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.

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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.priorartdoclib.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, is 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

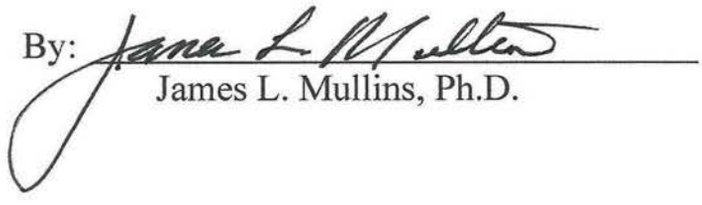
3 and its accessibility to a researcher. Further evidence of its public accessibility is it being listed as a reference in an article (Appendix 3E) published in 2010. It is, therefore, my conclusion that Document 3, the Dixon article, is an authentic document and was publicly accessible no later than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

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 silikonölarme 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 Injektabila 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 Montagekräfte 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 ermöglichen. 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 mm^2/s liegen sollte [3]. Die Monographie zu Polydimethylsiloxanen (PDMS) in

TechnoPharm 2, Nr. 4, 238–244 (2012)

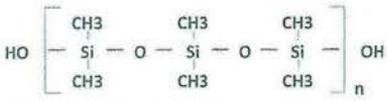


Abb. 1: Polydimethylsiloxan.

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 $(CH_3)_2\ SiCl_2$ 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 Trimethylchlorsilan Trimethylsiloxy-Endgruppen erhalten. Aus den entstehenden polydispersen Polymeren werden die kurzkettigen Moleküle durch Verdampfen entfernt, um einsatzfähiges 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 Oxydationsmitteln. Erst oberhalb von 130 °C kommt es zu Reaktionen wie Oxidation, Polymerisierung oder Depolymerisierung. Für das Molekül ist

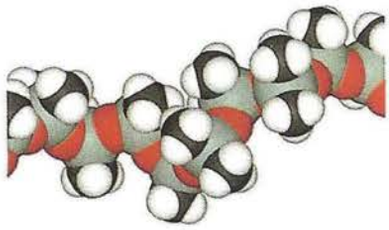


Abb. 2: Räumliche Darstellung Polydimethylsiloxan.

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 (1,63Å Si-O im Vergleich zu 1,43Å 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 Hohlnadelspritzen 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-

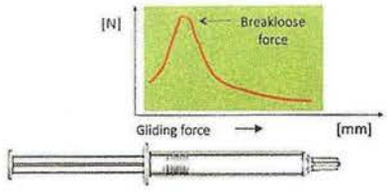


Abb. 3: Exemplarisches Kräfteprofil einer vorfüllbaren Spritze.

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 Kolbenstopfen 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 erreichen. Eine Erhöhung der Silikonölmenge ist aber wie schon gesagt mit einer Erhöhung der Anzahl von Silikonölpartikeln in der Lösung verbunden. Vor allem bei protein-basierten Medikamenten können unerwünschte Wechselwirkungen mit derartigen Silikonölpartikeln nicht ausgeschlossen werden. Subvisuelle Silikonölpartikel 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.

$$Si\text{-}O\text{-}Na + H_20 \leftrightarrow SiOH + NaOH$$

In basischen Umgebungen findet dagegen ein Ätzprozess statt.

$$2NaOH + (SiO_2)_X \rightarrow Na_2SiO_3 + H_2O$$

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

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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 (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.

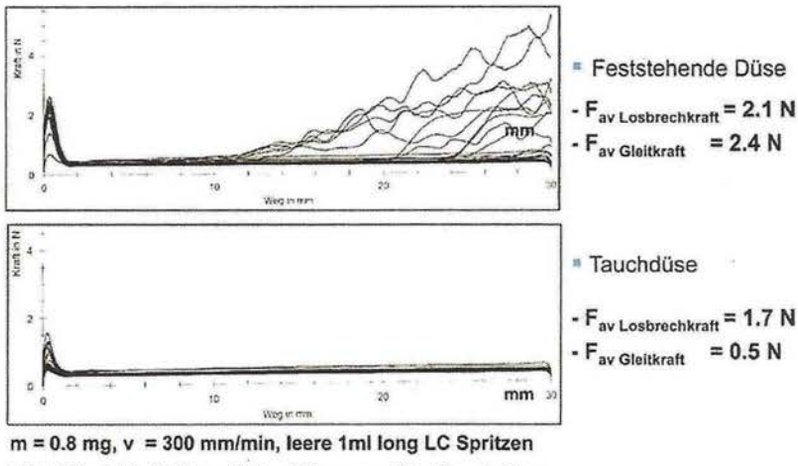

Abb. 4: Vergleich Kräfteprofil Tauchdüse gegen feststehende Düse.

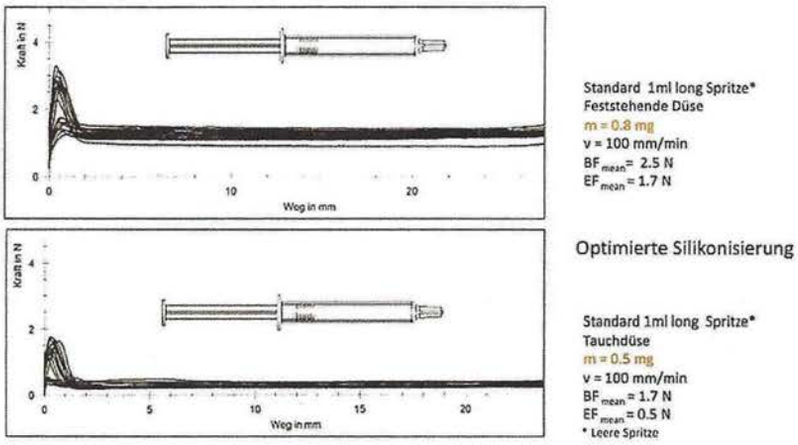

Abb. 5: Kräfteprofil nach optimierter Silikonisierung.

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 Molekülgewicht 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 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 – 1 000 nm.

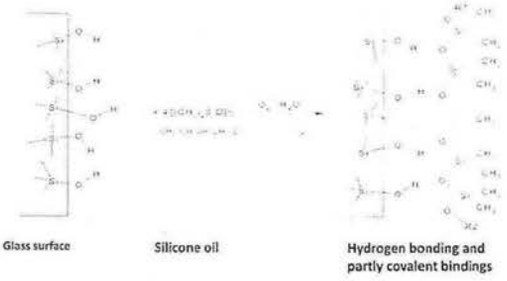

Abb. 6: Einbrennsilikonisierung.

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

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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 eingebrannten Beschichtung hingegen kann man dieses Phänomen nicht in dem Ausmaß beobachten (Abb. 7). Die Losbrechkraft bleibt über die Lagerzeit nahezu konstant.

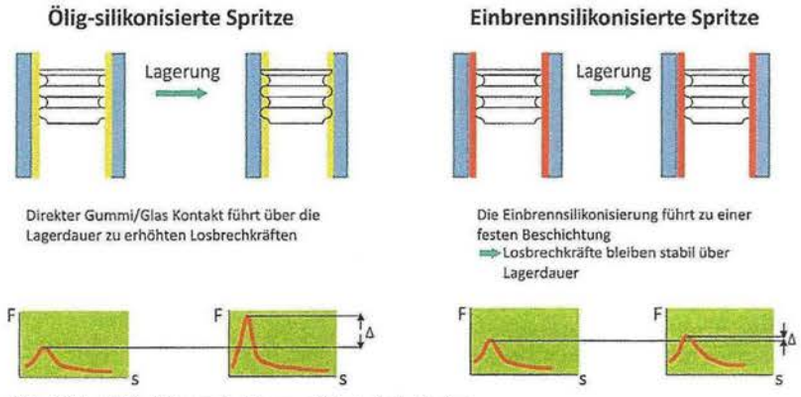

Abb. 7: Vergleich ölig- und einbrennsilikonisierte Spritze.

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

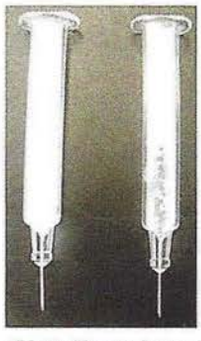

Abb. 8: Glasstaub-Test: links – Spritze mit Tauchdüse silikonisiert, rechts- Spritze mit feststehender Düse silikonisiert.

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

Abb. 9: Gleitkraftmessung.

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 Analysemethode 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

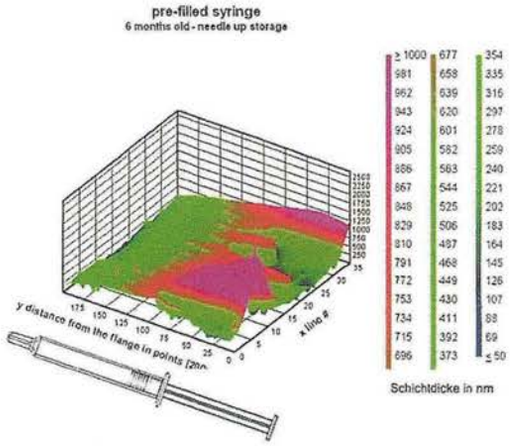

Abb. 10: Silikonölschichtdickenmessung mit dem Layer Explorer RapID (Quelle: Gerresheimer Buende).

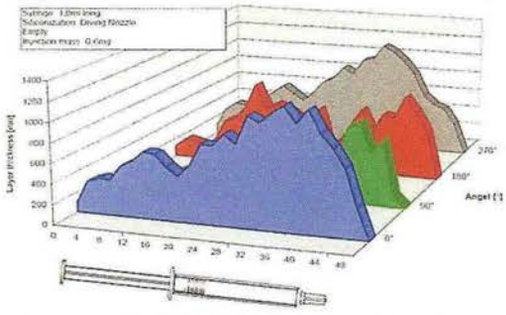

Abb. 10.1: Silikonölschichtdickenmessung mit dem Layer Explorer (Quelle: Gerresheimer Buende).

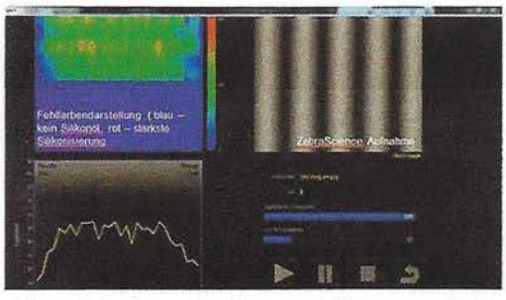

Abb. 11: ZebraScience Visualisierung Silikonisierung (Quelle: Gerresheimer Buende).

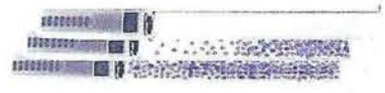

Abb. 11.1: Visualisierung der Spritzenkörpersilikonisierung (Zebra Science).

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 Silikonisierprozesses zu ermöglichen.

Ausblick

Beim Finishing von Glasspritzen geht der Trend zu silikonölreduzierten 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.

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Appendix 1B

Techno Pharm Fokus Verpackung

Die Silikonisierung von Spritzen

Trends, Methoden, Analyseverfahren

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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 silikonölarme 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 Injektabila 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 Montagekräfte 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 ermöglichen. 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 mm^2/s liegen sollte [3]. Die Monographie zu Polydimethylsiloxanen (PDMS) in

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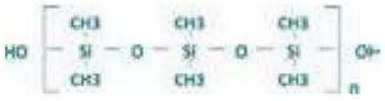

Abb. 1: Polydimethylsiloxan.

der USP [2] erlaubt hingegen die Verwendung von Silikonölen mit einer Viskosität von 20 bis 30000 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 1000 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 $(CH_3)_2\ SiCl_2$ 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 Trimethylchlorsilan Trimethylsiloxy-Endgruppen erhalten. Aus den entstehenden polydispersen Polymeren werden die kurzkettigen Moleküle durch Verdampfen entfernt, um einsatzfähiges 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 Oxydationsmitteln. 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 (1,63Å Si-O im Vergleich zu 1,43Å 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 Hohlnadelspritzen 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-

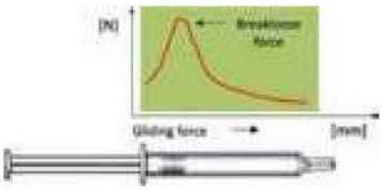

Abb. 3: Exemplarisches Kräfteprofil einer vorfüllbaren Spritze.

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 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 Kolbenstopfen 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 erreichen. Eine Erhöhung der Silikonölmenge ist aber wie schon gesagt mit einer Erhöhung der Anzahl von Silikonölpartikeln in der Lösung verbunden. Vor allem bei protein-basierten Medikamenten können unerwünschte Wechselwirkungen mit derartigen Silikonölpartikeln nicht ausgeschlossen werden. Subvisuelle Silikonölpartikel 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 1 mg/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.

$$Si\text{-}O\text{-}Na + H_20 \leftrightarrow SiOH + NaOH$$

In basischen Umgebungen findet dagegen ein Ätzprozess statt.

$$2NaOH + (SiO_2)_X \rightarrow Na_2SiO_3 + H_2O$$

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

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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 (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.

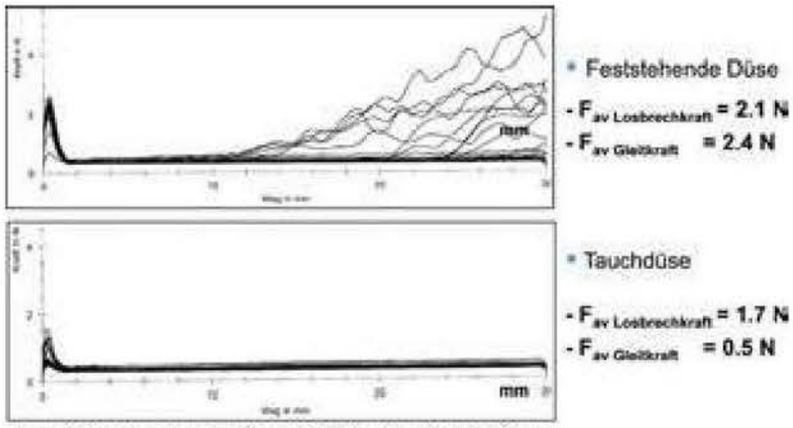

Abb. 4: Vergleich Kräfteprofil Tauchdüse gegen feststehende Düse.

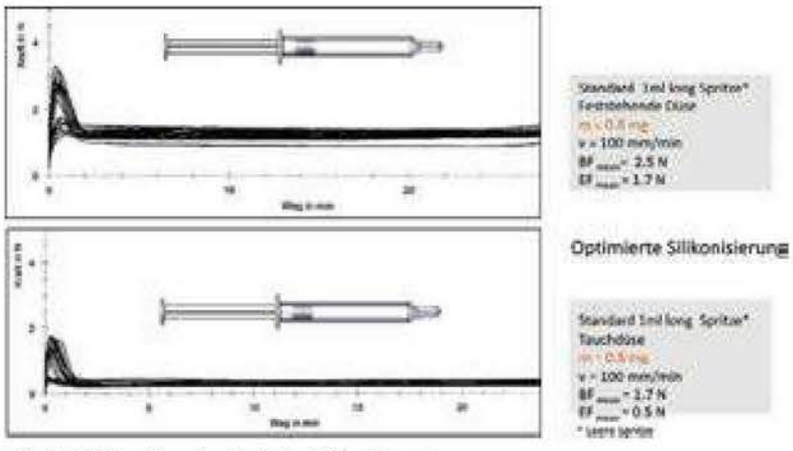

Abb. 5: Kräfteprofil nach optimierter Silikonisierung.

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 Molekülgewicht 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 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.

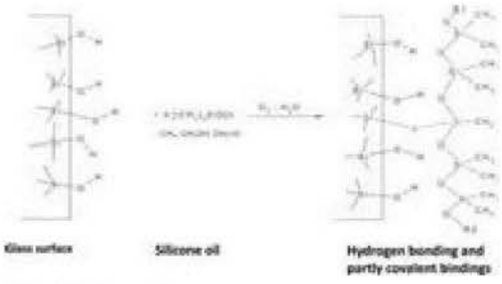

Abb. 6: Einbrennsilikonisierung.

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

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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 eingebrannten Beschichtung hingegen kann man dieses Phänomen nicht in dem Ausmaß beobachten (Abb. 7). Die Losbrechkraft bleibt über die Lagerzeit nahezu konstant.

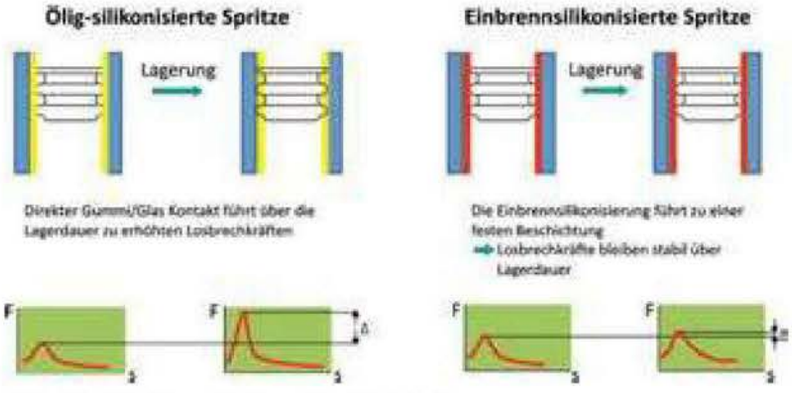

Abb. 7: Vergleich ölig- und einbrennsilikonisierte Spritze.

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

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

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

Abb. 9: Gleitkraftmessung.

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 Analysemethode 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

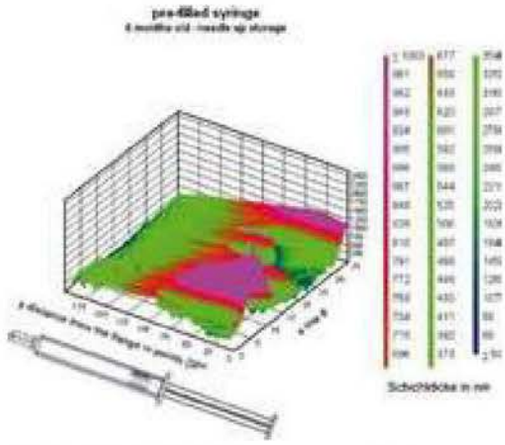

Abb. 10: Silikonölschichtdickenmessung mit dem Layer Explorer RapID (Quelle: Gerresheimer Buende).

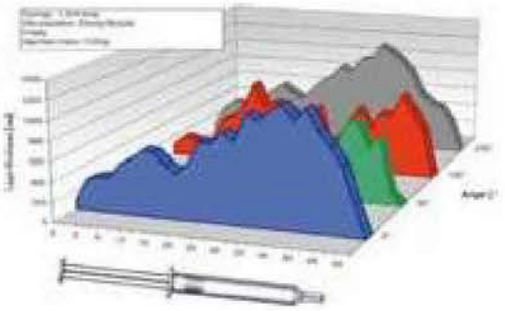

Abb. 10.1: Silikonölschichtdickenmessung mit dem Layer Explorer (Quelle: Gerresheimer Buende).

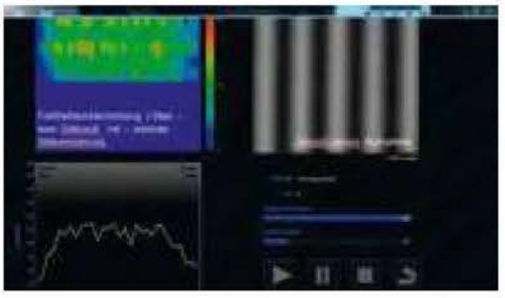

Abb. 11: ZebraScience Visualisierung Silikonisierung (Quelle: Gerresheimer Buende).

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 Silikonisierprozesses zu ermöglichen.

Ausblick

Beim Finishing von Glasspritzen geht der Trend zu silikonölreduzierten 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.

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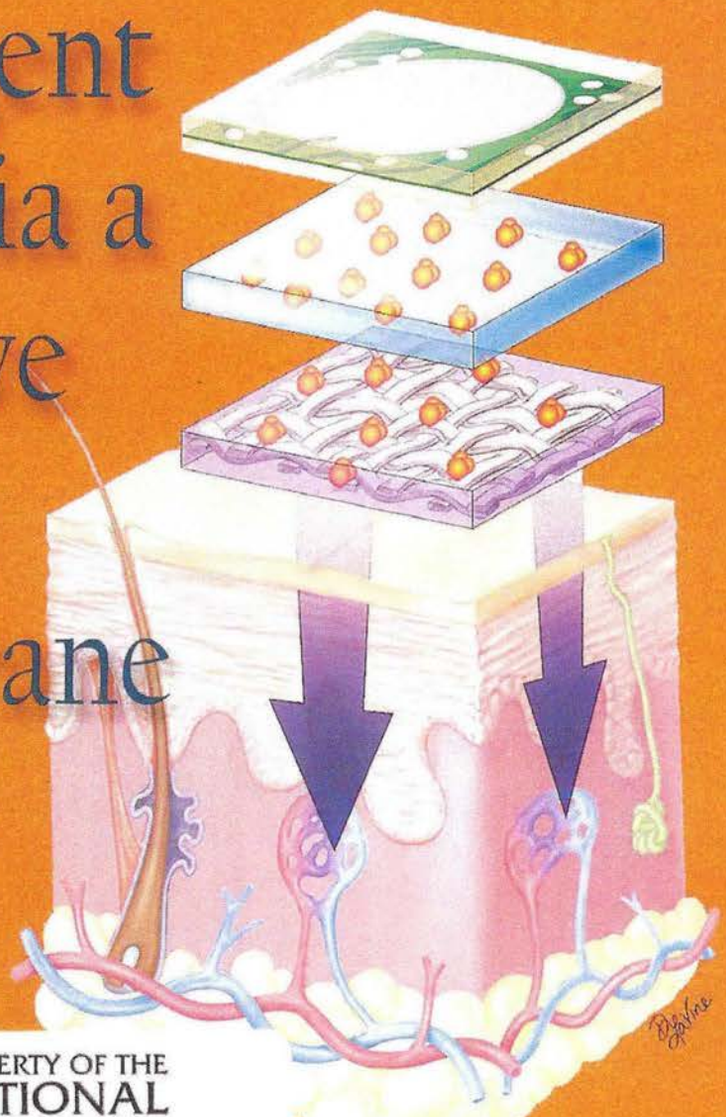

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Drug Delivery of Sensitive Biopharmaceuticals With Prefilled Syringes

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Successful, Sustained-Release Using a Selective Charged Ba… Membrane

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

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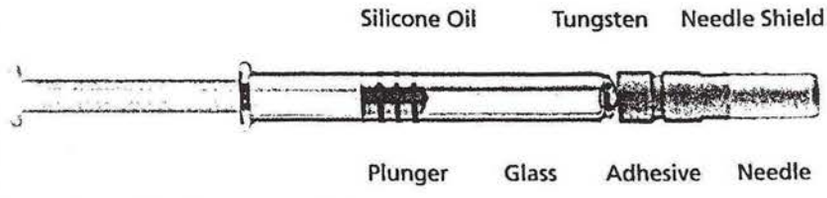
ADVANCED DELIVERY DEVICES

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]

FIGURE 1



Product Contact Materials in Syringe Systems

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 biotherapeutics is 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.[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

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.[19] Elevated pH might trigger oxidation and hydrolysis of biopharmaceuticals.

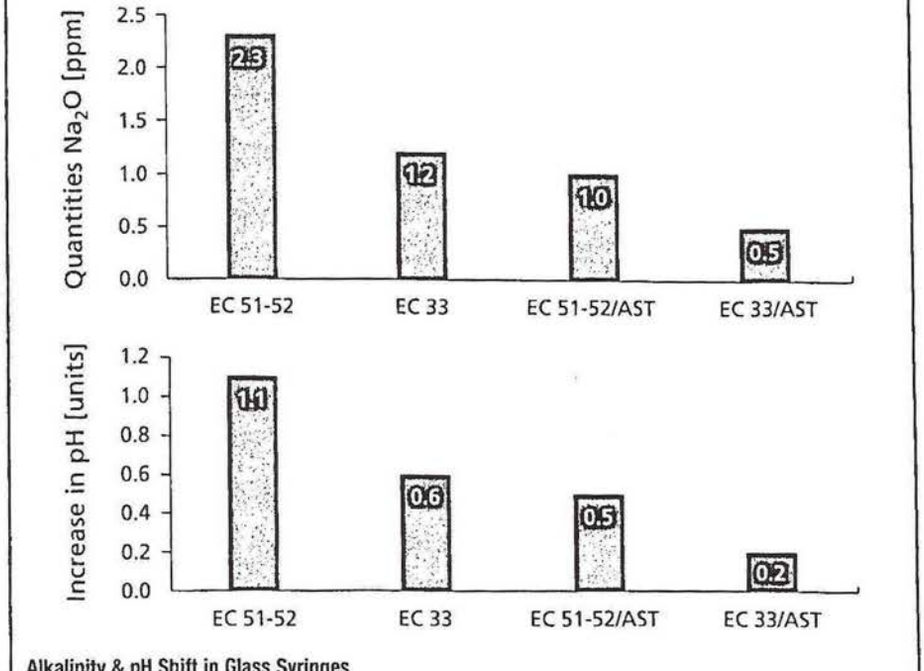
For the production of syringe barrels, glass tubing from Type I borosilicate glass according to USP, EP, and JP is used. Standard glass tubing has an extension coefficient of 51-52 and consists of 70% to 80% SiO_2, 15% B_2O_3/Al_2O_3, and up to 7% Na_2O. The role of sodium is to lower the forming temperatures 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-dest.: 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: $Na_2O + (NH_4)_2SO_4 \rightarrow Na_2SO_4 + 2\ NH_3 + H_2O$. 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

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^{n+} from stainless steel tanks used in manufacturing equipment) are known for similar interactions with active molecules.[1]

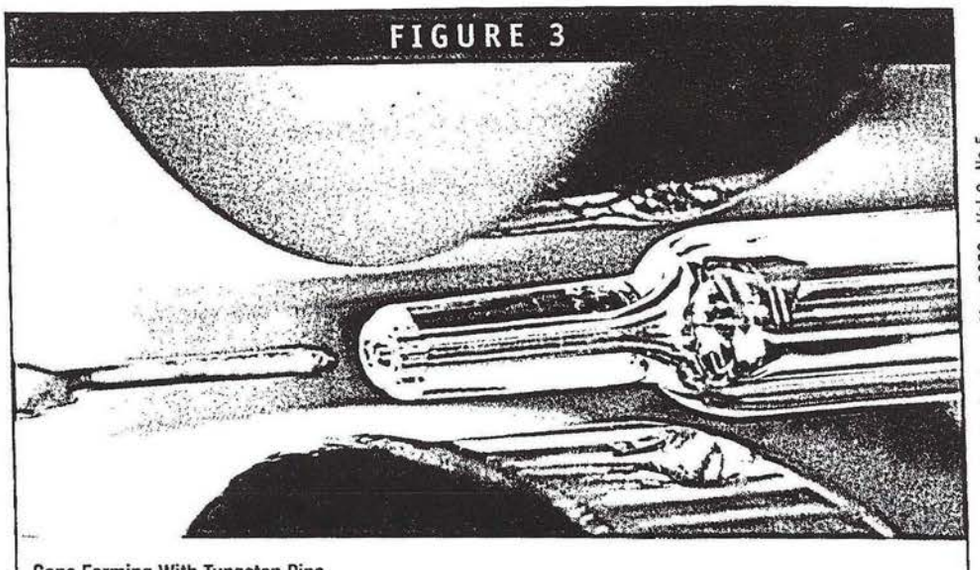
Prefilled syringes formed with tungsten pins contain trace amounts of tungsten compounds in the cone section, which is part of the product contact surface. Syringe filling processes with plunger placement under vacuum intensify the contact between active molecules and tungsten because air bubbles in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

FIGURE 3



Cone Forming With Tungsten Pins

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]

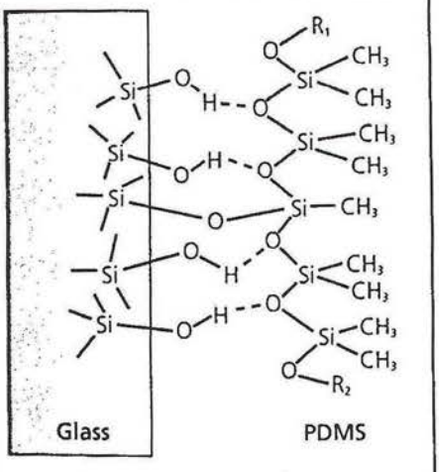
Prefilled syringes are containers and drug delivery systems at the same time. Functionality of these systems (viable activation and gliding forces of the plunger) is accomplished by siliconization. Silicone oils are viscous, inert materials with excellent characteristics as hydrophobic lubricants.[35,36] 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
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

FIGURE 4



Model of Silicone Coating

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.

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. ◆

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BIOGRAPHY

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Appendix 2B

Drug Delivery Technology

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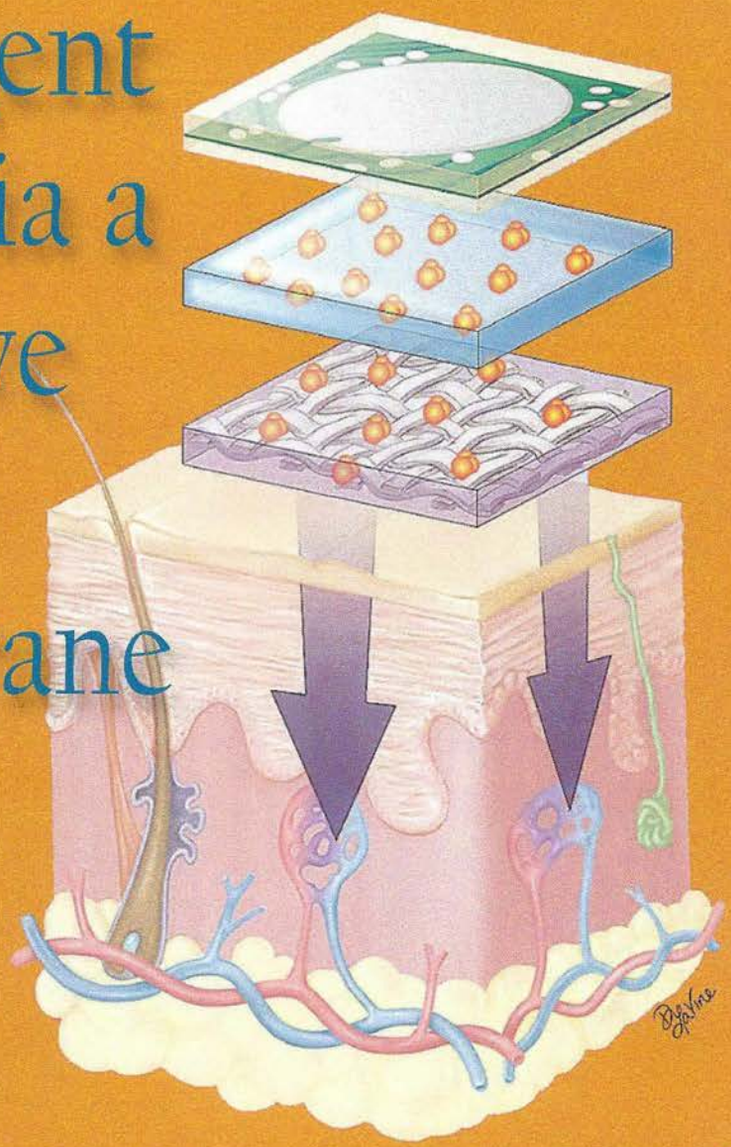
Intelligent TDD Via a Selective Barrier Membrane



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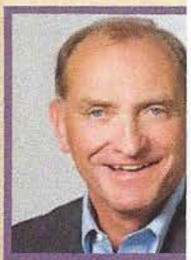

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Arno Fries, PhD
Drug Delivery of Sensitive Biopharmaceuticals

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

Cherng-ju Kim, PhD
A New Process Producing Biodegradable Microspheres of

Drug Delivery Technology

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Drug Delivery of Sensitive Biopharmaceuticals With Prefilled Syringes

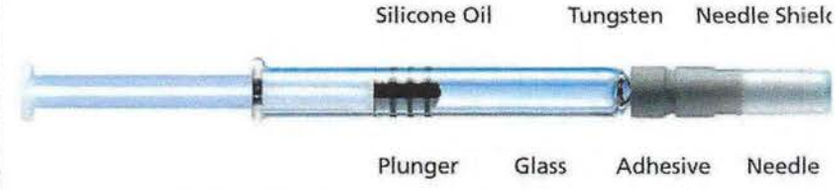
By: Arno Fries, PhD

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.

FIGURE 1



Product Contact Materials in Syringe Systems

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 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 ge 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

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.

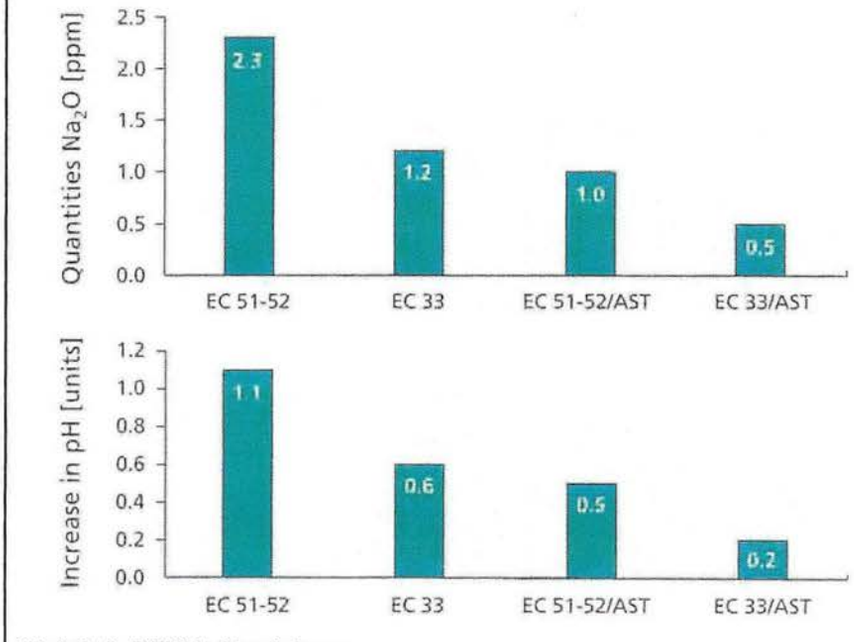
For the production of syringe barrels, glass tubing from Type I borosilicate glass according to USP, EP, and JP is used. Standard glass tubing has an extension coefficient of 51-52 and consists of 70% to 80% SiO_2, 15% B_2O_3/Al_2O_3, and up to 7% Na_2O. The role of sodium is to lower the forming temperatures 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-dest.: 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. Th 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 t 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: $Na_2O + (NH_4)_2SO_4 \rightarrow Na_2SO_4 + 2\ NH_3 + H_2O$. Removal c sodium sulfate is achieved downstream during washing of the syringe barrels and reduces significantly the amount of alkali ions on the gla surface.

FIGURE 2



Alkalinity & pH Shift in Glass Syringes

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 targeted

FIGURE 3

Cone Forming With Tungsten Pins

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]

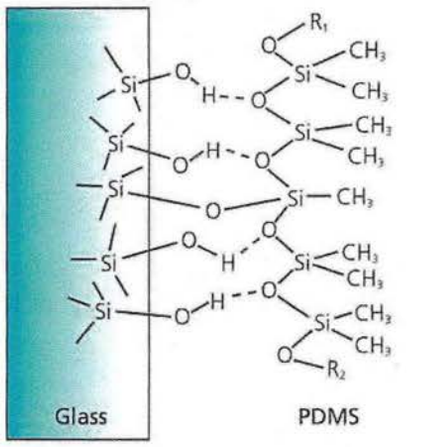
Prefilled syringes are containers and drug delivery systems at the same time. Functionality of these systems (viable activation and gliding forces of the plunger) is accomplished by siliconization. Silicone oils are viscous, inert materials with excellent characteristics as hydrophobic lubricants.[35,36] 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
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

FIGURE 4



Model of Silicone Coating

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

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

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. ◆

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Appendix 2E

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

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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.

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

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[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.

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 [MWCOs]) 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 ml mg^{-1} 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):

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

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):

$$DOL = \frac{A_{650} * MW}{[\text{protein}] * \varepsilon_{\text{dye}}}, \tag{2}$$

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 cm^{-1} 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-pentamethyl-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 NICOMP 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 FACSFlow 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 μg/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 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.

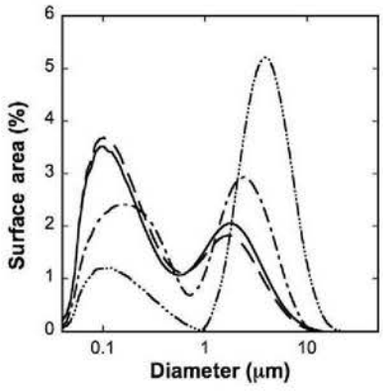

Fig.1. Surface area-weighted particle size distribution of silicone oil droplets in 10 mM sodium phosphate (pH 7.5) and 0.01% sodium azide buffer. The solid line represents excipient-free, the dashed line represents emulsions formed from solutions containing 0.03% polysorbate 20, the dash-dot-dash line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

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

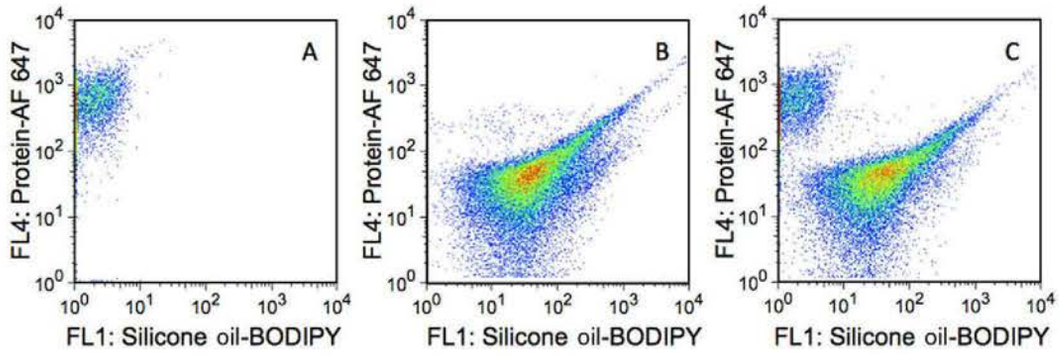

Fig.2. Detection of subvisible homogeneous protein aggregates: Fluorescence dot plots of 1- to 2-μm homogeneous BSA–AF 647 aggregates in the absence of silicone oil (A), silicone oil–BODIPY droplets with adsorbed BSA–AF 647 (B), and the mixture of homogeneous BSA–AF 647 aggregates and silicone oil–BODIPY 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.

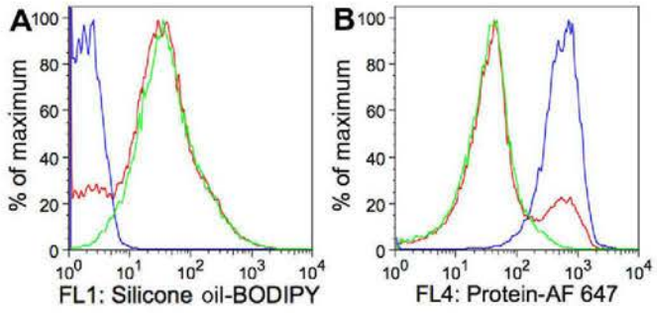

Fig.3. Fluorescence histograms of analyzed particles: BODIPY fluorescence (A) and AF 647 fluorescence (B). Histograms are representations of the data from Fig. 2, with the blue line representing the BSA–AF 647 aggregate suspension, the green line representing the silicone oil–BODIPY droplets with adsorbed 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.

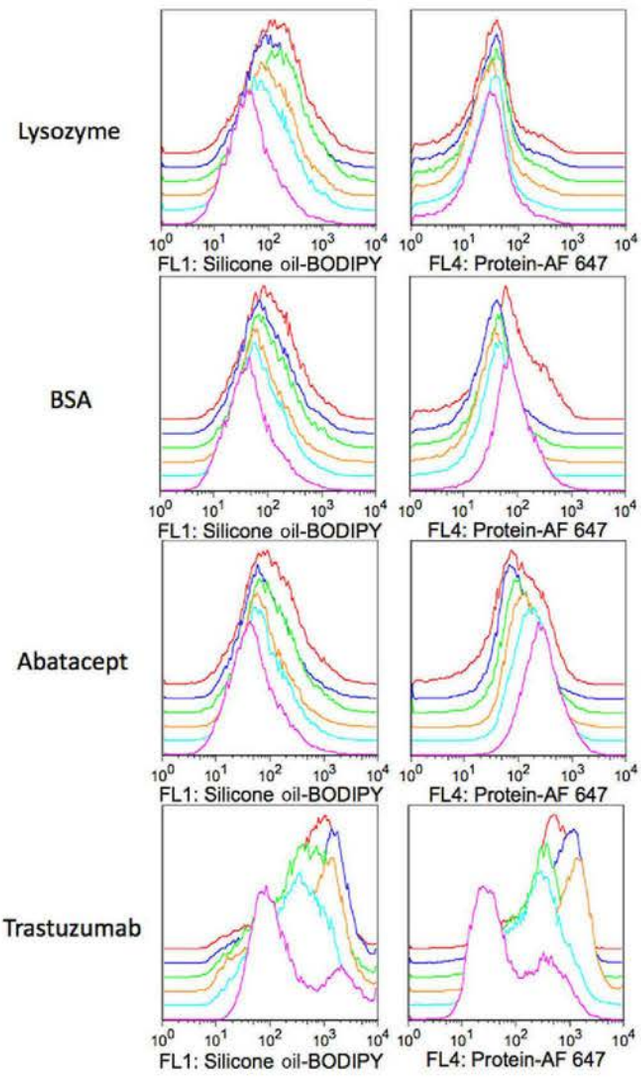

Fig.4. Flow cytometry analyses of protein–silicone oil mixtures in excipient-free formulations. Histograms illustrate particle BODIPY fluorescence intensities measured with the FL1 detector (530/30 nm, histograms on left) or AF 647 fluorescence intensities measured with the FL4 detector (661/16 nm, histograms 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).

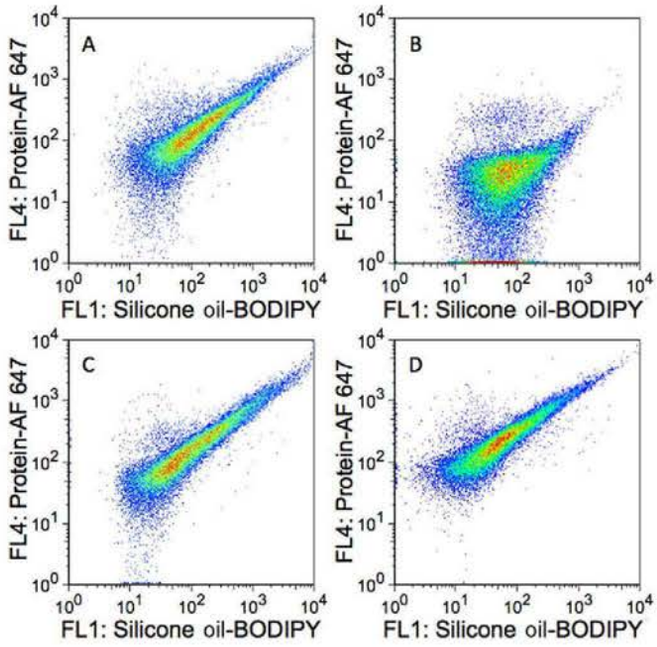

Fig.6. Effects of additives on the association of abatacept–AF 647 with silicone oil–BODIPY droplets. Dot plots represent silicone oil–BODIPY droplets coated with abatacept–AF 647 in formulations containing no additives (excipient-free) (A), 0.03% polysorbate 20 (B), 150 mM sodium chloride (C), and 250 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).

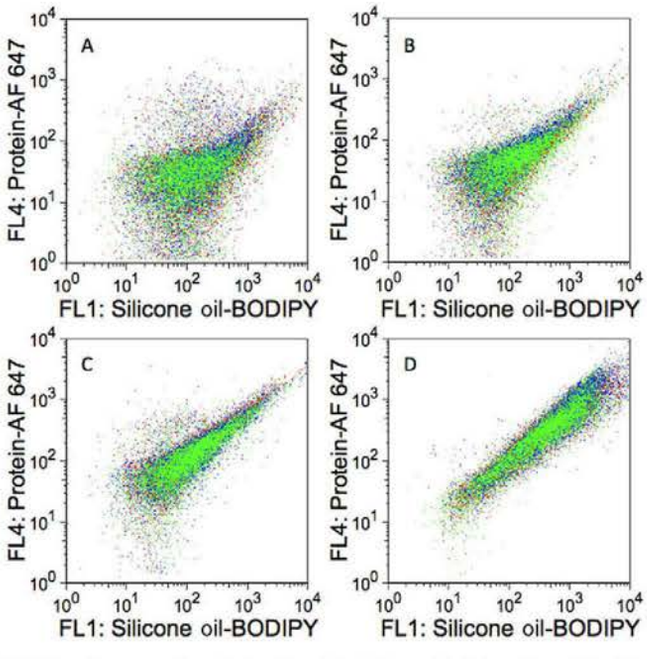

Fig.5. Sample-to-sample variation of protein–silicone oil mixtures in excipient-free formulations: Fluorescence dot plots of three separate samples of silicone oil–BODIPY droplets and lysozyme–AF 647 (A), BSA–AF 647 (B), abatacept–AF 647 (C), and trastuzumab–AF 647 (D). Each color represents a different 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.)

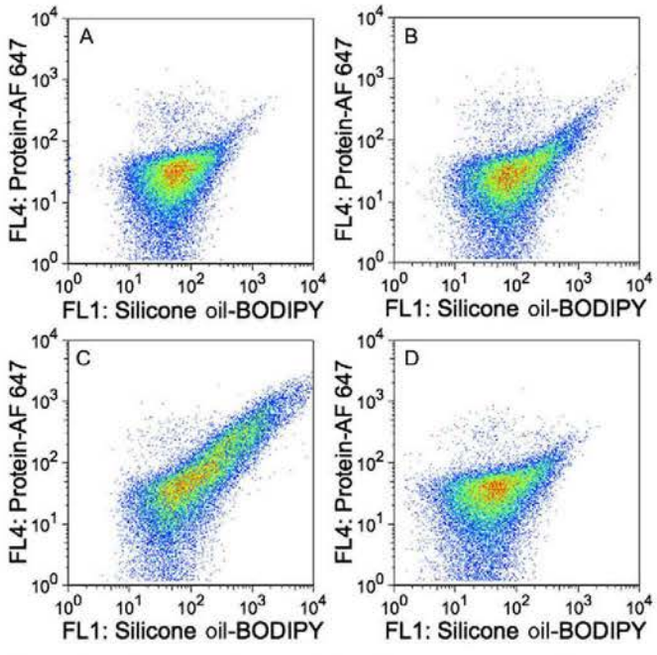

Fig.7. Effects of additives on the association of lysozyme–AF 647 with silicone oil–BODIPY droplets. Dot plots represent silicone oil–BODIPY droplets coated with abatacept–AF 647 in formulations containing no additives (excipient-free) (A), 0.03% polysorbate 20 (B), 150 mM sodium chloride (C), and 250 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).

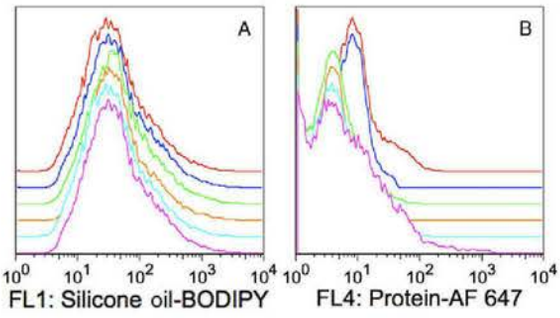

Fig.8. Flow cytometry analyses of trastuzumab–AF 647/silicone oil–BODIPY mixtures in 0.03% polysorbate 20 formulations. Histograms illustrate particle BODIPY fluorescence intensity measured with the FL1 detector (530/30 nm, histograms in panel A) and particle AF 647 fluorescence intensity measured with 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

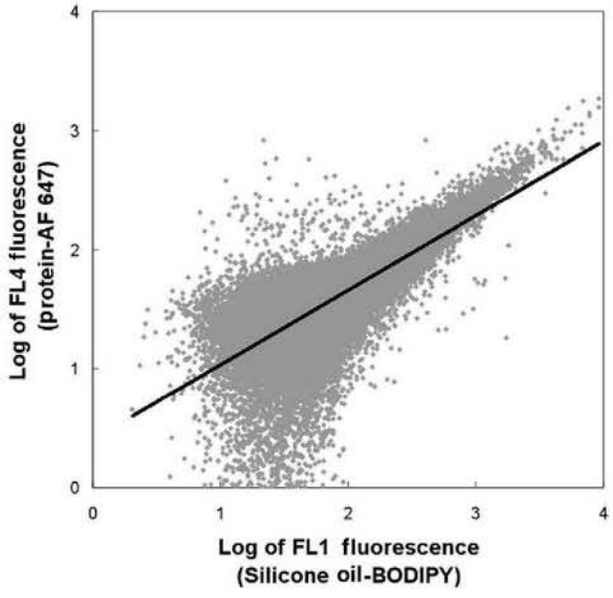

Fig.9. Slope analysis of BSA–AF 647 adsorbed onto silicone oil–BODIPY droplets. A slope of 0.65 was calculated from a linear regression of a log–log plot of FL4 fluorescence (protein–AF 647) versus FL1 fluorescence (silicone oil–BODIPY).

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 that 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

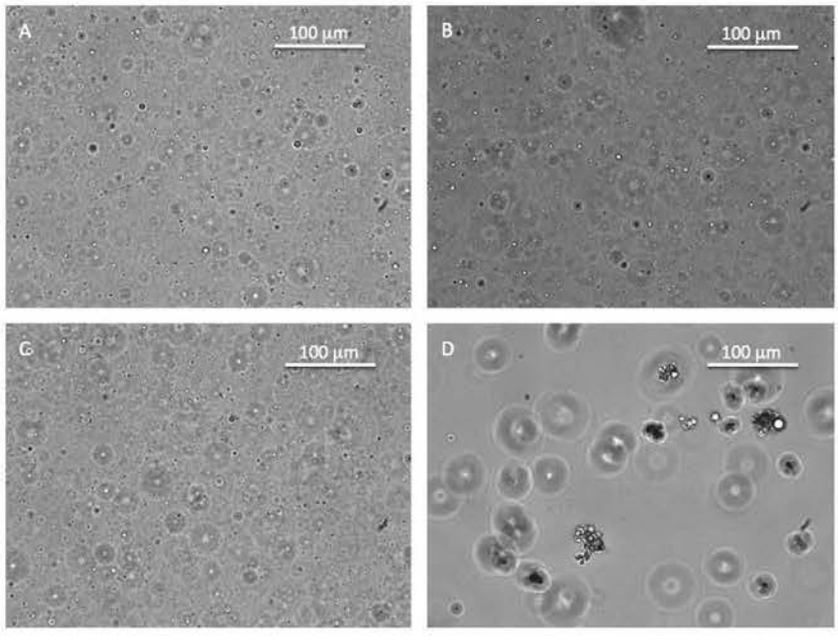

Fig. 10. Light microscopy images of silicone oil–BODIPY droplets coated with lysozyme–AF 647 (A), BSA–AF 647 (B), abatacept–AF 647 (C), and trastuzumab–AF 647 (D) at 200× magnification.

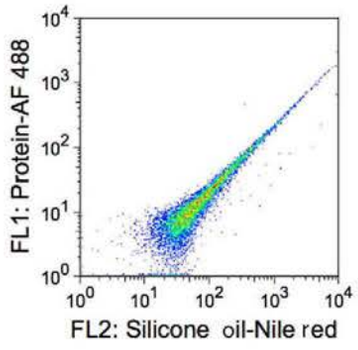

Fig.11. Spectral overlap of Nile red fluorescence from the FL2 detector into the FL1 detector on a BD FACScan instrument (Becton Dickinson, San Jose, CA, USA). The sample consisted of only silicone oil–Nile red droplets. (For interpretation of the references to color in this figure legend, the reader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

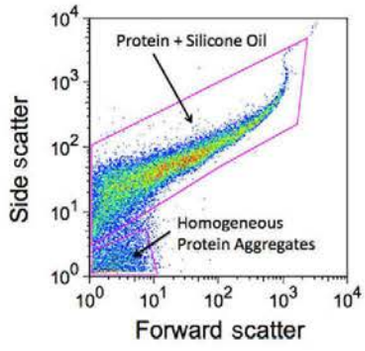

Fig.13. Light scatter dot plot of a mixture of homogeneous protein aggregates and silicone oil droplets with adsorbed protein. Light scattering measured at 90° (SSC) is plotted against low-angle light scattering (FSC) for the sample whose fluorescence is plotted in Fig. 2.

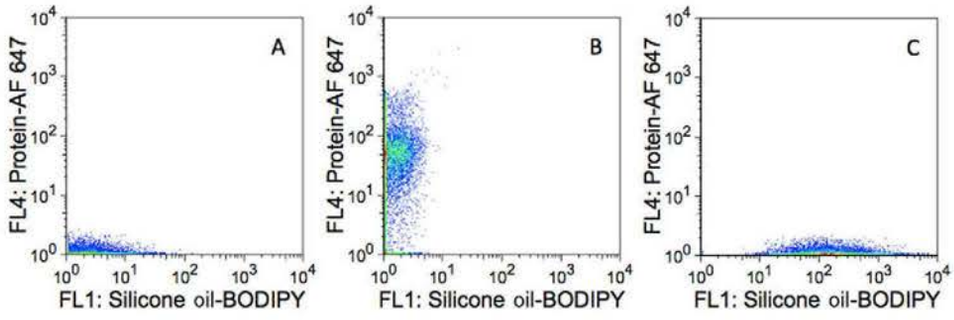

Fig.12. Tests for spectral emission overlap (optical spillover): Fluorescence dot plots of samples of unlabeled silicone oil and unlabeled protein (A), unlabeled silicone oil and BSA–AF 647 (B), and silicone oil–BODIPY droplets with unlabeled BSA (C).

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

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Appendix 3A

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Expert Opinion

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

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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

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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

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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, VEFG-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.

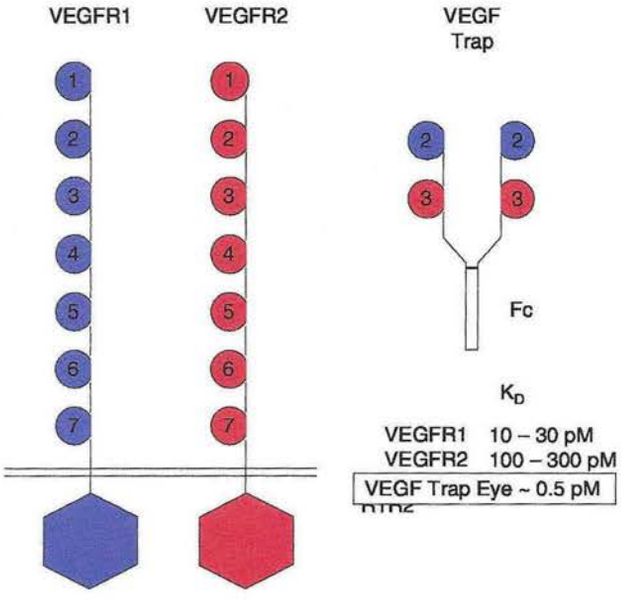

Figure 1. Schematic diagram of VEGF Trap-Eye, a fusion protein of binding domains of VEGF receptors-1 and -2 attached to the Fc fragment of human IgG.

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.

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Appendix 3B

Journal

Expert Opinion on Investigational Drugs >

Volume 18, 2009 - Issue 10

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

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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

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Appendix 3E

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REVIEW

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

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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 Schefler, 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

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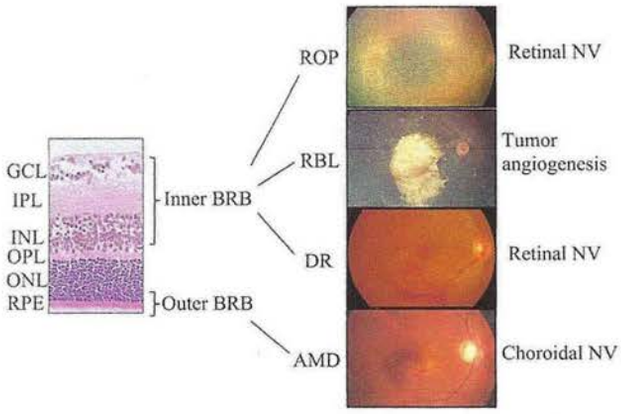

Fig. 1. The common causes of blindness have retinal neuropathies in common, and are due to angiogenesis and breakdown of inner or outer blood-retinal barriers. AMD, age-related macular degeneration; BRB, blood-retinal barrier; DR, diabetic retinopathy; GCL, ganglion cell layer; INL, inner nuclear layer; 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: nonproliferative 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 Schefler, 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, barriergenesis, 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 Schefler, 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, barriergenesis, and neurogenesis.

The roles of VEGF in angiogenesis-related blindness

VEGF not only induces angiogenesis, but also, it causes vascular permeability (Weis and Cheresh, 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; Kvanta 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 1α (HIF-1α), 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-1α 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 (PlGF). 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). PlGF, one member of the VEGF family, binds to the VEGF receptor-1, and inhibition of PlGF reduced the response to VEGF, leading to impaired angiogenesis (Carmeliet et al., 2001). Activation of the PlGF/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-1β, IL-6, IL-8, and TNF-α, were reported to be elevated (Sapieha et al., 2010). IL-1β is considered to be involved in the survival of retinal neurons and in angiogenesis via HIF-1α 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 aresult 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 (GlaxoSimthKline 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-1β, 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-PDGF 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 α5β1 integrin inhibitors were developed: chimeric antibody for α5β1 integrin (Volociximab, Ophthotech Corp.) and a small peptide α5β1 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 PDGRF; 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-1β	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	α5β1 integrin	Phase I
JSM6427	Jerini AG, Berlin, Germany	Small peptide	α5β1 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.

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