.

Syringe barrels from Type I borosilicate glass with extension coefficient 51-52 (EC 51-52) contain on average 2.3 ppm residual sodium oxide on the interior surface. Quantitative analysis is achieved by flame atom emission spectrometry according to ISO 4802-2.²³ When the barrels are manufactured from Type I borosilicate glass of extension coefficient 33 (EC 33), analysis shows a significantly reduced sodium oxide level of 1.2 ppm. This result is in line with data according to EP testing by equivalence titration with 0.01 M hydrochloric acid (EC 33: 0.46 ml HCl, EC 51-52: 0.90 ml HCl) and pH measurement with pH meter (EC 33: pH 6.1, EC 51-52: pH 6.6, aqua bi-dist.: pH 5.5).²⁴ Syringes from EC 33 glass increase the pH value of aqueous solutions by 0.6 units, whereas EC 51-52 glass barrels increase the pH by

1.1 units.²⁵ These data reflect that EC 33 glass tubing contains lower quantities of sodium oxide (4%) than EC 51-52 glass.

Syringe barrels produced from EC 51-52 glass and treated with ammonium sulfate (AST) contain on average 1.0 ppm sodium oxide in accordance to ISO 4802-2 testing. EP titration (AST: 0.44 ml HCl, untreated barrels: 0.90 ml HCl) and pH measurement (AST: pH 6.0, untreated barrels: pH 6.6) confirm this result. The increase in pH of surface-treated barrels is 0.5 units, which is 0.6 units lower than in untreated barrels. For ammonium sulfate treatment, dosing pumps are used to spray an aqueous solution of the agent onto the inner surface of syringe barrels. During the annealing step of the syringe manufacturing process, residual sodium oxide is converted under heat into the much better water-soluble sodium sulfate as follows: Na₂O + (NH₄)₂SO₄ → Na₂SO₄ + 2 NH₃ + H₂O. Removal of sodium sulfate is achieved downstream during washing of the syringe barrels and reduces significantly the amount of alkali ions on the glass surface.



This material was copied
at the NLM and may be

ADVANCED DELIVERY DEVICES

Quantitative analysis according to ISO 4802-2 shows that syringes manufactured from EC 33 glass and treated with ammonium sulfate contain merely 0.5 ppm residual sodium oxide on the interior surface. This is 78% lower than in untreated syringes from EC 51-52 glass. The pH of aqueous solutions in barrels from EC 33 tubing that are ammonium sulfate treated increases by 0.2 units, a decrease of 82% compared to standard syringes. The combination of both methods (EC 33 glass and AST) effects the strongest reduction of alkali leachables. This provides an efficient strategy to control alkalinity and pH-related interactions between sensitive biopharmaceuticals and glass-based prefilled syringes.

TUNGSTEN LEACHABLES

Transition metals are known as a cause for instability of sensitive products.¹ Tungsten can undergo interactions with protein therapeutics, leading to oxidation, aggregation, and degradation.²⁶⁻³⁰

In manufacturing processes of glass syringes, tungsten metal is commonly used due to its heat resistance. Pins from this material are keeping the bore open while the cone is being mechanically shaped with forming wheels (Figure 3).

Tungsten is well characterized and stands out among all metals with the highest melting point (3,422°C), the highest tensile strength at elevated temperatures, and the lowest vapor pressure.³¹ Even though tungsten is very wear-resistant, the metal is prone to oxidation under the conditions of syringe forming with temperatures up to 1,250°C. On the surface of tungsten pins, tungsten (IV) oxide (WO₂) can be formed at temperatures under 400°C and tungsten (VI) oxide (WO₃) between 500°C and 800°C. In aqueous solution, tungsten (VI) oxide produces a mixture of soluble mono, oligo, and polytungstates, which are stabilized at low pH.^{32,33} These large anions are highly charged species. They can interact with bipolar protein molecules through electrostatic attraction and induce formation of

colloidal solutions and aggregates.^{32,33} Tungsten compounds can also react with hydrocarbons to organometallic complexes and with molecules containing donor atoms to chelate complexes under formation of O-W-O, O-W-S, O-W-N, S-W-N, and S-W-S bonds. The metal and its compounds are also known as heterogeneous and homogeneous catalysts that convert high quantities of substrates through non-stoichiometric reactions.³⁴ Other metal leachables occasionally found in drug products (eg, Fe³⁺, Ni²⁺, and Mn²⁺ from stainless steel tanks used in manufacturing equipment) are known for similar interactions with active molecules.¹

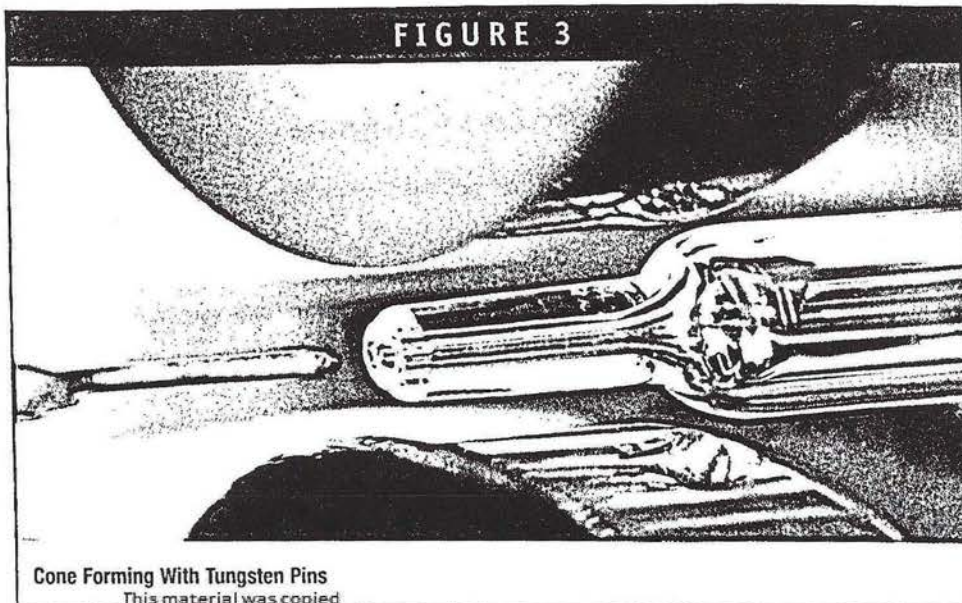
Prefilled syringes formed with tungsten pins contain trace amounts of tungsten compounds in the cone section, which is part of the product contact surface. Syringe filling processes with plunger placement under vacuum intensify the contact between active molecules and tungsten because air bubbles in the cone of the syringe are pulled out.³⁵

Proprietary methods for the extraction of tungsten from syringe barrels and the subsequent quantitative physicochemical analysis have been developed. Extractable tungsten concentrations are typically below 500 ppb and can be lower than 100 ppb, depending on manufacturing cycle and washing process. Staked needle syringes contain the lowest amount of extractable tungsten as most

of the bore is covered by the needle.

Incorporation of tungsten in syringe barrels can be further reduced by controlling the abrasion of tungsten pins or through substitution of tungsten with other materials. Wear of forming pins can be lowered by horizontal barrel-forming technology. This manufacturing process is using lower temperatures compared to vertical-forming techniques. Other methods are directed at controlling the physical properties of the forming pins. As a substitute for tungsten, alloys from group 9-10 transition metals can be employed. This approach allows tungsten-free syringe forming. However, intake of material from substitute pins into syringe barrels cannot be ruled out. Some biopharmaceutical companies prefer the use of tungsten pins because potential effects of tungsten on their products are better understood than for most other transition metals. Forming pins from non-metallic materials and alternative techniques of syringe forming are at an experimental stage.

To evaluate product stability of biopharmaceutical formulations, spiking studies in early phase development with material extracted from used tungsten (metal) pins are recommended. Subsequent stability studies in prefilled syringes verify the preliminary data and specify accepted tungsten (metal) levels. Advanced manufacturing methods for prefilled syringes together with targeted



ADVANCED DELIVERY DEVICES

stability studies ensure that interactions of highly sensitive biomolecules with tungsten are prevented.

SILICONE LUBRICANTS

Even though silicone oil is inert toward most drug products, interactions with sensitive biopharmaceuticals have been observed. Such incompatibilities include aggregation, deformation, and inactivation of native protein structures.^{7,27}

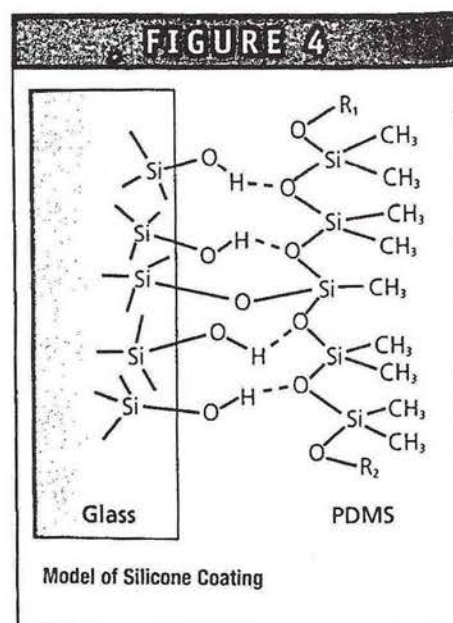
Prefilled syringes are containers and drug delivery systems at the same time. Functionality of these systems (viable activation and gliding forces of the plunger) is accomplished by siliconization. Silicone oils are viscous, inert materials with excellent characteristics as hydrophobic lubricants.^{35,36} They consist of a mixture of polydimethylsiloxane (PDMS) molecules with Si-O chains, which vary in length and number of OH groups. This molecular structure determines how silicone oil layers are adsorbed onto glass surfaces and the distribution, thickness, composition, and uniformity of the layers. In established manufacturing processes on the lines of syringe suppliers, biopharmaceutical companies, and CMOs, syringes are oily siliconized by spraying 0.4- to 1.0-mg silicone oil (eg, Dow Corning 360, Medical Fluid) into the barrels.

Advanced siliconization technology has been developed to lower the level of free (non-bound) silicone oil in prefilled syringes. The baked siliconization method uses emulsions of silicone oil (eg, Dow Corning 365, 35% Dimethicone NF Emulsion, diluted in HPW) sprayed into syringe barrels followed by heat treatment in a tunnel. Proprietary techniques and downstream washing processes vary depending on syringe supplier. Critical quality attributes of the siliconization process are controlled through the settings of siliconization pump and nozzle, the volume flow of silicone spray and air, the concentration of the silicone oil emulsion and tunnel temperature, speed, and length. This technology alters the nature

of the lubricant in the following way.^{37,38} Heat-induced polymerization reactions reduce fractions of low molecular weight from the silicone oil. Removal of water enables the lubricant to spread out evenly over the glass surface and creates a thin, uniform film. Mono-layers of the lubricant are affixed to the glass surface. The interactions between polydimethylsiloxane and molecules from the glass surface range from van der Waals forces to covalent Si-O bonds. This means thermal fixation processes convert silicone oil into Si(R)O coating as illustrated in Figure 4.³⁹ The thickness of silicone oil layers on the glass surface can be measured by reflectometry. A comparative study using cartridges as glass containers found for oily siliconization a layer thickness with a mean of 232.67 nm and for baked siliconization of 76.83 nm.³⁹

Parenteral biopharmaceutical products vary widely in nature. The sensitivity of the active substance, the viscosity of the formulation, the drug delivery system, and its mode of operation (eg, prefilled syringes either manually or driven by autoinjector device) determine the principal requirements. Silicone coatings of prefilled syringes can be customized to meet specific needs. Variation of process parameters adapts the characteristics of the siliconization. Best results are obtained when syringe manufacturer (supplier) and biopharmaceutical company (end user) partner and work along the following project steps:

1. End user: Specification of accepted silicone oil levels and system functionality
2. Supplier: Development of baked siliconization process for the specified attributes
3. Supplier: Manufacturing of customized baked silicone syringes in sample quantities



4. End user: QC testing of the samples, filled-syringe stability studies, and evaluation
5. Supplier: Scale-up, process validation, and industrial manufacturing of the syringes

Fundamental understanding of the design space of baked siliconization allows the syringe manufacturer to derive relevant process parameters from the specified quality attributes of the syringes. A range of syringe samples are produced through custom-engineered processes. Quality inspection and initial stability studies with the set of samples determine which silicone coating is ideal for purpose.

A case study has demonstrated how customization of baked silicone coatings facilitates stability of sensitive molecules in prefilled syringes (eg, vaccine candidate in biopharmaceutical development). The study has deepened the insight into the relationship between siliconization parameters and critical quality attributes.⁴⁰ The amount of extractable silicone oil could be reduced below the detection limit (0.03 mg) of ICP-AES according to EN ISO 11885.

This material was copied
at the NLM and may be

ADVANCED DELIVERY DEVICES

With low levels of lubricant quantity, the specified syringe functionality was fulfilled (plunger gliding forces in the range of 5 to 10 N).

Close partnerships between biopharmaceutical companies and syringe suppliers are instrumental in controlling the impact of product contact materials on sensitive biotherapeutics. Principal requirements regarding drug delivery systems are ideally defined and specified in an early phase of biopharmaceutical development. Manufacturing processes and quality attributes of prefilled syringes can be custom-engineered according to these needs.

SUMMARY

In today's biopharmaceutical market, products are exposed to fierce competition. The role of drug delivery strategies to differentiate products is growing. A number of biopharmaceuticals has already been commercialized in the prefilled syringe platform. However, syringe systems are sources of potential incompatibilities with sensitive molecules. The prefilled syringe industry has therefore engineered manufacturing processes that mitigate stability risks from alkali ions, tungsten, and silicone lubricants. Advanced methods for surface treatment to control pH, tungsten-reduced forming techniques, and baked silicization processes to immobilize silicone oil have been developed. Syringe manufacturers have established expertise in material science and process technology to understand biopharmaceutical requirements. Evolving needs of highly sensitive pipeline products can be met with customized drug delivery systems. Current technology allows biopharmaceutical companies to exploit the benefits of prefilled syringes and realize the full potential of their products. ♦

REFERENCES

- Allain L, Wang Q. Impact of package leachables on the stability of pharmaceutical products. *Am Pharm Rev*. 2007;10(4):38-44.
- Nicholas K. Extractables and leachables determination: a systematic approach to select and qualify a container closure system for a pharmaceutical product. *Am Pharm Rev*. 2006;9(3):21-27.
- Markovic I. CDH-R case studies of leaching problems in biotech products. *Int Pharm Qual*. 2008;2(2):25-26.
- DeGrazio FL. Parenteral packaging concerns for biotech drug products. *Drug Delivery Technology*. 2006;6(5):44-49.
- Jenke D. Suitability-for-use considerations for prefilled syringes: drug delivery. *Pharm Technol*. 2008;30:s33.
- Markovic I. Evaluation of safety and quality impact of extractable and leachable substances in therapeutic biologic protein products: a risk-based perspective. *Expert Opin Drug Saf*. 2007;6(5):487-491.
- Jones LS, Kaufmann A, Middaugh CR. Silicone oil induced aggregation of proteins. *J Pharm Sci*. 2005;94(4):918-927.
- Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS Journal*. 2006;8(3):1501-1507.
- Das T, Nema S. Protein particulate issues in biologics development. *Am Pharm Rev*. 2008;11(4):52-57.
- Scott C. Formulation development: making the medicine. *BioProcess Internat*. 2006;4(3):42-57.
- Romacker M, Schoenkecht T, Forster R. The rise of prefilled syringes from niche product to primary container of choice: a short history. *ONdrugDelivery*. 2008;4:4-5.
- Romacker M. The role of the prefilled syringe in a changing pharmaceutical world. *Scrip World Pharm News*. 2004;10(supplement):10-12.
- Harrison B, Rios M. Big shot, developments in prefilled syringes. *Pharm Technol*. 2007;31(3):50-60.
- Overcashier DL, Chan EK, Hsu CC. Technical considerations in the development of prefilled syringes for protein products. *Am Pharm Rev*. 2006;9(7):77-83.
- Eakins MN. The design and construction of prefilled syringes. *Am Pharm Rev*. 2007;10(6):47-51.
- Fries A. Neue Erkenntnisse über den Markt von Fertigspritzen. *Pharm Ind*. 2008;70(8):929-933.
- Fries A, Lingenberg B. New market insight into prefilled syringes and closure systems: primary data from patients, nurses, physicians, and industry experts. *ONdrugDelivery*. 2008;4:39-41.
- Greystone Associates. Prefilled syringes: drugs, devices and delivery strategies. *Market Study*; 2006.
- Frost & Sullivan. US Pharmaceutical Packaging Markets. *Market Study*; 2005.
- MIFOS Gesellschaft für Meinungsforschung und Beratung im Gesundheitswesen. *Market Study*; 2007.
- Vogel W. *Glass Chemistry*. Springer Verlag, Berlin and Heidelberg, 2nd Edition; 1994.
- Wlecke M. *Technical Study*. Unpublished Results; 2008.
- ISO 4802-2. *Glassware: Hydrolytic Resistance of the Interior Surfaces of Glass Containers, Part 2: Determination by Flame Spectrometry and Classification*. 1st Edition; 1988.
- European Pharmacopoeia. *Glass Containers for Pharmaceutical Use*. 30201, 303-308; 2005.
- Fries A, Schoenkecht T. Controlling the alkali release in glass based prefilled syringes. Poster presented at the AAPS Annual Meeting and Exposition, San Diego, CA, November 13, 2007.
- Osterberg RE. Potential toxicity of extractables and leachables in drug products. *Am Pharm Rev*. 2005;8(2):64-67.
- Markovic I. Challenges associated with extractable and/or leachable substances in therapeutic biologic protein products. *Am Pharm Rev*. 2006;9(6):20-27.
- Rosenberg AS, Worobee AS. A risk-based approach to immunogenicity concerns of therapeutic protein products, part 2: considering host-specific and product-specific factors impacting immunogenicity. *BioPharm Int*. 2004;17(12):34-40.
- Faulkner E. Container closure interactions with biopharmaceutical parenteral products. Paper presented at the ICB Annual Formulation Strategies for Protein Therapeutics Conference, San Francisco, CA, November 6-8, 2006.
- Lee H. Tungsten leaching from prefilled syringes and impact on protein aggregation. Poster presented at the PDA Extractables/Leachables Forum, Bethesda, MD, November 6-8, 2007.
- Lassner E, Schubert WD. *Tungsten: properties, chemistry, technology of the element, alloys and chemical compounds*. 1st ed. Kluwer Academic Plenum Publishers; New York, NY; 1999.
- Hecker J. Prefilled syringe, automejector quality assurance explored at FDA/industry forum. *Pharma Biotechnol Quality Control (The Gold Sheet)*. 2007;41(1):1-4.
- Swob R. Tungsten, prefilled syringes and protein aggregation. Paper presented at the PDA Universe of Prefilled Syringes and Injection Devices Conference, Bethesda, MD, October 23, 2006.
- International Tungsten Industry Association. *Tungsten Uses, Chemical Applications, Catalysts*. Website visited January 2009; www.tia.org.uk.
- Tomasek W. *Silicones: synthesis, properties and applications*. *PolymERIC Mater Encyc*. 1996;10:7706-7711.
- Curtis J. *Medical Applications of Silicones*. *Biomaterials Sciences*. 2nd ed. Elsevier Pub: New York, NY; 2004.
- Mumby T, Schurreit I, Surmann P. The fate of silicone oil during heat-curing glass silicization: changes in molecular parameters analyzed by size exclusion and high temperature gas chromatography. *PDA J Pharm Sci Technol*. 2000;54(5):383-397.
- Mumby T. *Fibremsilikonisierung bei Pharmazeutischen Glaspäckmitteln*. In: *Analytische Studien eines Produktionsprozesses*. PhD Thesis, Humboldt University, Berlin; 1999.
- Lankers M. Optimization Study of Baked-in Silicization in Cartridges. Paper presented at the PDA Interest Group Meeting, Prefilled Syringes, Berlin, Germany, December 2, 2008.
- Fries A, Khandke L, Malone R. Customization of Prefilled Syringe Systems for Biopharmaceutical Development: Case Study. Paper presented at the PDA Universe of Prefilled Syringes and Injection Devices Conference, Berlin, Germany, November 28, 2007.

BIOGRAPHY



Dr. Arno Fries is Head of Sales, USA Syringes and Director, Product Management, Syringes in the Gerresheimer Group. He joined the company in January 2006 and

is currently responsible for the North American syringe business of Gerresheimer Buende GmbH and global product management of syringe systems in the Gerresheimer Tubular Glass Division. He has 11 years of experience in leading sales and marketing positions in the life science industry and 4 years as a scientist at international research institutes in Japan and the UK. He earned his PhD in Chemistry from the University of Wuerzburg, Germany, and his degree in business administration from the IDB Institute in Frankfurt, Germany. Dr. Fries is a member of the Parenteral Drug Association and the Alexander von Humboldt Foundation. Contact: Gerresheimer Buende GmbH, Erich-Martens-Strasse 26-32, 32257 Buende, Germany; Phone +49.5223.164.401; a.fries@gerresheimer.com.

This material was copied
at the NIM and may be

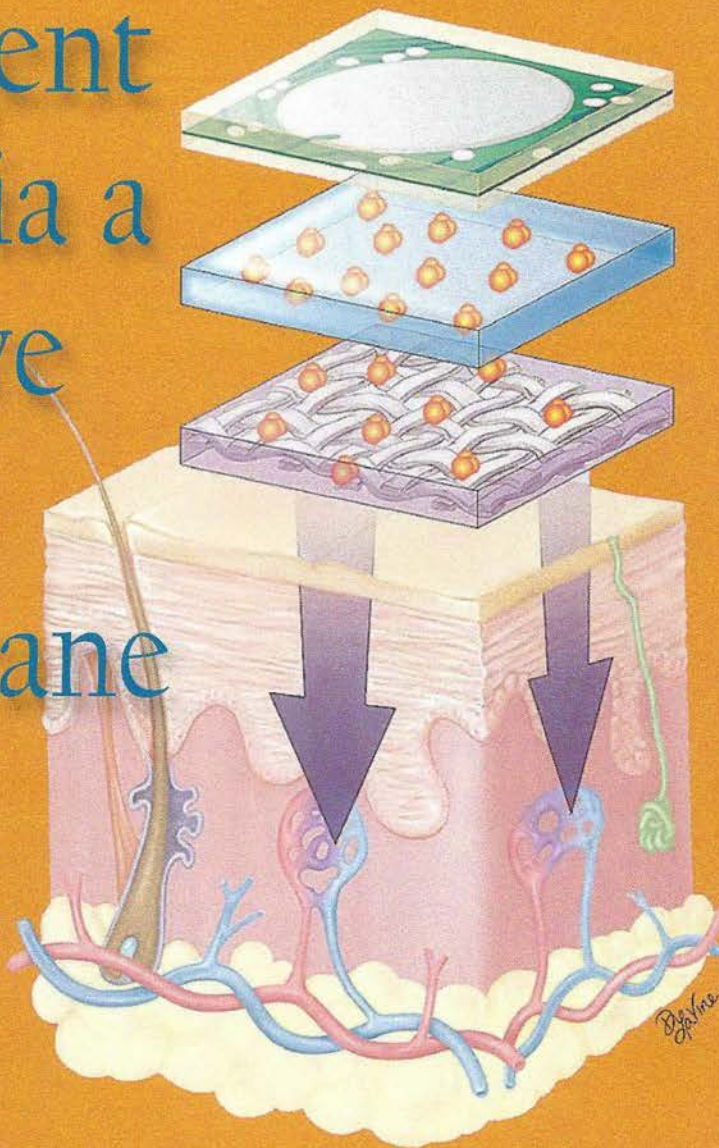
Appendix 2B

Drug Delivery Technology

May 2009 Vol 9 No 5

www.drugdeliverytech.com

Intelligent TDD Via a Selective Barrier Membrane



IN THIS ISSUE



INTERVIEW WITH
SYMIX TECHNOLOGY
PRESIDENT

RICHARD BOEHM

Characterization Services

Derek G. Hennecke, PhD

Inhaled Formulations

M.W. Samaha, PhD

Biopharmaceutical Development

Dave Mead, PhD

Particle Size Distribution

Philo Morse, MS

FEATURING

SPECIALTY PHARMACEUTICALS

Protein/Peptide Manufacturing

Cindy H. Dubin

Winning Partnerships

The science & business of drug development in specialty pharma, biotechnology, and drug delivery



Arno Fries, PhD

Drug Delivery of Sensitive Biopharmaceuticals



Emma A. Durand

Successful, Sustained-Release Using a Selective Charged Barrier



Cherng-ju Kim, PhD

A New Process Producing Biodegradable Microspheres of

Regeneron Exhibit 1033.056

Azopharma™
Product Development Group Inc.

The Total Product Development Company™

**Get the most out of your resources
with a single outsourcing partner!**

Azopharma Product Development Group offers bundled services from key sections of the drug development process including the Preclinical, CMC and Clinical phases that extend your financial resources by maximizing communication and minimizing downtime! Contact Azopharma to develop a comprehensive solution for your product development needs.

AZOPHARMA PRODUCT DEVELOPMENT GROUP OF COMPANIES:

Azopharma™
Contract Pharmaceutical Services

Integrated product development including synthesis, analysis, formulation and CTM manufacturing for all dosage forms

AVIVOCLIN™
Clinical Services

Human clinical pharmacology and monitoring services for Phase I-III clinical trials.

AniClin™
Preclinical Services

Preclinical services in support of early product development.

954-241-4340 • development@azopdogroup.com • www.azopdogroup.com/DPD

Drug Delivery Technology **FREE**
SUBSCRIPTION
OFFER

Keep abreast of the marketplace with the industry's only publication entirely devoted to drug delivery and development.

Go to www.drugdeliverytech.com
and click on Subscription Services

Once on the website you can also subscribe
to our eNewsletter!

For more information contact Ralph Vitaro at 973-299-1200
or e-mail him at rvitaro@drugdeliverytech.com

Drug Delivery

Technology

May 2009 Vol 9 No 5

PUBLISHER/PRESIDENT
Ralph Vitaro

EXECUTIVE EDITORIAL DIRECTOR
Dan Marino, MSc
dmarino@drugdeliverytech.com

CREATIVE DIRECTOR
Shalamar Q. Eagel

CONTROLLER
Debbie Carrillo

CONTRIBUTING EDITORS
Cindy H. Dubin
Debra Bingham
Jason McKinnie

TECHNICAL OPERATIONS
Mark Newland

EDITORIAL SUPPORT
Nicholas D. Vitaro

ADMINISTRATIVE SUPPORT
Kathleen Kenny

Corporate/Editorial Office
219 Changebridge Road, Montville, NJ 07045
Tel: (973)299-1200
Fax: (973) 299-7937
www.drugdeliverytech.com

Advertising Sales Offices

East & Midwest

Victoria Geis - Account Executive
Cheryl S. Stratos - Account Executive
103 Oronoco Street, Suite 200
Alexandria, VA 22314
Tel: (703) 212-7735
Fax: (703) 548-3733
E-mail: vgeis@drugdeliverytech.com
E-mail: cstratos@drugdeliverytech.com

West Coast

Warren De Graff
Western Regional Manager
818 5th Avenue, Suite 301
San Rafael, CA 94901
Tel: (415) 721-0644
Fax: (415) 721-0665
E-mail: wjdegraff@drugdeliverytech.com

International

Ralph Vitaro
219 Changebridge Road
Montville, NJ 07045
Tel: (973) 299-1200
Fax: (973) 299-7937
E-mail: rvitaro@drugdeliverytech.com

Mailing List Rental

Candy Brecht
Tel: (703) 706-0383
Fax: (703) 549-6057
E-mail: cbrecht@mgilists.com

All editorial submissions are handled with reasonable care, but the publishers assume no responsibility for the of artwork, photographs, or manuscripts. Every precaution is taken to ensure accuracy, but publishers cannot responsibility for the accuracy of information supplied herein or for any opinion expressed. Drug Delivery Tech (ISSN 1944-818X) is published 10 times in 2009, January, February, March, April, May, June, July/August, Sept, October, and November/December by Drug Delivery Technology LLC, 219 Changebridge Road, Montville NJ 07045. Subscription rates: \$99.00 for 1 year in the United States, Canada, and Mexico; \$153.00 for 1 year outside the U.S., Canada, and Mexico. All subscriptions are payable in US funds, drawn on US banks. Send payment to: Drug Delivery Technology LLC subscription Department, 219 Changebridge Road, Montville NJ 07045. Single copies (pr. \$15.00, US, Canada, and Mexico; \$24.00 in all other countries. Add \$5.00 per order for shipping and handling. Periodicals Postage Paid at Montville, NJ 07045-9998 and additional mailing offices. Postmaster: please send address changes to Drug Delivery Technology, 219 Changebridge Road, Montville NJ 07045. All rights reserved under the International and Pan-American Copyright Conventions. All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means, electronic or mechanical, including photocopy, recording, or information storage and retrieval system, without written permission from the publisher. Authorization to photocopy items for internal or personal use, or the internal or personal use of specific clients, is granted by Drug Delivery Technology LLC for libraries and other users registered with the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923. Phone: (978) 750-8400. Fax: (978) 750-4744.

Regeneron Exhibit 1033.057

ADVANCED DELIVERY DEVICES

Drug Delivery of Sensitive Biopharmaceuticals With Prefilled Syringes

By: Arno Fries, PhD

Recombinant proteins, monoclonal antibodies, and other biopharmaceuticals offer medication for life-threatening diseases. However, these products consist of sensitive molecules. Among the causes for chemical and physical instability are leachables in container closure systems.¹⁻⁵ Interactions of leached contaminants with therapeutic proteins can result in aggregation, particulate formation, and loss of native protein tertiary structures.^{6,7} Even small fractions of aggregated proteins might reduce biological activity and enhance immunogenicity.⁸ For these reasons, strategies to prevent aggregation pathways and monitor aggregate levels in biopharmaceutical formulations are important elements of product development.⁹

BIOMOLECULES RAISE THE BAR

Strength, efficacy, and safety of active molecules are closely related to their chemical and physical properties. Most biopharmaceuticals are more sensitive toward product contact materials from container closure systems than small molecules. The difference can be attributed to several reasons.^{3,10} Biomolecules contain, due to their large size, a high number of functional groups that are prone to react with other compounds. This opens a wide range of pathways for undesirable reactions with leachables. In addition, the stability of biopharmaceutical products hinges on the three-dimensional orientation of the molecules (eg, native folding



of proteins). Biopharmaceuticals are primarily administered as injectables, and liquid formulations increase the risk posed by leachables. Because these products often contain the active molecule in low concentrations, trace amounts of contaminants might interact with the whole quantity.

PREFILLED SYRINGES

Both for the ultimate end-users and biopharmaceutical companies, prefilled syringes offer advantages over traditional container systems.¹¹⁻¹⁵ Medical staff and patients prefer ready-to-use injection solutions in syringes because they are convenient and prevent medication errors. The industry is utilizing these benefits with life cycle strategies to gain competitive advantages and increase market shares.^{16,17} When molecules are expensive to manufacture, prefilled syringes increase revenues and earnings as they reduce product overfill compared to vials. Due to these benefits, the use of prefilled syringes grows at double-digit rates. The trend is predicted to continue over the coming years.^{18,19} However, for stability reasons, a number of biopharmaceutical

commercialized in vials as lyophilized formulations. This means the advantages of ready-to-use injection solutions in prefilled syringes are not leveraged.

THE PROCESS IS THE PRODUCT

Container closure compatibility is a regulatory requirement to protect the potency, efficacy, and safety of therapeutics. In glass-based syringe systems, a range of materials get in immediate contact with active ingredients: silicone oil, tungsten, closure, plunger, glass, and (for staked needle syringes) adhesive and needle (Figure 1). The fact that closures are considered product contact materials is reflected by change control procedures in the biopharmaceutical industry. When the rubber formulation of an established needle shield is modified by the supplier, 93.3% of the companies run complete stability studies.²⁰

An evolving trend among biopharmaceutical companies is to enter closer partnerships with syringe suppliers and to scrutinize all aspects of their processes. The paradigm from biopharmaceutical

Consultant level information and analysis on companies, pipeline/products, technologies, deals, acquisitions and venture capital investments in pharma/biotechnology, specialty pharma and drug delivery.



Innovation in
Pharmaceutical
Knowledge Management



www.pharmacircle.com 

Comprehensive, Innovative, Updated daily, Easy access and use

- Global pipeline and product information searchable by indication, phase, mechanism of action, route of administration and formulation type (180+ categories). Product information integrates sales, partnerships, deals, news, approval/market launch dates, clinical trials, labels, patents and exclusivity information.
- Powerful search engine that filters product and pipeline data by molecule properties (solubility, MW, food effect, first pass, bioavailability by route, BCS class...) as well as product attributes (dose, route of delivery, formulation type, frequency of administration, excipients, exclusivity...). Results are transferrable to Excel tables and interactive charts.
- Searchable FDA product module integrates most sought out content from Drugs@FDA (approval package/history, route, dosage forms, therapeutic equivalents), Electronic Orange Book (market and patent exclusivity), NDC (packaging info), Excipient data base (amount of excipients used in each dosage form) and Product Labels (non-PDF labels).
- Detailed and up-to-date analysis of drug delivery technologies with specific applications to products and pipeline.

Check out the free and comprehensive Contract Pharma data base which provides a multitude of search criteria and a module for side by side comparison of company services.



(760) 436 1199

or contact@pharmacircle.com

Regeneron Exhibit 1033.059

ADVANCED DELIVERY DEVICES

manufacturing, "the process is the product," is being transferred to the production of prefilled syringes. The rationale behind this shift in attention is that all substances used during glass cutting, forming, printing, needle staking, washing, silicization, assembly, packaging, and sterilization are potential contact materials with sensitive biomolecules. Biopharmaceutical companies want to catalog these materials and understand how syringe suppliers control their processes.

The following outlines recent advances in the field of prefilled syringes. Strategies to mitigate stability risks for sensitive biopharmaceuticals are discussed. Special focus is placed on alkalinity, tungsten, and silicone oil as sources of incompatibilities.

pH RANGE

When sensitive products are applied in glass syringes, the pH value of the formulation needs to be considered.¹⁰ Elevated pH might trigger oxidation and hydrolysis of biopharmaceuticals.

For the production of syringe barrels, glass tubing from Type I borosilicate glass according to USP, EP, and JP is used. Standard glass tubing has an extension coefficient of 51-52 and consists of 70% to 80% SiO₂, 15% B₂O₃/Al₂O₃, and up to 7% Na₂O. The role of sodium is to lower the forming temperatures of glass to 1,000°C to 1,200°C, a prerequisite for industrial converting processes. Glass is a well-characterized material, and Type I borosilicate has excellent hydrolytic resistance.²¹ However, the material is being heated during the syringe manufacturing cycle, and at temperatures above 800°C, sodium cations are migrating from inside the glass barrel to the surface. Each single syringe-forming step increases the quantity of sodium oxide on the glass surface by 15% to 30%.²² When an aqueous formulation is filled and stored in a syringe, sodium cations are being leached from the glass surface into solution. This causes in unbuffered solutions an increase in pH.

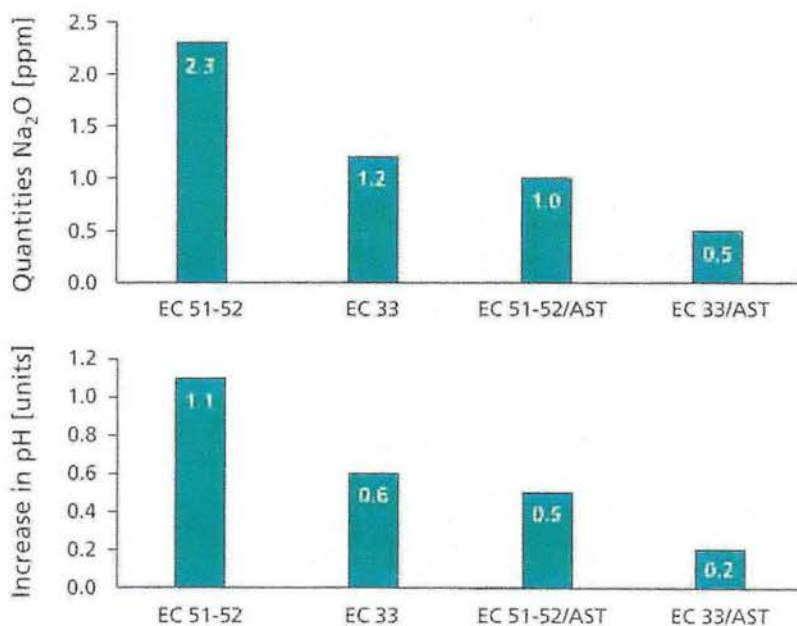
How can alkali ion leachables in glass syringes be reduced? The principal strategies are use of glass material with lower sodium content, treatment of the glass surface, and a combination of both. Figure 2 compares analytical results with 1-ml long Luer cone syringes manufactured with these methods.

Syringe barrels from Type I borosilicate glass with extension coefficient 51-52 (EC 51-52) contain on average 2.3 ppm residual sodium oxide on the interior surface. Quantitative analysis is achieved by flame atom emission spectrometry according to ISO 4802-2.²³ When the barrels are manufactured from Type I borosilicate glass of extension coefficient 33 (EC 33), analysis shows a significantly reduced sodium oxide level of 1.2 ppm. This result is in line with data according to EP testing by equivalence titration with 0.01 M hydrochloric acid (EC 33: 0.46 ml HCl, EC 51-52: 0.90 ml HCl) and pH measurement with pH meter (EC 33: pH 6.1, EC 51-52: pH 6.6, aqua bi-dist.: pH 5.5).²⁴ Syringes from EC 33 glass increase the pH value of aqueous solutions by 0.6 units, whereas EC 51-52 glass barrels increase the pH by

1.1 units.²⁵ These data reflect that EC 33 glass tubing contains lower quantities of sodium oxide (4%) than EC 51-52 glass.

Syringe barrels produced from EC 51-52 glass and treated with ammonium sulfate (AST) contain on average 1.0 ppm sodium oxide in accordance to ISO 4802-2 testing. EP titration (AST: 0.44 ml HCl, untreated barrels: 0.90 ml HCl) and pH measurement (AST: pH 6.0, untreated barrels: pH 6.6) confirm this result. The increase in pH of surface-treated barrels is 0.5 units, which is 0.6 units lower than in untreated barrels. For ammonium sulfate treatment, dosing pumps are used to spray an aqueous solution of the agent onto the inner surface of syringe barrels. During the annealing step of the syringe manufacturing process, residual sodium oxide is converted under heat into the much better water-soluble sodium sulfate as follows: Na₂O + (NH₄)₂SO₄ → Na₂SO₄ + 2 NH₃ + H₂O. Removal of sodium sulfate is achieved downstream during washing of the syringe barrels and reduces significantly the amount of alkali ions on the glass surface.

FIGURE 2



Alkalinity & pH Shift in Glass Syringes

ADVANCED DELIVERY DEVICES

Quantitative analysis according to ISO 4802-2 shows that syringes manufactured from EC 33 glass and treated with ammonium sulfate contain merely 0.5 ppm residual sodium oxide on the interior surface. This is 78% lower than in untreated syringes from EC 51-52 glass. The pH of aqueous solutions in barrels from EC 33 tubing that are ammonium sulfate treated increases by 0.2 units, a decrease of 82% compared to standard syringes. The combination of both methods (EC 33 glass and AST) effects the strongest reduction of alkali leachables. This provides an efficient strategy to control alkalinity and pH-related interactions between sensitive biopharmaceuticals and glass-based prefilled syringes.

TUNGSTEN LEACHABLES

Transition metals are known as a cause for instability of sensitive products.¹ Tungsten can undergo interactions with protein therapeutics, leading to oxidation, aggregation, and degradation.²⁶⁻³⁰

In manufacturing processes of glass syringes, tungsten metal is commonly used due to its heat resistance. Pins from this material are keeping the bore open while the cone is being mechanically shaped with forming wheels (Figure 3).

Tungsten is well characterized and stands out among all metals with the highest melting point (3,422°C), the highest tensile strength at elevated temperatures, and the lowest vapor pressure.³¹ Even though tungsten is very wear-resistant, the metal is prone to oxidation under the conditions of syringe forming with temperatures up to 1,250°C. On the surface of tungsten pins, tungsten (IV) oxide (WO_2) can be formed at temperatures under 400°C and tungsten (VI) oxide (WO_3) between 500°C and 800°C. In aqueous solution, tungsten (VI) oxide produces a mixture of soluble mono, oligo, and polytungstates, which are stabilized at low pH.^{32,33} These large anions are highly charged species. They can interact with bipolar protein molecules through electrostatic attraction and induce formation of

colloidal solutions and aggregates.^{32,33} Tungsten compounds can also react with hydrocarbons to organometallic complexes and with molecules containing donor atoms to chelate complexes under formation of O-W-O, O-W-S, O-W-N, S-W-N, and S-W-S bonds. The metal and its compounds are also known as heterogeneous and homogeneous catalysts that convert high quantities of substrates through non-stoichiometric reactions.³⁴ Other metal leachables occasionally found in drug products (eg, Fe^{3+} , Ni^{2+} , and Mn^{2+} from stainless steel tanks used in manufacturing equipment) are known for similar interactions with active molecules.¹

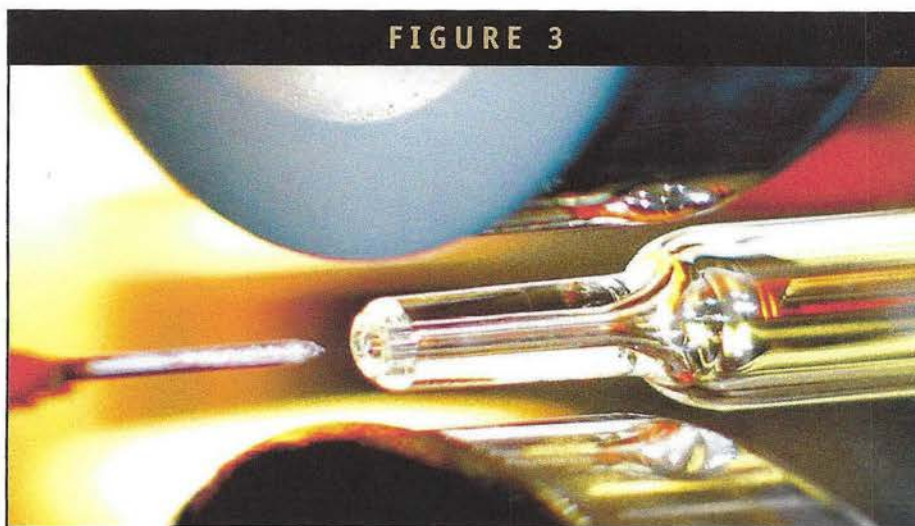
Prefilled syringes formed with tungsten pins contain trace amounts of tungsten compounds in the cone section, which is part of the product contact surface. Syringe filling processes with plunger placement under vacuum intensify the contact between active molecules and tungsten because air bubbles in the cone of the syringe are pulled out.³³

Proprietary methods for the extraction of tungsten from syringe barrels and the subsequent quantitative physicochemical analysis have been developed. Extractable tungsten concentrations are typically below 500 ppb and can be lower than 100 ppb, depending on manufacturing cycle and washing process. Staked needle syringes contain the lowest amount of extractable tungsten as most

of the bore is covered by the needle.

Incorporation of tungsten in syringe barrels can be further reduced by controlling the abrasion of tungsten pins or through substitution of tungsten with other materials. Wear of forming pins can be lowered by horizontal barrel-forming technology. This manufacturing process is using lower temperatures compared to vertical-forming techniques. Other methods are directed at controlling the physical properties of the forming pins. As a substitute for tungsten, alloys from group 9-10 transition metals can be employed. This approach allows tungsten-free syringe forming. However, intake of material from substitute pins into syringe barrels cannot be ruled out. Some biopharmaceutical companies prefer the use of tungsten pins because potential effects of tungsten on their products are better understood than for most other transition metals. Forming pins from non-metallic materials and alternative techniques syringe forming are at an experimental stage.

To evaluate product stability of biopharmaceutical formulations, spiking studies in early phase development with material extracted from used tungsten (metal) pins are recommended. Subsequent stability studies in prefilled syringes verify the preliminary data and specify accepted tungsten (metal) levels. Advanced manufacturing methods for prefilled syringes together with targete



Cone Forming With Tungsten Pins

ADVANCED DELIVERY DEVICES

stability studies ensure that interactions of highly sensitive biomolecules with tungsten are prevented.

SILICONE LUBRICANTS

Even though silicone oil is inert toward most drug products, interactions with sensitive biopharmaceuticals have been observed. Such incompatibilities include aggregation, deformation, and inactivation of native protein structures.^{7,27}

Prefilled syringes are containers and drug delivery systems at the same time. Functionality of these systems (viable activation and gliding forces of the plunger) is accomplished by siliconization. Silicone oils are viscous, inert materials with excellent characteristics as hydrophobic lubricants.^{35,36} They consist of a mixture of polydimethylsiloxane (PDMS) molecules with Si-O chains, which vary in length and number of OH groups. This molecular structure determines how silicone oil layers are adsorbed onto glass surfaces and the distribution, thickness, composition, and uniformity of the layers. In established manufacturing processes on the lines of syringe suppliers, biopharmaceutical companies, and CMOs, syringes are oily siliconized by spraying 0.4- to 1.0-mg silicone oil (eg, Dow Corning 360, Medical Fluid) into the barrels.

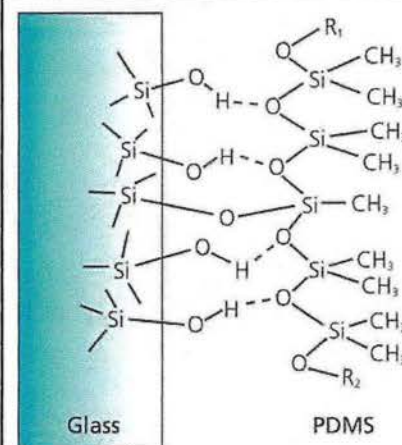
Advanced siliconization technology has been developed to lower the level of free (non-bound) silicone oil in prefilled syringes. The baked siliconization method uses emulsions of silicone oil (eg, Dow Corning 365, 35% Dimethicone NF Emulsion, diluted in HPW) sprayed into syringe barrels followed by heat treatment in a tunnel. Proprietary techniques and downstream washing processes vary depending on syringe supplier. Critical quality attributes of the siliconization process are controlled through the settings of siliconization pump and nozzle, the volume flow of silicone spray and air, the concentration of the silicone oil emulsion and tunnel temperature, speed, and length. This technology alters the nature

of the lubricant in the following way.^{37,38} Heat-induced polymerization reactions reduce fractions of low molecular weight from the silicone oil. Removal of water enables the lubricant to spread out evenly over the glass surface and creates a thin, uniform film. Mono-layers of the lubricant are affixed to the glass surface. The interactions between polydimethylsiloxane and molecules from the glass surface range from van der Waals forces to covalent Si-O bonds. This means thermal fixation processes convert silicone oil into Si(R)O coating as illustrated in Figure 4.³⁸ The thickness of silicone oil layers on the glass surface can be measured by reflectometry. A comparative study using cartridges as glass containers found for oily siliconization a layer thickness with a mean of 232.67 nm and for baked siliconization of 76.83 nm.³⁹

Parenteral biopharmaceutical products vary widely in nature. The sensitivity of the active substance, the viscosity of the formulation, the drug delivery system, and its mode of operation (eg, prefilled syringes either manually or driven by autoinjector device) determine the principal requirements. Silicone coatings of prefilled syringes can be customized to meet specific needs. Variation of process parameters adapts the characteristics of the siliconization. Best results are obtained when syringe manufacturer (supplier) and biopharmaceutical company (end user) partner and work along the following project steps:

1. End user: Specification of accepted silicone oil levels and system functionality
2. Supplier: Development of baked siliconization process for the specified attributes
3. Supplier: Manufacturing of customized baked silicone syringes in sample quantities

FIGURE 4



Model of Silicone Coating

4. End user: QC testing of the samples, filled-syringe stability studies, and evaluation
5. Supplier: Scale-up, process validation, and industrial manufacturing of the syringes

Fundamental understanding of the design space of baked siliconization allows the syringe manufacturer to derive relevant process parameters from the specified quality attributes of the syringes. A range of syringe samples are produced through custom-engineered processes. Quality inspection and initial stability studies with the samples determine which silicone coating is ideal for purpose.

A case study has demonstrated how customization of baked silicone coatings facilitates stability of sensitive molecules in prefilled syringes (eg, vaccine candidate in biopharmaceutical development). The study has deepened the insight into the relationship between siliconization parameters and critical quality attributes.⁴⁰ The amount of extractable silicone oil could be reduced below the detection limit (0.03 mg) of ICP-AES according to EN ISO 11885.

ADVANCED DELIVERY DEVICES

With low levels of lubricant quantity, the specified syringe functionality was fulfilled (plunger gliding forces in the range of 5 to 10 N).

Close partnerships between biopharmaceutical companies and syringe suppliers are instrumental in controlling the impact of product contact materials on sensitive biotherapeutics. Principal requirements regarding drug delivery systems are ideally defined and specified in an early phase of biopharmaceutical development. Manufacturing processes and quality attributes of prefilled syringes can be custom-engineered according to these needs.

SUMMARY

In today's biopharmaceutical market, products are exposed to fierce competition. The role of drug delivery strategies to differentiate products is growing. A number of biopharmaceuticals has already been commercialized in the prefilled syringe platform. However, syringe systems are sources of potential incompatibilities with sensitive molecules. The prefilled syringe industry has therefore engineered manufacturing processes that mitigate stability risks from alkali ions, tungsten, and silicone lubricants. Advanced methods for surface treatment to control pH, tungsten-reduced forming techniques, and baked siliconization processes to immobilize silicone oil have been developed. Syringe manufacturers have established expertise in material science and process technology to understand biopharmaceutical requirements. Evolving needs of highly sensitive pipeline products can be met with customized drug delivery systems. Current technology allows biopharmaceutical companies to exploit the benefits of prefilled syringes and realize the full potential of their products. ♦

REFERENCES

- Allain L, Wang Q. Impact of package leachables on the stability of pharmaceutical products. *Am Pharm Rev*. 2007;10(4):38-44.
- Nicholas K. Extractables and leachables determination: a systematic approach to select and qualify a container closure system for a pharmaceutical product. *Am Pharm Rev*. 2006;9(3):21-27.
- Markovic I. CDER case studies of leaching problems in biotech products. *Int Pharm Qual*. 2008;2(2):25-26.
- DeGrazio FL. Parenteral packaging concerns for biotech drug products. *Drug Delivery Technology*. 2006;6(5):44-49.
- Jenke D. Suitability-for-use considerations for prefilled syringes: drug delivery. *Pharm Technol*. 2008;30-333.
- Markovic I. Evaluation of safety and quality impact of extractable and leachable substances in therapeutic biologic protein products: a risk-based perspective. *Expert Opin Drug Saf*. 2007;6(5):487-491.
- Jones LS, Kaufmann A, Middaugh CR. Silicone oil induced aggregation of proteins. *J Pharm Sci*. 2005;94(4):918-927.
- Rosenberg AS. Effects of protein aggregates, an immunologic perspective. *AAPS Journal*. 2006;8(3):E501-E507.
- Das T, Nema S. Protein particulate issues in biologics development. *Am Pharm Rev*. 2008;11(4):52-57.
- Scott C. Formulation development: making the medicine. *BioProcess Internat*. 2006;4(3):42-57.
- Romack M, Schoenknecht T, Forster R. The rise of prefilled syringes from niche product to primary container of choice: a short history. *ONdrugDelivery*. 2008;4:4-5.
- Romack M. The role of the prefilled syringe in a changing pharmaceutical world. *Scrip World Pharm News*. 2004;10(supplement):10-12.
- Harrison B, Rios M. Big shot, developments in prefilled syringes. *Pharm Technol*. 2007;31(3):50-60.
- Overcashier DE, Chan EK, Hsu CC. Technical considerations in the development of prefilled syringes for protein products. *Am Pharm Rev*. 2006;9(7):77-83.
- Eakins MN. The design and construction of prefilled syringes. *Am Pharm Rev*. 2007;10(6):47-51.
- Fries A. Neue Erkenntnisse über den Markt von Fertigspritzen. *Pharm Ind*. 2008;70(8):929-933.
- Fries A, Lingenberg B. New market insight into prefilled syringes and closure systems: primary data from patients, nurses, physicians, and industry experts. *ONdrugDelivery*. 2008;4:39-41.
- Greystone Associates. Prefilled syringes: drugs, devices and delivery strategies. *Market Study*; 2006.
- Frost & Sullivan. US Pharmaceutical Packaging Markets. *Market Study*; 2005.
- MEFOS Gesellschaft für Meinungsforschung und Beratung im Gesundheitswesen. *Market Study*; 2007.
- Vogel W. *Glass Chemistry*. Springer Verlag, Berlin and Heidelberg, 2nd Edition; 1994.
- Wlecke M. *Technical Study*. Unpublished Results; 2008.
- ISO 4802-2. *Glassware: Hydrolytic Resistance of the Interior Surfaces of Glass Containers, Part 2: Determination by Flame Spectrometry and Classification*. 1st Edition; 1988.
- European Pharmacopoeia. *Glass Containers for Pharmaceutical Use*. 30201, 303-308; 2005.
- Fries A, Schoenknecht T. Controlling the alkali release in glass based prefilled syringes. Poster presented at the AAPS Annual Meeting and Exposition, San Diego, CA, November 13, 2007.
- Osterberg RE. Potential toxicity of extractables and leachables in drug products. *Am Pharm Rev*. 2005;8(2):64-67.
- Markovic I. Challenges associated with extractable and/or leachable substances in therapeutic biologic protein products. *Am Pharm Rev*. 2006;9(6):20-27.
- Rosenberg AS, Wrobec AS. A risk-based approach to immunogenicity concerns of therapeutic protein products, part 2: considering host-specific and product-specific factors impacting immunogenicity. *BioPharm Int*. 2004;17(12):34-40.
- Faulkner E. Container closure interactions with biopharmaceutical parenteral products. Paper presented at the ICB Annual Formulation Strategies for Protein Therapeutics Conference, San Francisco, CA, November 6-8, 2006.
- Lee H. Tungsten leaching from prefilled syringes and impact on protein aggregation. Poster presented at the PDA Extractables/Leachables Forum, Bethesda, MD, November 6-8, 2007.
- Lassner E, Schubert WD. *Tungsten: properties, chemistry, technology of the element, alloys and chemical compounds*. 1st ed. Kluwer Academic/Plenum Publishers; New York, NY; 1999.
- Hecker J. Prefilled syringe, autoinjector quality assurance explored at FDA/Industry forum. *Pharma Biotechnol Quality Control (The Gold Sheet)* 2007;41(1):1-4.
- Swift R. Tungsten, prefilled syringes and protein aggregation. Paper presented at the PDA Universe of Prefilled Syringes and Injection Devices Conference, Bethesda, MD, October 23, 2006.
- International Tungsten Industry Association. *Tungsten Uses*. Chemical

- Applications, Catalysts. Website visited January 2009: www.tita.org.uk.
- Tomanek W. Silicones: synthesis, properties and applications. *Polymeric Mater Encyc*. 1996;10:7706-7711.
 - Curtis J. *Medical Applications of Silicones*. Biomaterials Sciences, 2nd ed. Elsevier Pub: New York, NY; 2004.
 - Mundry T, Schurreit T, Surrmann P. The fate of silicone oil during heat-cured glass siliconization: changes in molecular parameters analyzed by size exclusion and high temperature gas chromatography. *PDA J Pharm Sci Technol*. 2000;54(5):383-397.
 - Mundry T. Einbremsilikonisierung bei Pharmazeutischen Glaspackmitteln. I Analytische Studien eines Produktionsprozesses. PhD Thesis, Humboldt University, Berlin; 1999.
 - Lankers M. Optimization Study of Baked-in Siliconization in Cartridges. Paper presented at the PDA Interest Group Meeting, Prefilled Syringes, Berlin, Germany, December 2, 2008.
 - Fries A, Khandke L, Malone R. Customization of Prefilled Syringe System for Biopharmaceutical Development: Case Study. Paper presented at the PDA Universe of Prefilled Syringes and Injection Devices Conference, Berlin, Germany, November 28, 2007.

BIOGRAPHY



Dr. Arno Fries is Head of Sales, USA Syringes and Director, Product Management, Syringes in the Gerresheimer Group. He joined the company in January 2006 and

is currently responsible for the North American syringe business of Gerresheimer Buende GmbH and global product management of syringe systems in the Gerresheimer Tubular Glass Division. He has 11 years of experience in leading sales and marketing positions in the life science industry and 4 years as a scientist at international research institutes in Japan and the UK. He earned his PhD in Chemistry from the University of Wuerzburg, Germany, and his degree in business administration from the IDB Institute in Frankfurt, Germany. Dr. Fries is a member of the Parenteral Drug Association and the Alexander von Humboldt Foundation. Contact: Gerresheimer Buende GmbH, Erich-Martens-Strasse 26-32, 32257 Buende, Germany; Phone +49.5223.164.401; a.fries@gerresheimer.com.

Appendix 2C



Search WorldCat

Search

[Advanced Search](#) [Find a Library](#)

[<< Return to Search Results](#)

[Cite/Export](#)

[Print](#)

[E-mail](#)

[Share](#)

[Permalink](#)

[Add to list](#)

[Add tags](#)

[Write a review](#)

Rate this item: 1 2 3 4 5

Drug delivery technology.

Publisher: Montville, NJ : Drug Delivery Technology, LLC

Edition/Format: Journal, magazine : Periodical : English [View all editions and formats](#)

Rating: (not yet rated) 0 with reviews - Be the first.

Subjects: [Drug delivery systems -- Periodicals.](#)
[Drug Delivery Systems.](#)
[Biological Transport.](#)
[View all subjects](#)

More like this [User lists](#) [Similar Items](#)

Search this publication for other articles with the following words:

Get a Copy

[Find a copy in the library.](#)

Find a copy online

Links to this item

drugdeliverytech.com

Find a copy in the library

Enter your location:

Submit a complete postal address for best results.



Displaying libraries 1-6 out of 59 for all 5 editions (8600 Rockville Pike, Bethesda, MD 20894, USA)

Show libraries holding [just this edition](#)

[<< First](#) [< Prev](#) 1 [2](#) [3](#) [Next >](#) [Last >>](#)

Library	Held formats	Distance	
1. National Library of Medicine Bethesda, MD 20894 United States	Journal / Magazine / Newspaper	1 mile MAP IT	Library info Add to favorites
2. Library of Congress Washington, DC 20540 United States	Journal / Magazine / Newspaper	9 miles MAP IT	Library info Ask a librarian Add to favorites
3. Shenandoah University Alson H. Smith, Jr. Library Winchester, VA 22601 United States	Journal / Magazine / Newspaper	58 miles MAP IT	Library info Add to favorites
4. Bridgewater College Library at Flory Hall	Journal / Magazine / Newspaper	110 miles MAP IT	Library info Add to favorites

Bridgewater, VA 22812 United States

5.	College of Staten Island, CUNY Staten Island, NY 10314 United States	 Journal / Magazine / Newspaper	192 miles MAP IT	Library info Ask a librarian Add to favorites
6.	Brooklyn College Brooklyn, NY 11210 United States	 Journal / Magazine / Newspaper	202 miles MAP IT	Library info Ask a librarian Add to favorites

<< First < Prev 1 2 3 Next > Last >>

Details

Genre/Form: Periodicals

Additional Physical Format: Online version:
Drug delivery technology (Online)
(DLC) 2008215769
(OCoLC)232115072

Material Type: Periodical, Internet resource

Document Type: Journal / Magazine / Newspaper, Internet Resource

ISSN: 1537-2898

OCLC Number: 48060225

Description: volumes : illustrations

Other Titles: Drug delivery technology

Reviews

User-contributed reviews

[Add a review](#) and share your thoughts with other readers. Be the first.

Tags

[Add tags](#) for "Drug delivery technology.". Be the first.

Similar Items

Related Subjects: (6)

- [Drug delivery systems -- Periodicals.](#)
- [Drug Delivery Systems.](#)
- [Biological Transport.](#)
- [Technology, Pharmaceutical.](#)
- [Drug Industry.](#)
- [Drug delivery systems.](#)

User lists with this item (1)

[CUH2A periodicals](#) (83 items)

by [MTuftsCUH2A](#)

 [Linked Data](#)

Appendix 2D



- Search
- Headings
- Titles
- Library Information
- Other Databases
- Help
- Home

Collections of the National Library of Medicine

Search Request: Advanced Menu = ("1537 2898")[in ISSN]

Search Results: Displaying 1 of 1 records

← Previous Next →

- Summary View
- Detailed View
- Table of Contents
- MARC View
- More Like This

Drug delivery technology.

Title: Drug delivery technology.

Description: v. : ill.

Coverage: Began in 2001; ceased with v. 10, no. 9 (Nov.-Dec. 2010).

Publisher: Montville, NJ : Drug Delivery Technology, LLC

Continued By: [Drug development & delivery](#)

ISSN: 1537-2898

Electronic Links: <http://www.drugdeliverytech.com>

NLM Unique ID: 101134826

Other ID Numbers: (OCoLC)48060225

Location: General Collection

Call Number: W1 DR514BK

Availability: v.2(2002)-10(2010)

Status: Available

Other Locations: [Find Other Libraries That Own This](#)

Get it NLM

← Previous Next →

Options for Print, Save or E-mail	
Select Download Format:	<input type="text" value="Full record with holdings"/> ▼ <input type="button" value="Print/Save"/>
E-mail Full record(s) to:	<input type="text"/> <input type="button" value="E-mail"/>

- Search
- Headings
- Titles
- Library Information
- Other Databases
- Help
- Home

[Copyright](#), [Privacy](#), [Accessibility](#)

U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894

[National Institutes of Health](#), [Health & Human Services](#)

[Contact NLM](#)

Appendix 2E



Flow cytometry: A promising technique for the study of silicone oil-induced particulate formation in protein formulations

D. Brett Ludwig^a, Joseph T. Trotter^b, John P. Gabrielson^a, John F. Carpenter^c, Theodore W. Randolph^{a,*}

^aUniversity of Colorado Center for Pharmaceutical Biotechnology, Department of Chemical and Biological Engineering, University of Colorado, Boulder, CO 80309, USA

^bCytometry and Advanced Technology Group, BD Biosciences, Becton Dickinson, San Diego, CA 92121, USA

^cUniversity of Colorado Center for Pharmaceutical Biotechnology, Department of Pharmaceutical Sciences, University of Colorado Health Sciences Center, Aurora, CO 80045, USA

ARTICLE INFO

Article history:

Received 19 August 2010

Received in revised form 30 November 2010

Accepted 6 December 2010

Available online 10 December 2010

Keywords:

Protein aggregation

Adsorption

Silicone oil

Formulation

Fluorescence

Flow cytometry

ABSTRACT

Subvisible particles in formulations intended for parenteral administration are of concern in the biopharmaceutical industry. However, monitoring and control of subvisible particulates can be complicated by formulation components, such as the silicone oil used for the lubrication of prefilled syringes, and it is difficult to differentiate microdroplets of silicone oil from particles formed by aggregated protein. In this study, we demonstrate the ability of flow cytometry to resolve mixtures comprising subvisible bovine serum albumin (BSA) aggregate particles and silicone oil emulsion droplets with adsorbed BSA. Flow cytometry was also used to investigate the effects of silicone oil emulsions on the stability of BSA, lysozyme, abatacept, and trastuzumab formulations containing surfactant, sodium chloride, or sucrose. To aid in particle characterization, the fluorescence detection capabilities of flow cytometry were exploited by staining silicone oil with BODIPY 493/503 and model proteins with Alexa Fluor 647. Flow cytometric analyses revealed that silicone oil emulsions induced the loss of soluble protein via protein adsorption onto the silicone oil droplet surface. The addition of surfactant prevented protein from adsorbing onto the surface of silicone oil droplets. There was minimal formation of homogeneous protein aggregates due to exposure to silicone oil droplets, although oil droplets with surface-adsorbed trastuzumab exhibited flocculation. The results of this study demonstrate the utility of flow cytometry as an analytical tool for monitoring the effects of subvisible silicone oil droplets on the stability of protein formulations.

© 2010 Elsevier Inc. All rights reserved.

Currently, subvisible particles in formulations of therapeutic proteins are attracting substantial scrutiny [1]. Published reports [2–4] have shown abundant levels of subvisible particles in formulations that meet current regulatory guidelines [5]. The presence of these particles is of concern because they may provide potential nucleation sites for protein aggregation, a principal degradation pathway for a number of protein therapeutics [6,7]. Furthermore, protein aggregates have been associated with undesirable immunogenic responses in patients receiving therapeutic proteins [8–10]. Despite the importance of detection and monitoring of subvisible particles, detection and characterization of particles in this size range present formidable analytical challenges for current methods.

Monitoring and control of subvisible protein particulates can be complicated by formulation components. For example, many proteins are now being formulated in prefilled glass syringes. To allow smooth plunger movement, these syringes typically are lubricated with silicone oil, which is sprayed onto the interior surfaces of the syringe during the syringe manufacturing process [11]. Although

the solubility of silicone oil in typical protein formulations is quite low [12], silicone oil may be present in the formulation in the form of an emulsion. Droplets of emulsified silicone oil may be detected by various optical techniques, but it is often difficult to distinguish silicone oil droplets from aggregates of protein.

The presence of emulsified silicone oil may result in increased rates of protein aggregation [13–17]. Conversely, we recently reported that proteins may adsorb to the surfaces of oil droplets, changing the kinetic stability of silicone oil emulsions [18]. To determine whether subvisible particles within a formulation are composed of protein, silicone oil, or protein adsorbed onto oil, it would be advantageous to use a technique that can simultaneously monitor particle size distributions and particle compositions.

One technique that has long been used in the field of cell biology is fluorescence-activated cell sorting (FACS),¹ often referred to as flow cytometry. Flow cytometry combines light scattering from

¹ Abbreviations used: FACS, fluorescence-activated cell sorting; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DCM, dichloromethane; MWCO, molecular weight cutoff; AF 647, Alexa Fluor 647; DLS, dynamic light scattering; FSC, forward scattering; SSC, side scattering; FITC, fluorescein isothiocyanate; AF 488, Alexa Fluor 488.

* Corresponding author.

E-mail address: theodore.randolph@colorado.edu (T.W. Randolph).

particles and light emission from fluorochromic molecules to generate specific multiparameter data sets for particles in the range of 1–100 μm [19,20]. Using hydrodynamic focusing techniques, flow cytometers are capable of counting and measuring the light scattering and fluorescence emission of thousands of individual particles per second. The high-throughput capability of flow cytometry, along with its ability to characterize individual particles as part of a large sample set, makes it a promising technique for use in the study of subvisible particles in protein formulations.

In the current study, we explored the use of flow cytometry as a method for the detection and characterization of subvisible particles in silicone oil-contaminated formulations of lysozyme, bovine serum albumin (BSA), abatacept, and trastuzumab. We used fluorescently labeled proteins and fluorescently stained silicone oil to show that flow cytometry has the ability to discriminate between homogeneous protein aggregates and heterogeneous particles made up of silicone oil and protein. Furthermore, flow cytometry analyses of our model systems provide evidence of protein adsorption onto silicone oil droplets, monolayer versus multilayer protein adsorption, and particle flocculation.

Materials and methods

Materials

Chicken egg white lysozyme (Fisher Bioreagents), BSA (Fisher Bioreagents), abatacept (Orencia, Bristol–Myers Squibb), and trastuzumab (Herceptin, Genentech) were obtained in lyophilized form. All buffer salts (sodium phosphate monobasic, sodium phosphate dibasic, and sodium acetate), excipients (polysorbate 20, sodium chloride, and sucrose), and solvents (dimethyl sulfoxide [DMSO] and dichloromethane [DCM]) were reagent grade or higher. Silicone oil (Dow Corning 360, 1000 cSt) was of medical grade. Solutions were prepared with filtered distilled deionized water (Nanopure II, Barnstead International, Dubuque, IA, USA).

Preparation of stock solutions

Lysozyme, BSA, and abatacept were reconstituted and dialyzed (Pierce Slide-A-Lyzer, 3500 and 10,000 molecular weight cutoffs [MWCs]) into 10 mM sodium phosphate (pH 7.5) and 0.01% sodium azide. Trastuzumab was reconstituted and dialyzed into 10 mM sodium acetate (pH 5.0) and 0.01% sodium azide. Abatacept and trastuzumab were reconstituted into a buffer similar to that which would result after reconstitution of the formulations using instructions from the respective product inserts [21,22]. The lysozyme and BSA reconstitution conditions were chosen such that lysozyme would carry a net positive charge and BSA would carry a net negative charge.

Protein concentrations were determined based on extinction coefficients of 2.63, 0.667, 1.01, and 1.4 $\text{ml mg}^{-1} \text{cm}^{-1}$ for lysozyme [23], BSA [24], abatacept [25], and trastuzumab [26], respectively, at 280 nm using a PerkinElmer Lambda 35 spectrophotometer (Wellesley, MA, USA).

Fluorescent labeling

Protein molecules were chemically labeled with Alexa Fluor 647 (AF 647, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol (MP 00143, Amine-Reactive Probes, Invitrogen). Following the labeling reaction, protein concentrations and degrees of labeling were determined using absorbance measurements at 280 and 650 nm. To determine protein concentrations, protein absorbance values at 280 nm were calculated according to Eq. (1):

$$A_{\text{protein}} = A_{280} - A_{650} (CF), \quad (1)$$

where *CF* represents the correction factor for fluorescent dye absorbance equal to 0.03 (MP 00143, Amine-Reactive Probes). Protein concentrations were then determined using extinction coefficients mentioned previously. Degrees of labeling (*DOL*) were determined according to Eq. (2):

$$DOL = \frac{A_{650} * MW}{[\text{protein}] * \epsilon_{\text{dye}}}, \quad (2)$$

where *MW* is the molecular weight of the protein, ϵ_{dye} represents the extinction coefficient of the dye at 650 nm equal to 239,000 $\text{cm}^{-1} \text{M}^{-1}$ (MP 00143, Amine-Reactive Probes), and protein concentration is in milligrams per milliliter (mg/ml). Lysozyme, BSA, abatacept, and trastuzumab had *DOL* values of 1, 3, 4, and 7, respectively.

Subsequent to determination of protein concentration and *DOL* measurements, samples were concentrated to approximately 20-mg/ml protein concentrations using Centricon YM-3 centrifugal filters (Millipore, Billerica, MA).

Silicone oil was stained with 4,4-difluoro-1,3,5,7,8-penta-methyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, Invitrogen). BODIPY dye was chosen for its nonpolar structure and previous use as a tracer for oil and other nonpolar lipids (BODIPY 493/503, product insert). To facilitate the staining, solutions of both the BODIPY dye and silicone oil were prepared in mutually miscible solvents. BODIPY was dissolved in DMSO at a concentration of 2.5 mg/ml, whereas 10 ml of silicone oil was dissolved in 20 ml of dichloromethane (DCM). After both the dye and silicone oil were completely dissolved, 400 μl of the BODIPY–DMSO solution was added to the silicone oil–DCM solution and mixed for 1 h. DMSO and DCM were then removed using a Laborota 4000 rotary evaporator (Heidolph Brinkmann, Elk Grove Village, IL, USA).

Silicone oil emulsion preparation

Silicone oil-in-aqueous buffer emulsions (~ 0.5 – 1.0% , v/v) were created by a combination of mechanical mixing and high-pressure homogenization. A 50-ml suspension of 4% (v/v) silicone oil in buffer was prepared by combining BODIPY-stained silicone oil and buffer in a stainless steel cylinder and mixing at room temperature with a 20-mm shaft rotor/stator (VirTishear Mechanical Homogenizer, VirTis) for 5 min at 5000 rpm. Immediately thereafter, the silicone oil-in-buffer suspension was passed five times through a high-pressure homogenizer (Emulsiflex C5 Homogenizer, Avestin) at a pressure of 50 MPa. The final emulsion, containing less than 1% (v/v) silicone oil, was collected in a 50-ml polypropylene centrifuge tube. The difference between the initial amount of silicone oil added and that present in the final emulsion was due to separation, or creaming, of the silicone oil in the sample chamber prior to passage through the emulsifier.

Given that the formulation additives chosen for this study could significantly affect the emulsification process, appropriate amounts of polysorbate 20, sodium chloride, and sucrose were added after emulsion formation to a standardized emulsion prepared in deionized water to obtain final excipient concentrations of 0.03% (w/v), 150 mM (0.9%, w/v), and 250 mM (8.6%, w/v), respectively. Mixtures were gently swirled until the excipients completely dissolved, and then the solutions were allowed to equilibrate for 1 h before being used in experiments.

Silicone oil droplet size

Silicone oil droplet size distributions in the emulsions were measured by laser diffraction analysis using a Beckman Coulter LS230 Laser Diffraction Particle Size Analyzer (Fullerton, CA, USA). Particle size was calculated assuming Mie scattering from

spherical particles using a value of 1.4046 for the refractive index of the silicone oil [27].

Detection of subvisible particles with flow cytometry

Fluorescently labeled protein aggregates of subvisible size were created by agitating 1-mg/ml samples of BSA labeled with AF 647 (BSA-AF 647) in 10 mM sodium phosphate (pH 7.5) and 0.01% sodium azide buffer. Here 0.5-ml samples in 1.5-ml polypropylene microcentrifuge tubes (Fisher Scientific, Hampton, NH, USA) were placed horizontally on a Lab-Line titer plate shaker (Barnstead International) and agitated at approximately 1000 rpm for 72 h at room temperature. Aggregate size was determined using a NI-COMP 380/ZLS (Particle Sizing System, Santa Barbara, CA, USA) dynamic light scattering (DLS) instrument. This procedure generated BSA aggregates with a mean intensity-weighted diameter of 1.8 μm (data not shown).

The suspension of labeled BSA aggregates was then analyzed using a BD FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm blue air-cooled argon laser and 635-nm red diode laser, four fluorescence detectors (FL1 530/30, FL2 585/42, FL3 670LP, and FL4 661/16), and two 488-nm light scattering detectors (low-angle forward scattering [FSC] and 90° side scattering [SSC]). To observe whether or not the technique could detect aggregates in the absence of silicone oil, 5000 particles from the aggregate suspension were analyzed using the fluorescent signals detected by the FL1 (silicone oil-BODIPY) and FL4 (protein-AF 647) detectors. All flow cytometry data sets were collected using BD FACSFlo sheath fluid and the low sample flow instrument option. All analyses of flow cytometry data were performed using FlowJo 8.8.6 (Tree Star, Ashland, OR, USA).

To ascertain the ability of flow cytometry to detect silicone oil droplets in protein formulations, a BODIPY-stained silicone oil emulsion was mixed with BSA-AF 647 and the resulting suspension was analyzed using the FACSCalibur instrument. FL1 and FL4 detector signals associated with 30,000 particles were used for this analysis.

To explore the ability of flow cytometry to resolve populations of homogeneous protein aggregates from silicone oil droplets with adsorbed protein, 250 μl of BSA-AF aggregate suspension was mixed with 250 μl of an emulsion consisting of silicone oil-BODIPY droplets with adsorbed BSA-AF 647. FL1 and FL4 signals from 30,000 particles were used for this analysis.

Silicone oil effects on protein formulation stability

Samples with a final protein concentration of 200 $\mu\text{g}/\text{ml}$ were created by combining appropriate amounts of protein solution with stock emulsion to a final silicone oil concentration of 0.5–1.0% (v/v) in 5-ml round-bottom polystyrene tubes (BD Biosciences, San Jose, CA, USA). Sample sets consisted of three separate samples of each protein in four different formulation conditions.

After varying periods of incubation (1 h, 8 h, 24 h, 72 h, 168 h [1 week], and 336 h [2 weeks]) at room temperature, samples were examined using flow cytometry for the presence of protein aggregates and for silicone oil droplets associated with protein. A total of 30,000 events were collected for each analysis.

Results

Silicone oil droplet size

Representative silicone oil droplet size distributions for stock emulsions are shown in Fig. 1. Surface area-weighted droplet size distributions of all emulsions were bimodal, with particle sizes

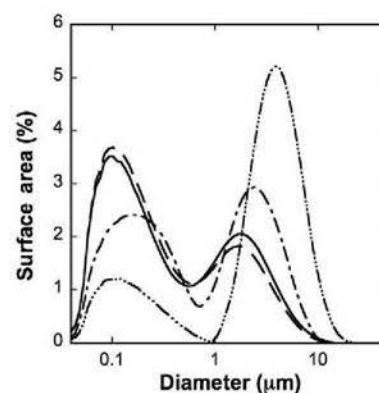


Fig. 1. Surface area-weighted particle size distribution of silicone oil droplets in 10 mM sodium phosphate (pH 7.5) and 0.01% sodium azide buffer. The solid line represents excipient-free, the dashed line represents emulsions formed from solutions containing 0.03% polysorbate 20, the dash-dot-dash line represents emulsions formed from solutions containing 250 mM sucrose, and the dash-dot-dot-dash line represents emulsions formed from solutions containing 150 mM sodium chloride. Data represent the arithmetic means of three replicate samples.

ranging from nanometers to microns. The two main populations were centered around 100 nm and 5 μm . The effects of the addition of various excipients on the silicone oil droplet size in silicone oil-in-aqueous buffer emulsions ranged from minimal to considerable (Fig. 1). The addition of 0.03% polysorbate 20 did not significantly change the silicone oil droplet size distribution compared with that of the excipient-free emulsion, whereas the addition of 250 mM sucrose or 150 mM sodium chloride shifted the distribution toward a larger silicone oil droplet size.

Detection of subvisible particles with flow cytometry

Flow cytometry analysis of the suspensions of aggregated, AF 647-labeled BSA reported a population of particles with considerable fluorescence around 661 nm, the wavelength associated with AF 647 fluorescence, and minimal fluorescence near 530 nm, the wavelength corresponding to BODIPY fluorescence (Fig. 2A).

Multiparameter analysis of a mixture of silicone oil-BODIPY droplets and BSA-AF 647 (nonagitated) revealed a population of particles with fluorescence characteristic of both AF 647 and BODIPY, indicative of protein associated with silicone oil (i.e., adsorbed onto the surface) (Fig. 2B). However, there was no evidence of the presence of homogeneous protein aggregates.

An examination of a mixture of the BSA-AF 647 agitated sample containing aggregates and the silicone oil-BODIPY droplets coated with BSA-AF 647 (nonagitated) showed two well-resolved populations of particles: one population exhibiting considerable AF 647 fluorescence and little BODIPY fluorescence (homogeneous protein aggregates) and another group made up of particles exhibiting both AF 647 and BODIPY fluorescence (presumably protein adsorbed onto silicone oil droplets) (Fig. 2C). These differences were also observed when looking at particle BODIPY fluorescence and AF 647 fluorescence separately (Fig. 3).

Silicone oil effects on protein formulation stability

To further explore the ability of flow cytometry to detect and characterize subvisible particles in protein formulations and to investigate the effects of silicone oil droplets on formulation stability, each of the experimental protein formulations was added to its respective BODIPY-stained silicone oil emulsion. For example, BSA-AF 647 in 10 mM phosphate (pH 7.5) and 150 mM sodium chloride was added to an emulsion of silicone oil-BODIPY droplets

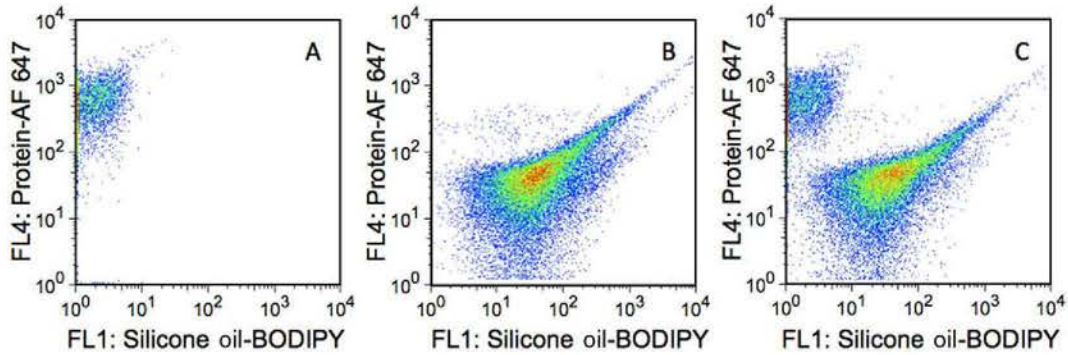


Fig. 2. Detection of subvisible homogeneous protein aggregates: Fluorescence dot plots of 1- to 2- μm homogeneous BSA-AF 647 aggregates in the absence of silicone oil (A), silicone oil-BODIPY droplets with adsorbed BSA-AF 647 (B), and the mixture of homogeneous BSA-AF 647 aggregates and silicone oil-BODIPY droplets with adsorbed BSA-AF 647 (C). The abscissas represent fluorescence intensities measured with the FL1 detector (530/30 nm), whereas the ordinates represent fluorescence intensities measured with the FL4 detector (661/16 nm). Panel A represents approximately 5000 collected events, whereas panels B and C each represent approximately 30,000 collected events.

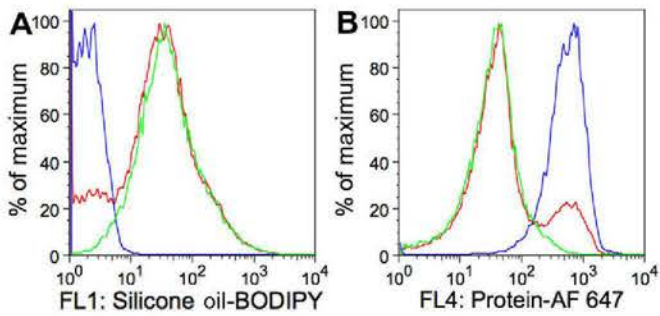


Fig. 3. Fluorescence histograms of analyzed particles: BODIPY fluorescence (A) and AF 647 fluorescence (B). Histograms are representations of the data from Fig. 2, with the blue line representing the BSA-AF 647 aggregate suspension, the green line representing the silicone oil-BODIPY droplets with adsorbed BSA-AF 647, and the red line representing the mixture of the BSA-AF 647 aggregate suspension and silicone oil-BODIPY droplets with adsorbed BSA-AF 647. The abscissas represent fluorescence intensity measured with the FL1 detector (530/30 nm) or FL4 detector (661/16 nm), whereas the ordinate represents number of events normalized to the maximum number of events recorded for any single fluorescence intensity (% maximum events).

in 10 mM phosphate (pH 7.5) and 150 mM sodium chloride. To examine the effects of prolonged silicone oil exposure, samples were incubated and analyzed at defined time points over a 2-week period.

For each protein, Fig. 4 shows a representative analysis of a protein-silicone oil mixture in an excipient-free formulation. For samples of lysozyme-AF 647, BSA-AF 647, and abatacept-AF 647 mixed with silicone oil-BODIPY droplets, histograms of BODIPY fluorescence showed a unimodal distribution of particles with a significant amount of BODIPY fluorescence characteristic of particles containing silicone oil. Similarly, histograms of AF 647 fluorescence showed a unimodal distribution of particles with significant AF 647 fluorescence, indicative of particles associated with protein. These unimodal distributions of BODIPY and AF 647 fluorescence persisted over 2 weeks of incubation (Fig. 4). After these 2 weeks of incubation, AF 647 histograms for the BSA-AF 647 and abatacept-AF 647 formulations showed a broader distribution than that at the earlier time points, with an increase in particles with higher AF 647 fluorescence reflecting increased levels of protein (Fig. 4).

For the samples of trastuzumab-AF 647 mixed with silicone oil-BODIPY droplets, histograms of BODIPY and AF 647 showed bimodal distributions (Fig. 4). As incubation time increased, the distributions shifted to reflect populations of particles with increased AF 647 and BODIPY fluorescence, indicative of particles consisting of increased levels of both silicone oil and protein.

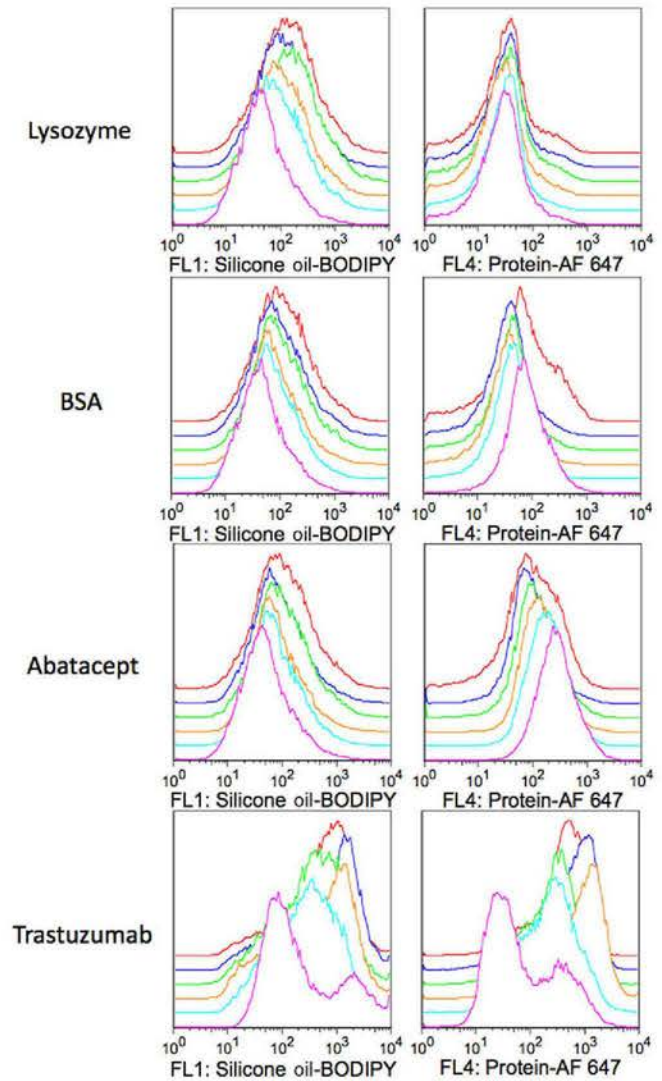


Fig. 4. Flow cytometry analyses of protein-silicone oil mixtures in excipient-free formulations. Histograms illustrate particle BODIPY fluorescence intensities measured with the FL1 detector (530/30 nm, histograms on left) or AF 647 fluorescence intensities measured with the FL4 detector (661/16 nm, histograms on right) versus percentage maximum events. For each panel, in histograms ordered from the lower most curve, pink, light blue, orange, green, dark blue, and red histograms represent samples incubated for 1 h, 8 h, 24 h, 72 h, 168 h (1 week), and 336 h (2 weeks), respectively. Histograms are offset for clarity, and each histogram represents approximately 30,000 events.

For each of the protein–silicone oil mixtures, the characteristic particle BODIPY and AF 647 fluorescence intensities were consistent for triplicate samples. The overlay of the fluorescence dot plots from three separate samples in Fig. 5 illustrates the reproducibility.

Similar to the excipient-free formulations, flow cytometry analyses of formulations containing 0.03% polysorbate 20, 150 mM sodium chloride, or 250 mM sucrose showed no evidence of a significant amount of homogeneous protein aggregates (data not shown). Although the tested formulation additives did not appear to affect the formation of homogeneous protein aggregates in formulations mixed with silicone oil–BODIPY emulsions, the addition of 0.03% polysorbate 20 had a noticeable effect on AF 647 particle fluorescence for BSA–AF 647, abatacept–AF 647, and trastuzumab–AF 647 formulations mixed with silicone oil–BODIPY emulsions. Fig. 6 illustrates this effect for abatacept. The AF 647 fluorescence increases with BODIPY fluorescence at a similar rate in the excipient-free (Fig. 6A), 150 mM sodium chloride (Fig. 6C), and 250 mM sucrose (Fig. 6D) formulations. However, the AF 647 fluorescence does not significantly increase with increasing BODIPY fluorescence in the 0.03% polysorbate 20 formulation (Fig. 6B). This suggests that the polysorbate 20 decreased the amount of protein adsorbed to the silicone oil. The addition of 0.03% polysorbate 20 to lysozyme–AF 647 formulations did not result in such dramatic effects (Fig. 7).

Furthermore, the addition of 0.03% polysorbate 20 to trastuzumab–AF 647 formulations mixed with silicone oil–BODIPY emulsions not only resulted in reduced AF 647 fluorescence (Fig. 8B) but also resulted in unimodal BODIPY fluorescence histograms (Fig. 8A) instead of the bimodal histograms seen in the excipient-free formulations (Fig. 4). The unimodal distribution for the BODIPY histogram was not seen when 150 mM sodium chloride or 250 mM sucrose was added to trastuzumab–AF 647 formulations mixed with silicone oil–BODIPY emulsions (data not shown).

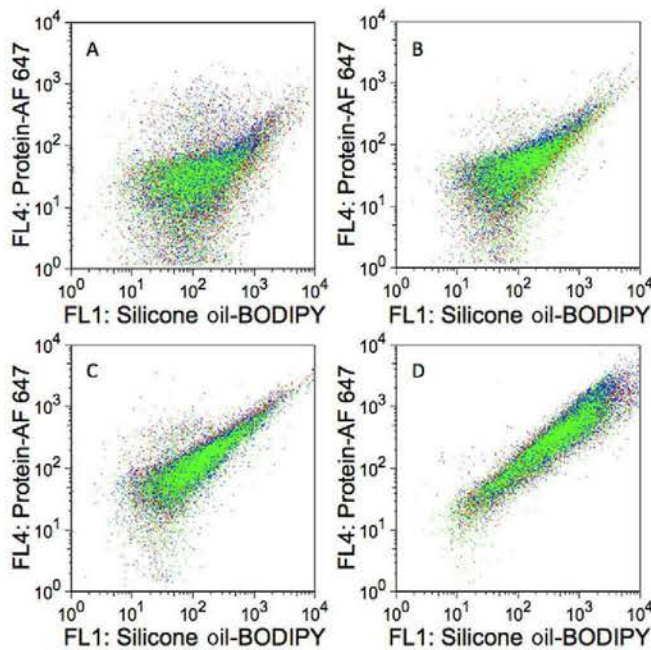


Fig. 5. Sample-to-sample variation of protein–silicone oil mixtures in excipient-free formulations: Fluorescence dot plots of three separate samples of silicone oil–BODIPY droplets and lysozyme–AF 647 (A), BSA–AF 647 (B), abatacept–AF 647 (C), and trastuzumab–AF 647 (D). Each color represents a different sample. Each sample was incubated for 72 h, and each dot plot represents approximately 30,000 events. The abscissas represent fluorescence intensities measured with the FL1 detector (530/30 nm), whereas the ordinates represent fluorescence intensities measured with the FL4 detector (661/16 nm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

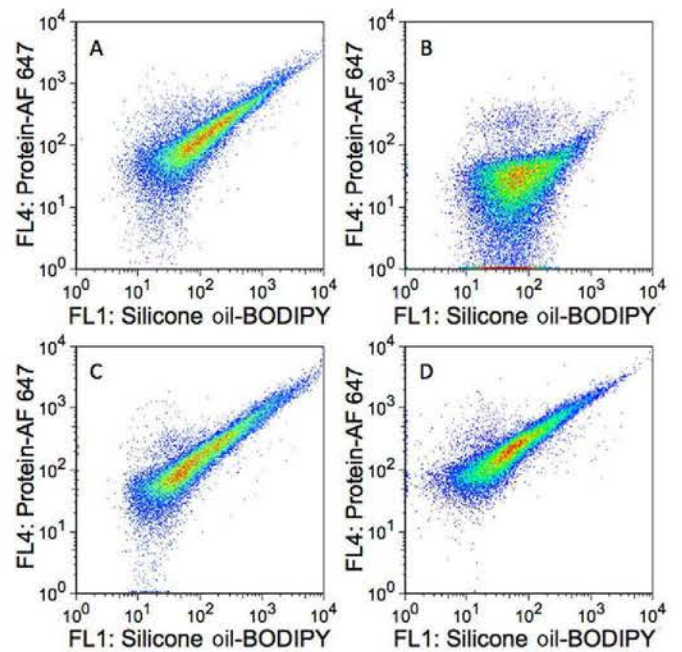


Fig. 6. Effects of additives on the association of abatacept–AF 647 with silicone oil–BODIPY droplets. Dot plots represent silicone oil–BODIPY droplets coated with abatacept–AF 647 in formulations containing no additives (excipient-free) (A), 0.03% polysorbate 20 (B), 150 mM sodium chloride (C), and 250 mM sucrose (D). Each sample was incubated for 8 h, and each dot plot represents approximately 30,000 events. The abscissas represent fluorescence intensities measured with the FL1 detector (530/30 nm), whereas the ordinates represent fluorescence intensities measured with the FL4 detector (661/16 nm).

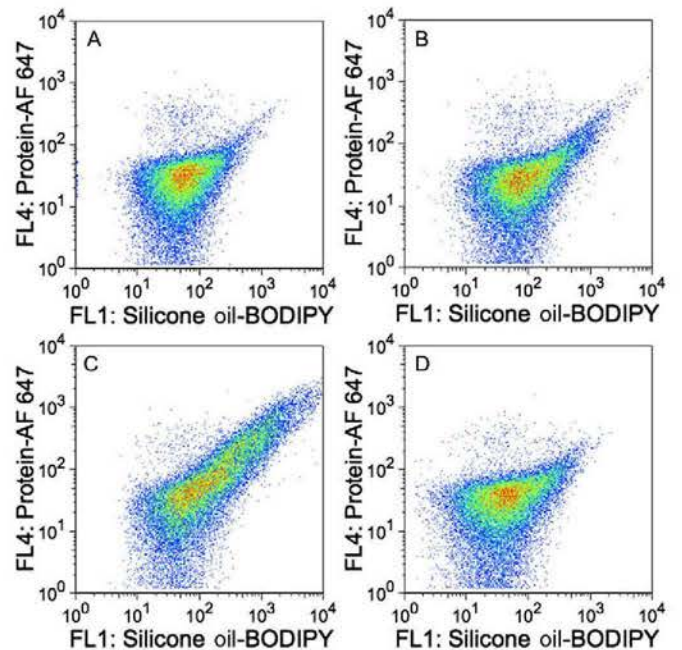


Fig. 7. Effects of additives on the association of lysozyme–AF 647 with silicone oil–BODIPY droplets. Dot plots represent silicone oil–BODIPY droplets coated with abatacept–AF 647 in formulations containing no additives (excipient-free) (A), 0.03% polysorbate 20 (B), 150 mM sodium chloride (C), and 250 mM sucrose (D). Each sample was incubated for 8 h, and each dot plot represents approximately 30,000 events. The abscissas represent fluorescence intensities measured with the FL1 detector (530/30 nm), whereas the ordinates represent fluorescence intensities measured with the FL4 detector (661/16 nm).

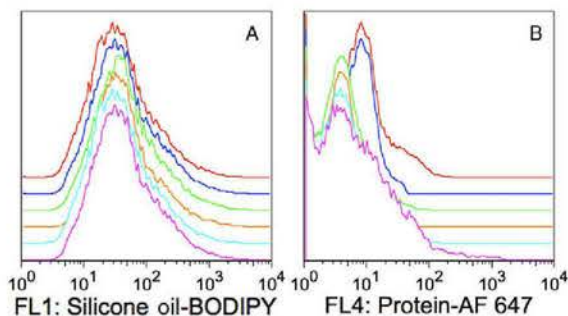


Fig. 8. Flow cytometry analyses of trastuzumab-AF 647/silicone oil-BODIPY mixtures in 0.03% polysorbate 20 formulations. Histograms illustrate particle BODIPY fluorescence intensity measured with the FL1 detector (530/30 nm, histograms in panel A) and particle AF 647 fluorescence intensity measured with the FL4 detector (661/16 nm, histograms in panel B) plotted versus percentage maximum events. For each panel, in histograms ordered from the lower most curve, pink, light blue, orange, green, dark blue, and red histograms represent samples incubated for 1 h, 8 h, 24 h, 72 h, 168 h (1 week), and 336 h (2 weeks), respectively. Traces are offset for clarity, and each histogram represents approximately 30,000 events.

Discussion

Flow cytometric detection of subvisible particles

Laser diffraction particle size analysis of each of the stock silicone oil emulsions exhibited a bimodal particle size distribution, with a population of particles of a size near 100 nm and another population from 1 to 10 μm (Fig. 1). Despite the bimodal distribution of silicone oil droplets measured using laser diffraction (Fig. 1), the majority of flow cytometry analyses resulted in a single distribution of particles. A likely reason for this discrepancy is that the FACSCalibur (along with most other commercially available flow cytometers) was designed primarily for intact cell analyses. In most cell preparations, submicron particles consist mostly of debris and therefore are irrelevant; as a result, nanometer-sized particles, such as the smaller size distribution of silicone oil droplets, tend to fall below the instrument's lower size limit of detection [19]. This lack of sensitivity to smaller particles illustrates one of the drawbacks encountered using standard commercially available flow cytometry instruments for the detection and study of subvisible particles. This apparent limitation in most cell-based standard instruments can be overcome by using a modified optical design for the purpose of detecting very small particles, and particles as small as 1 nm have been analyzed using flow cytometry [28].

Most flow cytometry instruments can detect micron-sized subvisible particles (Fig. 2). DLS determined the homogeneous protein aggregates from the stock BSA-AF 647 suspension to be 1.8 μm . Whereas particles of this size border on the threshold of detection for most commercially available flow cytometers, the FACSCalibur used for this study appeared to efficiently detect the protein aggregate particles (Fig. 2A).

Although the detection of subvisible homogeneous protein aggregates or protein adsorbed to silicone oil droplets separately is straightforward, resolution of a mixture of these particles can be difficult. The ability of a flow cytometer to detect and resolve particles by fluorescence is largely dependent on the fluorescence detection efficiency of the detector, optical background, and electronic noise [29]. To ensure optimal performance, the instrument must be properly characterized and proper quality control procedures must be employed to verify performance. (For a detailed explanation of instrument characterization, refer to the BD Biosciences webinar in Ref. [30].) Another consideration when maximizing sensitivity is the number of events being analyzed per second (event count rate). Although most modern flow cytometers are capable of counting thousands of events per second, experiments

run with lower flow and count rates are more likely to avoid particle coincidence, resulting in better resolution.

Silicone oil effects on protein formulation stability

For all of the formulations studied, there was no evidence of homogeneous protein aggregates. All of the detected particles exhibited both AF 647 and BODIPY fluorescence, demonstrating that the particles consisted of both protein and silicone oil. A likely reason for these findings is slow desorption kinetics of protein from the silicone oil-water interface. Studies have shown that whereas protein adsorption at liquid interfaces is thermodynamically reversible, the slow desorption kinetics would make it appear to be an irreversible process [31,32], a claim supported by the experimental results of this study and previous work [18].

Closer inspection of plots of AF 647 versus BODIPY fluorescence revealed more detailed information about the relationship between the protein and silicone oil droplets. Because BODIPY was dispersed uniformly throughout the silicone oil, BODIPY fluorescence intensity is expected to be proportional to the silicone oil volume. In contrast, AF 647 fluorescence intensity is proportional to the amount of protein on the surface of silicone oil droplets. Thus, assuming spherical droplets with uniform protein coatings, the slope of a log-log plot of AF 647 fluorescence intensity versus BODIPY fluorescence intensity is expected to exhibit a slope of 2/3. Alternatively, the slope of a log-log plot of AF 647 fluorescence intensity versus BODIPY fluorescence intensity would approach 1 if small oil droplets coalesced to form larger droplets without desorbing their respective protein layers. Data from flow cytometry analyses were exported to Microsoft Excel, and a linear regression was performed on log-log plots of AF 647 fluorescence intensity versus BODIPY fluorescence intensity. A representative linear regression of one of these analyses is shown in Fig. 9, and all of the analyses are summarized in Table 1.

For samples of BSA-AF 647 or abatacept-AF 647 mixed with silicone oil-BODIPY emulsions in excipient-free formulations, linear regression analyses resulted in slopes near 2/3, consistent with protein adsorption onto the silicone oil droplet surface. Similar analyses of samples containing 250 mM sucrose resulted in slopes

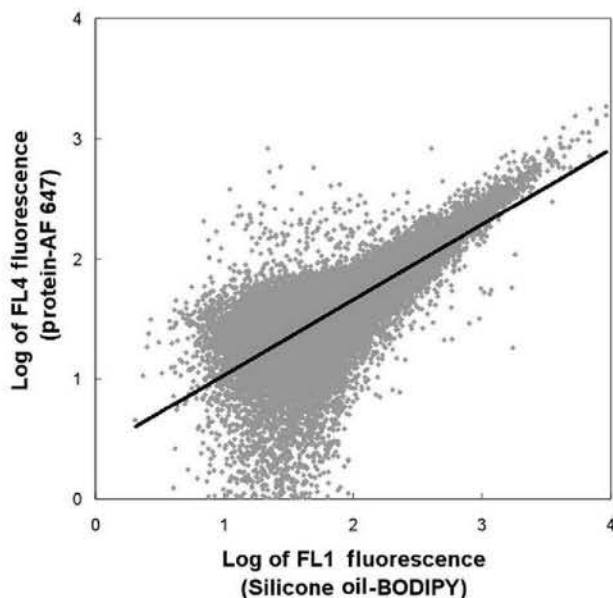


Fig. 9. Slope analysis of BSA-AF 647 adsorbed onto silicone oil-BODIPY droplets. A slope of 0.65 was calculated from a linear regression of a log-log plot of FL4 fluorescence (protein-AF 647) versus FL1 fluorescence (silicone oil-BODIPY).

Table 1
Relationship between AF 647 and BODIPY fluorescence.

Protein	Formulation additive			
	Excipient-free	0.03% polysorbate 20	150 mM sodium chloride	250 mM sucrose
Lysozyme–AF 647	0.50 ± 0.14	0.51 ± 0.11	0.79 ± 0.09	0.47 ± 0.08
BSA–AF 647	0.66 ± 0.05	0.44 ± 0.07	0.73 ± 0.05	0.59 ± 0.02
Abatacept–AF 647	0.68 ± 0.03	0.47 ± 0.06	0.74 ± 0.04	0.61 ± 0.03
Trastuzumab–AF 647	0.78 ± 0.03	0.33 ± 0.18	0.84 ± 0.02	0.80 ± 0.05

Note: The relationship is illustrated by the slope calculated from linear regressions of log–log plots of the FL1 fluorescence (silicone oil–BODIPY) versus FL4 fluorescence (protein–AF 647). Each value represents the average slope of 18 different linear regressions (three replicate samples for each of six time points). The reported values are means ± standard deviations.

slightly lower than 2/3, whereas the slopes calculated for samples containing 150 mM sodium chloride were slightly higher than 2/3.

For lysozyme–AF 647 formulations mixed with silicone oil–BODIPY emulsions, the slope of a plot of the logarithm of the AF 647 fluorescence plotted versus the logarithm of the BODIPY fluorescence was lower than 2/3 for formulations containing no additives or 250 mM sucrose. Likewise, formulations containing polysorbate 20 showed slopes lower than 2/3. Thus, for these formulations, the apparent protein surface coverage of the larger particles, when normalized by the volume of silicone oil, was less than that of the smaller particles. The cause of this phenomenon remains unclear.

We used light microscopy to probe whether flocculation might explain the behavior of trastuzumab–AF 647/silicone oil–BODIPY formulations where we observed relatively high slopes of log–log plots of AF 647 fluorescence versus BODIPY fluorescence. Particles were imaged using an Eclipse TE2000-S inverted optical microscope (Nikon Instruments, Melville, NY, USA) with a CoolSNAP ES charge-coupled device (CCD) camera (Photometrics, Tucson, AZ, USA). Images of particles from lysozyme–AF 647, BSA–AF 647, and abatacept–AF 647 formulations mixed with silicone oil–BODIPY emulsions showed separated individual droplets, whereas images of particles from the trastuzumab–AF 647 formulation mixed with silicone oil–BODIPY droplets showed large floccules of smaller droplets (Fig. 10). Thus, droplet flocculation is a plausible explanation for slopes higher than 2/3 observed for trastuzumab formulations (Table 1).

Fluorescent labels

For multicolor flow cytometry analyses, the choice of fluorescent labels warrants some consideration because most fluorescent materials emit over a fairly broad range of wavelengths. Although a fluorescent label may have an emission maximum near or in the range of a specific flow cytometry detector, the possibility remains that the label will also emit in the range of another detector. For example, 9-diethylamino-5H-benzo[α]phenoxazine-5-one (Nile red) dye is a polarity-sensitive fluorophore used to probe hydrophobic surfaces [33]. With its ability to be excited using a 488-nm laser (standard for most flow cytometers) and its emission maximum of 628 nm [34] (suitable for the FL2 585/42 BD FACSCalibur detector), Nile red would seem to be an ideal dye with which to stain silicone oil for flow cytometry analysis. However, Nile red has a broad emission spectrum that ranges from less than 600 nm to more than 700 nm depending on the environment. The spillover of the Nile red fluorescence emission into other detectors can lead to decreased sensitivity and improper data interpretation by significantly increasing the optical background.

Fig. 11 shows an example of Nile red fluorescence spillover into the FL1 530/30 detector often used to detect fluorescein isothiocyanate (FITC)- or Alexa Fluor 488 (AF 488)-conjugated materials. Even though the sample contained no FITC or AF 488 fluorophores, the FL1 detector registers a considerable signal because of the spillover of Nile red fluorescence. If an experiment were performed using a protein labeled with AF 488 and silicone oil stained with

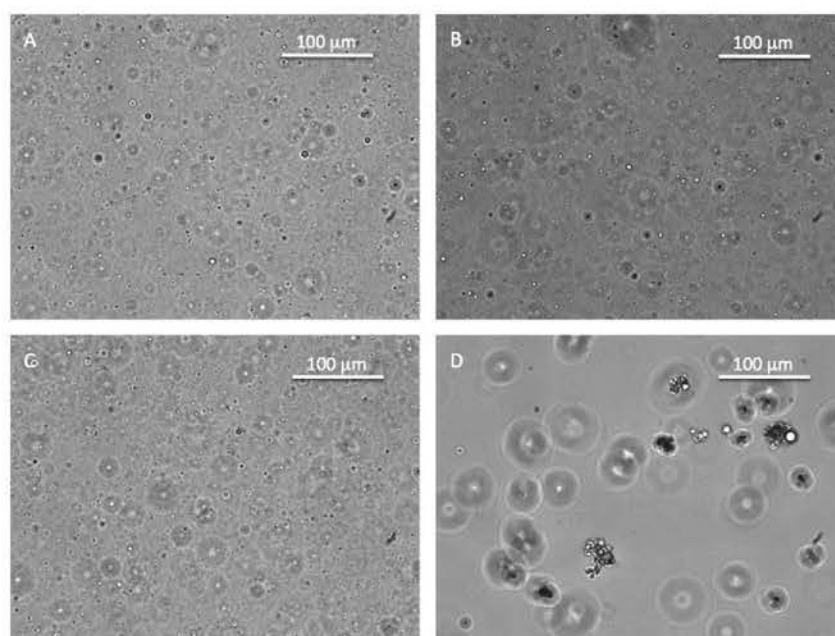


Fig. 10. Light microscopy images of silicone oil–BODIPY droplets coated with lysozyme–AF 647 (A), BSA–AF 647 (B), abatacept–AF 647 (C), and trastuzumab–AF 647 (D) at 200× magnification.

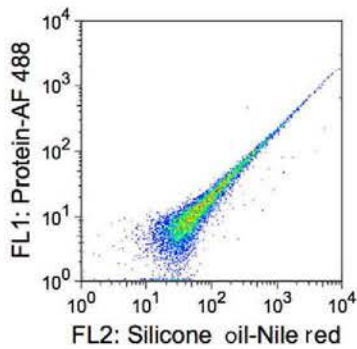


Fig. 11. Spectral overlap of Nile red fluorescence from the FL2 detector into the FL1 detector on a BD FACScan instrument (Becton Dickinson, San Jose, CA, USA). The sample consisted of only silicone oil–Nile red droplets. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nile red, interpretation of the FL1 data would be difficult or impossible because Nile red contributes enormous optical background in FL1 and would dramatically decrease the sensitivity to any AF 488-labeled protein. Flow cytometry analysis software does have the ability to compensate (correct) for spillover. Good general practice for flow cytometry experiments is to eliminate or minimize spillover whenever possible because compensation essentially translocates population medians while preserving the measured variance, which in this case would be large. Therefore, large optical background contributions result in much larger population coefficients of variation (broad populations) after compensation, and it becomes desirable to pick fluorescent labels with emissions that have minimal overlap for multicolor flow cytometry experiments. This is not difficult with the multiple excitation and detection capabilities of modern instruments. For this work, AF 647 and BODIPY 493/503 were adequate fluorescent label choices for flow cytometry analysis because neither label's fluorescence contributes significant optical background into the other's detector (Fig. 12).

For this study, fluorescent labeling was used to aid in the characterization of particles as either homogeneous protein aggregates or silicone droplets with adsorbed protein. However, conclusions from experiments of fluorescently labeled systems can have limitations, particularly when applying findings to the unlabeled systems. Labeling a molecule with a fluorescent marker modifies the properties of the molecule, and this may change intra- and intermolecular interactions.

For example, Alexa Fluor dyes carry a negative charge [35]; therefore, labeling a protein with one or more Alex Fluor molecules results in a molecule with a lower charge, and this could change electrostatic interactions. In this study, mixtures of trastuzumab–AF 647 with silicone oil–BODIPY emulsions in excipient-free formulations exhibited behavior consistent with flocculation. How-

ever, flocculation was not observed in previous work using unlabeled trastuzumab and unlabeled emulsion [18]. A likely reason for this discrepancy is modulation of electrostatic interactions due to fluorescent labeling. At the formulation pH used for both studies, trastuzumab would be expected to have a positive charge ($pI = 9.2$) [36]. Labeling the protein with AF 647 decreases the molecular charge, thereby dampening electrostatic repulsion, and this, subsequent to adsorption onto the surface of silicone oil droplets, may allow droplet flocculation. Attempts to measure the zeta potential of particles in fluorescently labeled systems were unsuccessful because the light source wavelength (633 nm) used by commercially available instruments also excites the AF 647 fluorophore.

One option to avoid these complications is to use intrinsic system properties for characterization analysis. Forward angle light scatter is strongly influenced by particle size and refractive index, whereas side scattering, in addition to being size related, tends to emphasize particle granularity or internal particle structure [28]. Previous work has shown the ability of flow cytometry to resolve populations of granulocytes, monocytes, and lymphocytes without the use of fluorescent labels [28].

For this work, a plot of FSC versus SSC for the mixture of the agitated sample containing BSA–AF 647 aggregates and silicone–BODIPY droplets with adsorbed BSA–AF 647 (nonagitated) resulted in two populations of particles (Fig. 13). These particles were characterized as homogeneous protein aggregates or silicone oil droplets with adsorbed protein based on gates from AF 647 fluorescence versus BODIPY fluorescence dot plots (Fig. 2C). Although the populations are not as well resolved as the corresponding groups seen in the fluorescence dot plot of the same sample (Fig. 2C), the scatter plot does illustrate the possibility of resolving particles without the use of extrinsic properties.

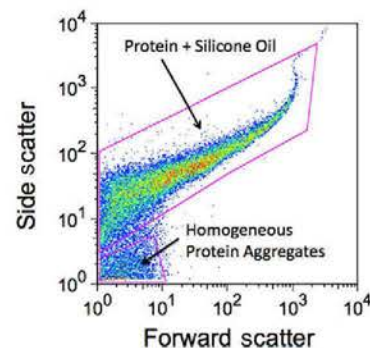


Fig. 13. Light scatter dot plot of a mixture of homogeneous protein aggregates and silicone oil droplets with adsorbed protein. Light scattering measured at 90° (SSC) is plotted against low-angle light scattering (FSC) for the sample whose fluorescence is plotted in Fig. 2.

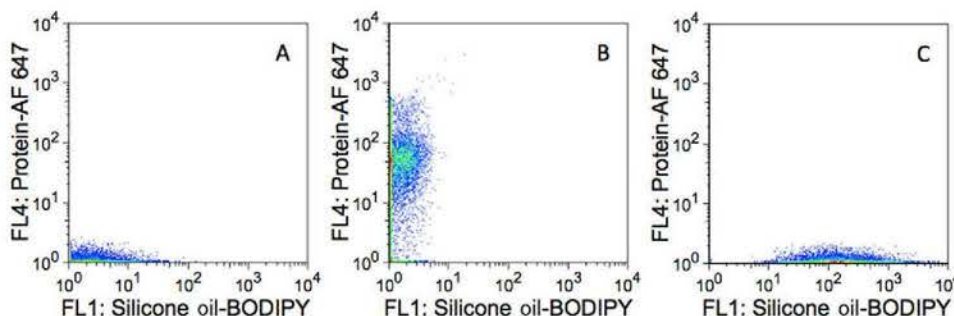


Fig. 12. Tests for spectral emission overlap (optical spillover): Fluorescence dot plots of samples of unlabeled silicone oil and unlabeled protein (A), unlabeled silicone oil and BSA–AF 647 (B), and silicone oil–BODIPY droplets with unlabeled BSA (C).

Conclusion

This study has demonstrated the utility of flow cytometry as an analytical tool for the study of subvisible particles in protein formulations. In a matter of seconds, flow cytometry can measure the optical properties of thousands of different particles, making it a high-throughput technique for the study of particle suspensions. Furthermore, flow cytometry can provide insight into how formulation additives affect protein–silicone oil interactions, making it a potentially useful formulation screening tool.

Acknowledgments

We thank Brent Palmer and Michelle Dsouza from the Clinical Immunology Flow Cytometry/Cell Sorting facility at the University of Colorado at Denver for their assistance while using their facilities. Funding provided by NIH T32 GM008732 (to DBL) and Becton Dickinson & Co.

References

- [1] J.F. Carpenter, T.W. Randolph, W. Jiskoot, D.J. Crommelin, C.R. Middaugh, G. Winter, Y.X. Fan, S. Kirshner, D. Verthelyi, S. Kozlowski, K.A. Clouse, P.G. Swann, A. Rosenberg, B. Cherney, Overlooking subvisible particles in therapeutic protein products: gaps that may compromise product quality, *J. Pharm. Sci.* 98 (2009) 1201–1205.
- [2] B.A. Kerwin, M.J. Akers, I. Apostol, C. Moore-Einsel, J.E. Etter, E. Hess, J. Lippincott, J. Levine, A.J. Mathews, P. Revilla-Sharp, R. Schubert, D.L. Looker, Acute and long-term stability studies of deoxy hemoglobin and characterization of ascorbate-induced modifications, *J. Pharm. Sci.* 88 (1999) 79–88.
- [3] A. Hawe, W. Friess, Stabilization of a hydrophobic recombinant cytokine by human serum albumin, *J. Pharm. Sci.* 96 (2007) 2987–2999.
- [4] A.K. Tyagi, T.W. Randolph, A. Dong, K.M. Maloney, C. Hitscherich Jr., J.F. Carpenter, IgG particle formation during filling pump operation: a case study of heterogeneous nucleation on stainless steel nanoparticles, *J. Pharm. Sci.* 98 (2009) 94–104.
- [5] U.S. Pharmacopeia, <788> Particulate Matter in Injections, U.S. Pharmacopeia–National Formulary, 2006.
- [6] E.Y. Chi, S. Krishnan, B.S. Kendrick, B.S. Chang, J.F. Carpenter, T.W. Randolph, Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor, *Protein Sci.* 12 (2003) 903–913.
- [7] E.Y. Chi, J. Weickmann, J.F. Carpenter, M.C. Manning, T.W. Randolph, Heterogeneous nucleation-controlled particulate formation of recombinant human platelet-activating factor acetylhydrolase in pharmaceutical formulation, *J. Pharm. Sci.* 94 (2005) 256–274.
- [8] E. Koren, L.A. Zuckerman, A.R. Mire-Sluis, Immune responses to therapeutic proteins in humans: clinical significance, assessment, and prediction, *Curr. Pharm. Biotechnol.* 3 (2002) 349–360.
- [9] J. Li, C. Yang, Y. Xia, A. Bertino, J. Glaspy, M. Roberts, D.J. Kuter, Thrombocytopenia caused by the development of antibodies to thrombopoietin, *Blood* 98 (2001) 3241–3248.
- [10] H. Schellekens, Immunogenicity of therapeutic proteins: clinical implications and future prospects, *Clin. Ther.* 24 (2002) 1720–1740. discussion on p. 1719.
- [11] A. Fries, Drug delivery of sensitive biopharmaceuticals with prefilled syringes, *Drug Deliv. Technol.* 9 (2009) 22–27.
- [12] S. Varaprath, C.L. Frye, J. Hamelink, Aqueous solubility of permethylsiloxanes (silicones), *Environ. Toxicol. Chem.* 15 (1996) 1263–1265.
- [13] E.A. Chantelau, M. Berger, Pollution of insulin with silicone oil, a hazard of disposable plastic syringes, *Lancet* 1 (1985) 1459.
- [14] E. Chantelau, M. Berger, B. Bohlken, Silicone oil released from disposable insulin syringes, *Diabetes Care* 9 (1986) 672–673.
- [15] R.K. Bernstein, Clouding and deactivation of clear (regular) human insulin: association with silicone oil from disposable syringes?, *Diabetes Care* 10 (1987) 786–787.
- [16] L.S. Jones, A. Kaufmann, C.R. Middaugh, Silicone oil induced aggregation of proteins, *J. Pharm. Sci.* 94 (2005) 918–927.
- [17] R. Thirumangalathu, S. Krishnan, M.S. Ricci, D.N. Brems, T.W. Randolph, J.F. Carpenter, Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution, *J. Pharm. Sci.* 9 (2009) 3167–3181.
- [18] D.B. Ludwig, J.F. Carpenter, J.B. Hamel, T.W. Randolph, Protein adsorption and excipient effects on kinetic stability of silicone oil emulsions, *J. Pharm. Sci.* 99 (2010) 1721–1733.
- [19] H.M. Shapiro, *Practical Flow Cytometry*, Wiley–Liss, New York, 1995.
- [20] Becton Dickinson, *Introduction to Flow Cytometry Web-Based Training*, Becton Dickinson, 2005.
- [21] Bristol–Myers Squibb, Oencia [product insert], Bristol–Myers Squibb, Princeton, NJ, 2005.
- [22] Genentech, Herceptin [product insert], Genentech, San Francisco, CA, 2005.
- [23] K. Hamaguchi, A. Kurono, Structure of muramidase (lysozyme): I. The effect of guanidine hydrochloride on muramidase, *J. Biochem.* 54 (1963) 111–122.
- [24] T. Peters, *All about Albumin: Biochemistry, Genetics, and Medical Applications*, Academic Press, San Diego, 1995.
- [25] Bristol–Myers Squibb, Oencia [product monograph], Bristol–Myers Squibb Canada, Montreal, 2009.
- [26] A. Cirstoiu-Hapca, L. Bossy-Nobs, F. Buchegger, R. Gurny, F. Delie, Differential tumor cell targeting of anti-HER2 (Herceptin) and anti-CD20 (Mabthera) coupled nanoparticles, *Int. J. Pharm.* 331 (2007) 190–196.
- [27] Dow Corning, Dow Corning 360 Medical Fluid [product information], Dow Corning, Midland, MI, 2009.
- [28] L.S. Cram, Flow cytometry: an overview, *Methods Cell Sci.* 24 (2002) 1–9.
- [29] J.C. Wood, Fundamental flow cytometer properties governing sensitivity and resolution, *Cytometry* 33 (1998) 260–266.
- [30] J. Trotter, The digital flow cytometer: performing instrument characterization for optimal setup, in: *BD Biosciences Webinar Series*, Becton Dickinson, 2006.
- [31] E. Dickinson, D.J. McClements, *Advances in Food Colloids*, Blackie Academic & Professional, London, 1996.
- [32] V.B. Fainerman, R. Miller, J.K. Ferri, H. Watzke, M.E. Leser, M. Michel, Reversibility and irreversibility of adsorption of surfactants and proteins at liquid interfaces, *Adv. Colloid Interface Sci.* 123–126 (2006) 163–171.
- [33] M.M. Davis, H.B. Hetzer, Titrimetric and equilibrium studies using indicators related to Nile blue A, *Anal. Chem.* 38 (1966) 451–461.
- [34] H. Du, R.C.A. Fuh, J.Z. Li, L.A. Corkan, J.S. Lindsey, PhotochemCAD: a computer-aided design and research tool in photochemistry, *Photochem. Photobiol.* 68 (1998) 141–142.
- [35] N. Panchuk-Voloshina, R.P. Haugland, J. Bishop-Stewart, M.K. Bhalgat, P.J. Millard, F. Mao, W.Y. Leung, Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates, *J. Histochem. Cytochem.* 47 (1999) 1179–1188.
- [36] H. Wiig, C.C. Gyenge, O. Tenstad, The interstitial distribution of macromolecules in rat tumours is influenced by the negatively charged matrix components, *J. Physiol.* 567 (2005) 557–567.

Appendix 3A

Expert opinion on investigational drugs.
v. 18, no. 10 (Oct. 2009)
General Collection
W1 EX52M
2009-10-20 09:53:24

Expert Opinion

on Investigational Drugs

EDITORIAL

- Phospholipase A2 inhibitors in the treatment of atherosclerosis: a new approach moves forward in the clinic
Keith E Suckling

DISCONTINUED DRUGS PERSPECTIVE

- Discontinued psychiatric drugs in 2008
Victoria D Kirchoff, Ha TT Nguyen, Joanna K Soczynska, Hanna O Woldeyohannes & Roger S McIntyre

REVIEWS

- Investigational drugs targeting FLT3 for leukemia
Celalettin Ustun, David L DeRemer, Anand P Jillella & Kapil N Bhalla
- Targeting IL-6 in the treatment of inflammatory and autoimmune diseases
Changhai Ding, Flavia Cicuttini, Jun Li & Graeme Jones
- Current data of targeted therapies for the treatment of triple-negative advanced breast cancer: empiricism or evidence-based?
Fausto Petrelli, Mary Cabiddu, Mara Ghilardi & Sandro Barni
- Current progress in the pharmacological therapy of fibromyalgia
Srinivas G Rao
- Glucose lowering and anti-atherogenic effects of incretin-based therapies: GLP-1 analogues and DPP-4-inhibitors
Manfredi Rizzo, Ali A Rizvi, Giatgen A Spinas, Giovam Battista Rini & Kaspar Bernels
- Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease
Rosanne M Seguin & Nicolay Ferrari
- Histamine H₂ and H₁ receptors as novel drug targets
Ekaterini Tiligada, Evangelia Zampeli, Kerstin Sander & Holger Stark

DRUG EVALUATIONS

- Agomelatine
Steven L Dubovsky & Calvert Warren
- AVE8062
Angelo Delmonte & Cristiana Sessa
- Cediranib
Jörg Dietrich, Daphne Wang & Tracy T Batchelor
- Clofarabine
Keeran Sampat, Hagop Kantarjian & Gautam Borthakur
- Fospropofol disodium
Jörg Fechner, Harald Ihmsen, Christian Jelezscov & Jürgen Schüttler
- VEGF Trap-Eye
James A Dixon, Scott CN Oliver, Jeffrey L Olson & Naresh Mandava

Expert Opinion on Investigational Drugs is indexed by Chemical Abstracts (CODEN: EOIDER), Current Contents/Life Sciences, EMBASE/Excerpta Medica, Index Medicus/Medline, ISI Alerting Services and Science Citations Index-Expanded. 2008 ISI Impact Factor 4.058

PROPERTY OF THE
NATIONAL
LIBRARY OF
MEDICINE



informa
PHARMACEUTICAL SCIENCE

Expert Opinion on Investigational Drugs

Forthcoming articles

- Investigational HDAC8 inhibitors for neuroblastoma
- Novel investigational Adenosine A2A receptor antagonists for Parkinson's disease
- Investigating the role of platelet derived growth factor (PDGF) as a potential drug therapy in bone formation and fracture healing.
- Monotherapy versus combination therapy for the treatment of chronic hepatitis B
- Discontinued oncology drugs 2008
- Lurasidone
- Vilazodone, a novel dual-acting serotonergic antidepressant for managing major depression

Editorial

- 1425 Phospholipase A2 inhibitors in the treatment of atherosclerosis: a new approach moves forward in the clinic
KE Suckling

Discontinued Drugs Perspective

- 1431 Discontinued psychiatric drugs in 2008
VD Kirchoff, HTT Nguyen, JK Soczynska, HO Woldeyohannes & RS McIntyre

Reviews

- 1445 Investigational drugs targeting FLT3 for leukemia
C Ustun, DL DeRemer, AP Jillella & KN Bhalla
- 1457 Targeting IL-6 in the treatment of inflammatory and autoimmune diseases
C Ding, F Cicuttini, J Li & G Jones
- 1467 Current data of targeted therapies for the treatment of triple-negative advanced breast cancer: empiricism or evidence-based?
F Petrelli, M Cabiddu, M Ghilardi & S Barni
- 1479 Current progress in the pharmacological therapy of fibromyalgia
SG Rao
- 1495 Glucose lowering and anti-atherogenic effects of incretin-based therapies: GLP-1 analogues and DPP-4-inhibitors
M Rizzo, AA Rizvi, GA Spinaz, GB Rini & K Berneis

- 1505 Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease
RM Séguin & N Ferrari
- 1519 Histamine H₃ and H₄ receptors as novel drug targets
E Tiligada, E Zampeli, K Sander & H Stark

Drug Evaluations

- 1533 Agomelatine, a melatonin agonist with antidepressant properties
SL Dubovsky & C Warren
- 1541 AVE8062: a new combretastatin derivative vascular disrupting agent
A Delmonte & C Sessa
- 1549 Cediranib: profile of a novel anti-angiogenic agent in patients with glioblastoma
J Dietrich, D Wang & TT Batchelor
- 1559 Clofarabine: emerging role in leukemias
K Sampat, H Kantarjian & G Borthakur
- 1565 Fospropofol disodium, a water-soluble prodrug of the intravenous anesthetic propofol (2,6-diisopropylphenol)
J Fechner, H Ihmsen, C Jeleazcov & J Schüttler
- 1573 VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration
JA Dixon, SCN Oliver, JL Olson & N Mandava

informa
healthcare

Expert Opinion on Investigational Drugs is grateful and indebted to the reviewers of all the above articles

2009 © Informa UK Ltd ISSN 1354-3784
This material was copied
at the NLM and may be
Subject US Copyright Laws

Regeneron Exhibit 1033.083

Editorial Office

Informa Healthcare, Telephone House,
69-77 Paul Street, London, EC2A 4LQ, UK

Tel: +44 (0)20 7017 7650
Fax: +44 (0)20 7017 7667
E-mail: expertopin@informa.com
Web: www.expertopin.com

Commissioning Editor: Fizah Khan
Production Editor: Sumayya Patel
Original Research Editor: Elizabeth Knowles
Drug Evaluations Editor: Elizabeth Knowles
Publisher: Anna Heinink

Editor-in-Chief: Dimitri P Mikhailidis
MSc, MD, FACB, FACA, FPPM, FRCP, FRCPath
Royal Free Hospital and University College
Medical School, London, UK

Senior Advisory Panel

Chairman: Fitzgerald JD: Matera Medica, UK
Abou-Gharbia M: Wyeth-Ayerst, USA
Baldwin JJ: Pharmacopeia, USA
Buckel P: Boehringer Mannheim, Germany
Duncan WAM: RW Johnson, USA
Evans C: Chiroscience, UK
Gillis S: Corixa, USA
Harrap K: Inst. Cancer Research, UK
Hoyer D: Novartis, Switzerland
Humphrey PPA: GlaxoSmithKline, UK
Krogsgaard Thomsen M: Novo, Denmark
Moncada S: The Cruciform Project, UK
Morich F-J: Bayer, Germany
Okazaki H: Takeda, Japan
Paioni R: Novartis, Switzerland
Poste G: Health Technology Networks, USA
Ringrose PS: Bristol-Myers Squibb, USA
Ross BC: GlaxoSmithKline, UK
Setoyama O: Chugai, Japan
Sugino Y: Takeda, Japan
Timmermanns P: DuPont, USA
Weston A: Univ. Manchester, UK

Section Editorial Board

Pulmonary-Allergy, Dermatological, Gastrointestinal & Arthritis

Bacon K, Braddock M, Farthing MJ, Foster M,
Norris A, Richards IM, Roomans GM, Snell N,
Taylor PC, Warne P

Anti-infectives

Blondeau J, Bryskier A, Buckheit R, Chopra I,
Clerici M, Georgopapadakou N, Neamati N,
Ryder N, Turik M

Endocrine & Metabolic

Brandi ML, Colca J, Dujovne C,
Fuller P, Kowluru R, McCormack J, Proietto J,
Whitfield J

Central & Peripheral Nervous Systems

Grundemar L, Gurwitz D, Hill RG, Kloog Y,
Palacios JM, Panetta JA, Schachter S,
Tamminga C, Tariot P, Wood M

Cardiovascular & Renal

Barton M, Danser AHJ, Herbert J-M,
Holvoet P, Papatsoris AG, Ruffolo RR,
von Haehling S, Winkelmann BR

Oncologic

Denny WA, Kelland L, Koutsilieris M,
Papatsoris AG, Papavassiliou AG, Sawyer TK,
Udayan D, Williams R, Zhang WW

Expert Opinion on Investigational Drugs

Aims and scope

Expert Opinion on Investigational Drugs is a MEDLINE-indexed, peer-reviewed, international journal publishing review articles and original papers on drugs in preclinical and early stage clinical development, providing expert opinion on the scope for future development.

The Editors welcome:

- *Reviews* covering preclinical through to Phase II data on drugs or drug classes for specific indications, and their potential impact on future treatment strategies
- *Drug Evaluations* reviewing the clinical and pharmacological data on a particular drug
- *Original Research* papers reporting the results of clinical investigations on agents that are in Phase I and II clinical trials

The audience consists of scientists, managers and decision-makers in the pharmaceutical industry, and others closely involved in R&D.

Editorial policy

Articles published in *Expert Opinion on Investigational Drugs* are commissioned by the Editor in collaboration with the Section Editorial Board. Authors who wish to contribute should contact the Editor. All reviews and drug evaluations are subject to peer-review.

Editorial boards

The Senior Advisory Panel and Section Editorial Board are composed of Senior Scientists involved in drug research and development. The Senior Advisory Panel is responsible for the development of the journal, while the Section Editors are responsible for selecting authors and topics for review to ensure comprehensive coverage of subjects in each therapeutic area.

Citations

Expert Opinion on Investigational Drugs is indexed by:

Chemical Abstracts (CODEN EOIDER) Current Contents®/Life Sciences
EMBASE/Excerpta Medica ISI Alerting Services (2008 Impact Factor 4.058)
Index Medicus/Medline Science Citations Index-Expanded

Copyright and disclaimer

Conditions of sale - *Expert Opinion on Investigational Drugs* may be circulated only to those members of staff who are employed at the site at which the subscription is taken out. Readers are reminded that, under internationally agreed copyright legislation, photocopying of copyright materials is prohibited other than on a limited basis for personal use. This means that making copies of any article published in *Expert Opinion on Investigational Drugs* is a breach of the law and can be prosecuted.

Whilst every effort is made by the publishers and advisory board to ensure that no inaccurate or misleading data, opinions or statements appear in this journal, they wish to make it clear that the data and opinions appearing herein are the responsibility of the contributor concerned. Accordingly, the publishers, advisory board, editors and their respective employees, officers and agents accept no liability whatsoever for the consequences of any inaccurate or misleading data, opinions or statements. Approved product information should always be reviewed prior to prescribing.

Expert Opinion

1. Introduction
2. Background
3. Conclusion
4. Expert opinion

VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration

James A Dixon, Scott CN Oliver[†], Jeffrey L Olson & Naresh Mandava
*University of Colorado Denver, Rocky Mountain Lions Eye Institute, Department of Ophthalmology,
1675 North Aurora Court, PO Box 6510, Mail Stop F-731, Aurora, CO 80045-2500, USA*

Background: Age-related macular degeneration (AMD) affects > 14 million individuals worldwide. Although 90% of patients with AMD have the dry form, neovascular AMD accounts for the vast majority of patients who develop legal blindness. Until recently, few treatment options existed for treatment of neovascular AMD. The advent of anti-VEGF therapy has significantly improved the safe and effective treatment of neovascular AMD. In addition to two anti-VEGF drugs currently in widespread use, ranibizumab and bevacizumab, a number of medications that interrupt angiogenesis are currently under investigation. One promising new drug is aflibercept (VEGF Trap-Eye), a fusion protein that blocks all isoforms of VEGF-A and placental growth factors-1 and -2. **Objective:** To review the current literature and clinical trial data regarding VEGF Trap-Eye for the treatment of neovascular AMD. **Methods:** Literature review. **Results/conclusion:** VEGF Trap-Eye is a novel anti-VEGF therapy, with Phase I and II trial data indicating safety, tolerability and efficacy for the treatment of neovascular AMD. Two Phase III clinical trials (VIEW-1 and VIEW-2) comparing VEGF Trap-Eye to ranibizumab are currently continuing and will provide vital insight into the clinical applicability of this drug.

Keywords: aflibercept, AMD, angiogenesis, neovascularization, VEGF, VEGF inhibition, VEGF Trap

Expert Opin. Investig. Drugs (2009) 18(10):1573-1580

1. Introduction

Age-related macular degeneration (AMD) affects > 1.75 million individuals in the US and it is estimated that by 2020 this number will increase to almost 3 million [1]. Worldwide, AMD is estimated to affect 14 million people [2]. While the vast majority of patients suffering from AMD have the dry form, ~ 80 – 90% of patients who develop severe vision loss have the neovascular or 'wet' form of the disease [3]. Until recently, healthcare professionals had few options when it came to treating neovascular AMD. For many years, subfoveal choroidal neovascularization (CNV) was treated with argon laser therapy according to guidelines from the Macular Photocoagulation Study [4-12]. This treatment, in the setting of subfoveal disease, was unsatisfactory for a number of reasons, including the limited benefits in visual stabilization and the high risk of inducing central vision deficits [13]. Treatment outcomes improved with the introduction of photodynamic therapy (PDT) which utilized a photosensitizing dye (verteporfin) to selectively target CNV. While more efficacious than previous treatments, patients receiving PDT failed to recover vision and continued to experience a decline in visual acuity [14] and the treatment was of questionable cost effectiveness [15].

The more recent development of agents that inhibit VEGF has largely supplanted these previous treatments. The pathogenesis of CNV in the setting of

informa
healthcare

AMD is complex; however, there is overwhelming evidence that VEGF is a predominant mediator in its genesis. VEGF receptors are expressed by a number of important cell types in the eye, including vascular endothelial cells, choroidal fibroblasts, retinal pigment epithelial cells and inflammatory cells attracted by hypoxia [16-19]. Higher levels of VEGF expression have been demonstrated in animal models [20,21] and human studies of eyes with AMD [17,22-24] and antagonism of VEGF in both settings have definitively demonstrated inhibition of neovascularization and vascular permeability. VEGF-A is the predominant member of the VEGF family targeted by drugs currently in widespread use; however, the group is also comprised of VEGF-B, VEGF-C, VEGF-D and placental growth factors-1 and -2.

Systemic administration of bevacizumab is effective against neovascular AMD; however, systemic complications limit its use [25]. Accordingly, all anti-VEGF agents for neovascular AMD are administered only by intravitreal injection. The two largest studies examining anti-VEGF therapy, the MARINA [26] and the ANCHOR [27,28] trials, were randomized, controlled, double-masked Phase III clinical trials that together evaluated monthly ranibizumab for the treatment of all types of neovascular AMD. In both trials, 94% of patients with neovascular AMD lost fewer than 15 letters of visual acuity at 12 and 24 months when treated with ranibizumab. Surprisingly, as many as 40% of patients in the two trials improved by > 15 letters from baseline at 2 years. Ranibizumab received the FDA approval for all types of neovascular AMD in 2006. Based on the results of these two landmark studies, anti-VEGF therapies for neovascular AMD have largely replaced previous treatment modalities.

2. Background

2.1 Overview of the market (unmet needs, competitor compounds/in clinical development)

By far the most commonly used anti-VEGF drugs currently in use for neovascular AMD are ranibizumab and bevacizumab. Pegaptanib was the first anti-VEGF drug approved by the FDA for the treatment of AMD; however, it proved less efficacious than current treatments [13] (possibly due to its selective binding of VEGF-165) and is no longer widely used in most countries. Ranibizumab is the only drug in widespread use currently approved by the FDA for treatment of neovascular AMD and is by far the most extensively studied [26,27,29,30]. It is a recombinant monoclonal antibody fragment with a high binding affinity for all isotypes of VEGF-A. Bevacizumab, currently being used off-label for the treatment of AMD in the US, is a humanized whole antibody to VEGF-A used in oncology regimens that also binds all isotypes of VEGF-A. Although ranibizumab has been shown to have a higher affinity for VEGF-A, it is not clear if ranibizumab has superior efficacy to bevacizumab. Retrospective and small randomized studies have suggested similar efficacy profiles [31,32]. The Comparisons of Age-Related

Macular Degeneration Treatment Trial (CATT) is a 2-year, multi-centered, randomized clinical trial comparing ranibizumab and bevacizumab for neovascular AMD. Enrollment began in February 2008. Despite the off-label status of bevacizumab, it continues to be a popular treatment choice in the US because of the significantly reduced price of treatment (\$ 50 – 100 for bevacizumab versus \$ 2000 for ranibizumab (2008 pricing)).

As previously mentioned, the MARINA [26] and the ANCHOR [27,28] trials examined the efficacy of ranibizumab when administered monthly. The time and financial burden of monthly injections has led to the initiation of studies to examine the efficacy of alternative dosing schedules. In the PIER study [30], patients initially received monthly injections of ranibizumab for 3 months followed by quarterly injections. Although patient visual acuities actually improved at 3 months, during the quarterly dosing segment visual acuity returned to baseline. The PrONTO study [29] looked at as needed (p.r.n.) dosing of ranibizumab after three consecutive monthly doses. The need for further injections was made on the basis of recurrent CNV as evidenced by worsening vision, retinal thickening on ocular coherence tomography (OCT) or abnormalities on fluorescein angiogram (FA). At 2 years of follow up, 78% of patients had maintained vision and vision had improved by > 3 lines in 43% of patients with an average of five injections a year. These later studies seem to indicate that quarterly dosing is associated with poorer outcomes but it may be possible to extend the time between injections if the patient is frequently monitored. However, even with the p.r.n. dosing utilized in the PrONTO study, patients are still required to make monthly visits to the office with frequent and expensive testing.

The development of new drugs for neovascular AMD has thus focused on both improving efficacy and extending duration of action. Most new compounds in development are targeted toward inhibition of various steps in the VEGF signaling pathway. There are a number of drugs in development that inhibit the downstream tyrosine kinase cascade activated by the binding of VEGF with its receptor (VEGFR). Vatalanib is an oral formulation that binds to all three VEGFRs and has recently completed Phase I/II study as adjuvant to PDT and ranibizumab [33]. Topical tyrosine kinase inhibitors currently undergoing Phase II clinical studies include pazopanib [34] and TG100801 [35]. Another approach utilizes siRNA to silence genes which express proteins involved in angiogenesis. Bevasiranib, an siRNA that targets VEGF-A mRNA, showed encouraging Phase I and II data, but the Phase III trial was halted in March 2009 for projected failure to meet the primary end point [36]. An extra antiangiogenic target being developed is pigment epithelium-derived factor (PEDF), a potent inhibitor of new vessel growth. AdGVPEDF.11D uses an adenovector to deliver the PEDF gene to target cells, resulting in the local production of PEDF in the treated eye. AdGVPEDF.11D has recently completed Phase I clinical trials [37]. Another

recently discovered alternative pathway for decreasing angiogenesis involves inhibition of nicotinic acetylcholine receptors. ATG3 (mecamylamine), a topical formulation that inhibits the nicotinic acetylcholine receptors, has shown promising results in animal and Phase I trials and is currently undergoing a Phase II study [25].

2.2 Introduction to compound

VEGF Trap-Eye is a novel anti-VEGF drug currently in commercial development for the treatment of neovascular AMD by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY, USA) in the US and in collaboration with Bayer HealthCare (Leverkusen, Germany) in global markets. Structurally, VEGF Trap-Eye is a fusion protein of key binding domains of human VEGFR-1 and -2 combined with a human IgG Fc fragment (Figure 1). Functionally, VEGF Trap-Eye acts as a receptor decoy with high affinity for all VEGF isoforms, binding more tightly than their native receptors. Unlike anti-VEGF drugs currently in use, VEGF Trap-Eye is designed to inhibit placental growth factors-1 and -2 in addition to all isoforms of VEGF-A.

2.3 Chemistry

VEGF Trap-Eye and aflibercept (the oncology product) have the same molecular structure, but there are substantial differences between the preparation of the purified drug product and their formulations. Both aflibercept and VEGF Trap-Eye are manufactured in bioreactors from industry standard Chinese hamster ovary cells that overexpress the fusion protein. However, VEGF Trap-Eye undergoes further purification steps during manufacturing to minimize risk of irritation to the eye. VEGF Trap-Eye is also formulated with different buffers and at different concentrations (for buffers in common) suitable for the comfortable, non-irritating, direct injection into the eye.

2.4 Pharmacodynamics

The aflibercept dose that is administered in oncology settings is either 4 mg/kg every 2 weeks or 6 mg/kg every 3 weeks, which corresponds to 2 mg/(kg week) with either schedule. The highest intravitreal dose being used in pivotal trials for VEGF Trap-Eye is 2 mg/month, which corresponds to at least a 280-fold lower potential systemic exposure than in the oncology setting. Early trials with aflibercept administered intravenously for AMD indicated that doses of 0.3 mg/kg (21 mg total) were inadequate to fully capture systemic VEGF. Thus, the low intravitreal dose of 2 mg allows for extended blocking of VEGF in the eye, but would be predicted to give negligible systemic activity as it will be rapidly bound to VEGF and inactivated.

2.5 Pharmacokinetics and metabolism

Aflibercept is cleared from circulation through two pathways: by binding to VEGF to form an inactive VEGF–aflibercept complex and by Fc-receptor or pinocytotic mediated pathways

that end in proteolysis, which are presumed to be similar to pathways that metabolize antibodies. At very high doses, free aflibercept has a terminal half-life of ~ 17 days in the circulation. The half-life of human intravitreal doses is unknown. Intravitreal primate doses of ranibizumab have a half-life of ~ 3 days [38]. At low blood levels, clearance of free aflibercept is rapid as a result of binding to VEGF with picomolar affinity [39].

2.6 Clinical efficacy

2.6.1 Phase I

A Phase I, randomized, double-blind, placebo-controlled trial of intravenous aflibercept (oncology formulation) was completed in 25 patients with AMD. Although systemic aflibercept did demonstrate a dose-dependent decrease in retinal thickness, the study was halted due to concerns of dose-dependent toxicity when one patient developed hypertension and another proteinuria [40].

The safety, tolerability and biological activity of intravitreal VEGF Trap-Eye in treatment of neovascular AMD was evaluated in the two-part Clinical Evaluation of Anti-angiogenesis in the Retina-1 (CLEAR-IT-1) study [41]. The first part was a sequential cohort dose-escalation study in which 21 patients were monitored for safety, changes in foveal thickness on OCT, best corrected visual acuity (BCVA) and lesion size on FA for 6 weeks. No adverse systemic or ocular events were noted and visual acuity remained stable or improved ≥ 3 lines in 95% of patients with a mean increase in BCVA of 4.6 letters at 6 weeks [42]. Patients showed substantially decreased foveal thickness [41].

In the second part, 30 patients received a single intravitreal injection of either 0.5 or 4 mg of VEGF Trap-Eye and were followed for 8 weeks. All patients were evaluated for their rates of retreatment, changes in BCVA, foveal thickness as well as change in total lesion size and area of CNV. Patients had ETDRS (Early Treatment of Diabetic Retinopathy Study) BCVA ranging from 20/40 to 20/320 with any angiographic subtype of CNV at baseline. No serious adverse events or ocular inflammation was identified during the study. At 8 weeks, the mean decrease in retinal thickness in the low dose group was 63.7 μm compared to 175 μm for the high dose group. Of the first 24 patients to complete the study, 11 out of 12 patients in the 0.5 mg dose group required retreatment in a median of 64 days, compared with 4 out of 12 in the 4 mg dose group who required retreatment in a median of 69 days [43].

VEGF Trap-Eye has also undergone a small open-label safety study for the treatment of diabetic macular edema (DME) [44]. The drug was administered as a single 4 mg intravitreal injection to five patients with longstanding diabetes and several previous treatments for DME. The single injection resulted in a median decrease of central macular thickness measured by OCT of 79 μm . BCVA increased by 9 letters at 4 weeks and regressed to a 3 letter improvement at 6 weeks.

VEGF Trap-Eye

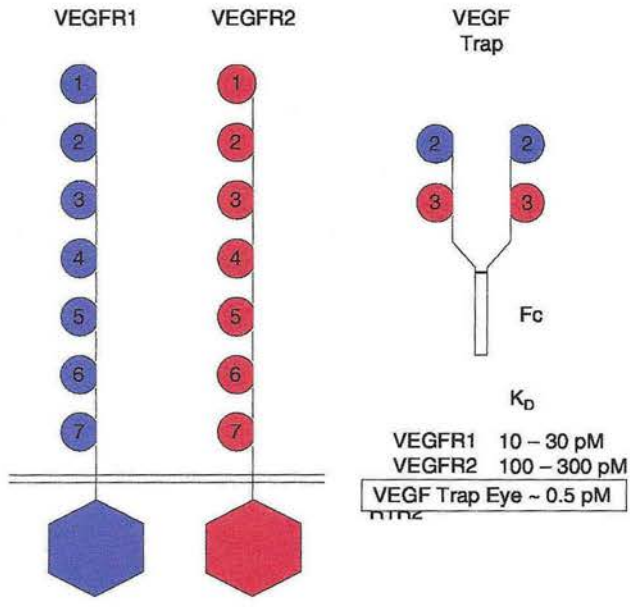


Figure 1. Schematic diagram of VEGF Trap-Eye, a fusion protein of binding domains of VEGF receptors-1 and -2 attached to the Fc fragment of human IgG.

2.6.2 Phase II

CLEAR-IT-2 trial [45] was a prospective, randomized, multi-center, controlled dose- and interval-ranging Phase II trial in which 157 patients were randomized to five dose groups and treated with VEGF Trap-Eye in one eye. The mean age of the group was 78.2 years and all angiographic subtypes of CNV were represented at baseline. The mean ETDRS BCVA in letters at baseline was 56. Two groups received monthly doses of either 0.5 or 2.0 mg for 12 weeks (at weeks 0, 4, 8 and 12) and three groups received quarterly doses of either 0.5, 2.0 or 4.0 mg for 12 weeks (at weeks 0 and 12). Following this fixed dosing period, patients were treated with the same dose of VEGF Trap-Eye on a p.r.n. basis. Criteria for re-dosing included an increase in central retinal thickness of ≥ 100 μm by OCT, a loss of ≥ 5 ETDRS letters in conjunction with recurrent fluid by OCT, persistent fluid as indicated by OCT, new onset classic neovascularization, new or persistent leak on FA or new macular subretinal hemorrhage.

Patients initially treated with 2.0 or 0.5 mg of VEGF Trap-Eye monthly achieved mean improvements of 9.0 ($p < 0.0001$) and 5.4 ($p < 0.085$) ETDRS letters with 29 and 19% gaining, respectively, ≥ 15 ETDRS letters at 52 weeks. During the p.r.n. dosing period, patients initially dosed on a 2.0 mg monthly schedule received an average of 1.6 more injections and those initially dosed on a 0.5 mg monthly schedule received an average of 2.5 injections. The median time to first reinjection in all groups was 110 days and 19% of patients required no more injections at week 52. Patients in these two monthly dosing groups also displayed mean decreases in

retinal thickness versus baseline of 143 μm ($p < 0.0001$) in the 2.0 mg group and 125 μm ($p < 0.0001$) in the 0.5 mg group at 52 weeks as measured by OCT [45].

Patients in the three quarterly dosing groups also showed mean improvements in BCVA and retinal thickness; however, they were generally not as profound as the monthly injection group [45].

2.6.3 Phase III

A two part Phase III trial of VEGF Trap-Eye was initiated in August of 2007. The first part, VIEW 1 (VEGF Trap: Investigation of Efficacy and safety in Wet age-related macular degeneration) [46] will enroll ~ 1200 patients with neovascular AMD in the US and Canada. This non-inferiority study will evaluate the safety and efficacy of intravitreal VEGF Trap-Eye at doses of 0.5 and 2.0 mg administered at 4-week dosing intervals and 2.0 mg at an 8 week dosing interval (following three monthly doses), compared with 0.5 mg of ranibizumab administered every 4 weeks. After the first year of the study, patients will enter a second year of p.r.n. dosing evaluation. The VIEW 2 [47] study has a similar study design and is currently enrolling patients in Europe, Asia Pacific, Japan and Latin America. In both trials, the primary outcome will be the proportion of patients who maintain vision at week 52 (defined as a loss of < 15 ETDRS letters).

2.7 Safety and tolerability

Based on Phase II study data, VEGF Trap-Eye seems to be generally well tolerated with no serious drug-related adverse events. In the 157 patients enrolled in CLEAR-IT 2 trial, there was one reported case of culture-negative endophthalmitis not deemed to be related to the study drug. There were also two deaths (one from pre-existing pulmonary hypertension and one from pancreatic carcinoma) and one arterial thromboembolic event (in a patient with a history of previous stroke) that occurred during the study period, but no serious systemic adverse events were deemed related to VEGF Trap-Eye administration. The most common adverse events reported in the study included conjunctival hemorrhage (38.2%), transient increased intraocular pressure (18.5%), refraction disorder (15.9%), retinal hemorrhage (14.6%), subjective visual acuity loss (13.4%), vitreous detachment (11.5%) and eye pain (9.6%) [45].

3. Conclusion

Anti-VEGF therapy has vastly improved the treatment of neovascular AMD in terms of both safety and efficacy. The ANCHOR [26] and MARINA [27,28] trials have established ranibizumab as an effective therapy when dosed monthly. It has been shown to stabilize vision in 94% of patients and in almost 40% of patients vision will actually improve by 3 or more lines. However, the monthly dosing schedules used in these trials present a financial and time burden to patients and healthcare practitioners. The more recent PIER [30] and

PrONTO [29] trials have shown that ranibizumab is less effective when dosed quarterly, but it may be possible to extend the time between injections when patients are followed closely with frequent examinations and ancillary testing. The most effective dosing regimen and monitoring program for anti-VEGF therapy has yet to be firmly established but new treatments are aimed at extending and improving on the efficacy of ranibizumab. VEGF Trap-Eye differs from established anti-VEGF therapies in its higher binding affinity for VEGF-A and its blockage of placental growth factors-1 and -2. Phase I data demonstrated acceptable safety and tolerability of VEGF Trap-Eye in the treatment of neovascular AMD. In Phase II study data, patients dosed in a similar fashion to the PrONTO trial demonstrated stabilization of their vision that was similar to previous studies of ranibizumab at 1 year. Of the greatest interest, patients dosed at 2.0 mg during the initial monthly dosing period required 1.6 injections on average during the p.r.n. dosing phase. While this number is difficult to compare directly to the number of injections required during the p.r.n. phase of the PrONTO ranibizumab study, it is promising. A direct comparison of the efficacy of VEGF Trap-Eye versus ranibizumab will be possible with the completion of two Phase III trials, the VIEW-1 and -2 studies.

4. Expert opinion

The advent of anti-VEGF therapy for treatment of neovascular AMD has revolutionized therapy for a common blinding disease. Before the development of pegaptanib, ranibizumab and bevacizumab, the diagnosis of neovascular AMD portended a prognosis of nearly universal decline in vision, and frequently loss of useful vision in the affected eye.

Current treatment regimens with either ranibizumab or bevacizumab now afford stabilization of vision in > 90% of patients, with significant vision gain in one-third of all patients treated. There have been no significant, proven adverse systemic effects with the intraocular use of either drug. However, limitations of current therapy include the need for frequent intraocular injections, as often as monthly, without a defined stopping point. Each injection subjects patients to risks of cataract, intraocular inflammation, retinal detachment and endophthalmitis. A significant time and financial burden falls on patients during their treatment course.

Desirable attributes for emerging therapies for neovascular AMD include higher visual improvement rates and decreased dosing intervals. For other indications, time-release delivery methods have met with some success, including the following agents: intraocular steroids, including polymeric fluocinolone and dexamethasone, lasting 3 years and 6 months, respectively [48-50], and for a single biologically active cytokine, ciliary neurotrophic factor, which is released for a period greater than 1 year by encapsulated, bioengineered, implanted cells [51]. While efforts are underway to develop

encapsulated cell technology for sustained-release anti-VEGF therapy, no investigational drugs or devices have progressed yet to clinical trial enrollment.

VEGF Trap-Eye represents the most promising anti-VEGF investigational drug that is currently in Phase III trial. VEGF Trap-Eye, a decoy VEGF receptor protein, binds all isoforms of free VEGF with high affinity, in addition to placental growth factor. In contrast to current anti-VEGF antibodies, which are rapidly cleared, the VEGF-VEGF Trap complex is relatively inert, and is degraded more slowly. Due to its high binding affinity and the ability to safely inject high doses into the eye, VEGF Trap-Eye may have longer duration of effect in the eye. Two Phase III studies in wet AMD, VIEW 1 and VIEW 2, are currently under way and seek to compare monthly ranibizumab to monthly or bimonthly VEGF Trap-Eye.

Data from the Phase II study with VEGF Trap-Eye were positive and the results from the non-inferiority Phase III trials will establish its efficacy versus ranibizumab. Its adoption into clinical practice will depend on efficacy at 4 and 8 week intervals. If effective at 4 week intervals only, VEGF Trap-Eye will be adopted into clinical practice if it offers a competitive price advantage over ranibizumab. If effective at 8 week intervals, VEGF Trap-Eye offers the opportunity to significantly reduce treatment burden on patients and physicians, and would probably find wide acceptance. The second p.r.n. dosing stage of the Phase III trial will also provide insight into whether VEGF Trap-Eye offers longer duration of treatment effectiveness than ranibizumab.

Data from the VIEW-1 and VIEW-2 trials will need to be interpreted by clinicians in the context of emerging adjuvant therapies that may extend the time between anti-VEGF therapy injections. Many clinicians now treat patients with anti-VEGF therapies in combination with verteporfin PDT. Randomized, open-label studies and one large retrospective case series database seem to indicate lower retreatment rates and improved visual outcomes when compared with monotherapy [52-55]. As a result, at least two prospective, randomized trials are currently underway to further examine combination verteporfin PDT and anti-VEGF treatments [56,57]. An extra combination treatment currently under study is the use of epiretinal brachytherapy with Strontium-90 combined with bevacizumab. A recently published small pilot study showed good safety and efficacy with a single application of epiretinal radiation and two bevacizumab injections after 12 months [58]. A larger, multi-center Phase III trial is underway [59].

Anti-VEGF agents are currently only approved for the treatment of exudative AMD. The multifactorial nature of DME, including non-VEGF mediated causes such as pericyte and endothelial cell damage and tractional mechanisms, has made treatment of this condition difficult using current modalities. Clinical studies are underway with anti-VEGF agents in DME and retinal vein occlusion. VEGF Trap-Eye is under Phase II investigation in DME and Phase III investigation in central retinal vein occlusion. The

FDA approval of VEGF Trap-Eye for these indications would significantly add to the ophthalmologists' armamentarium for treatment of retinal vascular disease.

Eventually, injectable agents targeting the VEGF pathway may be supplanted by implantable devices that deliver polymer-bound drug or manufacture the protein *in vivo*. Further therapies for neovascular AMD such as targeted radiation may confer extra treatment benefit. In the meantime, VEGF Trap-Eye is a

promising investigational drug that, if approved, will improve ophthalmologists' ability to treat neovascular AMD.

Declaration of interest

SCN Oliver is a clinical investigator for Genentech and Alcon. JL Olson and N Mandava are clinical investigators for Genentech, Regeneron and Alcon.

Bibliography

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

- Friedman DS, O'Colmain BJ, Munoz B, et al. Prevalence of age-related macular degeneration in the United States. *Arch Ophthalmol* 2004;122(4):564-72
- Magnitude and causes of visual impairment [fact sheet no. 282]. Available from: <http://www.who.int/mediacentre/factsheets/fs282/en/> [Accessed 1 Sep 2008]
- Congdon N, O'Colmain B, Klaver CC, et al. Causes and prevalence of visual impairment among adults in the United States. *Arch Ophthalmol* 2004;122(4):477-85
- Argon laser photocoagulation for neovascular maculopathy. Three-year results from randomized clinical trials. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1986;104(5):694-701
- A seminal paper in its era, regarding the use of laser photocoagulation for neovascular AMD.
- Krypton laser photocoagulation for neovascular lesions of age-related macular degeneration. Results of a randomized clinical trial. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1990;108(6):816-24
- Subfoveal neovascular lesions in age-related macular degeneration. Guidelines for evaluation and treatment in the macular photocoagulation study. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1991;109(9):1242-57
- Laser photocoagulation of subfoveal recurrent neovascular lesions in age-related macular degeneration. Results of a randomized clinical trial. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1991;109(9):1232-41
- Laser photocoagulation of subfoveal neovascular lesions in age-related macular degeneration. Results of a randomized clinical trial. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1991;109(9):1220-31
- Laser photocoagulation of subfoveal neovascular lesions of age-related macular degeneration. Updated findings from two clinical trials. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1993;111(9):1200-9
- Laser photocoagulation for juxtafoveal choroidal neovascularization. Five-year results from randomized clinical trials. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1994;112(4):500-9
- Occult choroidal neovascularization. Influence on visual outcome in patients with age-related macular degeneration. Macular Photocoagulation Study Group. *Arch Ophthalmol* 1996;114(4):400-12
- Fine SL, Hawkins B, Maguire M. Macular photocoagulation study. *Arch Ophthalmol* 1984;102(11):1583
- D'Amico DJ, Masonson HN, Patel M, et al. Pegaptanib sodium for neovascular age-related macular degeneration: two-year safety results of the two prospective, multicenter, controlled clinical trials. *Ophthalmology* 2006;113(6):992-1001, e1006
- The first anti-VEGF agent proven to be effective for neovascular AMD was pegaptanib.
- Kaiser PK. Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: 5-year results of two randomized clinical trials with an open-label extension: TAP report no. 8. *Graefes Arch Clin Exp Ophthalmol* 2006;244(9):1132-42
- Wormald R, Evans J, Smeeth L, Henshaw K. Photodynamic therapy for neovascular age-related macular degeneration. *Cochrane Database Syst Rev* 2007;(3):CD002030
- Kvanta A. Ocular angiogenesis: the role of growth factors. *Acta Ophthalmol Scand* 2006;84(3):282-8
- Shima DT, Adamis AP, Ferrara N, et al. Hypoxic induction of endothelial cell growth factors in retinal cells: identification and characterization of vascular endothelial growth factor (VEGF) as the mitogen. *Mol Med* 1995;1(2):182-93
- Vinore SA, Xiao WH, Aslam S, et al. Implication of the hypoxia response element of the Vegf promoter in mouse models of retinal and choroidal neovascularization, but not retinal vascular development. *J Cell Physiol* 2006;206(3):749-58
- Zhou J, Pham L, Zhang N, et al. Neutrophils promote experimental choroidal neovascularization. *Mol Vis* 2005;11:414-24
- Ishibashi T, Hata Y, Yoshikawa H, et al. Expression of vascular endothelial growth factor in experimental choroidal neovascularization. *Graefes Arch Clin Exp Ophthalmol* 1997;235(3):159-67
- Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. *Arch Ophthalmol* 2002;120(3):338-46
- Frank RN, Amin RH, Elliott D, et al. Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. *Am J Ophthalmol* 1996;122(3):393-403
- Kliffen M, Sharma HS, Mooy CM, et al. Increased expression of angiogenic growth factors in age-related maculopathy. *Br J Ophthalmol* 1997;81(2):154-62
- Kvanta A, Alverve PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. *Invest Ophthalmol Vis Sci* 1996;37(9):1929-34
- Moshfeghi AA, Rosenfeld PJ, Puliafito CA, et al. Systemic bevacizumab (Avastin) therapy for neovascular age-related macular

- degeneration: twenty-four-week results of an uncontrolled open-label clinical study. *Ophthalmology* 2006;113(11):2002 e2001-12
- **Data from this publication heralded the advent of the bevacizumab versus ranibizumab era for treatment of neovascular AMD.**
26. Rosenfeld PJ, Brown DM, Heier JS, et al. Ranibizumab for neovascular age-related macular degeneration. *N Engl J Med* 2006;355(14):1419-31
 - **The MARINA study was a pivotal trial leading to the approval of ranibizumab for neovascular AMD.**
 27. Brown DM, Kaiser PK, Michels M, et al. Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N Engl J Med* 2006;355(14):1432-44
 - **The ANCHOR study was pivotal in obtaining the FDA approval of ranibizumab for neovascular AMD.**
 28. Brown DM, Michels M, Kaiser PK, et al. Ranibizumab versus verteporfin photodynamic therapy for neovascular age-related macular degeneration: two-year results of the ANCHOR study. *Ophthalmology* 2009;116(1):57-65, e55
 29. Fung AE, Lalwani GA, Rosenfeld PJ, et al. An optical coherence tomography-guided, variable dosing regimen with intravitreal ranibizumab (Lucentis) for neovascular age-related macular degeneration. *Am J Ophthalmol* 2007;143(4):566-83
 - **Variable dosing of ranibizumab for neovascular AMD is now the norm in many practices; this was the first large retrospective of a variable approach.**
 30. Regillo CD, Brown DM, Abraham P, et al. Randomized, double-masked, sham-controlled trial of ranibizumab for neovascular age-related macular degeneration: PIER Study year 1. *Am J Ophthalmol* 2008;145(2):239-48
 31. Ladas ID, Karagiannis DA, Rouvas AA, et al. Safety of repeat intravitreal injections of bevacizumab versus ranibizumab: our experience after 2,000 injections. *Retina* 2009;29(3):313-18
 32. Moreno SF, Paloma JB. Therapeutic anti-VEGF in age-related macular degeneration: ranibizumab and bevacizumab controversy. *Br J Ophthalmol* 2008;92(6):866-7
 33. Safety and Efficacy of Oral PTK787 in Patients with Subfoveal Choroidal Neovascularization Secondary to Age-Related Macular Degeneration (AMD) [ClinicalTrials.gov identifier: NCT00138632] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00138632> [Accessed 19 Sep 2008]
 34. A study to evaluate to safety, tolerability and PK of pazopanib eye drops in healthy adult and elderly subjects. [ClinicalTrials.gov identifier: NCT00733304] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00733304> [Accessed 19 Sep 2008]
 35. Open-Label, Pilot Study of TG100801 in Patients With Choroidal Neovascularization Due to AMD [ClinicalTrials.gov identifier: NCT00509548] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00509548> [Accessed 19 Sep 2008]
 36. Safety & Efficacy Study Evaluating the Combination of Bevasiranib & Lucentis Therapy in Wet AMD (CARBON) [ClinicalTrials.gov identifier: NCT00557791] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00557791> [Accessed 19 Sep 2008]
 37. Study of AdGVPEDE11D in Neovascular Age-Related Macular Degeneration (AMD) [ClinicalTrials.gov identifier: NCT00109499] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00109499> [Accessed 19 Sep 2008]
 38. Gaudreault J, Fei D, Rusit J, et al. Preclinical pharmacokinetics of Ranibizumab (rhuFabV2) after a single intravitreal administration. *Invest Ophthalmol Vis Sci* 2005;46(2):726-33
 39. Rudge JS, Holash J, Hylton D, et al. Inaugural article: VEGF Trap complex formation measures production rates of VEGF, providing a biomarker for predicting efficacious angiogenic blockade. *Proc Natl Acad Sci USA* 2007;104(47):18363-70
 40. Nguyen QD, Shah SM, Hafiz G, et al. A phase I trial of an IV-administered vascular endothelial growth factor trap for treatment in patients with choroidal neovascularization due to age-related macular degeneration. *Ophthalmology* 2006;113(9):1522 e1521-14
 41. Hariprasad S. Fluorescein angiographic and OCT outcomes of a phase I, dose-escalation, safety, tolerability and bioactivity study of intravitreal VEGF Trap in patients with neovascular AMD: The CLEAR-IT 1 Study. Paper presented at: Combined Meeting of the Club Jules Gonin and the Retina Society; Oct 15-20th, 2006; Cape Town, South Africa
 42. Nguyen Q, Hariprasad S, Browning D, et al. Interim results of a phase I, dose-escalation, safety, tolerability, and bioactivity study of intravitreal VEGF Trap in patients with neovascular AMD: The CLEAR-IT 1 Study [abstract no. E-2868]. Paper presented at: Combined Meeting of the Club Jules Gonin and the Retina Society; Oct 15-20th, 2006; Cape Town, South Africa
 43. Nguyen Q, for the CLEAR-IT 1 investigators. A randomized comparison of the safety, tolerability, and bioactivity study two dose levels of intravitreal VEGF trap in patients with neovascular age-related macular degeneration. Paper presented at: Retina Society Annual Scientific Meeting; Sep 27-30th, 2007; Boston, MA
 44. Do DV, Nguyen QD, Shah SM, et al. An exploratory study of the safety, tolerability and bioactivity of a single intravitreal injection of vascular endothelial growth factor Trap-Eye in patients with diabetic macular oedema. *Br J Ophthalmol* 2009;93(2):144-9
 45. VEGF Trap-Eye in Wet AMD. CLEAR-IT 2: Summary of One-Year Key Results. Paper presented at: Retinal Society Annual Scientific Meeting; Sep 28th, 2008; Scottsdale, AZ
 46. Double-Masked Study of Efficacy and Safety of IVT VEGF Trap-Eye in Subjects With Wet AMD (VIEW 1) [ClinicalTrials.gov identifier: NCT00509795] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00509795> [Accessed 28 Sep 2008]
 47. VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW 2). [ClinicalTrials.gov identifier: NCT00637377] ClinicalTrials.gov [online]. Available from: <http://clinicaltrials.gov/ct2/show/NCT00637377> [Accessed 28 Sep 2008]
 48. Jaffe GJ, Martin D, Callanan D, et al. Fluocinolone acetonide implant (Retisert) for noninfectious posterior uveitis: thirty-four-week results of a multicenter

- randomized clinical study. *Ophthalmology* 2006;113(6):1020-7
49. Kuppermann BD, Blumenkranz MS, Haller JA, et al. Randomized controlled study of an intravitreal dexamethasone drug delivery system in patients with persistent macular edema. *Arch Ophthalmol* 2007;125(3):309-17
 50. Williams GA, Haller JA, Kuppermann BD, et al. Dexamethasone posterior-segment drug delivery system in the treatment of macular edema resulting from uveitis or irvine-gass syndrome. *Am J Ophthalmol* 2009;147(6):1048-54, 1054.e1-2
 51. Sieving PA, Caruso RC, Tao W, et al. Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. *Proc Natl Acad Sci USA* 2006;103(10):3896-901
 52. Lazic R, Gabric N. Verteporfin therapy and intravitreal bevacizumab combined and alone in choroidal neovascularization due to age-related macular degeneration. *Ophthalmology* 2007;114:1179-85
 53. Gragoudas ES, Adamis AP, Cunningham ET, et al.; VEGF Inhibition Study in Ocular Neovascularization Clinical Trial Group. Pegaptanib for neovascular age-related macular degeneration. *N Engl J Med* 2004;351:2805-16
 54. Heier JS, Boyer DS, Ciulla TA, et al.; FOCUS Study Group. Ranibizumab combined with verteporfin photodynamic therapy in neovascular age-related macular degeneration: year 1 results of the FOCUS study. *Arch Ophthalmol* 2006;124:1532-42
 55. Kaiser PK; Registry of Visudyne AMD Therapy Writing Committee, Boyer DS, Garcia R, Hao Y, et al. Verteporfin photodynamic therapy combined with intravitreal bevacizumab for neovascular age-related macular degeneration. *Ophthalmology* 2009;116(4):747-55
 56. Efficacy/safety of verteporfin photodynamic therapy and ranibizumab compared with ranibizumab in patients with subfoveal choroidal neovascularization. [ClinicalTrials.gov identifier: NCT00436553] *ClinicalTrials.gov* [online]. Available from: [http://clinicaltrials.gov/ct2/show/ NCT00436553](http://clinicaltrials.gov/ct2/show/NCT00436553) [Accessed 28 Sep 2008]
 57. Reduced Fluence Visudyne-Anti-VEGF-Dexamethasone In Combination for AMD Lesions (RADICAL) [ClinicalTrials.gov identifier: NCT00492284] *ClinicalTrials.gov* [online]. Available from: [http:// clinicaltrials.gov/ct2/show/ NCT00492284](http://clinicaltrials.gov/ct2/show/NCT00492284) [Accessed 28 Sep 2008]
 58. Avila MP, Farah ME, Santos A, et al. Twelve-month short-term safety and visual-acuity results from a multicentre prospective study of epiretinal strontium-90 brachytherapy with bevacizumab for the treatment of subfoveal choroidal neovascularisation secondary to age-related macular degeneration. *Br J Ophthalmol* 2009;93(3):305-9
 59. Study of Strontium90 Beta Radiation With Lucentis to Treat Age-Related Macular Degeneration (CABERNET) [ClinicalTrials.gov identifier: NCT00454389] *ClinicalTrials.gov* [online]. Available from: [http:// clinicaltrials.gov/ct2/show/study/ NCT00454389?term=cabernet&rank=1& show_locs=Y](http://clinicaltrials.gov/ct2/show/study/NCT00454389?term=cabernet&rank=1&show_locs=Y) [Accessed 28 May 2009]

Affiliation

James A Dixon MD Resident in Ophthalmology,
 Scott CN Oliver[†] MD Assistant Professor of
 Ophthalmology,
 Jeffrey L Olson MD
 Assistant Professor of Ophthalmology &
 Naresh Mandava MD Professor and Chair of
 Ophthalmology
[†]Author for correspondence
 University of Colorado Denver,
 Rocky Mountain Lions Eye Institute,
 Department of Ophthalmology,
 1675 North Aurora Court, PO Box 6510,
 Mail Stop F-731, Aurora, CO 80045-2500, USA
 Tel: +1 720 848 5029; Fax: +1 720 848 5014;
 E-mail: scott.oliver@ucdenver.edu

Appendix 3B



Journal

Expert Opinion on Investigational Drugs >

Volume 18, 2009 - Issue 10

1213 73  0
Views CrossRef citations Altmetric

Drug Evaluations

VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration

James A Dixon , MD, Scott CN Oliver , MD, Jeffrey L Olson , MD & Naresh Mandava , MD

Pages 1573-1580 | Published online: 20 Aug 2009

 Download citation  <https://doi.org/10.1517/13543780903201684> Full Article Figures & data References Citations Metrics Reprints & Permissions

Get access

Abstract

Background: Age-related macular degeneration (AMD) affects > 14 million individuals worldwide. Although 90% of patients with AMD have the dry form, neovascular AMD accounts for the vast majority of patients who develop legal blindness. Until recently, few treatment options existed for treatment of neovascular AMD. The advent of anti-VEGF therapy has significantly improved the safe and effective treatment of neovascular AMD. In addition to two anti-VEGF drugs currently in widespread use, ranibizumab and bevacizumab, a number of medications that interrupt angiogenesis are currently under investigation. One promising new drug is aflibercept (VEGF Trap-Eye), a fusion protein that blocks all isoforms of VEGF-A and placental growth factors-1 and -2. *Objective:* To review the current literature and clinical trial data regarding VEGF Trap-Eye for the treatment of neovascular AMD. *Methods:* Literature review. *Results/conclusion:* VEGF Trap-Eye is a novel anti-VEGF therapy, with Phase I and II trial data indicating safety, tolerability and efficacy for the treatment of neovascular AMD. Two Phase III clinical trials (VIEW-1 and VIEW-2) comparing VEGF Trap-Eye to ranibizumab are currently continuing and will provide vital insight into the clinical applicability of this drug.

Keywords:: aflibercept, AMD, angiogenesis, neovascularization, VEGF, VEGF inhibition, VEGF Trap

People also read

Article

[Intravitreal aflibercept injection for neovascular \(wet\) age-related macular degeneration >](#)

Matthew Ohr et al.

Expert Opinion on Pharmacotherapy
Volume 13, 2012 - Issue 4

Published online: 17 Feb 2012

Review

[Current advances in the treatment of neovascular age-related macular degeneration >](#)

Victor M. Villegas et al.

Expert Opinion on Drug Delivery
Volume 14, 2017 - Issue 2

Published online: 2 Aug 2016

Review

[Current drug and molecular therapies for the treatment of atrophic age-related macular degeneration: phase I to phase III clinical development >](#)

Huiling Li et al.

Expert Opinion on Investigational Drugs
Volume 26, 2017 - Issue 10

Published online: 24 Aug 2017

Review article

Pharmacokinetics, pharmacodynamics and pre-clinical characteristics of ophthalmic drugs that bind VEGF >

Michael W Stewart

Expert Review of Clinical Pharmacology
Volume 7, 2014 - Issue 2

Published online: 14 Feb 2014

Article

Aflibercept (VEGF Trap-Eye) for the treatment of exudative age-related macular degeneration >

Michael W Stewart

Expert Review of Clinical Pharmacology
Volume 6, 2013 - Issue 2

Published online: 10 Jan 2014

Review article

A systematic review of the efficacy and safety outcomes of anti-VEGF agents used for treating neovascular age-related macular degeneration: comparison of ranibizumab and bevacizumab >

Paul Mitchell

Current Medical Research and Opinion
Volume 27, 2011 - Issue 7

<https://www.tandfonline.com/doi/abs/10.1517/13543780903201684>

Published online: 15 Jun 2011

Information for

Authors

Editors

Librarians

Societies

Open access

Overview

Open journals

Open Select

Cogent OA

Help and info

Help

FAQs

Newsroom

Contact us

Commercial services

Connect with Taylor & Francis



Copyright © 2018 Informa UK Limited Privacy policy & cookies Terms & conditions
Accessibility

Registered in England & Wales No. 3099067
5 Howick Place | London | SW1P 1WG

Appendix 3C



Search WorldCat

Search

[Advanced Search](#) [Find a Library](#)

[<< Return to Search Results](#) [Cite/Export](#) [Print](#) [E-mail](#) [Share](#) [Permalink](#)

[Add to list](#) [Add tags](#) [Write a review](#) Rate this item: 1 2 3 4 5

Expert opinion on investigational drugs.

Publisher: London : Ashley Publications ; [S.l.] : Informa, [200-]-

Edition/Format: eJournal/eMagazine : Document : Periodical : English [View all editions and formats](#)

Rating: (not yet rated) 0 with reviews - Be the first.

Subjects: [Drugs -- Periodicals -- Design.](#)
[Drugs.](#)

More like this [Similar Items](#)

Get a Copy

[Find a copy in the library](#)

Find a copy online

Links to this item

ashley-pub.com

Find a copy in the library

Enter your location: [Find libraries](#)

Submit a complete postal address for best results.

Displaying libraries 1-6 out of 181 for all 13 editions (8600 Rockville Pike, Bethesda, MD 20894, USA)

Show libraries holding [just this edition](#)

[<< First](#) [< Prev](#) 1 [2](#) [3](#) [Next >](#) [Last >>](#)

Library	Held formats	Distance	
1. National Library of Medicine Bethesda, MD 20894 United States	Journal / Magazine / Newspaper	1 mile MAP IT	Library info Add to favorites
2. Johns Hopkins Medical Institutions William H. Welch Medical Library Baltimore, MD 21205 United States	Journal / Magazine / Newspaper	34 miles MAP IT	Library info Ask a librarian Add to favorites
3. Virginia Commonwealth University VCU Libraries Richmond, VA 23284 United States	Journal / Magazine / Newspaper	102 miles MAP IT	Library info Ask a librarian Add to favorites
4. Richard Bland College Richard Bland College Library South Prince George, VA 23805 United States	Journal / Magazine / Newspaper	128 miles MAP IT	Library info Add to favorites
5. University of Pittsburgh Health Science Library	Journal / Magazine / Newspaper	182 miles MAP IT	Library info Ask a librarian

Pittsburgh, PA 15261 United States

[Add to favorites](#)

6.

[Icahn School of Medicine at Mount Sinai](#)
Gustave L. and Janet W. Levy Library
New York, NY 10029 United States



[Journal / Magazine / Newspaper](#)

208 miles
MAP IT

[Library info](#)
[Ask a librarian](#)
[Add to favorites](#)

<< First < Prev 1 2 3 Next > Last >>

- Details

Genre/Form: Periodicals

Additional Physical Format: Expert opinion on investigational drugs (NL-LeOCL)115836608

Material Type: Document, Periodical, Internet resource

Document Type: Internet Resource, Computer File, Journal / Magazine / Newspaper

ISSN: 1744-7658

OCLC Number: 819148809

- Reviews

User-contributed reviews

[Add a review](#) and share your thoughts with other readers. [Be the first.](#)

- Tags

[Add tags](#) for "Expert opinion on investigational drugs.". [Be the first.](#)

- Similar Items


Related Subjects: (2)

[Drugs -- Periodicals -- Design.](#)

[Drugs.](#)

± Linked Data

Appendix 3D



LocatorPlus

Search the Collections of the National Library of Medicine

Search	Headings	Titles	Library Information	Other Databases	Help	Home
--------	----------	--------	---------------------	-----------------	------	------

Collections of the National Library of Medicine

Search Request: Advanced Menu = (0967-8298)[in]

Search Results: Displaying 3 of 3 records

← Previous Next →

Summary View

Detailed View

Table of Contents

MARC View

More Like This

Expert opinion on investigational drugs.

Title Abbreviation: Expert Opin Investig Drugs

Title: Expert opinion on investigational drugs.

Description: v. : ill.

Coverage: Vol. 3, no. 2 (Feb. 1994)-

Publisher: London : Ashley Publications Ltd., c1994-

Latest Publisher: 2015- : Abingdon, Oxford : Taylor & Francis

Continues: [Current opinion in investigational drugs](#)

ISSN: 1354-3784 (Print)

1744-7658 (Electronic)

Electronic Links: <http://www.tandfonline.com/loi/ieid20>

In: Index medicus v9n1, Jan. 2000-

MEDLINE v9n1, Jan. 2000-

PubMed v9n1, Jan. 2000-

NLM Unique ID: 9434197

Other ID Numbers: (DNLM)SR0081220(s)

(OCoLC)30379431

Location: General Collection

Call Number: W1 EX52M

Availability: v.3:no.2(1994)-v.3:no.4(1994),v.3:no.6(1994)-

v.22:no.4(2013),v.22:no.7(2013)-v.23:no.9(2014),v.24(2015)-v.25(2016)

Status: Available

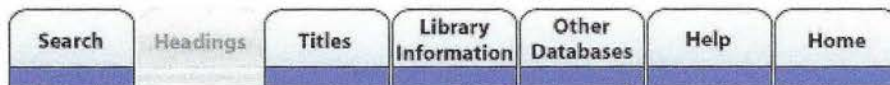
Other Locations: [Find Other Libraries That Own This](#)

Get it  NLM

← Previous Next →

Options for Print, Save or E-mail

Select Download Format: Full record with holdings ▼	Print/Save
E-mail Full record(s) to:	E-mail



[Copyright](#), [Privacy](#), [Accessibility](#)

U.S. National Library of Medicine, 8600 Rockville Pike, Bethesda, MD 20894

[National Institutes of Health](#), [Health & Human Services](#)

[Contact NLM](#)

Appendix 3E



REVIEW

How to Overcome Retinal Neuropathy: The Fight against Angiogenesis-related Blindness

Dong Hyun Jo^{1,2}, Jin Hyoung Kim², and Jeong Hun Kim^{1,2}

¹Department of Ophthalmology, College of Medicine, Seoul National University, Seoul 151-742, Korea and ²Fight against Angiogenesis-Related Blindness (FARB) laboratory, Department of Ophthalmology, College of Medicine, Seoul National University, Seoul 110-744, Korea

(Received August 9, 2010/Revised August 23, 2010/Accepted August 24, 2010)

The retina consists of neuronal cells of high metabolic activity that are supplied by an abundant vasculature. It is a main theme of ophthalmologic research, because retinopathies are common causes of blindness in all age groups: age-related macular degeneration in the elderly, diabetic retinopathy in the middle aged, and retinopathy of prematurity and retinoblastoma in children. Interestingly, angiogenesis underlies the pathogenesis of all these diseases, and breakdown of the blood-retinal barrier is also thought to play an important role before and throughout the process of new vessel formation. However, so far, most treatments have targeted angiogenesis only, especially vascular endothelial growth factor. Consideration of the restoration of the blood-retinal barrier should be required. In this review, we discuss the clinical manifestation, pathogenesis, and current treatment options for angiogenesis-related blindness. In addition, because of the recent introduction of novel strategies, we describe pathogenesis-based treatment options to treat angiogenesis-related blindness.

Key words: Angiogenesis-related blindness, Age-related macular degeneration, Diabetic retinopathy, Retinoblastoma, Retinopathy of prematurity

INTRODUCTION

The vertebrate retina is a 10-layered structure that includes 3 layers of nucleated cells and 2 plexiform layers. In the retina, photoreceptor cells and ganglion cells synapse with bipolar, horizontal, and amacrine cells, and through these synapses the responses to light by cone and rod cells are converted to electrical signals that are passed on to the brain via the optic nerve (Tian, 2004). The light-induced activation of photoreceptor cells is transmitted to bipolar and horizontal cells whose nuclei are in the inner nuclear layer and whose dendrites are in the outer plexiform layer. The other synaptic interactions occur in the inner plexiform layer between ganglion cells, amacrine

cells, and bipolar cells. In this point of view, retinal diseases are due to damage to the neuronal cells and their synapses, and therefore, can be considered as neuronal diseases.

There are many conditions that affect the retina: infection, ischemia, oxygen tension, tumors, and aging. Retinal diseases cause direct damage to visual signaling, and ultimately can induce blindness, and therefore, they are the main themes of much ophthalmologic research. The most common causes of blindness are retinopathies in all age groups: age-related macular degeneration (AMD) in the elderly, diabetic retinopathy (DR) in middle aged adults, and retinopathy of prematurity (ROP) and retinoblastoma in children (Aiello et al., 1994; Abramson and Scheffler, 2004). These most important retinal diseases have two major characteristics in common. First, pathologic angiogenesis lies at the pathogenesis of each of these diseases. Angiogenesis is a process that forms new blood vessels via sprouting from existing vessels (Folkman, 2006), and pathologic neovascularization leads to bleeding, exudate formation, and fibrovascular proliferation

Correspondence to: Jeong Hun Kim, M.D., FARB (Fight against Angiogenesis-Related Blindness) Laboratory, Department of Ophthalmology, Seoul National University College of Medicine and Seoul Artificial Eye Center, Clinical Research Institute, Seoul National University Hospital, Seoul 110-744, Korea
Tel: 82-2-2072-1560, Fax: 82-2-741-3187
E-mail: steph25@snu.ac.kr

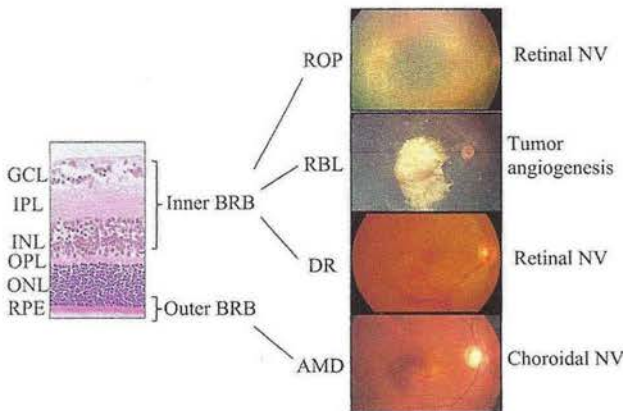


Fig. 1. The common causes of blindness have retinal neuropathies in common, and are due to angiogenesis and breakdown of inner or outer blood-retinal barriers. AMD, age-related macular degeneration; BRB, blood-retinal barrier; DR, diabetic retinopathy; GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer; ONL, outer nuclear layer; OPL, outer plexiform layer; RBL, retinoblastoma; ROP, retinopathy of prematurity; RPE, retinal pigment epithelium.

(Gariano and Gardner, 2005). Second, the disruption of the blood-retinal barrier (BRB) plays an important role before and throughout the process of new vessel formation (Kim et al., 2006). The BRB, a kind of blood-neural barrier, functions as a selective barrier between the central nervous and circulatory systems. In the retina, there are two kind of BRBs: the inner BRB consisting of endothelial cells of the retinal blood vessels, and the outer BRB formed by the retinal pigment epithelial (RPE) cells (Cunha-Vaz, 1976) (Fig. 1).

Although we have uncovered more about the detailed mechanisms of retinal neuropathies, current treatment has targeted angiogenesis only, especially vascular endothelial growth factor (VEGF). In this review, we discussed the clinical manifestation, pathogenesis, and current treatment options of angiogenesis-related blindness (ARB). In addition, because of the introduction of novel strategies, we discuss pathogenesis-based treatment options to treat ARB.

CLINICAL MANIFESTATIONS OF ANGIOGENESIS-RELATED BLINDNESS

Age-related macular degeneration

AMD is the leading cause of visual loss in the elderly (Gehrs et al., 2006). In the United States, approximately 1.75 million individuals are thought to suffer from AMD, and the overall prevalence of advanced AMD is estimated to be about 1.5% in the

population over 40 years old (Friedman et al., 2004). AMD is divided into non-neovascular AMD and neovascular AMD depending on the presence of choroidal neovascularization (Jager et al., 2008). Neovascular AMD represents only 10 to 15% of total AMD; however, most cases with severe visual loss result from Neovascular AMD (Ferris et al., 1984; Friedman et al., 2004). A review of epidemiologic studies from many countries indicated that age and family history are the most important risk factors for AMD. Smoking and hypertension were also associated with advanced AMD (Klein et al., 2004).

Choroidal neovascularization (CNV), a pathognomonic finding of neovascular AMD, becomes problematic after disruption of the RPE and Bruch's membrane, that is, after breakdown of the outer BRB. CNV invades into the subretinal space through the break in Bruch's membrane (de Jong, 2006). If left untreated, over days or several months, it advances to the formation of a fibrous scar, which is irreversible damage to the retinal neurons (Gehrs et al., 2006).

Diabetic retinopathy

Diabetic retinopathy (DR) is one of the most frequent microvascular complications of diabetes and the leading cause of blindness of middle aged individuals in both developed and developing countries (Frank, 2004). Diabetes affects about 7% of the US population, and the estimated prevalence of diabetic retinopathy is approximately 40% in adults with diabetes who are 40 years or older. Diabetes itself is the most important risk factor in DR, and roughly 50% of patients with DM for 25 years are finally afflicted by DR (Kempen et al., 2004).

Like AMD, and depending on the presence of neovascularization, DR is divided into two groups: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). PDR is a condition in which new vessels form, and these new vessels are weak, and apt to burst out into the vitreous humor?, which causes vitreous hemorrhage. More, fibrous proliferation of retinal cells, which is a sign of advanced PDR, can cause traction-induced retinal detachment (Penn et al., 2008).

Retinopathy of prematurity

ROP is the leading cause of blindness in neonatal infants, and between 10 and 18% of blindness are due to ROP in the developed world (Gilbert et al., 1997; Quiram and Capone, 2007). About 10% of infants are born preterm, and the incidence of ROP is approximately 70% among infants who are born weighing less than 1251 g. Generally, low birth weight and gesta-

tional age have been related to the severity and the occurrence of ROP. Data from two large clinical trials were very similar in the incidence and the risk factors for ROP, despite the fact that they were conducted about 20 years apart (Palmer et al., 1991; Good et al., 2005).

ROP is considered to be a biphasic disease: an initial phase of vessel loss and a subsequent phase of new vessel proliferation. From birth to a postmenstrual age of 30-32 weeks, vascular growth slows or ceases, and leads to nonvascularization of peripheral retina. The second phase of ROP begins around 32-34 weeks postmenstrual age, and is distinct from the first phase in that retinal neovascularization takes place (Chen and Smith, 2007).

Retinoblastoma

Retinoblastoma is the most common intraocular malignancy in childhood, affecting 1 in 15,000 children (Abramson and Scheffler, 2004). Via the break of the inner BRB and adjacent tissues, the tumor invades the choroid, the optic nerve, the optic chiasm, and the vessel, and metastasizes hematogenously to bone and bone marrow (Shields et al., 1993, 1994). In retinoblastoma, tumor angiogenesis is thought to begin in central regions of tumors, and spread to the tumor periphery (Pina et al., 2009).

PATHOGENESIS OF ANGIOGENESIS-RELATED BLINDNESS

Pathogenesis of angiogenesis, barrierogenesis, and neurogenesis

Four major retinal diseases share common characteristics. First, angiogenesis occurs during disease progression. Of course there are spatial discrepancies between diseases. ROP and DR affect retinal vessels that supply the inner layers of the retina (Sapieha et al., 2010), whereas, AMD affects choroidal vessels that supply the outer layers of the retina and the choroid. In retinoblastoma, angiogenesis within and adjacent to the tumors is important (Pina et al., 2009). Second, the BRB breakdown plays an important role in the course of the diseases. Because the BRB serves critical functions in the homeostasis of the retina, the breakdown of the inner and outer BRBs results in damage to the retina. Inner BRB breakdown occurs in the process of DR, ROP, and retinoblastoma development, and the breakage of the outer BRB is a distinctive feature in the progression of neovascular AMD (Kim et al., 2009a). Third, these 4 diseases finally cause damage to the neuronal cells via direct and indirect pathways (Abramson and Scheffler, 2004; Gehrs et al.,

2006; Chen and Smith, 2007; Davidson et al., 2007). In this view, ARB occurs due to abnormalities in three important processes for maintenance of physiologic status of the retina: angiogenesis, barrierogenesis, and neurogenesis.

The roles of VEGF in angiogenesis-related blindness

VEGF not only induces angiogenesis, but also, it causes vascular permeability (Weis and Cherech, 2005). That is, VEGF is involved in both angiogenesis and breakdown of the blood-retinal barrier. We repeatedly observed VEGF-induced increases in tube formation, migration (Kim et al., 2009a, 2009b), and permeability of retinal endothelial cells (Kim et al., 2009a, 2010). VEGF-mediated inner BRB breakdown might be mediated by VEGFR-2 activation (Kim et al., 2009a).

Many *in vivo* studies that analyzed ocular fluid or tissues showed that the levels of VEGF are elevated in ARB (Aiello et al., 1994; Kvantta et al., 1996; Lopez et al., 1996; Kliffen et al., 1997; Stitt et al., 1998; Funatsu et al., 2002; Sato et al., 2009).

The increased expression of VEGF is regulated by hypoxia-inducible factor 1 α (HIF-1 α), a transcription factor that binds to the hypoxia responsive element in the 5' region of the VEGF gene under hypoxic conditions (Pugh and Ratcliffe, 2003; Schofield and Ratcliffe, 2004). According to our previous reports, downregulation and destabilization of HIF-1 α protein induced decreases in expression of the VEGF gene (Kim et al., 2001; Oh et al., 2007), leading to inhibition of retinal and choroidal neovascularization (Kim et al., 2008, 2009b).

The roles of cytokines and growth factors besides VEGF in angiogenesis-related blindness

Other growth factors besides VEGF have received interest because VEGF inhibition can only lead to attenuation of new vessel growth, not regression of neovascularization (Brown et al., 2006; Tolentino, 2009). The growth factors that have attracted much attention are platelet-derived growth factor (PDGF) and placenta growth factor (PIGF). PDGF, which binds to PDGF receptors (PDGFR)-a and -b, is known to be involved in recruiting pericytes and smooth muscle cells, leading to vessel development and maturation (Hellberg et al., 2010). PIGF, one member of the VEGF family, binds to the VEGF receptor-1, and inhibition of PIGF reduced the response to VEGF, leading to impaired angiogenesis (Carmeliet et al., 2001). Activation of the PIGF/VEGF receptor-1 pathway led to alterations in RPE barrier function, indicating that

PlGF might also play a role in outer BRB maintenance (Miyamoto et al., 2008).

In ROP, limited transport of insulin-like growth factor-1 (IGF-1) and omega-3 polyunsaturated fatty acids (ω -3 PUFA) from the mother due to premature delivery leads to increased vulnerability to oxygen toxicity and subsequent hypoxia (Heidary et al., 2009). IGF-1 and ω -3 PUFA are well-known protective factors in developing retinal vasculatures (Hellstrom et al., 2001; Connor et al., 2007). In DR patients, hyperglycemia induces the formation of reactive oxygen species (ROS), leading to disturbed ocular hemodynamics (Abran et al., 1995), direct vascular damage (Sapieha et al., 2010), and increased production of vascular endothelial growth factor (VEGF) (El-Remessy et al., 2003).

Inflammation also contributed to the pathogenesis of retinal diseases leading to ARB. In DR, the levels of multiple inflammatory cytokines, including IL-1 β , IL-6, IL-8, and TNF- α , were reported to be elevated (Sapieha et al., 2010). IL-1 β is considered to be involved in the survival of retinal neurons and in angiogenesis via HIF-1 α activation (Jung et al., 2003; Abcouwer et al., 2008). TNF- α is also thought to play an important role in DR by inducing apoptosis of endothelial cells, which constitutes early vascular damage in the pathogenesis of DR (Joussen et al., 2009). In AMD, activation of the complement system seems to be associated with the integrity of RPE cells (Thurman et al., 2009), and this conclusion has come from genetic studies of patients with AMD which showed mutations in the complement factor H gene (Edwards et al., 2005).

CURRENT TREATMENT OPTIONS OF ANGIOGENESIS-RELATED BLINDNESS

Current treatment options for ARB are surgery, focal treatment such as laser photocoagulation, cryotherapy, thermotherapy, radiation therapy, and medical treatment (Table I). All of these treatment options

have a limitation in that only angiogenesis is targeted.

Surgical treatment

Surgery is done for complications of neovascularization such as vitreous hemorrhage and tractional retinal detachment from AMD, DR, and ROP. Vitrectomy is aimed at removal of media opacities, relief of tractional adhesions, and subsequent decreases in angiogenic cytokines and growth factors (Smiddy and Flynn, 1999; Haller et al., 2010). Scleral buckling is done to reattach the retina (Repka et al., 2006). Reattachment rates have been variable (range: 33 to 90%; Hubbard, 2008).

Retinoblastoma is an intraocular malignant tumor, so surgical treatment should be considered in the context of the removal of the primary tumor. Enucleation, en bloc removal of the eyeball, and substitution of a prosthesis for the eyeball, is the treatment of choice for localized retinoblastoma without invasion into adjacent tissues (Kim et al., 2004).

Focal treatment

Of focal treatments in ARB, laser photocoagulation is the mainstay option. Destruction of neuronal cells by high-energy laser beams reduced the need for a vascular supply to the peripheral retina, leading to the regression of neovascularization. With the same strategy, peripheral retinal ablation by cryotherapy has been done for ROP (Mantagos et al., 2009). Photodynamic therapy, another focal treatment for AMD, consists of intravenous injection of a photosensitizer verteporfin dye and projection of a low-energy laser (Bressler, 2001). As for retinoblastoma, radiation therapy, cryotherapy, laser photocoagulation, thermotherapy, and brachytherapy form the focal treatment options (Lin and O'Brien, 2009).

Medical treatment

As previously discussed, increased VEGF expression is involved in angiogenesis and BRB breakdown, and therefore has been regarded as the main target of

Table I. Current treatment options for age-related macular degeneration (AMD), diabetic retinopathy (DR), retinopathy of prematurity (ROP), and retinoblastoma

	AMD	DR	ROP	Retinoblastoma
Surgery	Scleral buckling, vitrectomy	Scleral buckling, vitrectomy	Scleral buckling, vitrectomy	Enucleation
Focal treatment	Laser photocoagulation, PDT	Laser photocoagulation, cryotherapy	Laser photocoagulation, cryotherapy	Radiation, laser photocoagulation, cryotherapy, thermotherapy
Medical treatment	Intravitreal injection of anti-VEGF antibody and steroid	Intravitreal injection of anti-VEGF antibody and steroid	Intravitreal injection of anti-VEGF antibody	Systemic chemotherapy

PDT, photodynamic therapy; VEGF, vascular endothelial growth factor.

medical treatment for ocular neovascular diseases. Currently used anti-VEGF antibodies in ophthalmology are pegatanib sodium (Macugen[®], Eyetech Inc.), ranibizumab (Lucentis[®], Genetech Inc.) and bevacizumab (Avastin[®], Genetech Inc.). The former 2 were approved for intravitreal injection, and the latter has been used off-label. However, many reports have shown significant efficacy of all three drugs (Ozkiris, 2010). In infants with ROP, the use of anti-VEGF antibody is restricted to therapy for aggressive and vascularly active forms of ROP (Rishi et al., 2009; Mintz-Hittner, 2010). In the context of tumor angiogenesis, there are few reports using anti-VEGF antibody in cell cultures or xenograft models (Lee et al., 2008). There have been no reports or ongoing trials in humans.

NOVEL STRATEGIES TO ANGIOGENESIS-RELATED BLINDNESS

So far, we have been able to treat only the conditions that result from angiogenesis by surgical or focal treatment. Anti-VEGF antibody has the same limitation in that it can only treat neovascularization, but it cannot prevent it. With the introduction of various novel strategies on development of ocular diseases, pathogenesis-guided treatments beyond anti-VEGF antibody have been suggested (Table II).

Using VEGF-dependent pathways

Inhibition of VEGF or VEGF receptors (VEGFR) are the popular mechanisms of drugs for ARB. VEGF Trap-Eye (Regeneron Pharmaceuticals Inc.) is a recombinant fusion protein combining binding sites of VEGFR1 and VEGFR2, and can bind to both VEGF and PlGF. A Phase III trial for AMD showed significant efficacy (Dixon et al., 2009; Kaiser, 2009). Drugs using RNA interference attracted attention. Small interfering RNAs (siRNA) inhibiting expression of VEGF (Bevasiranib, OPKO Health, Inc.) and VEGFR (Sirna-027, Allergan, Inc.) were developed, and phase III and II trials are ongoing, respectively (Shen et al., 2006; Beutel et al., 2009; Singerman, 2009).

There are also emerging drugs for targeting intracellular signal transduction, namely, inhibitors of receptor tyrosine kinase. PTK787 (Novartis) and Pazopanib (GlaxoSmithKline plc.) are inhibitors of VEGFR and PDGFR, and TG100801 (TargeGen, Inc.) is known to inhibit VEGFR, PDGFR, and fibroblast growth factor inhibitor. All 3 drugs are now in phase II clinical trials (Doukas et al., 2008; Mousa and Mousa, 2010).

Using VEGF independent pathway

Other growth factors and cytokines except VEGF are also targets of novel strategies. As for complement, cyclic peptide POT-4 (Potentia Pharmaceuticals, Inc.) and anti-C5 aptamer ARC1905 (Ophthotech Corp.) were developed and have undergone phase I trials (Ni and Hui, 2009). Inflammatory cytokines are utilized for development of new drugs as well. Human monoclonal antibody to IL-1 β , ACZ885 (Novartis), is a drug widely used in rheumatologic diseases. To assess safety and tolerability in AMD, a multicenter phase I study started and is in progress. Infliximab (Centocor Ortho Biotech Inc.), an inhibitor of TNF- α , showed efficacy with intravenous infusion in reducing macular edema for patients with type 2 diabetes in a pilot study of 4 patients (Sfikakis et al., 2005), and this agent is part of another pilot study on intravitreal injection. Anti-PDGFR aptamer E10030 (Ophthotech Corp.) is in a phase I study to establish the safety, tolerability, and pharmacokinetic profile of the drug.

Integrins are known to play a role in angiogenesis, and two $\alpha 5 \beta 1$ integrin inhibitors were developed: chimeric antibody for $\alpha 5 \beta 1$ integrin (Volociximab, Ophthotech Corp.) and a small peptide $\alpha 5 \beta 1$ integrin inhibitor, JSM6427 (Jerini AG). These drugs showed efficacy in preclinical studies, and phase I clinical trials are in progress (Ricart et al., 2008; Zahn et al., 2009).

Miscellaneous drugs are also under investigation. Anecortave acetate (Retaane[®], Alcon Inc.) is manufactured by modification of steroids, and presumably exerts its effect by decreasing extracellular matrix breakdown and inhibition of endothelial cell migration. It showed efficacy in a phase II study, and a phase III clinical trial is on the way (Slakter, 2006).

CONCLUSIONS AND FUTURE DIRECTIONS

ARB is the most common cause of blindness in all age groups, and creates a great economic burden in both developed and developing countries. Although both angiogenesis and breakdown of BRB bring about ARB, current treatment only targets angiogenesis, especially the VEGF pathway. Surgical and focal treatment are aimed at relief of complications from angiogenesis and reduction of the need for neovascularization. Anti-VEGF antibody also has same limitations in that it can only affect events after angiogenesis, not before new vessel formation.

As we have uncovered the pathogenesis of ARB, our treatment options have become more various: combination methods mixing conventional therapy with

Table II. Novel strategies to angiogenesis-related blindness

Agent	Company	Structure	Molecular target or mechanism of action	Drug development stage
Using VEGF dependent pathway				
VEGF Trap-Eye	Regeneron Pharmaceuticals Inc., Tarrytown, NY, USA	Fusion protein	All forms of VEGF-A and PlGF	Phase III
Bevasiranib (Cand5)	OPKO Health, Inc., Miami, FL, USA	siRNA	VEGF-A mRNA	Phase III
Sirna-027 (AGN211745)	Allergan, Inc., Irvine, CA, USA	siRNA	VEGF-R1 mRNA	Phase II
PTK787	Novartis, Basel, Switzerland	Enzyme inhibitor	VEGFR and PDGFR; receptor tyrosine kinase inhibitor	Phase II
TG100801	TargeGen, Inc., San Diego, CA, USA	Small molecule kinase inhibitor	Receptor tyrosine kinase inhibitor	Phase II
Pazopanib	GlaxoSmithKline plc., Middlesex, United Kingdom	Small molecule kinase inhibitor	VEGFR and PDGFR; receptor tyrosine kinase inhibitor	Phase II
Using VEGF independent pathway				
Anecortave acetate (Retaane [®])	Alcon Inc., Hünenberg, Switzerland	Modification from steroid	Decreasing ECM breakdown and inhibiting endothelial cell migration (?)	Phase III
PF-04523655	Pfizer Inc, New York, NY, USA; Quark Pharmaceuticals, Fremont, CA, USA	siRNA	RTP-801 gene	Phase II
POT-4	Potentia Pharmaceuticals, Inc., Louisville, KY, USA	Cyclic peptide	Complement inhibitor	Phase I
ARC1905	Ophthotech Corp., Princeton, NJ, USA	Anti-C5 aptamer	Complement C5	Phase I
ACZ885	Novartis	Human monoclonal antibody	Interleukin-1 β	Phase I
Infliximab	Centocor Ortho Biotech, Inc., Horsham, PA, USA	Humanized monoclonal antibody	TNF- α	Phase I
E10030	Ophthotech Corp.	Anti-PDGF aptamer	PDGF	Phase I
Sonepcizumab (LT1009)	Lpath, Inc., San Diego, CA, USA	Humanized monoclonal antibody	Sphingosine-1-phosphate	Phase I
AdGVPDEF	GenVec, Inc., Gaithersburg, MD, USA	Adenovirus vector	Increased expression of PEDF	Phase I
Volociximab	Ophthotech Corp.	Chimeric monoclonal antibody	α 5 β 1 integrin	Phase I
JSM6427	Jerini AG, Berlin, Germany	Small peptide	α 5 β 1 integrin	Phase I

ECM, extracellular matrix; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PEDF, pigment epithelium-derived factor; PlGF, Placental growth factor.

new drugs are extensively under clinical trials, and more target-specific treatments are being developed as monotherapy. As discussed in the Novel Strategies section, future therapy will target mechanisms upstream or downstream of the VEGF/VEGFR pathway, or other pathways, including growth factors other than VEGF and other inflammatory cytokines. And consideration of the restoration of blood-retinal barrier

should be required. The development of more target-specific and pathogenesis-based treatments can provide additions to our treatment options, and even measures to prevent ARB.

ACKNOWLEDGEMENTS

This study was supported by Mid-Career Research-

er Program (2010-0009050) through grant funded by MEST and by the Bio-Signal Analysis Technology Innovation Program (2010-0020645) of MEST/NRF.

REFERENCES

- Abcouwer, S. F., Shanmugam, S., Gomez, P. F., Shushanov, S., Barber, A. J., Lanoue, K. F., Quinn, P. G., Kester, M., and Gardner, T. W., Effect of IL-1beta on survival and energy metabolism of R28 and RGC-5 retinal neurons. *Invest. Ophthalmol. Vis. Sci.*, 49, 5581-5592 (2008).
- Abramson, D. H. and Scheffler, A. C., Update on retinoblastoma. *Retina*, 24, 828-848 (2004).
- Abran, D., Varma, D. R., and Chemtob, S., Increased thromboxane-mediated contractions of retinal vessels of newborn pigs to peroxides. *Am. J. Physiol.*, 268, H628-H632 (1995).
- Aiello, L. P., Avery, R. L., Arrigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thieme, H., Iwamoto, M. A., and Park, J. E., Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N. Engl. J. Med.*, 331, 1480-1487 (1994).
- Beutel, J., Rudolf, M., and Grisanti, S., Current and future therapies for age-related macular degeneration. *Expert Opin. Emerg. Drugs*, 14, 341-362 (2009).
- Bressler, N. M., Photodynamic therapy of subfoveal choroidal neovascularization in age-related macular degeneration with verteporfin: two-year results of 2 randomized clinical trials-tap report 2. *Arch. Ophthalmol.*, 119, 198-207 (2001).
- Brown, D. M., Kaiser, P. K., Michels, M., Soubrane, G., Heier, J. S., Kim, R. Y., Sy, J. P., and Schneider, S., Ranibizumab versus verteporfin for neovascular age-related macular degeneration. *N. Engl. J. Med.*, 355, 1432-1444 (2006).
- Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernelle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., Scholz, D., Acker, T., Djalma, T., Dewerchin, M., Noel, A., Stalmans, I., Barra, A., Blacher, S., Vandendriessche, T., Ponten, A., Eriksson, U., Plate, K. H., Foidart, J. M., Schaper, W., Charnock-jones, D. S., Hicklin, D. J., Herbert, J. M., Collen, D., and Persico, M. G., Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat. Med.*, 7, 575-583 (2001).
- Chen, J. and Smith, L. E., Retinopathy of prematurity. *Angiogenesis*, 10, 133-140 (2007).
- Connor, K. M., Sangiovanni, J. P., Lofqvist, C., Aderman, C. M., Chen, J., Higuchi, A., Hong, S., Pravda, E. A., Majchrzak, S., Carper, D., Hellstrom, A., Kang, J. X., Chew, E. Y., Salem, N. Jr., Serhan, C. N., and Smith, L. E., Increased dietary intake of omega-3-polyunsaturated fatty acids reduces pathological retinal angiogenesis. *Nat. Med.*, 13, 868-873 (2007).
- Cunha-Vaz, J. G., The blood-retinal barriers. *Doc. Ophthalmol.*, 41, 287-327 (1976).
- Davidson, J. A., Ciulla, T. A., McGill, J. B., Kles, K. A., and Anderson, P. W., How the diabetic eye loses vision. *Endocrine*, 32, 107-116 (2007).
- De Jong, P. T., Age-related macular degeneration. *N. Engl. J. Med.*, 355, 1474-1485 (2006).
- Dixon, J. A., Oliver, S. C., Olson, J. L., and Mandava, N., VEGF Trap-Eye for the treatment of neovascular age-related macular degeneration. *Expert Opin. Investig. Drugs*, 18, 1573-1580 (2009).
- Doukas, J., Mahesh, S., Umeda, N., Kachi, S., Akiyama, H., Yokoi, K., Cao, J., Chen, Z., Dellamary, L., Tam, B., Racanelli-layton, A., Hood, J., Martin, M., Noronha, G., Soll, R., and Campochiaro, P. A., Topical administration of a multi-targeted kinase inhibitor suppresses choroidal neovascularization and retinal edema. *J. Cell. Physiol.*, 216, 29-37 (2008).
- Edwards, A. O., Ritter, R. 3rd, Abel, K. J., Manning, A., Panhuysen, C., and Farrer, L. A., Complement factor H polymorphism and age-related macular degeneration. *Science*, 308, 421-424 (2005).
- El-Remessy, A. B., Abou-Mohamed, G., Caldwell, R. W., and Caldwell, R. B., High glucose-induced tyrosine nitration in endothelial cells: role of eNOS uncoupling and aldose reductase activation. *Invest. Ophthalmol. Vis. Sci.*, 44, 3135-3143 (2003).
- Ferris, F. L. 3rd, Fine, S. L., and Hyman, L., Age-related macular degeneration and blindness due to neovascular maculopathy. *Arch. Ophthalmol.*, 102, 1640-1642 (1984).
- Folkman, J., Angiogenesis. *Annu. Rev. Med.*, 57, 1-18 (2006).
- Frank, R. N., Diabetic retinopathy. *N. Engl. J. Med.*, 350, 48-58 (2004).
- Friedman, D. S., O'Colmain, B. J., Munoz, B., Tomany, S. C., McCarty, C., De Jong, P. T., Nemesure, B., Mitchell, P., and Kempen, J., Prevalence of age-related macular degeneration in the United States. *Arch. Ophthalmol.*, 122, 564-572 (2004).
- Funatsu, H., Yamashita, H., Noma, H., Mimura, T., Yamashita, T., and Hori, S., Increased levels of vascular endothelial growth factor and interleukin-6 in the aqueous humor of diabetics with macular edema. *Am. J. Ophthalmol.*, 133, 70-77 (2002).
- Gariano, R. F. and Gardner, T. W., Retinal angiogenesis in development and disease. *Nature*, 438, 960-966 (2005).
- Gehrs, K. M., Anderson, D. H., Johnson, L. V., and Hageman, G. S., Age-related macular degeneration-emerging pathogenetic and therapeutic concepts. *Ann. Med.*, 38, 450-471 (2006).
- Gilbert, C., Rahi, J., Eckstein, M., O'Sullivan, J., and Foster, A., Retinopathy of prematurity in middle-income countries. *Lancet*, 350, 12-14 (1997).
- Good, W. V., Hardy, R. J., Dobson, V., Palmer, E. A., Phelps, D. L., Quintos, M., and Tung, B., The incidence and course of retinopathy of prematurity: findings from the early

- treatment for retinopathy of prematurity study. *Pediatrics*, 116, 15-23 (2005).
- Haller, J. A., Qin, H., Apte, R. S., Beck, R. R., Bressler, N. M., Browning, D. J., Danis, R. P., Glassman, A. R., Googe, J. M., Kollman, C., Lauer, A. K., Peters, M. A., and Stockman, M. E., Vitrectomy outcomes in eyes with diabetic macular edema and vitreomacular traction. *Ophthalmology*, 117, 1087-1093 e3 (2010).
- Heidary, G., Vanderveen, D., and Smith, L. E., Retinopathy of prematurity: current concepts in molecular pathogenesis. *Semin. Ophthalmol.*, 24, 77-81 (2009).
- Hellberg, C., Ostman, A., and Heldin, C. H., PDGF and vessel maturation. *Recent Results Cancer Res.*, 180, 103-114 (2010).
- Hellstrom, A., Perruzzi, C., Ju, M., Engstrom, E., Hard, A. L., Liu, J. L., Albertsson-Wikland, K., Carlsson, B., Niklasson, A., Sjedell, L., Leroith, D., Senger, D. R., and Smith, L. E., Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinopathy of prematurity. *Proc. Natl. Acad. Sci. U.S.A.*, 98, 5804-5808 (2001).
- Hubbard, G. B. 3rd, Surgical management of retinopathy of prematurity. *Curr. Opin. Ophthalmol.*, 19, 384-390 (2008).
- Jager, R. D., Mieler, W. F., and Miller, J. W., Age-related macular degeneration. *N. Engl. J. Med.*, 358, 2606-2617 (2008).
- Joussen, A. M., Doehmen, S., Le, M. L., Koizumi, K., Radetzky, S., Krohne, T. U., Poulaki, V., Semkova, I., and Kociok, N., TNF-alpha mediated apoptosis plays an important role in the development of early diabetic retinopathy and long-term histopathological alterations. *Mol. Vis.*, 15, 1418-1428 (2009).
- Jung, Y. J., Isaacs, J. S., Lee, S., Trepel, J., and Neckers, L., IL-1beta-mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J.*, 17, 2115-2117 (2003).
- Kaiser, P. K., Vascular endothelial growth factor Trap-Eye for diabetic macular oedema. *Br. J. Ophthalmol.*, 93, 135-136 (2009).
- Kempner, J. H., O'Colmain, B. J., Leske, M. C., Haffner, S. M., Klein, R., Moss, S. E., Taylor, H. R., and Hamman, R. F., The prevalence of diabetic retinopathy among adults in the United States. *Arch. Ophthalmol.*, 122, 552-563 (2004).
- Kim, J. H., Khwarg, S. I., Choung, H. K., and Yu, Y. S., Management of porous polyethylene implant exposure in patients with retinoblastoma following enucleation. *Ophthalmic Surg. Lasers Imaging*, 35, 446-452 (2004).
- Kim, J. H., Park, J. A., Lee, S. W., Kim, W. J., Yu, Y. S., and Kim, K. W., Blood-neural barrier: intercellular communication at glio-vascular interface. *J. Biochem. Mol. Biol.*, 39, 339-345 (2006).
- Kim, J. H., Yu, Y. S., Shin, J. Y., Lee, H. Y., and Kim, K. W., Deguelin inhibits retinal neovascularization by down-regulation of HIF-1alpha in oxygen-induced retinopathy. *J. Cell. Mol. Med.*, 12, 2407-2415 (2008).
- Kim, J. H., Lee, Y. M., Ahn, E. M., Kim, K. W., and Yu, Y. S., Decursin inhibits VEGF-mediated inner blood-retinal barrier breakdown by suppression of VEGFR-2 activation. *J. Cereb. Blood Flow Metab.*, 29, 1559-1567 (2009a).
- Kim, J. H., Oh, M., Yu, Y. S., Kim, K. W., and Kwon, H. J., N-hydroxy-7-(2-naphthylthio) heptanamide inhibits retinal and choroidal angiogenesis. *Mol. Pharm.*, 6, 513-519 (2009b).
- Kim, J. H., Jun, H. O., Yu, Y. S., and Kim, K. W., Inhibition of protein kinase C delta attenuates blood-retinal barrier breakdown in diabetic retinopathy. *Am. J. Pathol.*, 176, 1517-1524 (2010).
- Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W., and Kim, K. W., Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat. Med.*, 7, 437-443 (2001).
- Klein, R., Peto, T., Bird, A., and Vannewkirk, M. R., The epidemiology of age-related macular degeneration. *Am. J. Ophthalmol.*, 137, 486-495 (2004).
- Kliffen, M., Sharma, H. S., Mooy, C. M., Kerkvliet, S., and De Jong, P. T., Increased expression of angiogenic growth factors in age-related maculopathy. *Br. J. Ophthalmol.*, 81, 154-162 (1997).
- Kvanta, A., Steen, B., and Seregard, S., Expression of vascular endothelial growth factor (VEGF) in retinoblastoma but not in posterior uveal melanoma. *Exp. Eye Res.*, 63, 511-518 (1996).
- Lee, S. Y., Kim, D. K., Cho, J. H., Koh, J. Y., and Yoon, Y. H., Inhibitory effect of bevacizumab on the angiogenesis and growth of retinoblastoma. *Arch. Ophthalmol.*, 126, 953-958 (2008).
- Lin, P. and O'Brien, J. M., Frontiers in the management of retinoblastoma. *Am. J. Ophthalmol.*, 148, 192-198 (2009).
- Lopez, P. F., Sippy, B. D., Lambert, H. M., Thach, A. B., and Hinton, D. R., Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. *Invest. Ophthalmol. Vis. Sci.*, 37, 855-868 (1996).
- Mantagos, I. S., Vanderveen, D. K., and Smith, L. E., Emerging treatments for retinopathy of prematurity. *Semin. Ophthalmol.*, 24, 82-86 (2009).
- Mintz-Hittner, H. A., Avastin as monotherapy for retinopathy of prematurity. *J. AAPOS*, 14, 2-3 (2010).
- Miyamoto, N., De Kozak, Y., Normand, N., Courtois, Y., Jeanny, J. C., Benezra, D., and Behar-Cohen, F., PIGF-1 and VEGFR-1 pathway regulation of the external epithelial hemato-ocular barrier. A model for retinal edema. *Ophthalmic Res.*, 40, 203-207 (2008).
- Mousa, S. A. and Mousa, S. S., Current status of vascular endothelial growth factor inhibition in age-related macular degeneration. *BioDrugs*, 24, 183-194 (2010).
- Ni, Z. and Hui, P., Emerging pharmacologic therapies for wet age-related macular degeneration. *Ophthalmologica*, 223, 401-410 (2009).

- Oh, S. H., Woo, J. K., Yazici, Y. D., Myers, J. N., Kim, W. Y., Jin, Q., Hong, S. S., Park, H. J., Suh, Y. G., Kim, K. W., Hong, W. K., and Lee, H. Y., Structural basis for depletion of heat shock protein 90 client proteins by deguelin. *J. Natl. Cancer Inst.*, 99, 949-961 (2007).
- Ozkiris, A., Anti-VEGF agents for age-related macular degeneration. *Expert Opin. Ther. Pat.*, 20, 103-118 (2010).
- Palmer, E. A., Flynn, J. T., Hardy, R. J., Phelps, D. L., Phillips, C. L., Schaffer, D. B., and Tung, B., Incidence and early course of retinopathy of prematurity. The Cryotherapy for Retinopathy of Prematurity Cooperative Group. *Ophthalmology*, 98, 1628-1640 (1991).
- Penn, J. S., Madan, A., Caldwell, R. B., Bartoli, M., Caldwell, R. W., and Hartnett, M. E., Vascular endothelial growth factor in eye disease. *Prog. Retin. Eye Res.*, 27, 331-371 (2008).
- Pina, Y., Boutrid, H., Scheffler, A., Dubovy, S., Feuer, W., Jockovich, M. E., and Murray, T. G., Blood vessel maturation in retinoblastoma tumors: spatial distribution of neovessels and mature vessels and its impact on ocular treatment. *Invest. Ophthalmol. Vis. Sci.*, 50, 1020-1024 (2009).
- Pugh, C. W. and Ratcliffe, P. J., Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat. Med.*, 9, 677-684 (2003).
- Quiram, P. A. and Capone, A. Jr., Current understanding and management of retinopathy of prematurity. *Curr. Opin. Ophthalmol.*, 18, 228-234 (2007).
- Repka, M. X., Tung, B., Good, W. V., Shapiro, M., Capone, A., Jr., Baker, J. D., Barr, C. C., Phelps, D. L., and Van Heuven, W. A., Outcome of eyes developing retinal detachment during the early treatment for retinopathy of prematurity study (ETROP). *Arch. Ophthalmol.*, 124, 24-30 (2006).
- Ricart, A. D., Tolcher, A. W., Liu, G., Holen, K., Schwartz, G., Albertini, M., Weiss, G., Yazji, S., Ng, C., and Wilding, G., Volociximab, a chimeric monoclonal antibody that specifically binds alpha5beta1 integrin: a phase I, pharmacokinetic, and biological correlative study. *Clin. Cancer Res.*, 14, 7924-7929 (2008).
- Rishi, E., Rishi, P., Ratra, D., and Bhende, M., Off-label use of bevacizumab in retinopathy of prematurity. *Retina*, 29, 284; author reply 284-285 (2009).
- Sapieha, P., Hamel, D., Shao, Z., Rivera, J. C., Zaniolo, K., Joyal, J. S., and Chemtob, S., Proliferative retinopathies: angiogenesis that blinds. *Int. J. Biochem. Cell Biol.*, 42, 5-12 (2010).
- Sato, T., Kusaka, S., Shimojo, H., and Fujikado, T., Simultaneous analyses of vitreous levels of 27 cytokines in eyes with retinopathy of prematurity. *Ophthalmology*, 116, 2165-2169 (2009).
- Schofield, C. J. and Ratcliffe, P. J., Oxygen sensing by HIF hydroxylases. *Nat. Rev. Mol. Cell Biol.*, 5, 343-354 (2004).
- Sfikakis, P. P., Markomichelakis, N., Theodossiadis, G. P., Grigoropoulos, V., Katsilambros, N., and Theodossiadis, P. G., Regression of sight-threatening macular edema in type 2 diabetes following treatment with the anti-tumor necrosis factor monoclonal antibody infliximab. *Diabetes Care*, 28, 445-447 (2005).
- Shen, J., Samul, R., Silva, R. L., Akiyama, H., Liu, H., Saishin, Y., Hackett, S. F., Zinnen, S., Kossen, K., Fosnaugh, K., Vargeese, C., Gomez, A., Bouhana, K., Aitchison, R., Pavco, P., and Campochiaro, P. A., Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Ther.*, 13, 225-234 (2006).
- Shields, C. L., Shields, J. A., Baez, K. A., Cater, J., and De Potter, P. V., Choroidal invasion of retinoblastoma: metastatic potential and clinical risk factors. *Br. J. Ophthalmol.*, 77, 544-548 (1993).
- Shields, C. L., Shields, J. A., Baez, K., Cater, J. R., and De Potter, P., Optic nerve invasion of retinoblastoma. Metastatic potential and clinical risk factors. *Cancer*, 73, 692-698 (1994).
- Singerman, L., Combination therapy using the small interfering RNA bevasiranib. *Retina*, 29, S49-S50 (2009).
- Slakter, J. S., Anecortave acetate for treating or preventing choroidal neovascularization. *Ophthalmol. Clin. North Am.*, 19, 373-380 (2006).
- Smiddy, W. E. and Flynn, H. W. Jr., Vitrectomy in the management of diabetic retinopathy. *Surv. Ophthalmol.*, 43, 491-507 (1999).
- Stitt, A. W., Simpson, D. A., Boockock, C., Gardiner, T. A., Murphy, G. M., and Archer, D. B., Expression of vascular endothelial growth factor (VEGF) and its receptors is regulated in eyes with intra-ocular tumours. *J. Pathol.*, 186, 306-312 (1998).
- Thurman, J. M., Renner, B., Kunchithapautham, K., Ferreira, V. P., Pangburn, M. K., Ablonczy, Z., Tomlinson, S., Holers, V. M., and Rohrer, B., Oxidative stress renders retinal pigment epithelial cells susceptible to complement-mediated injury. *J. Biol. Chem.*, 284, 16939-16947 (2009).
- Tian, N., Visual experience and maturation of retinal synaptic pathways. *Vision Res.*, 44, 3307-3316 (2004).
- Tolentino, M. J., Current molecular understanding and future treatment strategies for pathologic ocular neovascularization. *Curr. Mol. Med.*, 9, 973-981 (2009).
- Weis, S. M. and Cheresch, D. A., Pathophysiological consequences of VEGF-induced vascular permeability. *Nature*, 437, 497-504 (2005).
- Zahn, G., Vossmeier, D., Stragies, R., Wills, M., Wong, C. G., Loffler, K. U., Adamis, A. P., and Knolle, J., Preclinical evaluation of the novel small-molecule integrin alpha5beta1 inhibitor JSM6427 in monkey and rabbit models of choroidal neovascularization. *Arch. Ophthalmol.*, 127, 1329-1335 (2009).